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EUROPEAN ATOMIC ENERGY COMMUNITY - EURATOM

LABELLED PROTEINS IN TRACER STUDIES

PISA, Italy
January 17-19, 1966

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and Use of Labelled Proteins in Tracer Studies
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G. MILHAUD (Institut Pasteur, Paris)
J. SIRCHIS (Euratom, Brussels)

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LIST OF PARTICIPANTS

C. AMBROSINO	Istituto Chimica dell'Università, Corso Massimo d'Azeglio 48, Torino
A. ANDERER	Max-Planck Institut, Spemanstrasse 35, Tübingen
S. B. ANDERSEN	Dept. Clin. Physiol., Glostrup Hospital, Copenhagen
D. ANDREANI	Istituto Patologia Medica, Università, Roma
Y. ARNOULD	Université de Bruxelles, 115, Bld de Waterloo, Bruxelles 1
M. AUBERT	Clinique Médicale Universitaire, Lausanne-1012
M. AURELL	University of Göteborg, Sahlgrenska Sjukhuset, Goteborg SV
R. BARNER	F. Hoffmann — La Roche et C° AG, Basel
M. F. BECCHINI	Clinica Medica, Università, Pisa
R. BIANCHI	Clinica Medica, Università, Pisa
V. BOCCI	Università di Siena, Via Laterina 4, Siena
D. BRANDENBOURG	Technische Hochscule, Veltmanplatz, Aachen
H. BRAUMAN	Institut Bordet, 7, rue Héger Bordet, Bruxelles
K. BRUNFELDT	Niels Steensens Hospital, Gentofte
B. A. BURROWS	Boston Univ. Med. Center, 15, Stonkhton St., Boston 18, Mass.
R. CARDINAUD	Dept. de Biologie, C. E. N. Saclay, Gif-sur-Yvette
A. CENTI COLELLA	Istituto di Radiologia, Università, Roma
G. CESONI	SORIN, Saluggia
Y. COHEN	C. E. A., C. E. N. Saclay, B. P. n° 2, Gif-sur-Yvette
A. COMAR	C. E. A., C. E. N. Saclay, B. P. n° 2, Gif-sur-Yvette
N. CONTE	Istituto Semeiotica Medica, Università, Padova
P. M. COTES	National Institute for Medical Research — Mill Hill, London N. W. 7
J. COURSAGET	C. E. A., C. E. N. Saclay, B. P. n° 2, Gif-sur-Yvette
P. G. CROSIGNANI	Università di Milano, Via Commenda 12, Milano
H. DAS	Philips-Duphar-Hemdijk, Amsterdam
J. M. DECONINCK	Hôpital St. Pierre, Louvain
B. DELALOYE	Clinique Médicale Universitaire, 1000 Lausanne
C. DELCROIX	Lab. Central de Médecine Nucléaire, U. L. B., 20, rue Evers, Bruxelles 6
R. DE LUCA	Istituto Semeiotica Medica, Università, Napoli
W. A. DE VOOGD VAN DER STRAETEN	Cell Biology and Histology, Rijnsburgerweg, 10 Leiden
H. DITSCHUNEIT	I. Med. Univ. Klinik, Ludwig-Rehn Strasse, Frankfurt/Main
L. DONATO	Clinica Medica, Università, Pisa
H. DOORENBOS	HaydnLaan, Groningen

J. DUMONT	EURATOM, Bruxelles
H. DWORSCHAK	EURATOM, Bruxelles
P. W. DYKES	University of Birmingham, Medical School, Birmingham
R. P. EKINS	Institute of Nuclear Medicine, Middlesex Hospital, London W. 1
Ch. J. FALLAIS	C. E. N., Mol
G. FEDERIGHI	Clinica Medica, Università, Pisa
G. FEDERSPIL	Istituto Semeiotica Medica, Università, Padova
J. P. FELBER	Clinique Médicale Universitaire, Lausanne
P. A. FINOT	S. A. AFICO, La Tour De Peilz
P. FRANCHIMONT	Hôpital de Bavière, Liège
J. R. M. FRANCKSON	Université de Bruxelles, 115, Bld de Waterloo, Bruxelles 1
P. T. FRANGOPOL	Institute of Atomic Physics, P. O. Box 35, Bucharest
A. E. FREEDLENDER	Boston City Hospital, Boston
T. FREEMAN	National Institute for Medical Research — Mill Hill, London N. W. 7
I. GALATZEANU	Division of Research IAEA, Kärntnerring, Vienna I
M. GALLO	SORIN, Saluggia
A. GALSKOV	University of Copenhagen, Juliane Mariesvej 28, Copenhagen Ø
G. B. GERBER	C. E. N., Mol
J. S. GLOVER	The Radiochemical Centre, Amersham
A. H. GORDON	National Institute for Medical Research — Mill Hill, London N. W. 7
T. GOSZTONYI	National Atomic Energy Commission, Isotope Institute, Budapest
F. C. GREENWOOD	Imperial Cancer Research Fund — Lincoln's Inn Field, London W. C. 2
R. GUIDO	Istituto Semeiotica Medica, Università, Padova
A. HAGEN	C. F. Boeringer U. Soehne, Bahnhofstrasse 5, 8132 Tutzing
C. N. HALES	Department of Biochemistry, Tennis Court Road, Cambridge
L. G. HEDING	Novo Research Institute, Fuglebakkevej 115, Copenhagen N. V.
H. HERZMANN	Lindenberger Weg 70, Berlin-Brech
K. HØEDT-RASMUSSEN	Medical Department B, Bispebjerg Hospital Copenhagen N. V.
R. HOFER	II. Med. Klinik, Alserstr. 4, Wien 9
W. L. HUGHES	Dept. of Physiology, Tufts Medical School, Boston
R. ILLIG	University of Zürich, Steinwiesstr. 75, Zürich
P. JACQUES	Laboratoire de Chimie Physiologique, 6, Dekenstraat, Louvain
K. JØRGENSEN	Novo Research Institute, Fuglebakkevej 115, Copenhagen N. V.
P. G. KNUTSSON	AIEA/FAO, Kärntnerring, Vienna I
R. KÖDDING	Max-Planck Institut für Eiweissforschung-Schillerstr. 46, 8 München 15

W. KONIJNENDIJK	Pharmacological Laboratory, Bloemsingel 1, Groningen
H. KUNI	Institut f. Strahlenbiologie und Isotopenforschung-Lahustr, Marburg-355
C. LUCCHINI	Istituto di Gerontologia, Università, Firenze
R. LUNDSTRÖM	AB Atomenergi, Studsvik, Nyköping
A. LUYCKX	Hôpital de Bavière, Institut de Médecine, Liège
A. S. McFARLANE	National Institute for Medical Research — Mill Hill, London N. W. 7
W. MAIER-BORST	Deutsches Krebsforschungszentrum, 21, Berlinerstrasse 6900 Heidelberg
H. MAIER-HUESER	C. E. N. Saclay, Gif-sur-Yvette
P. F. MARTINI	Clinica Medica, Università, Torino
C. M. E. MATTHEWS	Hammersmith Hospital, Ducane Road, London W. 12
F. MELANI	Abt. Klin. Endokrinologie, I. Med. Univ. Klinik Ludwig-Rehn strasse 14, Frankfurt/Main
G. MENZINGER	Istituto Patologia Medica, Università, Roma
R. MICHEL	Collège de France, 11, place Marcellin-Berthelot, Paris 5^{e}
G. MILHAUD	Institut Pasteur, 28, rue du Docteur Roux, Paris 15^{e}
M. MILONE	Istituto di Chimica Generale e Inorganica, Torino
G. MONASTERIO	Clinica Medica, Università, Pisa
D. MOSSA	Istituto Chimico dell'Università, Corso Massimo d'Azeglio 48, Torino
F. MUHLBERGER	Bundesministerium f. Handel u. Wiederaufbau, Stübenring, 1 Wien 1
E. MULDER	University Hospital, Catharijnesingel 101, Utrecht
W. MULLIGAN	Dept. of Veterinary Physiology, University, Glasgow
E. NIEMANN	Farbwerke Hoechst AG, 623 Frankfurt/Main
R. NORBERG-STENBECK	King Gustaf V Research Institute, Stockholm 60
B. NOSSLIN	Malmö Allmanna Sjukhus, Malmö
E. OGRIS	2nd. Medical University Clinic, Garnisongasse 13, Wien 9
H. A. OOMS	Université de Bruxelles, 115, Bld de Waterloo, Bruxelles 1
P. ORSELLO	SORIN, Saluggia
P. A. OSINSKI	Hôpital St. Pierre, Louvain
G. F. PAGANO	Clinica Medica, Università, Cagliari
P. G. PAGANO	Corso Peschiera 311, Torino
R. PALUMBO	Clinica Medica Generale, Università, Perugia
P. PAVONI	Istituto Patologia Medica, Università, Roma
E. F. PFEIFFER	I. Medizinische Universitätsklinik, Ludwig-Rehn-Strasse 14, 6 Frankfurt/Main
F. PIGORINI	Istituto di Radiologia, Università, Roma
L. O. PLANTIN	King Gustaf V Research Institute, Stockholm

L. PONTONI	Istituto Semeiotica Medica, Università, Napoli
J. T. POTTS	National Heart Institute, Bethesda-Md 20014
W. F. R. POVER	Dept. Medical Biochemistry and Parmacology — Biology Building, University of Birmingham, Birmingham 13
H. J. QUABBE	Universitätskliniken, Spandauer Damm 130, 1 Berlin 19
D. V. REBOLLO GARRIDO	Dirección de quimica e isotopos, Sección de isotopos Av. Complutense 3, Madrid
E. REGOECZI	National Institute for Medical Research, Min Hill, London N. W. 7
W. D. REITSMA	Dept. of Internal Medicine, University Hospital, Groningen
C. REUGE	Centre National de Transfusion Sanguine, 6, rue A. Cabanel, Paris 15e
A. REUTER	C. E. N., Mol
N. RICCIONI	Clinica Medica, Università, Pisa
R. RIPA	Istituto Patologia Medica, Università, Ferrara
U. ROSA	SORIN, Saluggia
C. A. ROSSI	Istituto Chimica Biologica, Università, Pisa
A. ROSSI	SORIN, Saluggia
M. A. ROTHSCHILD	Manhattan V. A. Hospital, E. 24th St. and 1St. Av., New York, 10010
E. SAMOLS	Royal Free Hospital, Grays Inn Road, London W. C. 1
C. SCANDELLARI	Istituto Semeiotica Medica, Università, Padova
G. A. SCASSELLATI	SORIN, Saluggia
H. SCHATZ	2nd Medical University Clinic, Garnisongasse 13, Wien 9
K. E. SCHEER	Deutsches Krebsforschungszentrum, Berline Strasse 21, 6900 Heidelberg
G. SEGRE	Istituto di Farmacologia, Camerino
U. SENIN	Clinica Medica Generale, Università, Perugia
J. SIRCHIS	EURATOM, Bruxelles
P. SIZONENKO	Hôpital Saint-Vincent de Paul, 74 Ave. Denfert Rochereau, Paris 14e
U. STEWART	Abt. Klin. Endockrinologie I. Med. Univ. Klinik Ludwig-Rehn-Strasse, Frankfurt/Main
G. STRAUCH	Faculté de Médecine de Paris, 15, rue de l'Ecole-de-Médecine, Paris 6e
F. TEULINGS	Radiotherapeutic Institute, 297 Groene Hilledijk, Rotterdam 24
E. TRIANTAPHYLLIDIS	Faculté de Médecine, 43, rue des Sts Pères, Paris 6e
C. TRITTO	Clinica Medica, Università, Napoli
A. UZAN	Industrie Biologique française, 35, quai du Moulin de Cage, Genevilliers
H. VAN CAUWENBERGE	Hôpital de Bavière, Liège
S. VENTURA	Clinica Medica Generale, Università, Perugia
P. VESIN	31, rue de Moscou, Paris 8e

F. VITEK — Institute of Biophysics, Praha
G. VACCHELLI — SORIN, Saluggia
K. F. WEINGES — II. Med. Univ. Klinik u Poliklinik, 665 Homburg Saar
M. G. WOLDRING — University of Groningen, Oostersingel 59, Groningen
R. S. YALOW — Veterans Administration Hospital, 130 West Kingsbridge Road, New York 10468
E. ZIMMER — SORIN, Saluggia

PREFACE

by Professor A. CARRELLI,
Vice-President of the European Atomic Energy Community

Every noteworthy advance made by scientific research can be said to be always closely linked with a further development of our understanding. That is to say that the enrichment of our knowledge is essentially connected with some greater quantity of experimental data that we can gather by increasing our awareness of the external world; the larger body of information gathered then raises a broader uncertainty which drives the human mind, faced with more complicated problems, to formulate concepts of yet greater depth and significance.

The development of the optical microscope and, more recently the electronic microscope, to mention only two of the more important examples out of many, opened up immense and profoundly significant fields of research to biologists, but who would presume that this system had brought us to the furthest bounds of knowledge, especially in so complex a field as that of life processes ?

The recent advances in biochemistry, the achievements made in the field of physiology and genetics, have substantially added to our knowledge of the life processes, but problem after problem faces the inquirer each time another stage is reached on the difficult road of research.

And now, with Fermi's discoveries, which have led in recent times to the synthetizing of radioactive isotopes of every chemical element, a new field has opened up, offering marvellous promise to both biological and chemical research.

The presence, in a limited quantity of material, of radioactive substances endows that portion of material with peculiar electrical properties. Modern physical methods detect such properties with exceptional sensitivity. Minute traces of radioactive matter can be picked up, so that by introducing into the complex metabolism that takes place in the biological field a compound in which certain atoms are radioactive isotopes, that compound can be followed along the whole of its route by this highly sensitive method, providing the biologist with information that would be absolutely unthinkable with the customary methods.

Such, then, is the essential use of « artificial » radioactivity in biology, but at the very outset this ingenious method raises the problem of preparing and utilizing the compounds containing the labelled molecules. Of particular importance is the use of suitably labelled proteins. A new and highly intricate branch of research has thus grown up.

An idea of the complexity and importance of the questions this method has posed to researchers can be obtained from the pages of this book, which contains the numerous papers presented at the Pisa Colloquium.

This publication shows the intricacy and significance of this field of research in present-day biology, thus demonstrating the usefulness of these meetings, which bring together an ever-growing number of research scientists, who are tackling the complex phenomenon of life with vision and constantly encouraging prospects.

OPENING ADDRESS

by Professor G. MONASTERIO,
Director of the Medical Clinic and the Nuclear Medicine Center, Pisa

The use of radioactive labelled proteins has made it possible to obtain information of utmost importance both to the physiologist and to the clinician.

The interest for these tracers appears evident from their numerous applications to experimental and diagnostic investigations. Among the labelled proteins stands out the human serum albumin, which, tagged usually with iodine-131 and more recently with chromium-51, has found its way to a variety of applications.

The radioactive labelling, more often with iodine-131, of some proteic hormones has made it possible to study *in vivo* their metabolism and mechanism of action, to assess, under pathologic conditions, the nature of the different sensitivity of the organism to some of them, especially to insulin, and to approach the problem of their assay with radioimmunological methods.

Over the last few years the information derived from the use of labelled proteins has been piling up. Usually, γ-emitting radioisotopes have been chosen as labels for the proteins, since they allow their detection *in vivo* by external counting. However, the results obtained have been often conflicting even in the same group of workers and in spite of the accuracy of the employed technique.

The reasons for such discrepancies have been attributed to the alterations of the proteins during the labelling process and, sometimes, to the inadequacy of the analytical procedures which have been used. It is, of course, a fundamental prerequisite that the labelling process does not modify the physical and chemical properties of the molecules to such extent that the biological characteristics of the proteins be altered. Only if this requirement is satisfied, the labelled proteins may be considered as physiological tracers, i. e., it can be assumed that their behaviour is equal to that of the corresponding proteins of the organism.

This fundamental problem was the subject, some nine years ago, in November 1956, of a comprehensive and detailed discussion at a conference sponsored by the New York Academy of Sciences under the chairmanship of S. P. Masouredis. At that meeting, the first two communications were presented by W. L. Hughes and by A. S. McFarlane, whom we have the pleasure to have with us this morning.

Some of the technical problems which were set forth at that time have now been answered more or less completely, making thus possible to secure more reliable informations through the use of proteic tracers.

It was deeply felt, therefore, the need to bring again together the workers interested to these problems for a new extensive discussion. The initiative for the present meeting is merit of the European Atomic Energy Community under the auspices

of which the congress is being held. To the European Atomic Energy Community, I wish to express my thanks for choosing Pisa as the place for this conference and for assigning to the Medical Clinic of this University the task of organizing it.

The subject of the congress might seem far from the field of interests of an Institute of Internal Medicine, since the meeting is focused on technical problems more or less foreign to a physician. However, these problems have been raised, to a large extent, by the physician himself, since their solution is a necessary step for any methodological progress in the experimental and diagnostic investigation with radioactive tracers.

It is true, on the other hand, that the solution of the problems involved in labelling proteins is not to be expected from the physician or from any worker in a single branch of the basic sciences, since it demands, because of its complexity, the joint effort of people with different knowledge and experience.

It occurs quite often, in fact, that the fundamental problems for the physiologist, and, more generally, for the biologist, derive from the clinical observations which inspire the work of these researchers. The solution of these problems, however, as well as almost any progress in medicine, depends on the close cooperation of mathematicians, chemists, physicists and physicians, on the applications of experimental and analytical methods of the basic sciences, and on the full and timely usage of the means provided by an ever growing technology. In this regard electronic computers deserve special mention.

Owing to the numerous and complex attributions of an Institute of Internal Medicine, which include teaching, research, and care of patients, it would be necessary to set up, within such an Institute, largely independent departments where basic sciences, technology and clinical problems may be brought in contact with each other. It should thus be possible to solve several problems and to promote the progress in medicine. There is now a trend to accomplish this integration of different scientific disciplines in autonomous Institutes the attributions of which comprehend only research activities and exclude teaching responsibilities and care of patients. In these Institutes scientists of different background can work together in close cooperation entirely dedicated to research problems. It should thus be possible to develop new and more advanced methods, which are instrumental to any progress in the various fields of medicine.

Only through a working integration of mathematicians, chemists, physicists and physicians the growing gap between medicine and basic sciences may be bridged and clinical medicine may take up, on a larger and firmer ground, the characteristics of biological science which become it.

This congress, that brings together, for a few days and with a program of intensive talks, workers in clinical medicine and medical biology with those in fundamental disciplines, offers me the opportunity to stress the need for such cooperation and to point out the so often recurrent mistake of believing that the clinician attributions have nothing to do with such problems. In medicine, as I have already

remarked, the work of the researcher is the more fruitful the more is inspired to the direct clinical observation. Without the close cooperation of the clinician, the work of the isolated researcher is bound to become sterile, since most of the times is the former to raise the problems of actual interest. Furthermore, the clinician stimulates the researcher with his urgency for the solution of a problem and checks in man the validity of the results.

It is well known, on the other hand, that in other applied sciences and particularly in those of very recent origin such as the space sciences, the more difficult problems have been overcome only through a wide and close cooperation among different disciplines and the progress itself of the basic sciences and technology has been promoted by the need and urgency of solving these problems.

It is thus understandable the reason for which the clinician is so sensitive to the solution of the most various problems, even when the methods involved appear quite foreign to the domain of his competence and interests.

It is thus explained also the program of the congress and its attendance by many clinicians and researchers of clinical extraction. In fact, after the first and second session, biological aspects of labelling the proteins in general on and the plasma proteins in particular, the application to metabolic studies of these tracers, i.e., proteic hormones and plasma proteins, will be treated tomorrow, and finally, the discussion will center on the theoretical problems, the solution of which is essential for a correct analysis and interpretation of the results obtained with the proteic tracers.

In the act of giving my warm welcome to all the researchers here gathered even from distant countries, I wish to express them my personal thanks and those of my collaborators of the Medical Clinic of Pisa for having accepted the EURATOM invitation to participate actively to this congress. I wish also to thank Prof. Alessandro Faedo "Magnifico Rettore" of our University for offering us his generous hospitality.

I take the opportunity of this meeting to renew the feelings of my obligation to Euratom for having acknowledged with the organization of this congress both the paramount interest of these problems and in general of the applications of the atomic energy to the scientific research in medicine, and the work accomplished by the researchers of Pisa during the last three years under the Contract of Association with the Euratom and the Free University of Brussels.

I wish that the results of our proceedings may show the Euratom that some convictions periodically inspiring its policy, and in force of which medicine is considered the cinderella of the various applied sciences, are quite groundless. I express the hope, in fact, that the results so far obtained may prove the validity and productivity of the effort devoted to sponsor and promote research in this field.

As a matter of fact, setting aside any other considerations, it is just in this field of scientific investigation that, on the basis of the experience so far available, the fastest progresses with the minimum cost may be expected; it may be foreseen, the-

refore, the highest revenue of the means employed. It is in this field that the Euratom might reasonably pursue the design of attaining in a short time a position of prominence whereas, in other fields, where incomparably larger means are required, such a design would be extremely aleatory and in any case realizable only in a very long run.

FUNDAMENTAL ASPECTS OF PROTEIN LABELING

Chairman : W. L. HUGHES

THE CHEMICAL REQUIREMENTS OF A SATISFACTORY LABEL FOR PROTEINS

W. L. HUGHES (*)

Department of Physiology, Tufts Medical School, Boston, Massachusetts, USA

ABSTRACT

The labeling of proteins is discussed in general terms with emphasis on the advantages and limitations of various techniques. The practical advantages of iodination and some of the complications which affect interpretations are reviewed in the light of present information as to the mechanism of iodination and the role of the tyrosyl group in protein structure and function. Some new approaches to these problems are suggested.

Labeled proteins are used primarily as tracers for the purpose of understanding the function of the native unlabeled protein, and therefore should differ from the unlabeled protein by a minimal amount, or at least by an amount too small to be recognized by the biological system under investigation. The ideal label would therefore appear to be an isotope of an element contained in the molecule under study. In the case of proteins this would then usually be an isotope of carbon, hydrogen, nitrogen, or sulfur. Moreover, the isotope should occur in the protein as an atomic replacement linked in a normal way to the neighbouring atoms. If it is to be used to follow the fate of the protein, it must remain with the protein until the protein is destroyed, and if it is intended to follow the fate of a degradative fragment, it must likewise remain with the fragment.

To be sure, even isotopes of the same element are not handled identically or else present day methods of separating isotopes would be impossible. However, since isotopes differ only in mass, their chemical properties are different primarily in processes affected by mass such as diffusion or kinetic processes involving the transfer of an atom. Even in these instances the small difference in mass minimizes these effects for all the biologically important isotopes excepting those of hydrogen. In the case of hydrogen while large isotope effects may be observed in hydrogen transfer processes, when the isotope remains attached to the same organic grouping during a reaction process, its effects are minimal.

Protein molecules are still beyond the synthetic prowess of the chemist and so the incorporation of a label into the native protein must be done biosynthetically via a labeled intermediate as first developed by Hevesy [1]. These processes utilize the isotope inefficiently and demand the chemical isolation of the particular protein under study since all the proteins are labeled by the process. Furthermore, the data obtained may be difficult to interpret because of reutilization : that is, the label may transfer during the study to a new molecule of the same species which cannot

(*) The author is indebted to the U. S. Atomic Energy Commission for its continued support of his work on the preparation and exprimental use of labeled proteins.

be distinguished from the original molecule. In this case additional information concerning the extent of this back-reaction must be obtained [2]. In human experimentation the method is also complicated by the large radiation dose to which the subject is exposed.

Consequently, most labeled proteins are prepared *in vitro*. This might be done by appropriate exchange of a radioactive atom with its stable isotope in the protein as, for example, a triton for a proton. However, this procedure although it has proven useful in studies of protein structure [3] does not seem applicable to biological studies because proton exchange takes place under physiological conditions and therefore would continue to occur during a physiological study. Perhaps the exchange of labeled terminal amino acids might be carried out enzymatically. However, such groups could be subsequently lost by similar enzymatic action *in vivo*.

Other types of hydrogen exchange are possible and may prove valuable in the future. Thus oxytocin has been labeled by iodination followed by catalytic deiodination with tritium gas to restore biological activity as a tritiated hormone [4]. Also the Wilzbach technique has been applied to proteins [5]. In this process the protein is exposed to a large amount of tritium gas. Exchange of tritium with hydrogen in any position of the protein molecule can occur through radiation processes. These may involve either the production of energetic tritium atoms which exchange with a hydrogen in the protein or the production of protein radicals by the loss of hydrogen which can recapture a tritium. In any case successful exchanges are always accompanied by a variety of degradative changes so that the isolation of labeled but undamaged molecules is extremely difficult or probably impossible in the case of proteins containing thousands of bonds sensitive to rupture by the radiation.

For many purposes the introduction of new and foreign labeled groupings into the protein molecule has proven most practical. In every instance where this is done the effects on the original protein must be ascertained both at the chemical and at the biological level. Such precise re-assessment of the labeled molecule is frequently arduous. However, the advantages to be obtained in terms of practical results are usually worth the effort. An immediate advantage of these procedures over biosynthesis is that frequently the native protein is already available in a highly purified state. This obviates repetition at a micro level, of the usually involved procedures for isolation and characterization of the protein. In addition, as already pointed out, the isotopic yields are much better since only the desired protein is labeled.

The choice of a label will depend upon the nature of the protein as well as on the type of study to be undertaken. Fortunately, a wide variety of radioisotopes with different radiant energies and half-lives are available, so that the first consideration should be the chemical properties to be achieved. In principle, any introduced group must change the properties of the original molecule. However, the degree of change will be related to the degree of conformational alteration both of the protein as a whole and of its reactive site. One might predict the change in chemical and biological properties would be related to the mass and electrical charge

of the introduced group and to its position relative to the functional part of the protein. Since proteins contain thousands of atoms, the change in mass would appear trivial, and since the reactive site of a protein molecule usually appears to involve a small fraction of the molecule, the chances of a successful modification would seem good.

However, it must be remembered that the active site frequently contains some of the most reactive groups in the molecule so that finding a reagent which will not affect these groups may be very difficult. Furthermore, the identity of the functionally important groupings is still not known for most proteins, and in fact, modifications of the type we are discussing have proven most useful in discovering the functionally important groups. Returning to our principle of producing the smallest possible percentage change in the protein, the group introduced should be as small as possible, but more important it should not introduce new charge or change the charge distribution on the protein. Therefore, the introduction of a single atom would usually seem preferable and the halogens have proven most successful. We shall return to these shortly.

Of course, the atom introduced should remain attached to the protein throughout the study and hence a co-valent bond not attacked by the usual substances present in biological fluids would seem necessary. This requirement appears to rule out most metals for labeling purposes since they form ionic bonds which exchange readily with water. However, in certain favorable circumstances as with iron in hemoglobin or with manganese in an active site, as we shall see in the following papers, coordination may complex the metal sufficiently firmly for biological studies. Similarly, chromium can be used because its complexes in the valence 3 state are extremely sluggish. This too will be discussed in detail in succeeding papers. Some metal-carbon bonds would have sufficient stability as for example, the C-Hg bond. However, the preparation of these usually must be done in the absence of active hydrogen, a condition hard to achieve with a protein.

Among the non-metals useful in labeling, besides the seventh group of the periodic table, (the halogens), we might also consider the fourth, fifth, and sixth groups, represented by carbon, nitrogen, and oxygen. However, these elements present complications in that their stable valence states are 4, 3, and 2 respectively, requiring the introduction of several atoms to satisfy their valency and frequently leading to the introduction of a charged group into the protein. In the case of carbon, the smallest group to be introduced would be methyl (CH_3). Actually, this grouping is not larger than bromine but the usual manner of its introduction via methylating reagents involves the esterification of a carboxyl grouping (which changes the charge on the protein and forms a labile derivative) or to the replacement of an active hydrogen of a sulfhydryl, or imidazyl group. If the methylating reagent could be directed specifically to an amino group the changes produced should be minimal and a very useful derivative should result, although the long half-life of carbon-14 would preclude the possibility of obtaining the high specific activity necessary for some tracer studies.

Nitrogen and oxygen do not have useful radionuclides, but the elements below them in the periodic table, phosphorus and sulfur do. However, their stable valence states of + 5 and + 6 respectively, require the simultaneous introduction of several other atoms, usually oxygen. Thus, the hydroxyl groups of serine or threonine may be phosphorylated with $POCl_3$, but this introduces the charged -O-P $\overset{\overset{O}{\|}}{=}$ $(OH)_2$ group into the molecule. Perhaps the most useful phosphorous derivative has proven to be diisopropyl fluoro phosphate (DFP), which will attack a variety of active hydrogens but has found use as a specific reagent because it attacks the active site of certain esterases such as choline esterase and chymotrypsin much more rapidly. The stability of the bond formed is sufficient to permit the isolation of phosphorylated peptides upon hydrolysis of the inactivated enzyme [6]. This suggests that such labeled derivatives might also prove valuable in studying the metabolic fate of these proteins in the plasma similar to studies which have been carried out with DFP labeled erythrocytes. Chlorosulfonic acid has been used to label the hydroxyl groups. This introduces an acid sulfate group with its attendant charge and thus changes the properties of the protein, at least in its region. However, methane sulfonyl chloride should obviate this difficulty. This has been rarely used, but the corresponding benzene sulfonyl chloride as its para-iodo derivative (pipsyl chloride) has found wide use in labeling proteins for peptide sequence studies since the resulting sulfonamide bond resists acid hydrolysis [7].

Now we have progressed into the specific organic reagents for proteins and to attempt to even cite these would consume my remaining time so let us return to halogenation. Halogens form reasonably stable bonds with carbon in a variety of compounds either by the displacement of hydrogen or by addition to an unsaturated carbon-carbon bond. Fortunately many of these reactions are slow, and in aqueous solutions only a limited number of the more rapid reactions need be considered. The reactions of iodine with proteins were discussed by Ramachandran ten years ago [8] in an excellent review which needs few additions to bring it up to date. Since the halogens are increasingly reactive in the order iodine, bromine, chlorine, fluorine, the other halogens possess still broader spectra of reactions. In addition, they possess no isotopes of wide biological applicability, excepting ^{82}Br and so will not be discussed further here. (Astatine, (element 85), the last of the halogens has no stable isotopes and thus presents interesting problems with reactions at truly tracer concentrations. It has been possible to bind astatine to protein, although whether the reaction occurred with tyrosyl residues in an entirely analogous fashion to iodine was not clear[9].)

Iodine possesses about 2 dozen radio-nuclides and some of these shown in Figure 1 have found wide utility. The radio-isotope is usually obtained as the iodide with or without added carrier ^{127}I. If adequate care has been taken to exclude all stable iodine in its preparation the limiting specific activity must depend on its half-life. However, in practice other isotopes are always present limiting the specific activities which can be obtained [10].

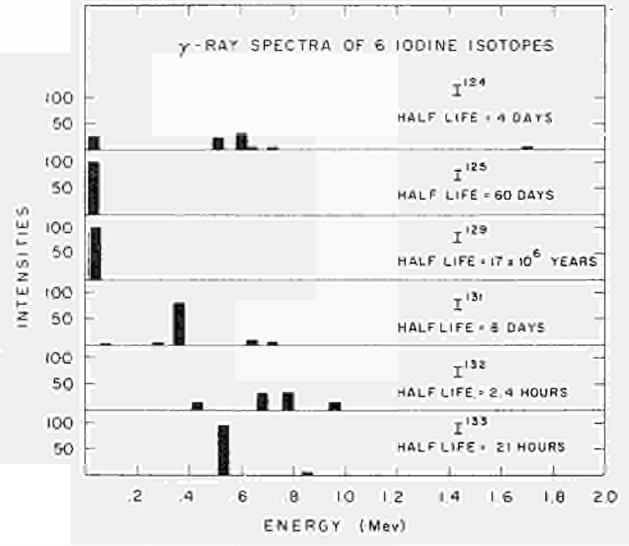

FIGURE 1.

Iodide, of course, cannot form stable linkage with protein. Protein labeling reported by the simple addition of radio-iodide may have involved higher oxidation states of iodine perhaps brought about by radiation-induced peroxides. Alternately the iodide may have reacted with radiation-induced protein radicals to form a covalent bond. In any case such radiation-induced events are rare and very inefficient. Therefore the first step in iodination is to convert the iodine to a higher oxidation state, usually of 0 or of $+1$. This may be most simply done by the addition of carrier iodine, or better of tri-iodide ion since iodine dissolves in water only to the extent of 1 milli-molar. The following equilibria then apply : (Figure 2).

I_3^-	$\rightleftarrows I_2 + I^-$	$K = 0.0013$
$I_2 + H_2O$	$\rightleftarrows H_2OI^+ + I^-$	$K = 1.2 \times 10^{-11}$
$I_2 + OH^-$	$\rightleftarrows IOH + I^-$	$K = 30$
HOI	$\rightarrow H^+ + OI^-$	$K = 10^{-11}$
$3OI^-$	$\rightarrow IO_3^- + 2I^-$	
$I_2 + RNH_2$	$\rightleftarrows RNHI + H^+ + I^-$	

FIGURE 2. — Equilibria of Iodine with Solvents

Chemists are still not in agreement as to the identity of the active iodinating species; iodine, hypoiodous acid, and hypoiodinium ion have all been implicated. The first reaction in any case can be used to control the rate of reaction since the concentration of free iodine is inversely related to the iodide concentration. Hypoiodous acid and hypoiodinium ion concentrations also depend upon that of iodide but in these cases a second iodide ion is produced in their formation so that the concen-

tration of these species depends inversely upon the square of the iodide concentration. Reaction 5 which is essentially irreversible in alkaline solution, acts to remove the iodinating species converting it to iodate and iodide. However, it occurs at an unappreciable rate below pH 10. The last reaction can occur with ammonia or the amino groups of proteins or with other nitrogenous bases such as sulfonamides or succinimide but is rapidly reversible and results in no permanent binding of iodine. However, like reaction 1, it may limit the rate of other iodinating reactions.

When an iodine solution containing these species is added to protein, additional reactions occur (Figure 3). These processes are either irreversible (or essentially so) in that the equilibrium is far to the right, as in the case of SH oxidation. The reactions with sulfhydryl always occur first. However, they do not result in stable bonding of iodine. Whenever geometrical factors permit, the third and fourth reactions of Figure 3 take place, liberating all of the iodine as iodide ion. In the case of monothiol proteins such as serum albumin, in which the formation of disulfide link is sterically difficult, only the third reaction occurs and two equivalents of iodine are consumed, but the sulfenyl iodide must hydrolyze rapidly, since the final product is devoid of iodine [11]. Consequently, in any labeling experiment

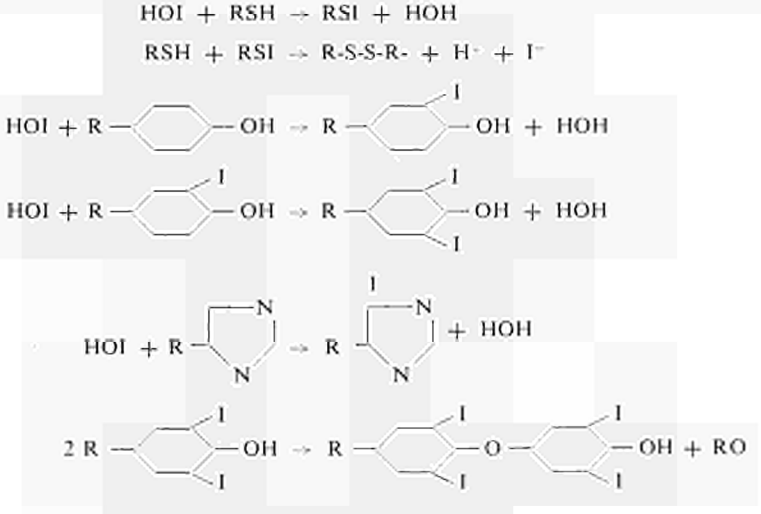

FIGURE 3. Iodine Reactions with Protein

the first iodine consumed, equivalent to the SH content of the solution, must be considered lost for purposes of labeling. The rate of SH oxidation depends inversely on the hydrogen and the iodide ion concentrations suggesting that either hypoiodous acid or thiolate anion is the active species. Aside from causing a poor yield in labeling, the oxidation of SH groups will of course be disastrous for proteins whose function requires a sulfhydryl group, and some method of protecting these groups should be developed. Metals can be readily reacted with SH groups but

do not protect against iodination. Alkylating reagents will protect but are difficult to remove. Cunningham and Nuenke have produced mixed disulfides by reacting the sulfenyl iodide reaction product of a protein with a small mercaptan [12]. This process might be used before labeling the protein and permit subsequent restoration of the sulfhydryl after iodination by dissociation of the disulfide bond with an excess of mercaptan.

The reaction with tyrosyl residues is the basis of all iodine tagging of proteins. The kinetics of this reaction have recently been reinvestigated with acetyl tyrosine by Mayberry *et al.* [13, 14] who prefer to write the reaction as illustrated in Figure 4. They assume the rate determining step is the second reaction involving the base catalyzed displacement of a proton from a quinoid form.

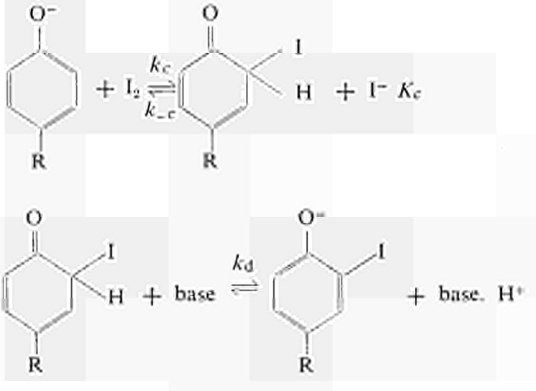

FIGURE 4

This mechanism is consistent with their data and explains the general base catalysis of the reaction, the dependence of the rate on the hydroxyl ion concentration, and on the square of the iodide concentration. Obviously, alternate mechanisms involving I^+ or HOI could also be formulated to fit the data. However, the requirement for a phenolate ion which is implied by the non-reactivity of O-substituted phenols makes hypoiodous acid difficult to accept. This mechanism also explains the labeling of iodotyrosines by exchange with molecular iodine, which has been shown to proceed more rapidly than iodination itself. Mayberry *et al.* [15] have also measured the rate of entry of the second iodine atom to form diiodoacetyl tyrosine and find it is 30 times slower than the first when the rate measurement is based on the concentration of the respective phenolate ions. Therefore at neutral pH where both tyrosine and its monoiodo derivative are present in the unionized form, the rates of iodination must be about equal since the phenolic pK's are 10.2 and 8.8 respectively. Consequently with incomplete iodination the presence of both mono and diiodo tyrosyl residues, which is frequently observed, is to be expected.

The mildest and best method of iodination is that using iodine in excess iodide (I_3^- reagent), and rates are readily controlled by the amount of iodide present. However, since all iodine atoms are equivalent, the fraction of iodine incorporated varies inversely with the excess of iodide used. To increase the fraction of iodine incorporated, iodine itself may be used with theoretical yield of 50 %, or ICl may be employed with a theoretical yield of 100 % [16]. However, any groups such as sulfhydryl, which are concomitantly oxidized, will decrease the yield proportionately. Therefore, procedures have been developed for regenerating any iodide to reactive iodine *. In recent work this is usually done by adding chloramine-T which presumably forms iodine by the following mechanism :

This reaction as developed by Glover *et al.* [17], also avoids the addition of carrier iodine and thus permits the labeling of proteins at the very high specific activities which are required for hormonal assay to be discussed later in this conference. A simple procedure for handling large amounts of radioactivity and assuring mixing has been reported by Reith and Brown [18]. This method can be performed rapidly and followed by the immediate addition of a radioprotective agent, such as serum albumin, to minimize radiation damage [35].

However, hidden changes may be produced in the protein by this reaction since part of the iodine formed may cycle between the reagent and the protein alternately reducing the reagent and oxidizing the protein. Such changes may be appreciable and still go undetected unless efforts are made to measure the amount of chloramine-T consumed as well as the iodine incorporated. In addition chloramine-T, or any other oxidant powerful enough to oxidize iodine, may attack the protein directly. The vigorous nature of these reagents has been observed by Koshland who was able not only to iodinate tyrosine and histidine but to destroy methionine and tryptophan in gamma globulin by exposure to an excess of iodine chloride at pH 9 and 5° C [19]. McFarlane has controlled the process by careful stoichiometric addition with rapid complete mixing (jet iodination) [20].

The presence in a given protein of tyrosyl residues of varying reactivity is frequently noted both by spectrophotometric studies and by iodination in which a portion of the residues remain unreacted even after vigorous treatment. This has been studied in greatest detail in ribonuclease by Scheraga *et al.* [21, 22] who believe they can identify spectrophotometrically 3 reactive and 3 inert or "buried" tyrosyls on the basis of differences in the dissociation of their phenolic hydroxyl groups. Since the complete amino acid sequence of ribonuclease is known, more detailed information can be obtained by peptide analysis of iodinated ribonuclease. Iodine

(*) In the next paper Dr. Rosa will describe a simple but elegant method in which he oxidizes the iodide to iodine in an electrolytic cell.

readily reacts with 3 of the 6 tyrosyl residues to give an enzymically active product and two of the unattacked tyrosines have been identified as residues # 25 and 97. This has been confirmed by Donovan [23].

Pressman *et al.* [24] have found that the terminal tyrosyl in chymotrypsin iodinates three times faster than the other tyrosyls in this molecule. Iodo-insulin has been the subject of many investigations, but since it will be discussed repeatedly in the next papers I will not mention it further here.

Pressman's group have also iodinated proteins in the presence of specific small interacting molecules, and found that the protein sites responsible for binding the small molecule were then protected and not altered by iodination. Thus, heavy iodination of serum albumin (44 atoms iodine) decreased the binding of p-iodobenzoate ions by 50 %, but this decrease could be prevented when iodination to the same extent took place in the presence of excess p-iodobenzoate. The presence of p-iodobenzoate did not protect the site binding methyl orange [25].

Pressman has also provided evidence that some antibody sites may contain essential tyrosyl residues since iodination decreased the affinity of the antibody for an anionic hapten containing a phenyl arsonic acid group. This decrease could be prevented if the hapten was present during iodination [26]. Koshland has confirmed this but also shown that this is not invariably so since iodination of an antibody to a hapten containing a cationic phenyl ammonium group was not protected by the presence of the hapten [27]. An explanation of these effects of course does not require the hapten to interact directly with the tyrosyl residue of the antibody. The mechanism might involve a reversible configurational change exposing a tyrosyl residue in the region of the active site, which is prevented by the presence of the hapten. Alternately, the presence of an additional negative charge at the antibody site might decrease the rate of iodination by depressing the ionization of an adjacent phenolic hydroxyl group or by preventing the approach of anions required for base catalysis of iodination.

In the iodination of proteins it is usually impossible to be certain that changes are absolutely limited to the tyrosyl residues, and therefore a recent study on biologically synthesized proteins in which tyrosine was replaced by 3 fluoro-tyrosine should be noted. The phenolic hydroxyl of 3 F-tyrosine (like 2 I-tyrosine) is appreciably more acidic than that of tyrosine. A tyrosine requiring mutant of Escherichia coli was grown on medium in which 3 F-tyrosine was substituted for tyrosine. While the growth rate was slower than in tyrosine medium, nevertheless multiplication did occur [28]. The β-galactosidase was studied in some detail both in terms of its enzymatic properties and its stability. The fluorinated enzyme was less stable towards urea or heat. This might be due to weaker hydrogen bonds involving the phenolic hydroxyl since fluorination would facilitate their ionization and dissociation. However, fluorine substitution had no effect on the Michaelis constant of the enzyme [29]. It will be interesting to learn what changes may have been produced in other enzymes. However, these could not have been too great since presumably all of the other enzymes in the organism must also have been active in order to permit

growth. Therefore, this much alteration of the phenolic pK must be generally permissible in protein structure without loss of function, and any case of protein inactivation due to the substitution of a single iodine into tyrosine is worthy of further investigation. If such cases are found, a critical comparison of the phenolic pK's of both 3-fluoro and 2-iodo substitution will be necessary. If these are comparable, then inactivation due to mono-iodo substitution would almost certainly implicate steric effects due to the size of the iodine atom. Steric effects have been seen in thyronine derivatives and used to outline certain spatial requirements at the target of thyroid hormone action [30].

Mono-iodo tyrosine has not been reported to be incorporated into proteins. However, this may be due to the specificity of the tyrosyl activating enzyme involved in synthesis and not to structural requirements of the proteins. Here too it would be interesting to see whether tyrosyl attached to transfer RNA could be iodinated and then incorporated into protein. In any event the lack of incorporation of iodotyrosine is important for turnover studies since it precludes the reutilization of the iodine of iodinated proteins following their catabolism.

In conclusion, while proteins labeled *in vivo* by metabolic processes would seem to represent the ideal for tracer work, the practical difficulties in their preparation, isolation, and use make *in vitro* labeling very attractive. Certain proteins can be labeled with iodine without loss of activity or function and perform comparably to those labeled *in vivo*. In addition, iodine labeled proteins are free from the problem of reutilization, which complicates studies with *in vivo* labeled materials. However, the adequacy of iodine labeling for each protein will depend on whether it contains essential groups labile to iodine. Such groups may be sulfhydryl or other easily oxidized groups or an essential reactive tyrosyl.

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DISCUSSION

K. BRUNFELDT (*Gentofte*) : In addition to the interesting survey given by Dr. Hughes, I should like to stress some points, which should be taken into consideration when using labeled proteins :

1) A change in structure of a protein due to the process of preparation. The weak antigenic properties demonstrated by species own proteins, as shown by various authors, indicate artefacts even in experiments with well characterized proteins.
2) Changes brought about by the labeling procedure due to the buffer system, use of oxydizing agents, urea, etc.
3) Modifications in the protein molecule due to the introduction of groups containing the isotope or the incorporation of the isotope itself.
4) Radiation damage when radioactive isotopes are used.
5) The possible effect of disintegrated molecules, if the radioactive isotope is a part of the molecule itself, for example ^{14}C in the backbone of a peptide structure.

No consideration seems to have been given to the use of stable isotopes and I should therefore very much appreciate to have your comments on this point. Finally, I should like to mention that a loss of biological activity does not necessarily indicate that the labeled group or its surroundings participate in the reaction by which the hormonal activity is caused. It may be so that the labeling will cause a reaction with other cell components hindering the labeled hormone from reaching the receptor system.

W. L. HUGHES (*Chairman*) : We ordinarily think of the iodine in tyrosine as stable. On the other hand, as I pointed out, iodinated oxytocin has been labeled with tritium by catalytic deiodination. Furthermore according to the mechanism of iodination which I presented, the reactions are basically reversible. So we must always anticipate the possibility that iodine can be lost from the protein. While this has been frequently postulated in the past, the evidence has been unconvincing. Dr. McFarlane has some new evidence that a small amount of iodine may be coming out of the molecule. Would you like to mention this now Dr. McFarlane ?

A. S. MCFARLANE (*London*) : It is usually accepted that iodine is very firmly bound to proteins but in some recent experiments it has emerged that in fact a steady dissociation is going on. This does not arise from enzymic or bacterial action and appears to be unavoidable. It proceeds in general at a higher rate with labeled fibrinogen that with albumins, but it is never more that about 0.5 % per day. This rate of dissociation would not cause any adverse affects in metabolic experiments with most small animals in which turnover rates are relatively high, but it could account for up to 10 % of the measured turnover rate of albumin in humans. On theoretical grounds iodotyrosines should not dissociate at such rates and the possibility cannot be excluded that the dissociation is of iodine less firmly bound to other amino acids.

V. Bocci (*Siena*) : I would like to stress several advantages of the Chloramine T procedure :

The labeling efficiency is of about 90 % and the iodine-131, which is firmly bound to the proteins, can be recovered almost quantitatively after proteolysis, as MIT. Soluble radioactivity is less than 1 % and raises up to about 1.5 % after 30 days storage at + 1° C.

DEAE cellulose chromatography, Sephadex G-200 filtration and electrophoresis on Pevikon block and starch gel show a similar distribution of labeled and unlabeled proteins. Thus, the presence of altered proteins detectable by these analyses is excluded.

The rat liver retention test and the rabbit total body radioactivity decay curve suggest a normal biological behaviour of homologous ^{131}I serum proteins. However as I previously reported (*Int. J. Appl. Rad. Isot.*, **15** : 449-456 [1964]; *Nature*, **203** : 888-889 [1964]) it is advisable to use the Chloramine T, controlling two factors : Protein Chloramine T ratio and the time of oxidation.

FACTORS AFFECTING PROTEINS IODINATION (*)

U. ROSA (+), G. F. PENNISI (+), G. A. SCASSELLATI (+), R. BIANCHI (°), G. FEDERIGHI (°), L. DONATO (°), C. A. ROSSI (•) and C. AMBROSINO (*)

(+) *SORIN, Nuclear Research Center, Saluggia, Italy.*
(°) *Nuclear Medicine Center, University of Pisa, Italy.*
(•) *Institute of Biological Chemistry, University of Pisa, Italy.*
(*) *Institute of Chemistry, University of Turin, Italy.*

ABSTRACT

The effects of various parameters involved in the iodination of proteins have been studied, with special reference to human serum albumin and insulin iodination.

Efforts have been made to separately evaluate the importance of the various steps involved in the preparation of a labelled protein in determining its final biological behaviour. Extraction and purification of the protein, rôle of -SH groups oxidation, type of labelling procedure employed, effect of the distribution of the labelling iodine within the molecule are considered and discussed.

Attempts are made to correlate the modifications induced by the above factors with the biological behaviour of labelled proteins.

INTRODUCTION

The use of labelled proteins as tracers in the study of the metabolic processes requires that the proteic tracer be identical in its biological behaviour to the corresponding native protein. In the course of a research program on the preparation of labelled proteins for metabolic studies, the effect of the progressive iodination on the biological behaviour of certain proteins was investigated. During this work, the specific results of which will be reported in other communications of this symposium, experiments were carried out in the attempt of separately defining the influence of the various factors, which intervene in the proteins iodination. This communication is substantially based on the results of such experiments.

An iodinated protein is the final product of a series of operations, the last of which is the iodination itself. Each of these operations may evidently interfere on the biological behaviour of the proteic tracer. The only way to answer the question whether the iodoprotein is or is not an adequate tracer of the native protein is then to study separately the contribution of the individual effects. In most cases, the isolated protein, before being iodinated, has already suffered the effects of different factors capable of affecting its biological properties. The common techniques of physico-chemical analysis may represent a first way of choice, allowing to eliminate the most grossly altered samples; but usually we lack adequate criteria in order to

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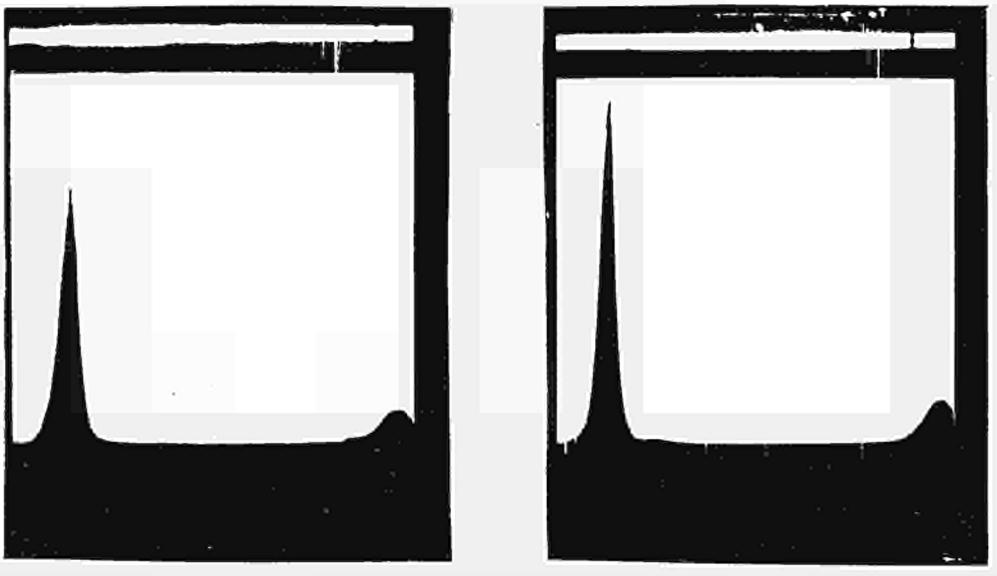
establish a priori whether the isolated protein represents an acceptable starting material. Under this respect, the case of human serum albumin (HSA) is a typical example. Several lots of HSA, prepared by different producers, were examined, in order to select a suitable sample for the labelling (Table I).

TABLE I. — SH groups/mole, metal content and fractional catabolic rate values, for some lots of human serum-albumin. -SH groups were titrated according to Benesch and Benesch [1], and to A. C. Allison [2]. Activation analyses were performed in RS1 Avogadro reactor, at a thermal flux of 1.10^{13} n/cm^2.sec^{-1} [3]. Catabolic rate values were calculated by the urine to plasma ratio method [4].

HSA lot.	SH groups/mole HSA		Metal content (neutron activation analysis) in p.p.m.					Catabolic rate after labelling up to 0.5-1 iodine atoms per mol.
	Ag-Tris	HOPh Hg pH2	Cu	Fe	Zn	Hg	Ag	
H D O	0.037 ∓ 0.001	—	15	80	95	0.3	0.6	14.91 ∓ 1.97
H D 1	—	—	—	—	—	—	—	15.33 ∓ 5.74
H D 8	0.101 ∓ 0.004	—	—	—	—	—	—	18.66 ∓ 2.61
H D 10	0.26 ∓ 0.001	0.21 ∓ 0.01	—	—	—	—	—	10.60 ∓ 0.91
H D 11	0.27 ∓ 0.005	—	11	95	105	0.1	0.4	11.44 ∓ 1.38
H D 12	0.184 ∓ 0.007	0.180 ∓ 0.003	17	98	150	0.1	0.5	10.96 ∓ 0.91
ISI 24	0.40 ∓ 0.02	0.42 ∓ 0.005	21	40	60	0.2	0.8	19.53 ∓ 3.59
Mann L 2435	0.47 ∓ 0.001	0.405 ∓ 0.05	18	24	12	0.2	0.8	16.1 ∓ 2.83

Titration with silver nitrate in TRIS buffer or phenyl-mercuric hydroxide at pH 2 revealed that in some of these lots the content in titratable-SH had been reduced to 20-30 % of the original value. The differences in -SH content per mole among the various lots of HSA are very likely to be attributed to oxidation phenomena. In fact, another supposition could be that a part of the -SH groups is not titratable, because it is blocked by metal impurities collected during the process of separation from the plasma; but this hypothesis can be rejected following the results of the neutron activation analysis, carried out on the various lots of serum albumin : the metal content is too low to account for the titration results. The samples of every lot were labelled under standard conditions, up to an iodination degree of about 0.5 iodine atoms per molecule. The catabolic rate of each preparation was measured in normal subjects.

The results show remarkable differences among the catabolic rate values of the HSA lots. Since we can reasonably presume that the iodination process, by itself, equally affects every lot, it is evident that the observed differences in the metabolic behaviour reveal differences already existing in the starting material. These differences could not be foreseen through the above mentioned analyses. In fact,



Lot ISI
$\overline{CR} = 19.5$

Lot HD11
$\overline{CR} = 11.5$

$\overline{CR}$ = mean catabolic rate on 7 days

FIGURE 1. — Tiselius electrophoresis of two lots of native human serum albumin. (On the left) lot ISI, having a mean fractional catabolic rate of 19.5 % (7 days). On the right, lot HD11, having a mean fractional catabolic rate of 11.5 % (7 days).

no correlation was found among the metabolic behaviour, the extent of sulphydryl oxidation, and the electrophoretic properties. All the samples were found to be homogeneous, according to the free boundary electrophoresis and the ultracentrifugation. Tiselius patterns of two HSA lots, having catabolic rate values of 11 % and 19 % respectively, do not show any noticeable difference (Figure 1).

Another factor, which could be able to affect the behaviour of HSA, is the pasteurization treatment, which is known to favour the formation of high molecular weight aggregates [5]. In Table II the fractional catabolic rate values are reported of two preparations of radioiodinated HSA, coming from batches HD12 and HD12 bis respectively; the second batch is an aliquot part of the first one, subjected to the pasteurization process. The two preparations were iodinated at a very low specific activity (4-6 μC/mg) with ^{131}I and ^{125}I respectively and injected into the same subject. No significant difference could be demonstrated in their metabolic behaviour.

Coming now to the iodination procedure, there is no doubt that the behaviour of a tagged protein depends on a large amount on the labelling technique. In the last years the traditional methods were abandoned and replaced by more sophisti-

TABLE II. — Effect of pasteurization, on the metabolic behaviour of Iodinated Human Serum Albumin

Lot	Id°	Catabolic rate % day^{-1} °°	Note
HD 12	0.68	10.96 ± 0.91	^{131}I-labelled, not pasteurized
H D 12 B	0.67	11.1 ± 1.01	^{125}I-labelled, pasteurized

° Iodination degree, iodine atoms/mole HSA.
°° Mean value over 7 days.

cated ones. This trend has been justified by the fact that it has been experimentally observed that some methods give rise to the formation of metabolically heterogeneous iodinated products. As a matter of fact, part of the discrepancies existing in the literature concerning iodinated HSA, are due to the use of different iodination techniques. In order to investigate separately the different factors which intervene during the iodination process, we have developed a special method which allows to operate under controlled conditions. Since the method has been already extensively described [6, 7] we shall only briefly recall the aspects of it which are relevant to the present studies.

Iodination takes place in an electrolytic cell, which is outlined in Figure 2. The protein is dissolved in a 9 °/$_{oo}$ NaCl solution containing potassium iodide, labelled with ^{131}I or ^{125}I. The anode is made of a platinum vessel of a large surface; the cathode is isolated by a cellophane membrane and the contact between cathode and anode is assured by NaCl solution. Polarization of the system gives rise to the formation of elemental iodine at the anode.

The rate of iodine formation depends on the current flowing in the electrolytic circuit. The current is adjustable through a power supply, which permits to feed the system with the wanted current value and thus to obtain the desired rate of formation of elemental iodine. Some experiments were performed in order to establish whether the conditions in which iodination takes place may affect the protein, independently from the effect of the iodine. An experiment was directed to assess the effect, on the titratable — SH of the HSA, of a 24 hrs exposure in the cell, in absence of KI, that is without formation of elemental iodine. No modification has been detected, allowing to conclude that the method itself does not show oxidative side effects. In order to investigate the effects of the iodination procedure on the metabolic behaviour of the HSA, the following experiment was carried out (Figure 3). A HSA preparation was divided in two aliquots. Both were electrolytically iodinated in standard conditions, up to an average content of 0.5 iodine atoms per molecule,

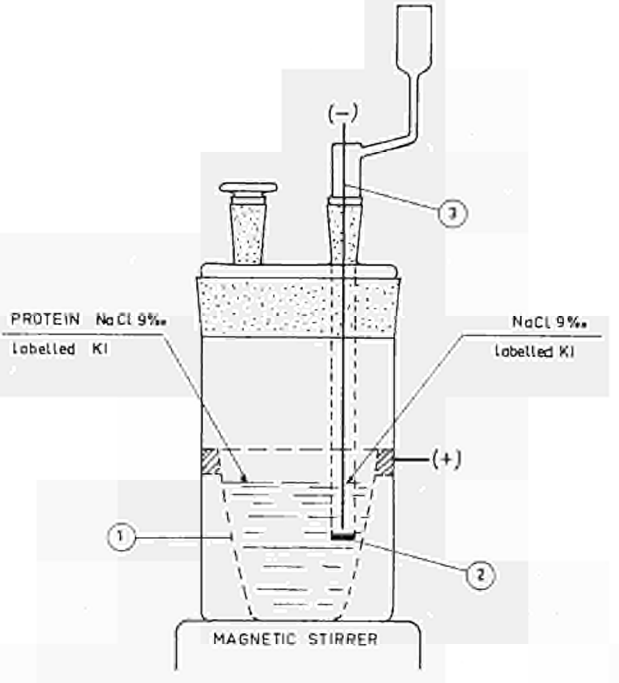


FIGURE 2. — Electrolytic cell for protein iodination.

using ^{131}I in one case and ^{125}I in the other. Both preparations were dialyzed against distilled water. The preparation iodinated with ^{125}I was then reintroduced into the cell, with a fresh solution of ^{125}I labelled KI, in presence of sodium thiosulphate. The latter competes with the protein towards elemental iodine, thus preventing the iodination of additional tyrosine residues. After 24 hrs of electrolysis, during which, in the absence of thiosulphate, the protein would have been iodinated up to an average iodine content of 24 iodine atoms per molecule, the solution was extracted and dialyzed.

The iodination degree was found to be unmodified. The two preparations were then injected into the same subject and their fractional catabolic rate was measured. The results are essentially identical for the two prepara ions, confirming that the method is "*per se*" devoid of effects on the protein metabolic behaviour. HSA is an adequate material for simultaneously studying the oxidation of definite functional groups and the iodination of tyrosine residues. In Figure 4 an experiment of progressive HSA iodination by electrolysis at 300 μA is reported; in the course of it we followed the two chemical processes due to the iodine, that is the oxidation of the sulphydryl groups and the substitution into the tyrosyl residues. On the abs-

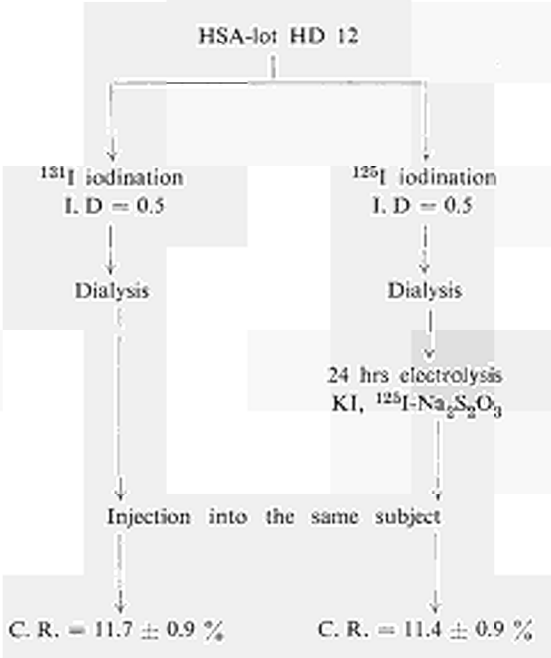


FIGURE 3. — Effect of the iodination procedure on the metabolic behaviour of HSA.

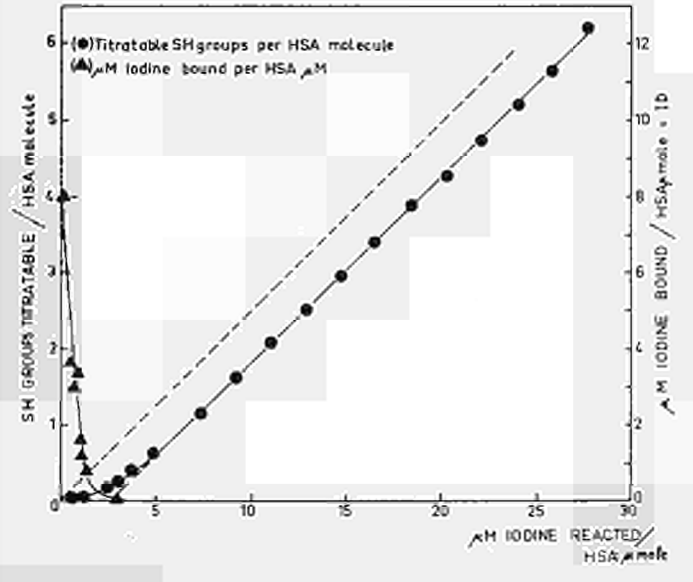


FIGURE 4. — Electrolytic iodination of human serum albumin at 0.3 mA : HSA conc. 10 mg/ml; KI, ^{125}I-labelled (200 μ C), $1.5.10^{-3}$ M; NaCl, 0.9 mg/ml. Total volume 10 ml.

cissas are reported the μM of elemental iodine formed in the solution at a constant rate, which is proportional to the current of 300 μ A. On the ordinates, the number of titratable -SH groups per HSA molecule is reported, on the left side; on the right side the μM of bound iodine per molecule of HSA, that is the iodination degree, are reported. Oxidation of the sulphydryl groups was followed by titrating aliquots of the solution by an amperometric system and by measuring at the same time, the amount of protein-bound iodine by paper electrophoresis. The slow rate of iodine formation permitted to follow easily the process. As one can see, the oxidation of the sulphydryls is completed, when the substitution into the tyrosyl residues begins.

This fact has been well known since a long time [8] : so that it seems surprising the claim of some Authors [9] that have reported to have iodinated HSA without -SH oxidation. On the contrary, -SH oxidation has to be accepted as an unavoidable preliminary effect, whenever the iodination of HSA is performed. Now, can this oxidative process interfere with the metabolic behaviour of the iodinated human serum albumin ? Some of the results obtained when comparing albumin batches (see Table I) may throw some light on this problem.

The iodination process of the various batches of native albumins has been proved to complete the oxidation of the -SH groups. Now, if the sulphydryl played really a primary rôle in affecting the metabolic behaviour of the albumin, the iodinated albumins, in which all the -SH groups have completely disappeared, should have to show, all, degradation rates of the same order. The abolishment of the -SH by the iodine would have, in this case, to efface the differences, which may exist initially among the different batches of serum albumin. Since, as we have seen, this does not agree with the experimental data, it would seem possible to conclude that the oxidation only of -SH groups, both during the isolation step and during the iodination, does not appreciably affect the degradation rate of serum albumin.

The second process, that is tyrosine substitution, proceeds according the stoicheiometry of the reaction between the tyrosine and the iodine, up to an iodination degree of 15-16 iodine atoms per molecule. If we look at the process of tyrosine iodination, we should be induced to consider it as an entirely isolated process from a chemical standpoint. However, an additional source of variability operates independently, along all the course of tyrosyne group iodination : this parameter is the actual distribution of iodine among and within the various molecules. This factor must necessarily have significant bearing on the properties of the iodinated protein, as judged from its metabolic behaviour.

A iodinated protein must be considered as a population of not homogeneous molecules, as far as their iodine content is concerned. In the case of HSA, Oncley [10] has proposed a distribution of binomial type. According to this hypothesis, preparations of HSA iodinated at an average substitution degree of 0.5 and 1 iodine atoms per molecule, would have the distributions reported in Figure 5.

In the upper side of the figure, the relative percentages of molecules containing 0, 1, 2, 3 and 4 iodine atoms per molecule are reported. As it can be seen, in serumalbumins at iodination degrees of 0.5 and 1, about 8 % and 27 % respectively of the

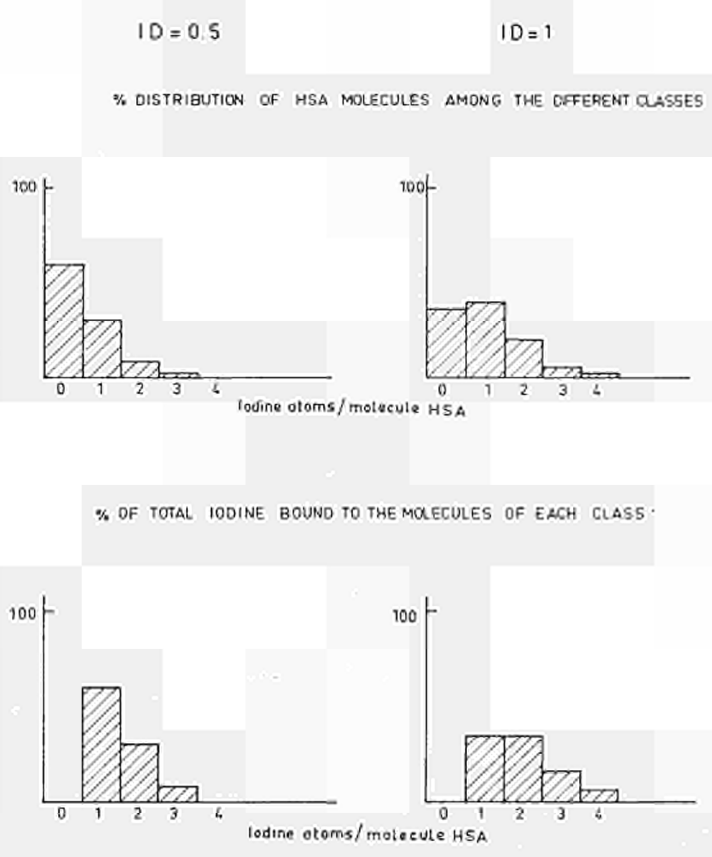


FIGURE 5. — Intermolecular distribution of iodine in iodinated HSA, following a binomial law.

molecules contain more than one iodine atom. When the labelled albumin is used as a metabolic tracer, only the molecules actually containing iodine are counted. In the lower part of the figure, the relative percentages of iodine in each class of iodinated molecules are reported. At iodination degrees 0.5 and 1, about 39 % and 60 % respectively of iodine is bound to molecules containing more than one iodine atom. Obviously there is no possibility of direct proof of the validity of this distribution hypothesis.

In an attempt to approach this problem, we have measured in preparations of iodinated HSA the amount of mono- and di-iodotyrosine residues, which must in some way be related to the distribution of the iodine.

In figure 6 the results are reported of two experiments of progressive iodination of HSA, performed at different formation rates of the elemental iodine. In one case, the current being kept constant at 300 μ A, the iodine developed at a rate of 0.09 μM/min; in the second case the current was 3 mA and the iodine formation rate was tenfold higher. The fraction of substituted tyrosines as di-iodo form in

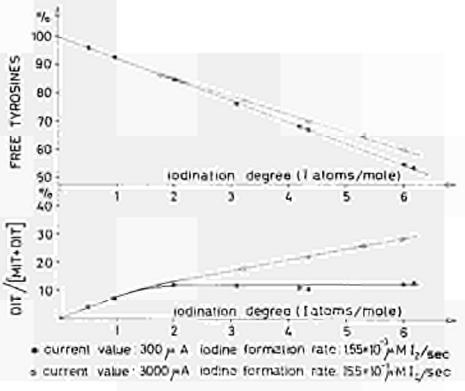


FIGURE 6. — See text.

preparations at the same iodination degree, varies according to the iodine formation rate. The preparations obtained with higher formation rates always contained relatively larger amount of DIT. On the contrary, the two curves are superposable at the low values of the iodination degree.

The results show that the rate of iodine formation definitely affects the distribution of the iodine. Several Authors have recommended a slow addition of the elemental iodine to the protein solution, with the aim of minimizing possible oxidative side effects. On the contrary, it is highly probable that the slow iodine addition rather than minimizing side effects leads to iodine distributions different from those obtained when the elemental iodine is rapidly added.

In figure 7 are reported the percentages of substituted residues as di-iodo form, found in preparations of iodinated HSA, obtained with different iodination methods. A comparison is made among iodoalbumins prepared according to three different techniques : iodide-iodate, iodine monochloride and electrolysis at two different iodine formation rates (0.3 and 3 mA). At a low iodination degree, the two chemically labelled preparations and the preparation electrolytically labelled at 3 mA do not differ from the one obtained at 0.3 mA, that is with a much slower rate of iodine formation. This is easily explained by the high number of available tyrosyl residues, that makes extremely unlikely the formation of di-iodinated residues at this level of iodination. On the contrary, at higher iodination degree, the 0.3 mA preparation definitely differs from the others, in which the iodine has been formed at a very fast rate. On the other hand, it cannot be excluded that differences in the iodine distribution may also occur at low iodination degrees. These will not be detected by using our empirical criterium of estimating the relative amount of mono- and di-iodo residues, because at this level only monoiodinated tyrosines are formed.

Under this respect, another point must be taken into account, that is the possibility of heterogeneities deriving not only from intermolecular, but also from intramolecular differences in iodine distribution. This means that molecules containing

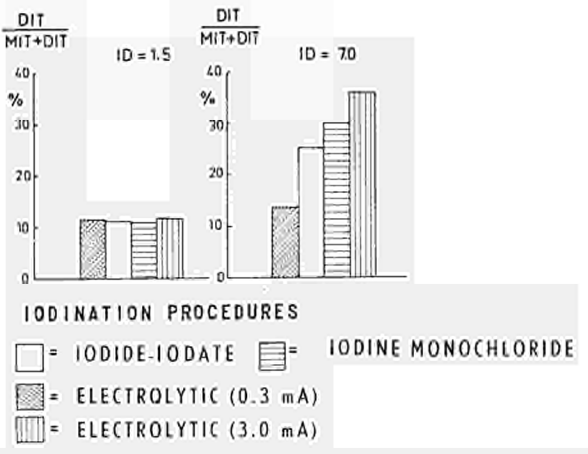


FIGURE 7. — Di-iodinated tyrosine residues found in preparations of ^{125}I-labelled human serum albumin, iodinated according to different procedures. The results are expressed as % of di-iodinated tyrosine residues (DIT), on the total number of iodinated tyrosine residues (MIT + DIT).

the same number of iodine atoms can differ from each other as far as the distribution of iodine among the tyrosyl residues within a single molecule is concerned.

Insulin provides a good example of this kind. As it is known, the insulin monomer contains four tyrosine residues, two in each chain. It has been proved that, at the same iodination degree, the distribution of iodine among the tyrosine residues of the A and B chains depends on the pH at which iodination takes place [11]. In figure 8 two preparations of iodinated insulin of the same iodination degree are compared : the former prepared at pH 1.5, according the Springell's method [11], the latter prepared at pH 7.6 by electrolysis. The preparations differ in their content of mono and di-iodinated residues, measured by total hydrolysis. But these differences are found also when the isolated A chains are analyzed. In the lower part of the figure the insulin primary structure, according to Sanger, is represented together with the distribution of iodine on the four tyrosine residues. These data were measured by complete fragmentation of the iodo insulin electrolytically prepared at pH 7.6; it is evident that these data should not agree with the total number of the mono and di-iodinated residues found both in A chain and in the whole molecule of insulin, iodinated at a pH 1.5. This latter must therefore have a different internal distribution.

It remains to be established whether a difference in the distribution may bring to different results, when the biological behaviour of the iodinated proteins is compared. Fraenkel-Conrat, in 1950 [14], noticed that two preparations of iodinated insulin, obtained with different methods, but having the same iodination degree, showed remarkable differences in their biological activity. Fraenkel-Conrat measured the number of mono and di-iodinated residues contained in the two prepa-

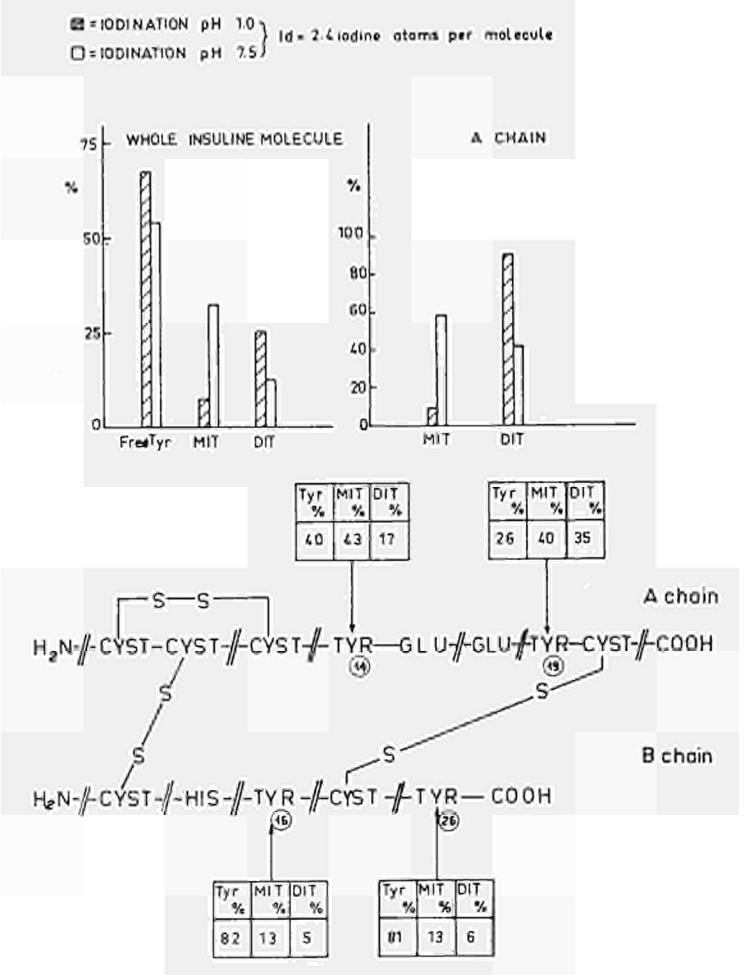


FIGURE 8. — % of tyrosine residues found under uniodinated form (Tyr) and under mono-(MIT) or di-iodinated form (DIT) in insulin preparations iodinated at pH 1.0 and 7.5, up to an iodination degree of 2.4 at. I/mol.

On the left, results of the hydrolysis performed on the iodinated insulins. On the right, results of the hydrolysis performed on the A chains, electrophoretically separated after cleavage of the inter-chains di-thio bonds [12]. In the tables, results of the hydrolysis performed on the four fragments A_1-A_{14}, A_{15}-A_{21}, B_1-B_{22}, B_{23}-B_{30}, each containing one tyrosine residue (see text).

rations and obtained different results, what let him presume that the differences in biological activity could be accounted for by differences in the iodine distribution.

In conclusion, the biological behaviour of an iodinated protein is largely dependent on the preparative conditions. A special care is required in controlling the various factors, which intervene in the iodination; as a general statement, the methods, in which strong oxidizing agents are avoided and the iodine is either introduced

in already oxidized form or generated under mild conditions should be preferred. Finally, the distribution factor, both at intermolecular and intramolecular level, should always be considered in evaluating experiments of progressive iodination, not only when they are performed to assess the adequacy of an iodinated protein as a metabolic tracer, but also when attempting to interpret the biological changes associated with the progressive iodination.

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DISCUSSION

W. L. HUGHES (*Chairman*) : Dr. Rosa has developed an important new procedure for iodinating proteins. However to better evaluate his process I would like to know :

1) The electro-chemical efficiency. How many faradays does it take to introduce one gram-atom of iodine ? If it takes many faradays, then what chemical changes are being produced by the rest of the current ?
2) What is the explanation for the variation of the ratio of MIT to DIT with current density? Could this be due to reaction of the protein adsorbed on the platinum anode ? Such adsorption might unfold the protein and permit easier iodination.

U. ROSA (*Saluggia*) : Unfortunately, due to the short time, it was not possible to give all the details of the method. The oxidation of the iodide ions to elemental iodine is quantitative in our process, that is we have a 100 % current yield. As it was shown in Figure 4, all the electrolysis current is consumed to generate iodine; with the exception of the first part of the process, iodine reacts stoicheiometrically with tyrosine residues.

R. S. YALOW (*New York*) : The degradation rate of 11.5 % per day is faster than the usual values reported for the best of the human serum albumin preparations. Can you compare your values directly with degradation rates of about 3.5 % total albumin per day and account for the difference ?

L. DONATO (*Pisa*) : The values reported by Dr. Rosa were expressed in % of the intravascular pool and not of the total protein mass. This probably explains the apparently high value, when compared to the 3.5 % figure, which refers to the total protein mass.

A. S. MACFARLANE (*London*) : With reference to Mrs. Yalow's question, I was quite happy about the catabolic rates which Dr. Rosa and his collaborators obtained for these human albumins. However the catabolic rate is not a particularly sensitive criterion of iodination. The equilibrium distribution between intra- and extravascular spaces as measured by extrapolating the plasma specific activity curve or the fraction of the injected dose which appears in the urine in the first day are both much more sensitive and I wonder whether Dr. Rosa has any data on either of these ?

L. DONATO : I do not have these data available now but the fractions of injected dose that appear in the urines at various times were calculated in all cases because they are required for calculation of the catabolic rate as fraction of the intravascular pool. These data are supplied in Dr. Bianchi's paper.

F. C. GREENWOOD (*London*) : Dr. Rosa and Dr. Hughes have mentioned Chloramine T as a strong oxidizing agent and postulated effects on a molecule during radioiodination. The radioiodination of proteins by the Chloramine T method was developed primarily for the preparation of labeled protein hormones for use *in vivo* radioimmunoassays (Greenwood, Hunter and Glover, 1963, *Biochem. J.*,

89 : 114). No effect of Chloramine T *per se* on the immunological properties of growth hormone was detected. The use of this method (Bocci, 1964, *Int. J. Applied Radioisotopes*, **15** : 449) for the preparation of labeled albumin for *in vivo* studies has been reported. An effect of Chloramine T *per se* on the biological properties in this situation may or may not be present. For radioimmunological work Chloramine T is no problem, but radioimmunoassays have other problems.

W. L. HUGHES (*Boston*) : I would like to make another comment relative to your distribution of mono- and diiodotyrosine which I thought was very interesting, that is this large difference of rate of iodination which you observed particularly electrolytically and not so much in the other procedures. I am wondering, could this perhaps be due to the fact that iodination might be taking place at the electrode? In this case lower current values would favour monoiodination as the diffusion process would have the time to renew the protein layer. If you are iodinating at slow rate you have more chance to diffuse at the electrode. In addition, a process taking place at the electrode could open up or expose tyrosine residues normally buried and so you could monoiodinate it.

U. ROSA : This point should be discussed in the light of some results, which will be reported at the next session, concerning our experiments on the metabolic behaviour of serumalbumins labeled at different values of iodination degree. We have found that preparations having nine or ten iodine atoms per mol. showed the same value of the catabolic rate as the preparations having 0.5 iodine atoms per mol. This could be tentatively considered a proof that the method by itself is not able to interfere on the conformation of the protein.

On the other hand, I agree with you that iodination probably takes place on the electrode; we have found that the surface of the anode plays a rôle in the process, in the sense that a too small anodic surface does not allow the process to take place.

CRITERIA FOR ASSESSING THE ADEQUACY OF IODINATED PROTEINS FOR METABOLIC STUDIES

T. FREEMAN

National Institute for Medical Research
Mill Hill, London, Great-Britain

ABSTRACT

Valid data can only be obtained from turnover experiments with labelled plasma proteins if the protein for labelling is of adequate purity and if it is known that the labelled protein is handled by the body in the same way as the native protein.

Evidence that the controlled introduction of limited amounts of iodine into the protein molecule does not produce denaturation has been provided by the comparison of the behaviour of iodine labelled proteins in animals with similar proteins labelled biosynthetically with ^{14}C, and also by a simultaneous comparison in a human analbuminaemic subject of the removal rate of unlabelled albumin with that of a trace dose of the same albumin labelled with ^{131}I.

However, proteins can be readily denatured during the procedures necessary for their isolation in a state of purity and because of variable factors in the labelling technique it is necessary to assess the quality of individual labelled preparations. Even for a human labelled protein this may be done in animals. Since grossly denatured proteins are rapidly removed by the reticuloendothelial cells the amount of activity retained in the RE system of the liver shortly after injection is an index of gross denaturation. Also, minor differences in the behaviour of labelled heterologous proteins can be detected by injecting them into tolerant animals.

Evidence of denaturation is also apparent after injecting labelled proteins into humans. A pure undenatured protein will give a constant rate of breakdown throughout the period of study; thus a falling catabolic rate indicates either denaturation or heterogeneity of the labelled protein. In particular, the breakdown rate, calculated from the urinary excretion rate of the label, should not be greater during the first 24 hours than on subsequent days.

It is essential in any valid tracer study of plasma protein metabolism to use labelled proteins that behave in the body identically with the native ones. Although protein molecules in general are essentially altered by the insertion of iodine provided certain precautions are taken this does not appear to affect seriously their physiological behaviour. In addition their use especially in the study of plasma protein metabolism, both in man and in animals, has three main advantages : firstly, the label is not reincorporated into protein once it is liberated by catabolism, provided, of course, that uptake of radioiodine by the thyroid is effectively blocked with large doses of stable iodine. Secondly the label remains firmly attached until the protein is catabolised, the iodine being rapidly excreted, and thirdly, labelling can be performed *in vitro* thus obtaining very high specific activities. On the

other hand the use of purified proteins and variabilities in labelling procedures make it easy to obtain denatured products which, after injection, give data which possibly bear little relationship to the behaviour of the native protein.

The validity of iodine as a trace label for plasma proteins has been demonstrated by a comparison in animals between proteins labelled biosynthetically with carbon-14 and similar proteins labelled with radioactive iodine [1, 2]. In addition, one of Bennhold's analbuminaemic patients was given a large infusion of unlabelled albumin and a trace quantity of the same protein labelled with radioactive iodine [3], and the specific activity of albumin was found to remain constant for many weeks while the albumin concentration fell steadily. Both these experiments demonstrate that the introduction of iodine under carefully controlled conditions [4] can provide a perfectly satisfactory product, but it will be necessary to check this for new proteins and new methods of labelling.

For satisfactory metabolic experiments using radioactive iodine labelled proteins the following criteria should be observed : 1) all the radioactivity in the injected material should be protein bound, 2) it would be bound to a single homogeneous protein, and 3) denatured labelled protein should not be present.

The first of these, namely that all the activity is protein bound, is easily examined, either by precipitating the protein usually with trichloroacetic acid and measuring the activity in the supernatant, or by separating large and small molecules by gel filtration. A simple column of Sephadex G 25 suffices for this, but the use of a gel bed of Sephadex G 100, 150, or 200, will give information both about the proportion of activity present as small molecules, and the homogeneity of the labelled protein. Good preparations give symmetrical peaks which become asymmetric in the presence of polymers.

The second requirement, namely that all the radioactivity should be bound to a single plasma protein, can also be examined by *in vitro* techniques. Although it is desirable to use pure proteins for labelling, this ideal is seldom attainable. Since some proteins have variable avidities for iodine a trace amount of contaminating protein may be associated with significant amounts of the total radioactivity. It is important to examine the distribution of radioactivity by techniques such as electrophoresis, gel filtration, etc. When contamination is present the labelled material together with unlabelled whole serum may be refractionated. Indeed it is sometimes necessary to iodinate a mixture of proteins, then to mix this with some fresh serum and re-isolate. In this way a single labelled protein may be obtained without ever freeing it completely from other proteins.

The third requirement, that the labelled plasma protein should be essentially undenatured, is not so easy to fulfil because of difficulty in recognising small proportions of denatured protein in the product. For instance, labelled proteins that are identical with the native ones by *in vitro* tests may nevertheless be catabolised more rapidly.

Altered proteins fall into two main groups; those in which denaturation is gross and which are rejected by the body within a few minutes; and those in which

the alteration is minor and which are retained but are catabolised more rapidly than the native protein.

Two animal tests have been devised for demonstrating denaturation in human plasma proteins [5]. The first takes advantage of the rapidity with which some foreign molecules are taken up by the reticulo-endothelial system of the liver. In a preliminary study trace amounts of soluble heat denatured labelled albumin [6] were injected into rats which were killed at intervals and the blood perfused out with physiological saline. Figure 1 shows total radioactivities in various organs and protein bound and free activities in the perfusate. The results demonstrate complete clearance of denatured protein from the blood by the liver with a survival time of the denatured molecules in the liver of only eight minutes. Between the eighth and fifteenth minute a high proportion of the denatured molecules were in the liver, though never 100 % of the dose because later protein molecules were arriving in the liver when iodine from the first had already been discharged into the blood stream. The proportion of the injected dose retained by the liver at about twelve minutes has been arbitrarily used as a measure of gross denaturation, deliberately heat-denatured albumins giving values in the region of 70 %, and good quality ones less than 1.5 %.

Minor alterations in protein molecules are more difficult to detect. Where a good quality preparation is available this may be compared with the suspect label-

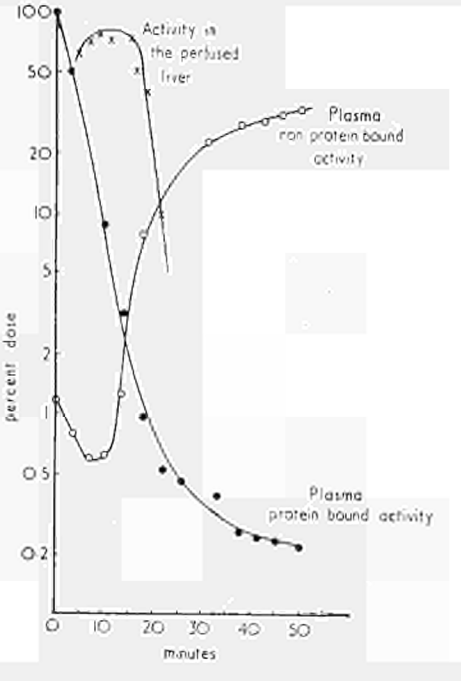

FIGURE 1. — Diagram illustrating the distribution of radioactivity after the intravenous injection of 1 mg of soluble heat denatured albumin into a rat

led protein and the use of two iodine isotopes permits this to be done in the same animal.

Figure 2 shows a comparison between an undenatured human albumin and a similar albumin altered in the process of isolation from plasma. Since immune rejection of labelled heterologous proteins would seriously interfere with this test after a few days a rabbit was used which had been made tolerant to human plasma at birth. Plasma activities relative to total protein concentrations are expressed as a percentage of the value for the first plasma sample. Total body activities for ^{131}I were measured directly in a ring of Geiger Muller tubes, and total body ^{125}I activities were derived indirectly from the urinary excretion. Catabolic rates were calculated from daily losses of total body activity related to the mean plasma radioactivity on the same day, no correction being made for delay in iodide excretion from the body iodide pool. These results showed that the suspect albumin was catabolised faster than the comparison human albumin, and also that its rate of catabolism decreased progressively presumably because some molecules have been more damaged than others.

The undenatured albumin used as a reference protein in this and subsequent experiments was supplied by Behringewerke, and was prepared by repeated salt precipitation. This albumin was used in Bennhold's case of analbuminaemia, and has been extensively examined *in vitro* and biologically in animals and humans. The labelled material contains less than 2.5 % of heterogenous protein, much of which is albumin polymer.

In the experiment shown in Figure 2, no correction was made for delay in iodide excretion caused by the iodide pool. When protein catabolism is rapid as in case of fibrinogen the quantity of activity retained in this pool can be high, so

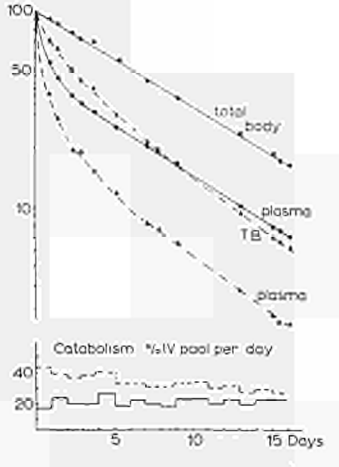

FIGURE 2. — Comparison in a tolerant rabbit of good quality human albumin, labelled with ^{131}I and a human albumin isolated (using Rivanol-ammonium sulphate and ether) labelled with ^{125}I.

that the urinary activity ceases to reflect closely the rate of iodide liberated by catabolism, and the total body activity can be considerably higher than the retained protein bound activity.

Since plasma iodide concentration is a balance between the rate of liberation of the label and iodide excretion, this value should remain constant during the experiment. The finding of high plasma non-protein bound activity shortly after injection indicates some denatured protein [7, 8, 9].

Although this type of double label experiment gives the clearest indication of denaturation the work involved is considerable, and a simpler test has been devised. In our experience the proportion of an injected human protein which is retained in a fully grown tolerant rabbit on a given day is remarkably constant. However, this proportion varies between rabbits, and for comparable results to be obtained an independently labelled reference protein must be used in the same animal within a short interval. If the survival of activity associated with the denatured protein is expressed as a fraction of that of the undenatured one (Figures 3 and 4) the results from the two animals are comparable. Figure 3 shows the total body activities obtained after the injection of undenatured iodinated human albumin and deliberately overiodinated. Figure 4 shows the same data, with the suspect protein expressed in relation to the undenatured albumin in two different rabbits. Figure 5 shows the relative retention of iodinated albumins subjected to different

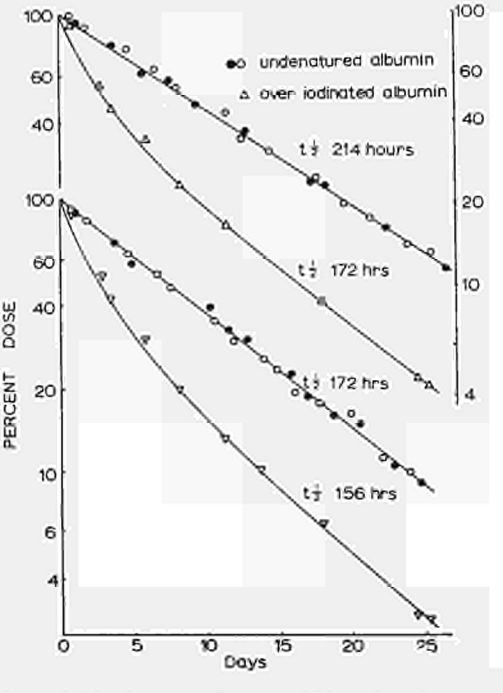

FIGURE 3. — Total body activities in two tolerant rabbits after the successive injections of two good quality labelled albumins and over iodinated albumin

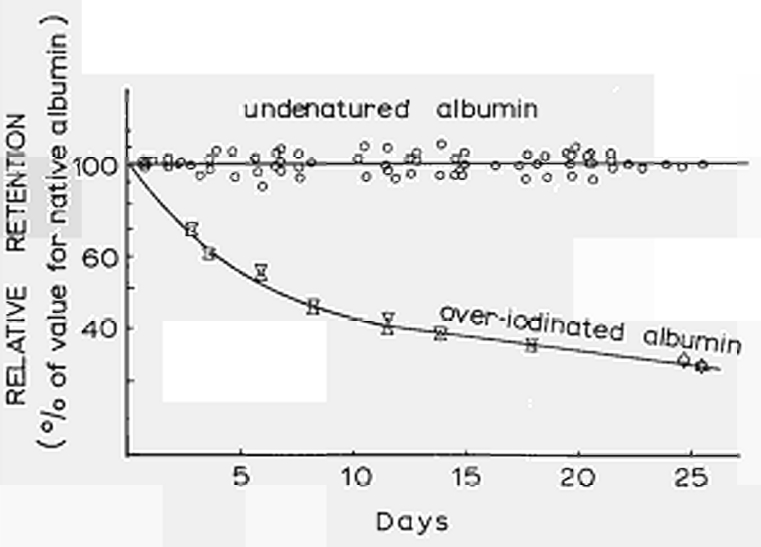

FIGURE 4. — The data from Figure 3; the denatured albumin is expressed as a per cent of the undenatured albumin

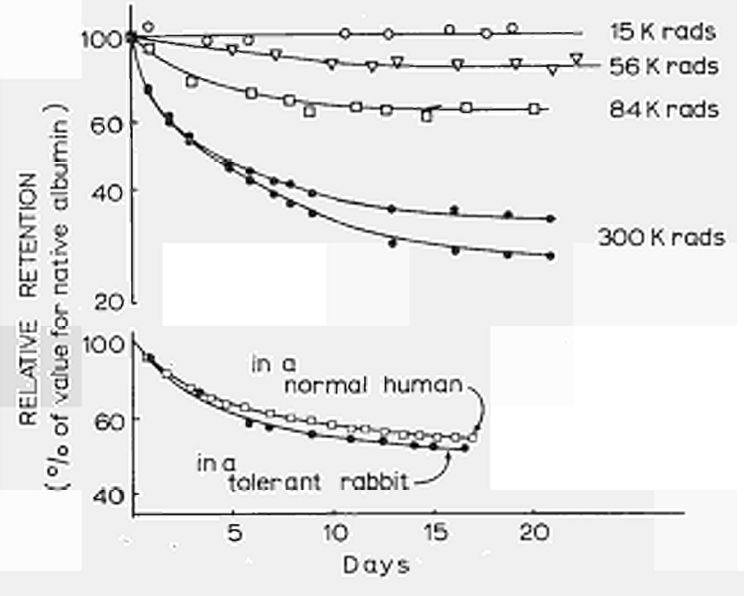

FIGURE 5. — Relative retentions of albumin subjected to different amounts of self irradiation at 2-3 mg/ml protein concentration

degrees of self irradiation [10, 11]. Also on this diagram is shown the relative retentions of the same protein in a tolerant rabbit and in a human subject. Figure 6 shows relative retentions obtained with albumins iodinated at various iodine levels. Also shown are the relative retentions of overiodinated human albumin in humans calculated from the work of Berson and Yalow [12]. Above 2.9 atoms per molecule the catabolic rate increased progressively and below a mean level of 1.5 atoms per molecule the catabolic rate was constant. In an experiment with a mean level of one atom of iodine to one hundred protein molecules a significant increase in catabolic rate was found. However, when the preparation was examined 12 % of the activity was found to be associated with a contaminating protein — presumably one with a higher avidity for iodine. This was removed by refractionating the labelled material and the resulting labelled albumin gave relative retentions identical with albumin labelled at one atom per molecule. This was also found by McFarlane in comparing very different levels of iodine substitution in rabbit albumin. The observation that albumin labelled at a mean substitution of 1.5 atoms showed no evidence of denaturation taken in conjunction with considerations of random distribution of the label indicated that albumin can bind more than one iodine without overt signs of denaturation. Other protein molecules, however, for instance haptoglobin, transferrin, arosomucoid, and fibrinogen can only be satisfactorily labelled at lower levels and it appears that no generalisation is possible regarding

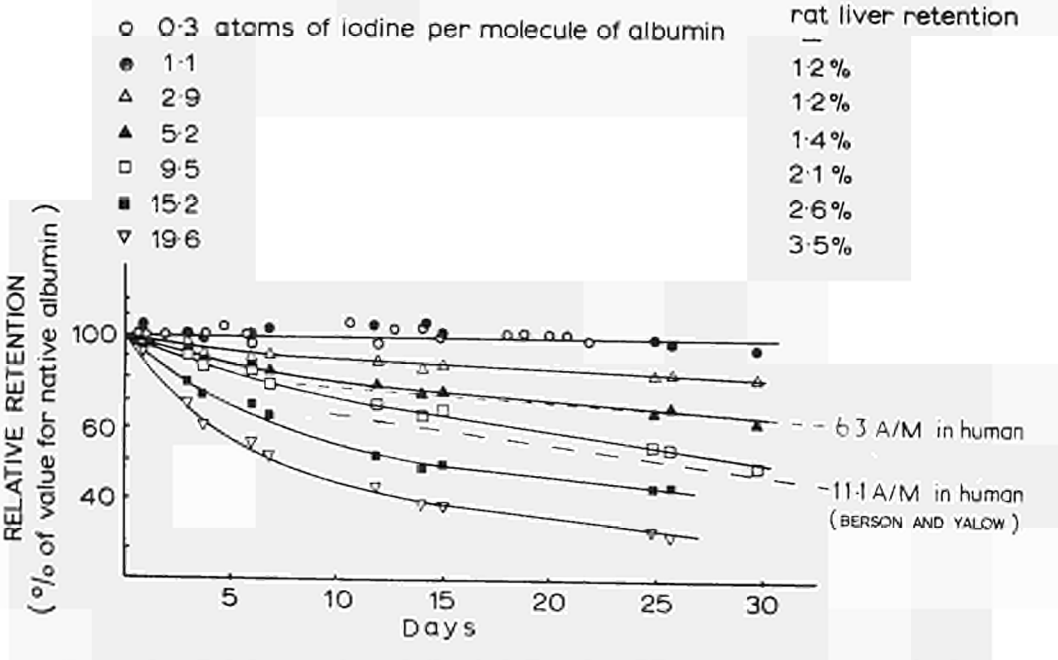

FIGURE 6. — Relative retentions of albumin iodinated to different mean atoms per molecule.

"physiological" levels of iodine substitution. Figure 7 shows the relative retention of albumins separated from plasma in a variety of ways. Each of these was labelled at a mean of less than one atom of iodine per molecule. In these and the types of denaturation discussed the retention curves all suggested the presence of a range of molecules with very different catabolic rates.

Examples of a constant difference in the elimination rates of two proteins are shown in Figures 8 and 9. Figure 8 shows the results obtained after injecting a mixture of totally denatured albumin and undenatured albumin, the retention rapidly dropping to 60 % and the behaviour thereafter being that of undenatured protein. Figure 9 shows the results obtained in comparing good quality human albumin in rabbits with undenatured rabbit albumin in rabbits, the small differences between rabbit and human albumin presumably accounting for the more rapid catabolism of the latter. The mechanism by which this accelerated catabolism is effected is unknown. Also in Figure 9 the behaviour of normal human transferrin was related in much the same way to that of normal human albumin in a human subject. In both cases catabolism was faster than that of the reference protein and the constancy of the rates was reflected by the linearity. This type of comparison between different proteins can only give useful information if the intra/extra vascular pool ratios and transfer rates are similar — as they are for transferrin and albumin.

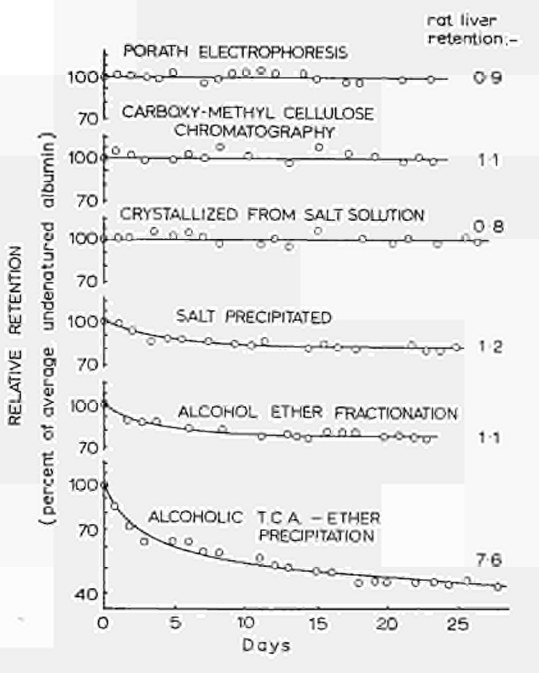

FIGURE 7. — Relative retention of albumins isolated by different techniques from plasma; all iodinated at below one atom per molecule

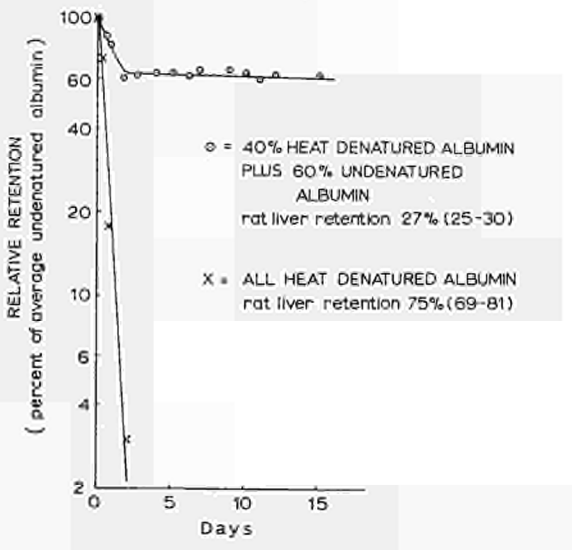

FIGURE 8. — Relative retention after the injection of a mixture of 40 % heat denatured albumin and 60 % undenatured albumin

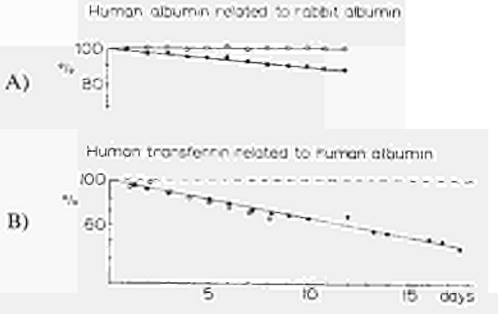

FIGURE 9. — A) Relative retention in rabbits relating good human albumin to good rabbit albumin.
B) Relative retention in two humans relating good transferrin to good albumin.
In both these experiments the straight line formed by the relative retention indicates that both proteins are catabolised at a uniform rate.

All the preceding data concern the comparison of a suspect protein with an undenatured one. Where this cannot be done, as for instance with a newly isolated protein, the only criterion available for the absence of traces of denatured protein is the finding of a constant rate of breakdown throughout the exepriment. Fortunately, experience shows that it is difficult to change albumin, gammaglobulin, transferrin, and haptoglobin in such a way that they are catabolised at a uniformly faster rate than the native proteins, though this may not be true for all proteins. It has also not proved possible to alter a protein so that it survives in the body lon-

ger than the native one, the only exception to this being possibly transferrin polymer. This protein forms polymers *in vitro* which can be isolated from the monomeric form and labelled. When these were injected into normal subjects together with distinctively labelled monomer the fractional catabolism of the polymeric form was always slower than the monomer.

To summarize, denaturation of protein molecules can be classified as two types, namely, gross denaturation is easily detectable (because the molecules are removed rapidly from the circulation) and a measure of which can be obtained from the retention in the rat liver, and denaturation which requires the use of a reference undenatured protein for its recognition. Where no protein that is known to be undernatured is available, the only absolute evidence for the absence of denaturation is the comparison with the same protein labelled biosynthetically with ^{14}C. However, because all the proteins studied have shown random degrees of alteration when damaged, the finding of a constant fractional catabolism throughout the experiment gives some presumptive evidence for the absence of denaturation. Catabolism can be calculated from urinary data alone only if the rate is relatively low. With more rapidly metabolised proteins it is essential to measure the level of non-protein bound activity in the plasma or to correct the urinary data for the presence of the iodide pool. I suggest that data of this type should be quoted in all papers on the metabolism of plasma proteins as evidence for the absence of denaturation.

Finally, as you will appreciate from published work, denaturation arising from faulty iodination or self-radiation damage can be easily avoided, but in my opinion the isolation of pure proteins in an undenatured state is not always so easy: and this may be by far the most common cause of misleading results in experiments using iodine labelled proteins.

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DISCUSSION

A. ANDERER (*Tübingen*) : I just want to look at the problem from an immunochemical point of view. If one uses substituted proteins even in tolerant animals, a breakage of the tolerance may be expected and a specific titer against the carrier moiety of these substituted proteins may be obtained. With reference to this, I will just mention the recent papers of Feldman ([1]). This indicates that iodinated proteins behave like substituted proteins, since it could be definitely proved that one has an antigenic specificity against the iodinated tyrosine residue in the protein ([2]). You interpret your results only in terms of denaturation, but could it not be possible that the different metabolic rate you find is due to breakage of tolerance and the regaining antigenicity of the protein sample ?

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2. For references see : Karl LANDSTEINER, «The specificity of serological reactions».

T. FREEMAN : I do not think so because these animals were used repeatedly. Time after time we got the same survival of albumin in the first experiment as we got in the last experiment, having done some 60 or 70 turnovers on the same rabbit. If this was due to breaking of tolerance I would have expected some change to occur between the first and the last experiment.

A. ANDERER : I think the change has not been so big. If you look at Feldman's experiments, some animals respond and some do not, but he finally could prove precipitating antiserum against the carrier moiety of the chemically substituted proteins. He used gammaglobulin.

T. FREEMAN : Well, they were made tolerant with unlabeled human plasma and when they were injected with iodinated albumin there was no immune brake in the body activity curve.

A. ANDERER : May be that is the difference. Feldman uses X-Ray irradiated animals.

W. L. HUGHES (*Chairman*) : Does the normal rabbit in the beginning has the same catabolic rate as the tolerant rabbit after it becomes immunized ?

T. FREEMAN : The rabbits were made tolerant at birth. The tolerance does not seem to change the rate of catabolism. Up to the moment of getting an immune brake, non-tolerant rabbits behave in the same way.

ÜBER DIE BEI DER REAKTION VON PROTEINEN MIT RADIOAKTIVEM JOD UND CHROM MARKIERTEN AMINOSÄUREN

E. NIEMANN

Farbwerke Hoechst AG, Frankfurt (Main)-Höchst, Deutschland

ZUSAMMENFASSUNG

Bei der Markierung von Proteinen mit Chrom und Jod werden diese Elemente von bestimmten, für sie spezifischen Aminosäuren gebunden. Es wurden Insulin, dessen isolierte Ketten A und B, sowie Glucagon und Albumin mit elementarem Jod markiert und dabei die angebotenen Jodmengen variiert. Das Verhältnis der in diesen Eiweißen gebildeten Jodtyrosine und -histidine ließ sich nach enzymatischer Hydrolyse der Eiweiße mit Hilfe papierelektrophoretischer und -chromatographischer Methoden quantitativ bestimmen. Im Insulin wird Tyrosin bevorzugt und erst bei hohem Jodangebot Histidin jodiert; dagegen bilden sich im Glucagon und der isolierten Insulinkette B schon bei der Substitution von ~ 1 Jodatom pro Molekül Eiweiß Jodhistidine neben Jodtyrosinen. Im Albumin bleibt in einem Jodierungsbereich bis zu 3 Atomen Jod pro Molekül das Verhältnis der Mono- und Dijodtyrosine im Gegensatz zu den anderen Proteinen angenähert gleich.

Die Chromierungsreaktion wurde am Beispiel des Albumins untersucht. Durch vergleichende Chromierungen von Albuminderivaten und durch die enzymatische Hydrolyse des Chromalbumins konnte bewiesen werden, daß Chrom über die freien Carboxylgruppen der Glutaminsäure des Proteinmoleküls gebunden wird.

ABSTRACT

Chromium and iodine atoms, in use for the labelling of proteins, are bound to definite amino acids which are specific for these elements. The following materials were labelled with elemental iodine varying the amount of iodine over a wide range : insulin, chains A and B of insulin, glucagon and albumin. After enzymatic hydrolysis, the ratio of iodotyrosines and iodohistidines formed was determined, using paper chromatography and electrophoresis. The hydrolysis of iodinated insulin yielded iodotyrosines; iodohistidines were formed only under higher degrees of iodination. In the case of glucagon and isolated chain B of insulin, iodohistidines appeared as well as iodotyrosines, even with an iodination degree of only one atom iodine per protein molecule. In contrast to the behaviour of other proteins, the ratio between the two iodotyrosines in iodinated albumin does not change with the degree of iodination, up to 3 atoms iodine per molecule.

Albumin was taken to study the reaction of trivalent chromium on proteins. Enzymatic hydrolysis of chromium labelled albumin showed the chromium to be bound to the free carboxyl groups of glutamic acid. For further confirmation the binding powers of albumin derivatives for chromium were examined.

Die Verteilung der Jodatome in jodmarkierten Proteinen ist durch die Struktur der Proteinmoleküle bedingt und außerdem von der Jodierungstechnik und der dabei zur Reaktion kommenden Jodmenge abhängig. Wir haben das Auftreten

bestimmter Jodaminosäuren bei der Jodmarkierung von Insulin, dessen isolierten Ketten A und B, Glucagon und Albumin untersucht und die Ergebnisse verglichen. Die Präparate waren nach folgendem Prinzip jodiert : Elementares Jod der geforderten spezifischen Aktivität wird in eine geringe Menge eines mit Wasser mischbaren organischen Lösungsmittels eindestilliert. Diese Lösung setzt man tropfenweise einer gepufferten Eiweißlösung geeigenten pH-Wertes zu. Man vermeidet somit die Einwirkung eines anderen Oxydationsmittels auf das Protein. Die Jodierung ist nach fünf Minuten beendet. Man erhält Reaktionsausbeuten bis zu 95 % der Theorie in bezug auf Jod. Insulin z. B. nimmt unter diesen Bedingungen bei entsprechendem Jodangebot noch neun Jodatome pro Molekül bei gleicher Reaktionsausbeute auf.

Die jodmarkierten Eiweiße lassen sich mit Pepsin, Chymotrypsin und Aminopeptidase, isoliert aus Nierenpartikeln (Pfleiderer u. Celliers [1]) spalten. Man kann dabei oft auf die Anwendung von Pepsin oder Chymotrypsin verzichten; dies ist jedoch im Einzelfall zu entscheiden. Für die Hydrolyse haben wir abweichend von den sonst üblichen optimalen Bedingungen die Temperatur auf 30 °C und den pH-Wert auf 7,4 festgesetzt, um eine Jodabspaltung aus den während der Reaktion freigesetzten Jodaminosäuren soweit wie möglich zu vermeiden.

Für die Auftrennung der jodmarkierten Aminosäuren in den verschiedenen Eiweißhydrolysaten sind die Papierchromatographie und die Papierelektrophorese eingesetzt worden. Abbildung 1 zeigt die elektrophoretische Trennung von Mono- und Dijodtyrosin, sowie Mono- und Dijodhistidin.

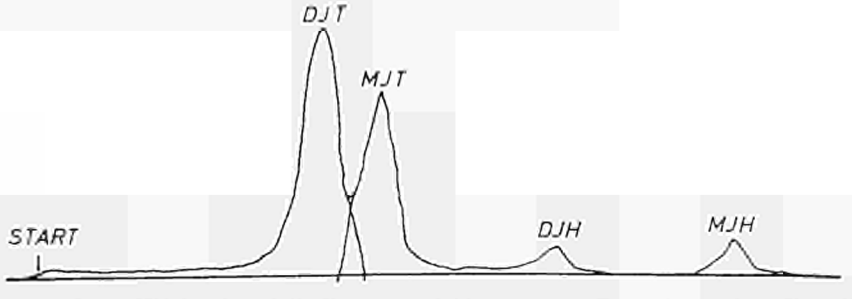


Abbildung 1. — Elektrophoretische Trennung jodierter Aminosäuren in Ameisensäure/Eisessig pH 2,0; 60 V/ cm; 2 Std.
(MJT = Monojodtyrosin, DJT = Dijodtyrosin,
MJH = Monojodhistidin, DJH = Dijodhistidin).

In Tabelle I ist die Aktivitätsverteilung in verschiedenen Jodinsulinhydrolysaten zusammengestellt. Bei einem Jodierungsgrad unter Eins lassen sich als Jodierungsprodukte nur Mono- und Dijodtyrosin nachweisen, wie auch De Zoeten u. De Bruin [2], sowie Rosa *et al* [3] gezeigt haben. Ein Insulinpräparat mit etwa drei Atomen Jod pro Molekül enthält außerdem Spuren jodierten Histidins. Die Hydrolyse der noch höher jodierten Präparate, die nun auch Dijodhistidin liefern, war nur schwierig auszuwerten, da das Protein enzymatisch nicht quantitativ gespalten

TABELLE I. — Aktivitätsverteilung in Jodinsulin-Hydrolysaten

Atome Jod pro Molekül Insulin	% der Gesamtaktivität					
	Monojod-Tyrosin	Dijod-Tyrosin	Monojod-Histidin	Dijod-Histidin	freies Jod	Protein-Rückstand
0,6	63	37	—	—	—	—
2,7	20	79	<1	—	—	—
6,1	10	70	4	3	3	10
8,1	3	60	6	7	4	20

TABELLE II. — Aktivitätsverteilung in enzymatischen Hydrolysaten jodmarkierter Proteine

	Atome Jod pro Molekül Protein	% der Gesamtaktivität					
		Monojod-Tyrosin	Dijod-Tyrosin	Monojod-Histidin	Dijod-Histidin	freies Jod	Protein-Rückstand
Kette A (oxyd.)	0,9	60	40	—	—	—	—
Kette B (oxyd.)	1,1	52	45	3	—	—	—
Glucagon	0,08	89	9	—	—	2	—
	0,7	75	14	7	2	2	—
	2,2	28	52	6	8	2	4
	3,6	11	62	6	11	2	8

TABELLE III. — Aktivitätsverteilung in Jodalbumin-Hydrolysaten

Atome Jod pro Molekül Protein	% der Gesamtaktivität		
	Monojod-Tyrosin	Dijod-Tyrosin	freies Jod
0,2	76	22	2
0,3	75	23	2
0,9	87	11	2
2,6	81	15	4

werden konnte und Dijodhistidin leicht in Monojodhistidin und Jod zerfällt. Die enzymatische Hydrolyse kann man durch Zugabe von Cystein, das die Disulfidbrücken des Insulins öffnet, quantitativ zu Ende führen, jedoch wird dann auch das Dijodhistidin zu Monojodhistidin reduziert. Untersucht man die jodierten Insulinketten oder jodmarkiertes Glucagon, kommt man zu dem in Tabelle II dargestellten Ergebnis. Sowohl in der Kette B als auch im Glucagon bildet sich Jodhistidin schon bei der Anlagerung von einem Jodatom pro Molekül Protein. In beiden Proteinen muß also das Histidin, bedingt durch die Struktur der Moleküle, leichter zugänglich sein als im Insulin. Die Aminosäuresequenz des Glucagons beginnt z. B. mit Histidin. Die bemerkenswert hohe Dijodhistidinbildung in Glucagon höheren Jodierungsgrades ist wahrscheinlich eine Funktion des bei der Jodierungsreaktion angewendeten pH-Wertes, wie aus an Histidin ausgeführten Versuchen geschlossen werden kann. Albumin zeigt im Gegensatz zu den bisher besprochenen Proteinen bei einem Jodierungsgrad von 0,2-2,5 Atomen Jod pro Molekül ein einheitliches Verteilungsbild u.z. 75-87 % der Gesamtaktivität als Monojodtyrosin und den Rest als Dijodtyrosin. Das bedeutet, 14-7 % der jodierten Aminosäuren sind Dijodtyrosi (Tabelle III).

Albumin reagiert — wie bekannt — unter einfachen Reaktionsbedingungen auch mit Kationen. In der medizinischen Diagnostik eingeführt ist insbesondere das Chromhumanalbumin-^{51}Cr (Waldmann [4]). In einem chrommarkierten Albumin ist die Bindung zwischen den dreiwertigen Chromionen und dem Eiweiß sehr

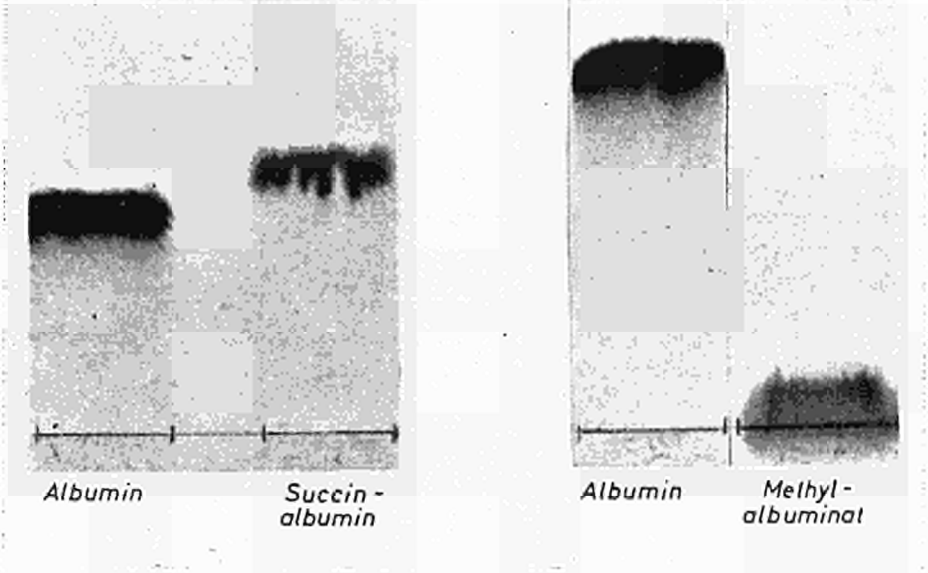


ABBILDUNG 2. — Vergleich der elektrophoretischen Wanderung von Albumin, Succinalbumin und Methylalbuminat bei pH 8,6.

stabil. Weder in saurem noch in alkalischem pH-Bereich verliert die Verbindung Chrom. Das Präparat ist mit $(NH_4)_2SO_4$ fällbar und lediglich unter Einwirkung von Trichloressigsäure lassen sich von dem markierten Eiweiß einige Prozent des gebundenen Chroms abspalten.

Die Reaktion des Albumins bei einem Angebot von einem Chromion pro Molekül Eiweiß verläuft in kurzer Zeit quantitativ. Erst bei höherem Chromangebot sinken die Reaktionsausbeuten. Sie können aber durch Verlängerung der Reaktionszeit oder Entfaltung der Albuminmoleküle mit Harnstoff wieder gesteigert werden. Polyacrylamid-, Papier- und Immunelektrophorese, sowie Untersuchungen in der Ultrazentrifuge haben gezeigt, daß die Proteinmoleküle durch die Chromierung vernetzt werden.

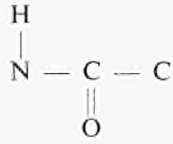
Für die Bindung zweiwertiger Metallionen an Proteine werden in der Literatur immer wieder die Aminosäuren Cystein, Histidin oder aber z. B. im Falle des Transferrins Tyrosin (Jones u. Perkins [5]) diskutiert. Zur Klärung der Chromalbuminbindung haben wir Chromalbumin-^{51}Cr enzymatisch hydrolysiert, außerdem Albumin durch spezifische Reaktionen verändert und an diesen Präparaten die Chromierbarkeit geprüft, um die für diese Reaktion funktionellen Gruppen der Aminosäuren festlegen zu können. Die Albuminderivate wurden zunächst durch ihr elektrophoretisches Verhalten bei pH 8,6 im Vergleich zum Humanalbumin charakterisiert. Beispiele sind in Abbildung 2 dargestellt. Weder durch die photooxydative Zerstörung des Histidins [6] noch durch die Jodierung des Tyrosins werden die Ausbeuten der Chromierungsreaktion herabgesetzt (Tabelle IV). Damit war die Beteiligung von Histidin und Tyrosin and der Bindung der Chromionen unwahrscheinlich. Die Jodierung, hier ausgeführt mit einem Angebot von zehn Mol Jod

TABELLE IV. — Chromierung verschiedener Derivate von Humanalbumin unter gleichen Reaktionsbedingungen

(Ansatz : 0,7 × 10^{-6} M Albumin; 1,6 × 10^{-6} M Chrom)

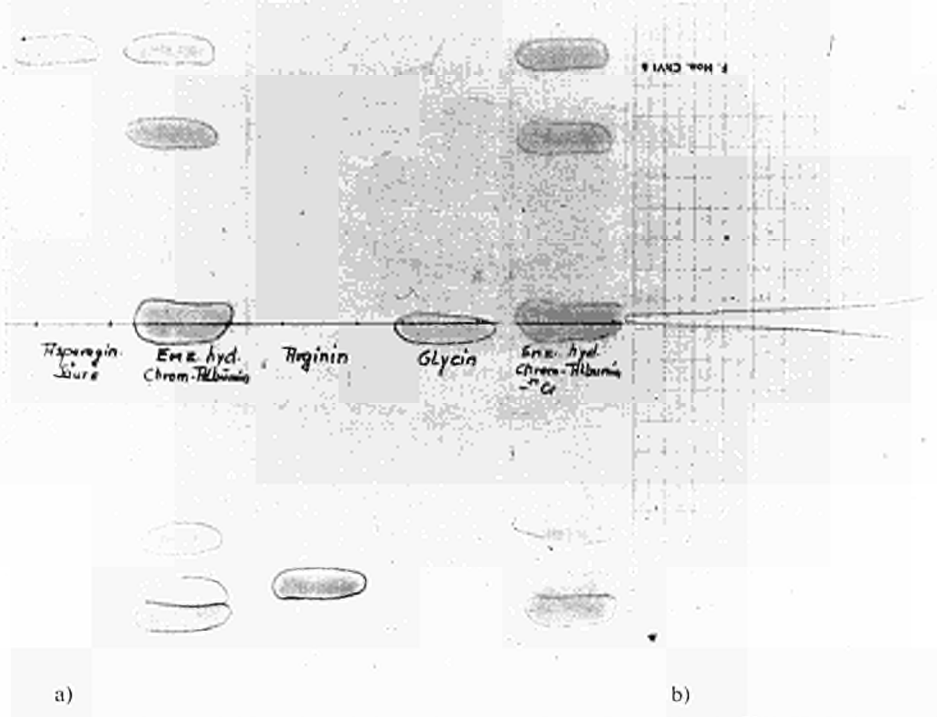
Veränderung des Albuminmoleküls	Chromierbarkeit im Vergleich zu Albumin
Photooxydation des Histidins	unverändert
Jodierung des Tyrosins	unverändert
Succinierung freier Aminogruppen mit Bernsteinsäureanhydrid	erhöht
Veresterung freier Carboxylgruppen mit Methanol/HCl	erniedrigt

pro Mol Albumin führt gleichzeitig zur Oxydation freier SH-Gruppen, so daß die Bindung des Chroms über Cystein ebenfalls nicht wahrscheinlich ist. Die Anlagerung von Bernsteinsäureanhydrid [7] führt zur Bildung neuer Carboxylgruppen



$$\mathrm{N-C(=O)-CH_2-CH_2-COOH}$$

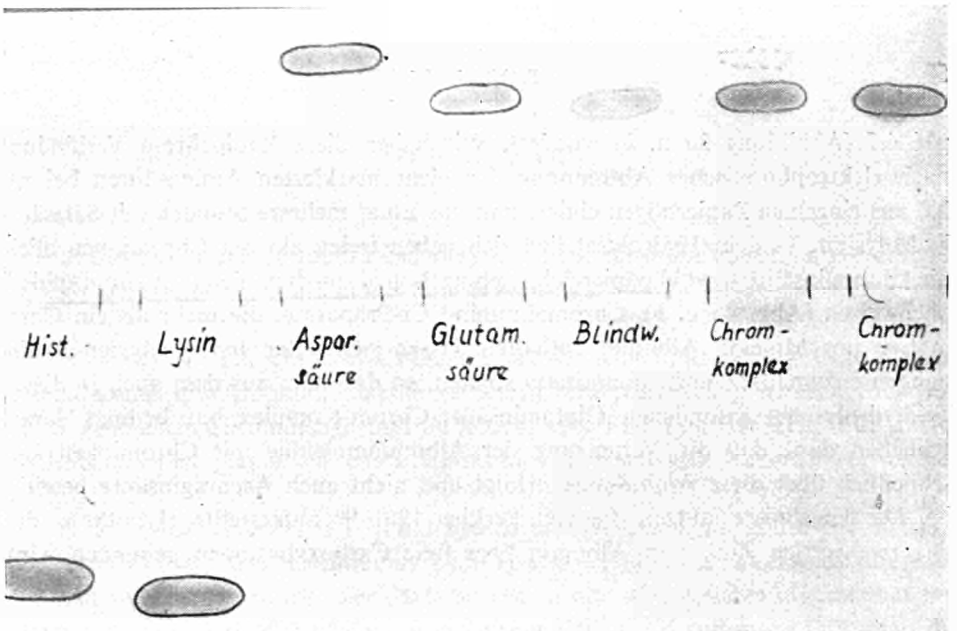
in dem Proteinverband. So veränderte Präparate binden mehr Chrom als das Albumin. Nach Veresterung der in dem Albuminmolekül vorhandenen freien Carboxylgruppen mit Methanol/HCl [8] beobachteten wir eine geringere Chrombindung. Hieraus folgt, daß die Chromierungsreaktion allein an den freien Carboxylgruppen abläuft.



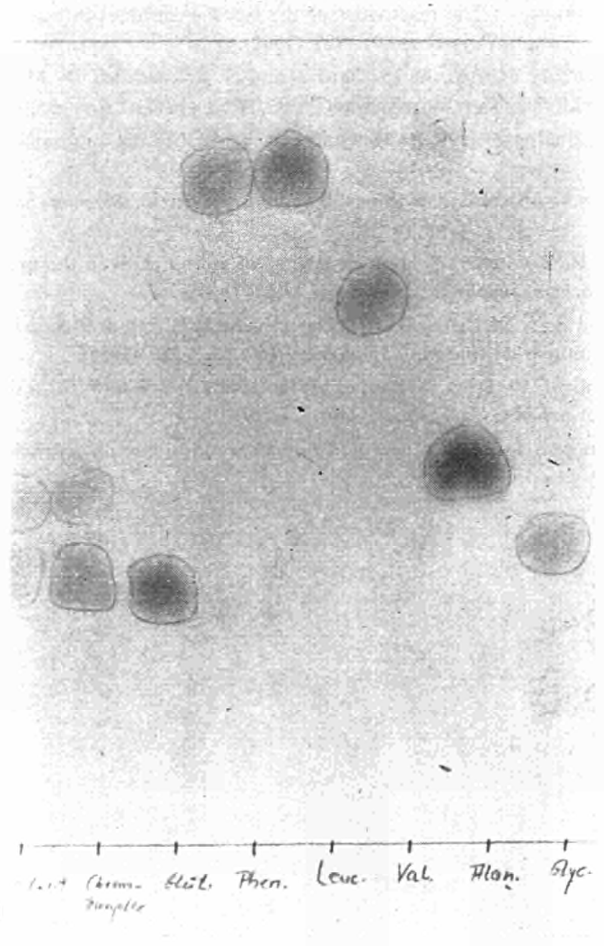
a) b)

ABBILDUNG 3. — Elektrophoretische Trennung des Chromalbumin-^{51}Cr-Hydrolysates in Pyridin/Eisessig pH 6,5; 50 V/cm; 1 Std.
a) Anfärbung der Aminosäuren mit Ninhydrin
b) Diagramm der Radioaktivität.

Die enzymatische Hydrolyse des Chromalbumins-^{51}Cr führte zu dem gleichen Ergebnis. Als Beispiel für die Untersuchung sei hier ein Albuminpräparat, das mit 0,2 Atomen Chrom pro Molekül Albumin vernetzt war, aufgeführt. Für die enzymatische Hydrolyse wurden Pepsin, Trypsin oder Chymotrypsin und Aminopeptidase verwendet. Die papierelektrophoretische Auftrennung des lyophilisierten Hydrolysates zeigte eine radioaktiv markierte Substanz, die weder bei pH 2,0 noch



a)



b)

ABBILDUNG 4. — Identifikation von Glutamisäure im Chromaminosäure-Hydrolysat

a) Elektrophorese pH 6,5; 50 V/cm; 1 Std.

b) Chromatographie in n-Butanol-Pyridin-Eisessig-Wasser (375 : 250 : 75 : 300).

pH 6,5 (Abbildung 3*a* u. *b*) wandert. Wir haben diese Radiochrom Verbindung nach elektrophoretischer Abtrennung der nicht markierten Aminosäuren bei pH 2,0 aus einzelnen Papierbögen eluiert und das Eluat mehrere Stunden mit Salzsäure hydrolysiert. In dem Hydrolysat ließ sich neben freien aktiven Chromionen allein die Glutaminsäure sowohl papierelektrophoretisch als auch papierchromatographisch nachweisen (Abb. 4*a* u. *b*). Chromalbumin-^{51}Cr-Präparate, die mehr als ein Chromatom pro Molekül Albumin enthalten, lassen sich unter den gegebenen Bedingungen enzymatisch nicht quantitativ spalten, so daß man aus dem auch in diesen Teilhydrolysaten gefundenen Glutaminsäure-Chrom-Komplex nur bedingt darauf schließen darf, daß die Vernetzung der Albuminmoleküle mit Chromionen ausschließlich über diese Aminosäure erfolgt und nicht auch Asparaginsäure beteiligt ist. Die Ergebnisse stützen die von Perkins 1961 [9] aufgestellte Hypothese, daß das zweiwertige Zink von Albumin über freie Carboxylgruppen gebunden wird.

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DISCUSSION

Y. COHEN (*Paris*) : Je voudrais savoir si l'orateur considère une albumine marquée au chrome comme une albumine non dénaturée ?

E. NIEMANN : Ich würde es als denaturiert bezeichnen, da bei der Chromierung des Albumins die Albuminmoleküle vernetzen. Die Vernetzung ist eine Funktion der angebotenen Chrommenge und außerdem eine Funktion der Zeit. Für biologische Studien ist, glaube ich, das Präparat nicht in wertvoller Weise zu verwenden.

E. REGOECZI (*London*) : I would like to ask Dr. Niemann about the distribution of MIT and DIT in albumin hydrolysates as obtained after iodinations at different substitution ratios. The data shown in one of the slides suggest that low iodination ratios (e.g. 0.2 atom per mol.) gave less MIT and more DIT than did higher ones. Can these observations be reconciled with the binomial distribution law for the iodine label in a solution of homogenous protein? Should not one expect the reverse to be true ?

E. NIEMANN : Ich wollte mit der Tabelle nur zeigen, dass wir in einer Versuchsreihe von 0,2 bis 2 Atomen Jod pro Molekül Albumin keinen signifikanten Unterschied gefunden haben. Es handelt sich hier um Schwankungsbreiten der Reaktion

LABELLING OF PROTEINS WITH TRITIUM

T. GOSZTONYI (°), J. MARTON (°), A. KOVACS (°), L. KOCSAR (•) and S. VIRAG (+)

(°) *National Atomic Energy Commission, Isotope Institute, Budapest, Hungary.*

(•) *National Research Institute for Radiobiology and Hygiene, Budapest, Hungary.*

(+) *IV. Department of Medicine, University of Budapest, Hungary.*

Abstract

Various methods of labelling proteins with tritium are found in the literature. A simple method was developed in our laboratories for the labelling of several proteins and precursors. Experiments were performed by bringing boron trifluoride complex of tritiated acetic acid into contact with the protein to be labelled and shaking the mixture. This procedure offered mild reaction conditions. Preparation of the reagent and labelling reactions are also simple and require no special apparatus. Labelling of human albumin, ovoalbumin, ribonucleo-proteides, somatotropin, insulin and beta-lipoproteide were investigated. Purity control and identification of the labelled products obtained were performed by immunoelectrophoresis. Specific activities obtainable, purity and biological activity of the products and comparison of the new method with other labelling procedures are discussed.

In the field of protein labelling various methods are known in the literature. The Wilzbach gas exposure technique [1], biosynthetic methods using labelled precursors [2] and synthetic methods for preparing labelled derivatives [3] from proteins have been used successfully. These methods are rather complicated and require special apparatus. In case of hydrogenolysis of iodinated peptides, a method developed by Roche *et al.* [4], the use of special equipment for handling tritrium gas is also necessary.

In our laboratoires a simple exchange reaction has been used for labelling organic compounds. This reaction is a modification of the method reported by Yavorsky and Gorin [5]. In the Yavorsky method the boron trifluoride complex of tritiated phosphoric acid is the reagent which is applicable for labelling organic compounds in a heterogeneous exchange reaction. The strongly acidic medium, however, causes decomposition in some cases. Therefore the application of a weaker acid-complex seemed to us to be more advantageous in labelling of some "acid-sensitive" compounds. The boron trifluoride complex of acetic acid was found to be useful for this purpose. Preparation of this reagent is very simple and can be performed in any chemical or biochemical laboratory without the need of any special equipment. It is a stable compound and can be purified by distillation. Exchange reactions by using the complex are simple too and can be effected by contacting or shaking the material to be labelled with the reagent. Sometimes acetic acid can be used as solvent and so the reaction takes place in homogeneous phase. The equi-

librium can be reached within a few hours at room temperature. No special purification of the labelled compound is necessary in general. Specific activities obtainable are naturally lower than those obtained by using the Yavorsky reagent.

As protein precursors some amino acids were treated with the reagent. It can be seen from the Table I that in labelling of amino acids a sufficient activity yield can be obtained. The rate of the exchange reaction was found to be markedly dependent on temperature. This is shown in Table II, where results obtained in labelling of DL-phenylalanine can be seen.

Preliminary experiments were performed with a few dipeptides. Glycyl-DL-alanine, glycyl-DL-leucine and glycyl-L-tyrosine (50 mg of each) were exposed to

TABLE I. — Labelling of amino acids with acetic acid-boron trifluoride reagent at room temperature for 20 hours

Compound (weight 5 g)	Reagent		Specific activity (mC/g)	Activity yield (%)
	weight (mg)	activity (mC)		
Glycine	1,000	10.9	0.14	0.64
DL-Alanine	1,000	10.9	0.13	0.60
DL-Valine	1,000	10.9	0.11	0.50
DL-Leucine	1,000	10.9	0.16	0.70
DL-Phenylalanine	1,000	131.2	2.00	0.75
L-Tryptophane	1,000	133.7	2.52	0.94
L-Tyrosine	1,000	133.7	0.91	0.34

TABLE II. — Temperature dependence of the exchange reaction in case of DL-phenylalanine

Time of reaction		Specific activity (mC/g)	Activity yield (%)
at 100 °C	at 24 °C		
0	24	2.00	0.76
2	22	1.34	0.51
4	20	1.52	0.58
6	18	1.87	0.71
12	12	2.83	1.08
20	4	3.75	1.43

Substrate : 500 mg.
Reagent : 1,000 mg (131.2 mC).

the reagent for a period of two hours. Chromatographic investigation of the products showed that no destruction of the peptide bonds occured during the labelling. Specific activities and activity yields are shown in Table III.

TABLE III. — Results of labelling of some dipeptides

Compound (weight 50 mg)	Reagent		Specific activity (mC/g)	Activity yield (%)
	weight (mg)	activity (mC)		
Glycyl-alanine	1,000	216	0.148	0.003
Glycyl-DL-leucine	1,000	216	0.148	0.005
Glycyl-L-tyrosine	1,000	216	0.456	0.010

All of the amino acids and dipeptides were chemically and radiochemically pure after labelling as proved by chromatography.

One of the "acid sensitive" compounds which could not be labelled by using the Yavorsky reagent, was cholesterol. Exchange reactions with the acetic acid-boron trifluoride reagent, however, resulted labelled cholesterol with very good activity yields and high chemical and radiochemical purity.

All of these results allowed us to hope that this reaction can serve as a simple method for labelling proteins.

β-lipoprotein isolated from rabbit blood was firstly investigated [6]. The compound in a 5 % sodium chloride solution was lyophylized and the protein-salt mixture (400 mg) was shaken with the reagent (3.7 g = 130 mC) for 24 hours at room temperature. Then the tritiated complex was decomposed by adding water with subsequent lyophylization. Labil tritium was washed out with distilled water by repeated lyophylization. The labelled product was used in the salt solution without any further purification. The solution had a specific activity of 0.7μC/ml. Immunoelectrophoresis and subsequent autoradiography (using Kodak AR-10 stripping film) showed that the labelled β-lipoprotein remained practically unaltered during the exchange reaction and shows the same behaviour in living organism as the unlabelled compound.

A ribonucleoprotein preparate was sucessfully labelled in the same way. The labelled product (1.8 μC/mg) shows only a slight degradation in immunoelectrophoretic and subsequent autoradiographic investigation.

The hormonal polypeptides, somatotropin and insulin, gave good results in preliminary experiments.

In labelling experiments of human albumin and ovoalbumin considerable or total denaturation of the proteins occured, so these experiments must be considered as unsuccessful.

These results show that the method is suitable for labelling of some proteins while others undergo decomposition under these circumstances. A more detailed investigation of the reaction is in progress.

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DISCUSSION

K. BRUNFELDT (*Gentofte*) : I should like to know whether you could give some information on the degree of destruction during the tritium labeling of insulin.

T. GOSZTONYI : I cannot say anything about this problem as our experiments in labeling insulin are only preliminary experiments. I can only say that after purification we could isolate labeled insulin but I cannot say anymore about degradation degree at present.

W. L. HUGHES (*Chairman*) : Can you say what position is labeled in the protein and was the lipid labeled too ?

T. GOSZTONYI : Not now, but we are dealing with this problem. It seems so that mainly the tertiary carbon hydrogen bonds, and especially the aromatic carbon hydrogen bonds, are affected in the labeling process.

On the other hand, I cannot say whether the lipids are labeled. Anyway in labeling of cholesterol very good labeling results can be obtained so we consider that both, protein and cholesterol perhaps could be labeled.

LABELLED PLASMA PROTEINS FOR METABOLIC STUDIES

Chairman : A. S. McFARLANE
National Institute for Medical Research, London, Great Britain

PRELIMINARY REMARKS BY THE CHAIRMAN

The first three papers in this session will deal with criteria for the preparation and proper use of individual labelled plasma proteins, namely albumin, γ-globulin and fibrinogen. These ought to be non-controversial, but we may get divergent expressions of opinion in the discussion of the last two which deal with the important physiological matter of regulation of synthesis and catabolism. In this connection I would ask all participants to make it quite clear whether they are referring to absolute or fractional rates. Synthesis rates are inferred from iodine data obtained in steady state conditions — as they must be — and what is actually measured is the slope of the plasma specific activity curve, so that the result is a fractional rate and one which refers to the total body pool. On the other hand when catabolism is measured from so-called U/P ratios the result is expressed as a fraction of the plasma pool. In discussions of regulation mechanisms it can be important to make a clear distinction between these two and indeed I would ask all speakers to refer as far as possible to what was actually measured without any assumptions about steady state conditions.

IODINATED ALBUMIN AS A METABOLIC TRACER (*)

R. BIANCHI (°), U. ROSA (•), G. FEDERIGHI (°), G. F. PENNISI (•), G. A. SCASSELLATI (•) and L. DONATO (°)

(°) *Medical Clinic and Center of Nuclear Medicine, University of Pisa, Italy.*

(•) *SORIN, Nuclear Research Center, Saluggia, Italy.*

Abstract

A selected preparation of HSA has been used to study the effect of progressive iodination on the biological behaviour of the labeled protein, as judged from its catabolic rate.

Iodination up to 24 iodine atoms per albumin molecule has been performed under standardized and controlled conditions. Preparations were tested for their catabolic rate level in normal humans. The amount of MIT (**) and DIT (**) residues at various degrees of iodination were determined.

The existence of a limiting value of iodination degree below which the catabolic rate is little affected by the degree of iodination was observed. This limiting value has been shown to vary widely with the conditions under which iodination is performed, and to be related to the internal distribution of iodine inside the molecule.

Introduction

The question whether iodinated albumin can be considered an adequate biological tracer has been matter of extensive studies. Most of the recent data tend to suggest that under appropriate labelling conditions ^{131}I-albumin may be considered to fulfil the requirements for tracer studies. However, some experiments with ^{35}S labelled proteins [1] seem to contradict this conclusion.

Although these latter results are largely open to criticism [2, 3] we thought advisable to approach the problem by directly studying the processes through which the progressive iodination modifies the biological behaviour of HSA (**).

Experiments attempting to correlate the process of iodination with the biological behaviour of HSA have so far received little attention and non-systematic contributions [4].

The proper use of this approach demands that the process of iodination be entirely isolated from other processes occurring simultaneously. A preliminary requirement to this sort of study was therefore the establishment of adequate conditions both from the point of view of the preparation of the tracer and of the measurement of the selected biological parameter.

(*) Work performed under Euratom Association Contract No. 026-63-4 BIAC and Euratom Contract No. 048-64-6 BIOI.

(**) MIT : monoiodotyrosine; DIT : diiodotyrosine;
HSA : human serum albumin.

Experiments Performed and Results

Labelling procedure. The preparation method for a study of progressive iodination should permit to iodinate proteins in the mildest possible conditions, such that tyrosine iodination be the only occurring reaction, without parallel processes going on simultaneously.

The method of electrolytic iodination seemed to us well suited for these purposes [5]. In fact it permits to carry out the iodination under controlled conditions, so that iodine is discharged at a preestablished rate, and it reacts stoicheiometically with the protein tyrosine [6].

Moreover, the method by itself has been shown to be devoid of effects on the protein [6].

Selection of albumin batch. On the basis of studies on different albumin batches that were reported in a previous paper [6], it was decided to select the preparations on the basis of their behaviour in humans, choosing the lot yielding the lowest value for fractional catabolic rate (FCR) at an iodination degree (I.D.) of 0.5-1 iodine atoms per molecule (i.a.m.).

Table I presents the level of the FCR for the selected albumin batch (A), iodinated at I.D. of 0.5-1, and measured in a group of 6 normal subjects.

Table I

Evaluation of Selected Albumin Batch (A)

Mean value and standard deviation of the fractional catabolic rate of a selected albumin batch, measured in 6 normal subjects, after iodination between 0.5 and 1.0 i.a.m.

Cases No.	Iodination degree	Catabolic rate % day^{-1}	Standard deviation
6	0.5-1	10.84	0.45

Variance analysis of the results. It is shown that the F values for intra- and inter-subjects variability (resp. days and subjects in the table) do not attain significant values.

Source of variation	Sum of squares	Degree of freedom	Mean square	F value
Days	13.2	5	2.64	*1.69*
Subjects	6.6	5	1.32	*0.85*
Error.	38.9	25	1.56	—

$F_{0.05} = 0.00$ $F_{0.01} = 9.47$

The FCR was determined from the urine-to-plasma ratio of activity.

The mean catabolic rate was 10.84 % per day and both intra- and inter-subjects variations are very small, despite the fact that all the values, including that of the first day, were taken into account. Both variations did not reach statistical significance as shown by the variance analysis, the results of which are also reported in the table.

These observations were performed over a six days period. This observation time is short when compared with the duration of albumin metabolic studies. However, it proved long enough to provide us meaningful informations concerning the FCR of the preparations.

To eliminate inter-subjects variability, in some cases two albumin preparations were injected into the same subject, labelled with ^{131}I and ^{125}I respectively.

Effect of progressive iodination. A series of preparations were obtained from the selected batch A, iodinated at increasing I.D., up to 26 i.a.m.

All the iodination procedures were carried out in the cold, and excess iodine removed by dialysis. Care was also taken to avoid effects due to self-irradiation by using very low specific activity in the case of ^{131}I (less than 10 μC/mg) and injecting all the preparations not later than 48 hours from labelling. The FCR of each preparation was measured in normal subject. Several points were measured with the double tracer technique using a preparation iodinated between 0.5 and 1 as reference.

In Figure 1 are reported the FCR values in the range of I.D. between 0.5 and 26 i.a.m. The two standard deviation ranges for preparations iodinated between 0.5 and 1 are also reported in the figure.

It is evident that up to an I.D. of the order of about 10 the average FCR does not exceed the two sigma range for the preparations iodinated between 0.5 and 1 i.a.m.

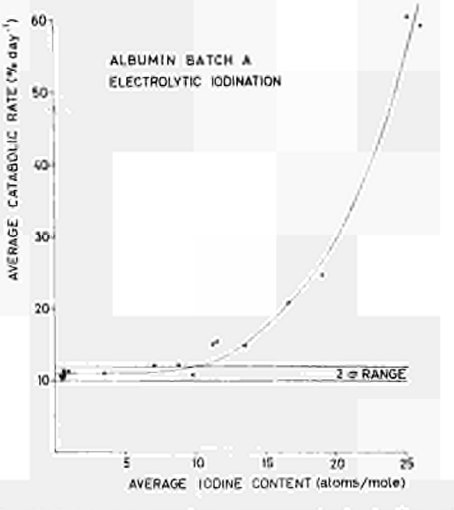


FIGURE 1. — Average FCR of HSA preparations progressively iodinated from 0.5 to 26 i.a.m. The 2σ range refers to the preparations iodinated between 0.5 and 1 i.a.m.

Above this level, FCR rises progressively to reach a value of the order of 60 % per day for an I.D. of about 24.

The electrophoretic behaviour of each preparation was also tested. Free boundary electrophoresis did not show substantial changes in homogeneity for preparations with iodine content ranging from 0.5 to 16 i.a.m.

Agar gel electrophoresis revealed only an increased mobility for increasing value of the I.D., without apparent inhomogeneity or trailing effects, as shown in Figure 2.

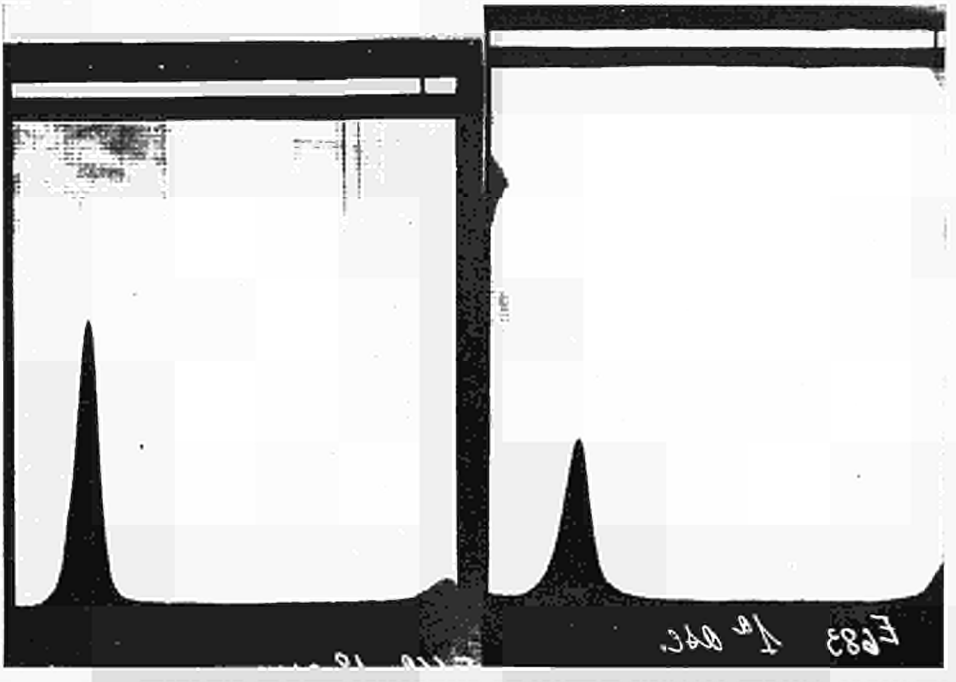
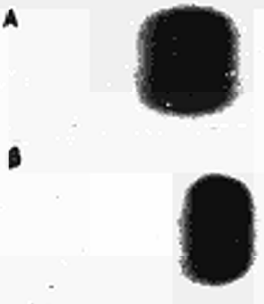


FIGURE 2. — TISELIUS ELECTROPHORESIS OF NATIVE HUMAN SERUM ALBUMIN (on the left) AND OVERIODINATED (16 at. I/mol) SERUM ALBUMIN (on the right).



ELECTROPHORESIS ON AGAR GEL OF :

A) Overiodinated serum albumin (16 at. I/mol).
B) Native serum albumin.

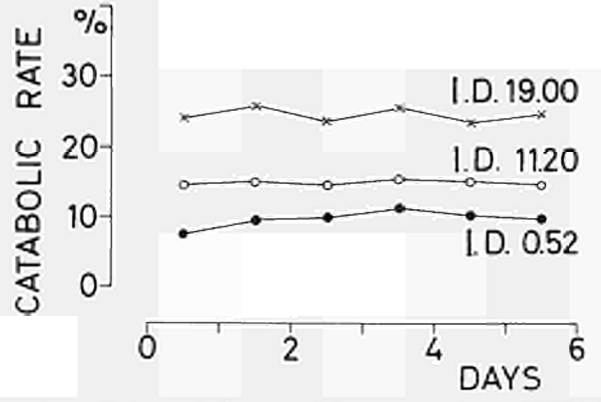


FIGURE 3. — Daily catabolic rates of three preparations of IHSA (batch A).

These findings rule out the occurrence of gross alteration products, a conclusion which is supported by the apparent constancy of the daily values of the FCR in each preparation, including those above the I.D. of 11. Examples of this pattern are shown in Figure 3.

These findings indicate that iodinated HSA preparations above an I.D. of 11 are handled as new species with FCR values higher than that of low iodinated preparations. However, this increase in the degradation rate for increasing iodine content, has nothing in common with the behaviour of grossly denatured preparations, in which the daily catabolic rate rapidly and continuously decreases in successive days.

Comparison with other methods of iodination. On the basis of the above findings we thought of interest to use the double tracer technique to compare the metabolic

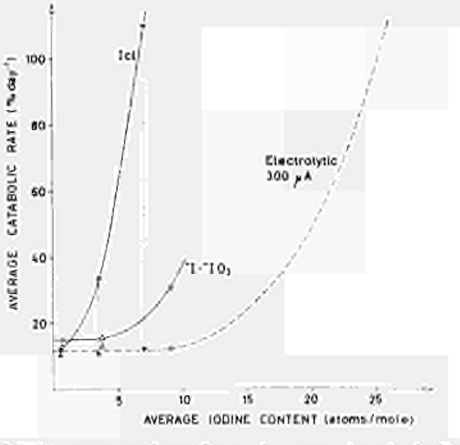


FIGURE 4. — FCR values of HSA preparations from the same batch, iodinated at increasing I.D. with different techniques.

The vertical lines connect values obtained in the same experiment, in which a double tracer technique (^{131}I and ^{125}I) was used to compare in the same subject the FCR of two preparations obtained from the same batch, at the same I.D., with different techniques. The dotted line is replotted from Figure 1.

behaviour observed with the electrolytic method with that of preparations obtained from the same batch using the iodide-iodate or the iodine-monochloride procedures [6]. In Figure 4 the results are compared at different I.D.

It is evident that at an I.D. between 0.5 and 1 i.a.m. the various methods show small differences, with the possible exception of somewhat higher levels for the iodide-iodate method. On the other hand, as soon as the I.D. rises above 2 or 3, while the electrolytically labelled proteins maintain a constant value of FCR, both the iodide-iodate and the iodine-monochloride labelled proteins, show increasingly higher values of FCR, and grossly inhomogeneous handling. An example is shown in Figure 5.

Comparison with other albumin batches. Besides the differences due to various iodination methods, similar differences could derive from the properties of the starting material.

Figure 6 presents the results of the FCR studies performed on a different batch of HSA (batch B) subjected to progressive iodination under the same conditions used for batch A.

The trend shown by this preparation is similar to that of batch A, with the only difference that the FCR starts to increase at I.D. of about 5-6 i.a.m. As for the previous batch, no substantial heterogeneities were observed, neither in the daily

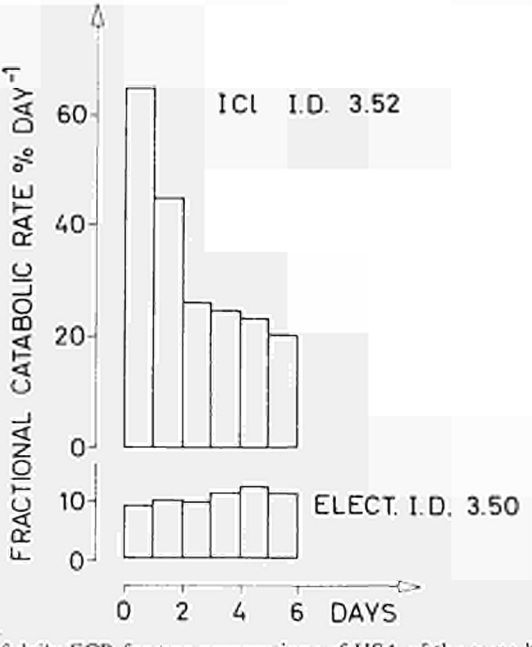


FIGURE 5. — Values of daily FCR for two preparations of HSA of the same batch (A) iodinated at the same I.D. and injected into the same subject. In the upper part the results of the preparation labelled with the ICl method (^{125}I), in the lower part these of the preparation electrolytically labelled (^{131}I).

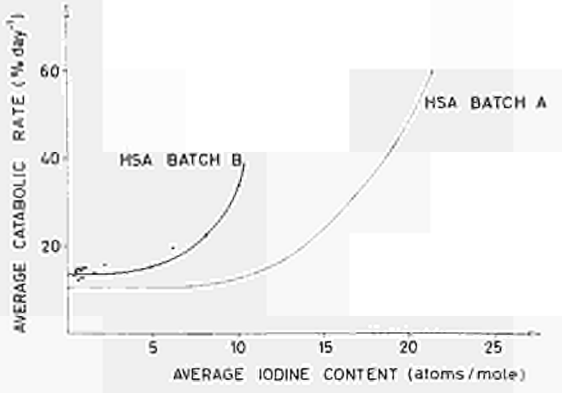


FIGURE 6. — Comparison of the FCR values of 2 batches of HSA submitted to progressive iodination. The line referring to batch A has been replotted from Figure 1.

values of FCR, nor in the electrophoretic behaviour. In this case also, the behaviour definitely differs from that of grossly altered preparations.

Intramolecular distribution of iodine. In the hypothesis that the explanation of the above findings could be related to the intramolecular distribution of iodine, the amount of mono-iodo and di-iodo tyrosine residues at various I.D. was measured on the preparations obtained from batch A.

The results are presented in Figure 7. It can be seen that the percentage of the actual free tyrosine residues decreases progressively to about 20 % of its original value, as the iodine content increases from 0 to 11 i.a.m. The remaining 20 % of free tyrosine disappears at a much slower rate, since the iodine content has to reach a level of 25 before free tyrosines disappear entirely.

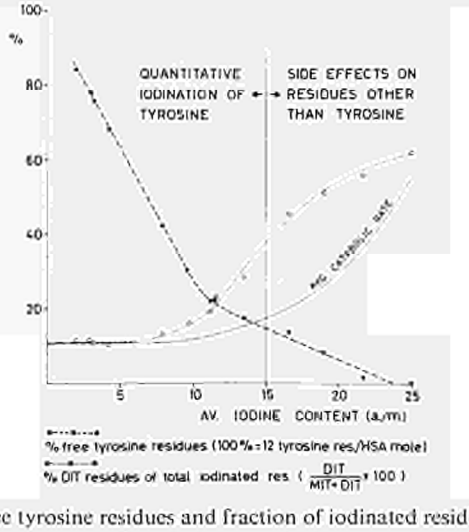


FIGURE 7. — Percent of free tyrosine residues and fraction of iodinated residues in DIT form, versus increasing I.D. in the selected albumin batch submitted to progressive iodination. The line describing the average catabolic rate has been replotted from Figure 1.

This indicates that most of the additional iodine which reacts with the protein, above an I.D. of 10-11 i.a.m., is used to transform mono-iodo into di-iodo derivates as shown by the increase of the latter.

On the other hand, effects other than iodination of tyrosine, probably involving histidine residues, appear above a level of 15. This explains why in this range DIT percentage rises at a slower rate than expected.

Discussion and Conclusions

When the behaviour of free tyrosines is compared with that of FCR of progressively iodinated albumin (both are shown in Figure 6) it seems possible to conclude that, with a proper choice of starting material and iodination conditions, most of the tyrosines residues in the protein can be substituted without appreciable changes of their catabolic rate.

We do not believe that the critical value, above which a deviation of FCR occurs, could be further displaced towards higher I.D. by acting on the various parameters. In fact, the conditions of this experiment can probably be considered as the mildest possible from the point of view of iodination : the rate of reaction and the amount of reacting iodine are minimal, the albumin concentration is maximal, and no side reactions occur. Further slowing of the rate of discharge of elemental iodine or increase of the albumin concentration did not result in appreciable differences of the observed FCR.

These observations permit to conclude that the isolated tyrosine iodination process, by itself, has to reach an I.D. of about 10-11 for affecting the metabolic behaviour of the protein.

The increase of FCR that occurs in the range from 10 to 15 i.a.m., within which side effects have not yet appeared, indicates that at this level the process of tyrosine substitution has determined a critical intramolecular condition, resulting in a modification of the metabolic behaviour of the protein.

On the other hand, the results of studies with other iodination procedures and with different albumin batches, indicate that a critical intramolecular condition can be attained at much lower iodination degrees. The higher probability of attaining a critical intramolecular condition at lower I.D., could be inherent in methods like the iodide-iodate and the iodine-monochloride techniques. On the other hand, the same result could be a consequence of changes occurring during isolation and purification of the protein.

All these data point to the importance of the distribution of iodine within the molecule, as a determinant of its metabolic fate.

Too little information is presently available to grant more than pure speculative reasoning on this subject. However, it seems now obvious to us that further investigation of the protein changes about the I.D. of 10 should provide valuable information of the mechanism by which iodine affects the behaviour of the protein.

As a side consequence, but relevant to the aim of this work, the demonstration that, in absence of side effects, iodination may reach a very high level before a critical intramolecular structure is attained, proves the adequacy of low iodinated albumin preparations as metabolic tracers.

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DISCUSSION

E. TRIANTAPHYLLIDIS (*Paris*) : Avez-vous injecté dans chaque cas la même quantité de radioactivité ou bien cette quantité variait-elle avec le degré d'ioduration ?

R. BIANCHI : The amount of injected radioactivity was about 50 microcuries in all cases.

E. TRIANTAPHYLLIDIS : Y-a-t-il une différence de mobilité électrophorétique entre l'albumine ordinaire et l'albumine faiblement iodée ?

U. ROSA (*Saluggia*) : Il y a une différence de mobilité négligeable entre l'albumine marquée à 0,5 atome d'iode/molécule et l'albumine native. Par contre nous avons remarqué des différences significatives dans le cas des albumines hautement iodées, notamment pour des taux d'iodination de l'ordre de 6 à 7 atomes d'iode par molécule.

M. A. ROTHSCHILD (*New York*) : Are your control curves followed for a long enough period to determinate the validity of the preparation ? Other studies have shown that iodination at the level you describe did result in alterations in the plasma decay curve. According to Berson and Yalow, these changes made their appearance only after the curves were followed for some weeks (Berson, S. A. and Yalow, R. S., Schreiber, S. S. and Post, J., *J. Clin. Inv.*, **33** : 746, 1953).

R. BIANCHI : As I have pointed out in my presentation, our experimental period was of short duration for practical purposes; on the other hand, the values of the fractional catabolic rate obtained by us, and expressed as percent of plasma albumin, are in excellent agreement with that of the various AA. who prolonged the experiment for 3 or 4 weeks.

It is well established that under steady state conditions the fraction of plasma radioiodinated albumin degraded per day is constant from the beginning of the study; our studies rely on this evidence and have confirmed it.

On the other hand if the fractional catabolic rate is expressed in percent of the total activity retained in the body it necessarily shows higher values in the first few days, progressively decreasing towards a constant value that is reached after some time. This pattern, to which I think Dr. Rothschild refers, is implicit in such a calculation method, which yields the rate of change of the tracer content in the entire body, and does not necessarily imply denaturation of the label.

BASIC ASPECTS OF GAMMAGLOBULIN METABOLISM

S. B. ANDERSEN

Department of Clinical Physiology, Glostrup Hospital, Copenhagen, Denmark

ABSTRACT

γG-globulin comprises far the greatest part of the protein in the gamma fraction of human serum. The present review is concerned with metabolic studies using ^{131}I-γG-globulin, with the evidence which can be presented for the validity of such studies, and how the homogeneity of the labelled protein can be controlled. Evidence is presented that γG-globulin from a kinetical point of view is catabolized intravascularly.

The catabolic pathways of γG-globulin are discussed and it is concluded that γG-globulin may be catabolized by a non-specific process in the gastrointestinal tract, liver and lungs. Finally it is emphasized that a satisfactory mathematical model must be used for the calculations and normal values for γG-globulin metabolism is given.

I have been requested to concentrate on the basic aspects of metabolic studies with iodinated gammaglobulin, and particularly to present evidence for the validity of such studies.

In the paper by Freeman (*) general criteria for evaluating the quality of iodine labelled plasma proteins were already given. I am going to apply these criteria to gammaglobulin and, in addition, to discuss where and how gammaglobulin is catabolized. Finally, I shall say a few words about calculation methods and give my own normal values for the metabolism of γG-globulin in man.

Freeman pointed out that valid data can be obtained only if the labelled protein is homogeneous metabolically. By this expression is meant that all the labelled molecules must have the same chance of being catabolized. None of them must have a greater tendency to be broken down than the others. Otherwise it would be impossible to analyze the kinetics of the molecules and the metabolic parameters could not be calculated.

Metabolic heterogeneity may be due either to impurity or to denaturation. The results of the earliest studies of gammaglobulin — and of some of the latest — are questionable on account of both these factors.

It is well known to day that the gamma fraction of human serum consists of the three immunoglobulins : γG-globulin, γM-globulin and γA-globulin. Cohen and Freeman [1] have shown that γM-globulin is catabolized much more rapidly than γG-globulin. A mixture of immunoglobulins, therefore, is heterogeneous metabolically and cannot be used in metabolic studies. A complete account of the metabolism of the gamma fraction should be based on knowledge of the metabo-

(*) These Proceedings, p. 31

lism of each of the immunoglobulins. However, γG-globulin is found in far the greatest concentration. Thus the metabolism of this immunoglobulin will represent the metabolism of the gamma fraction fairly well.

The purity of a labelled preparation of γG-globulin should be tested by several different physico-chemical methods, as for instance paper electrophoresis, immunoelectrophoresis, autoradiography and ultracentrifugation. But it is not enough to show that the protein is pure. Metabolic heterogeneity may also be due to denaturation, either on account of the fractionation method or on account of the iodination.

The ordinary Cohn fractionation with cold ethanol will always denature γG-globulin. This method should not be used to isolate γG-globulin for metabolic studies. Column chromatography on DEAE cellulose or DEAE Sephadex is much more gentle. We have used this method — which is quite simple — for the last five or six years with satisfactory results.

The iodination of the protein should not be too heavy, at most two or three atoms of iodine per molecule of γG-globulin — preferably less. It is essential to use a method in which strong organic solutions are not employed. We have used the iodine monochloride method [2] for a number of years. It is easy to use and has proven very satisfactory.

Control for metabolic heterogeneity

The question is now to demonstrate if the labelled γG-globulin is hetereogenous metabolically. Physico-chemical methods are usually not sensitive enough for demonstrating denaturation. They will only reveal severe changes in the structure of the protein molecules which will produce gross biological denaturation of the protein. The most sensitive physico-chemical test for denaturation is probably column chromatography on ion exchanger cellulose. Labelled γG-globulin should show the same pattern on DEAE cellulose as the genuine protein.

The best way of testing the labelled γG-globulin is to employ biological methods. In practice this can be done by evaluating the results of the turnover study. The excretion of radioactive iodide in the urine during the first 24 hours should not be greater than on subsequent days. Iodide liberated during catabolism of protein in the beginning of the study will have to fill up the iodide pool before any excretion can take place.

In addition, the ratio between urinary activity and plasma activity should be constant throughout the study. A falling ratio would indicate metabolic heterogeneity of the labelled protein.

An example is shown in Figure 1. The average urinary excretion is shown for 21 normal subjects. The daily value for urine/plasma ratio (U/P) has been expressed in per cent of the average for the whole period in order to calculate the average variation for all subjects. It is apparent that the excretion during the first day is low and that the U/P ratio is fairly constant throughout the study. A small devia-

tion from this pattern can be explained by the effect of the iodide pool, which I will discuss later.

We have one more rather simple biological test to our disposal, namely simultaneous determination of plasma volume with T-1824 and the labelled protein [3]. This can be done when the turnover determination is started. Denatured labelled

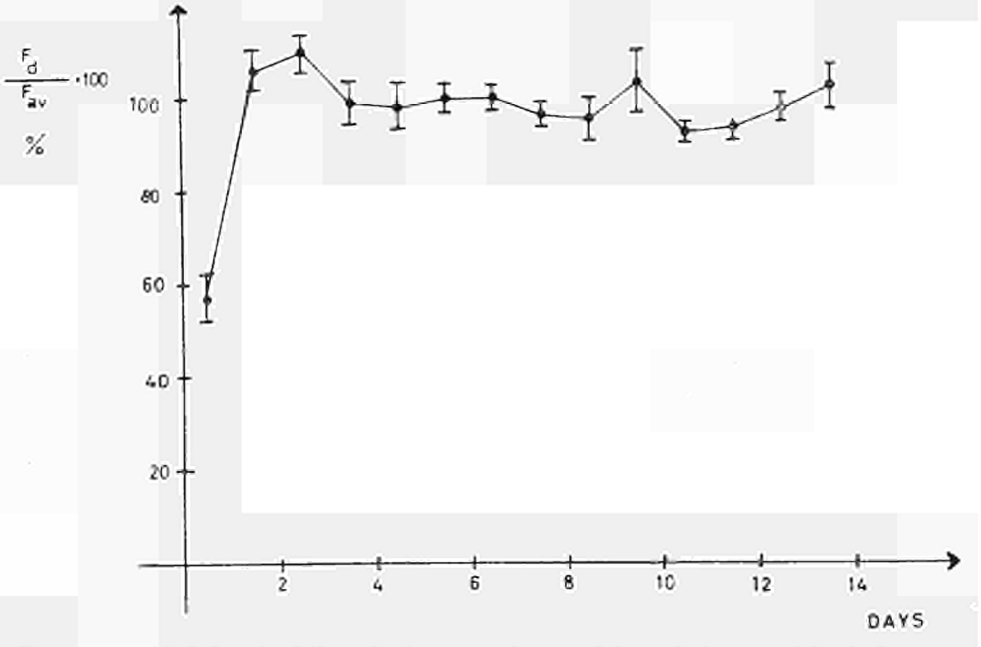

FIGURE 1. — Variations in daily ratios between urinary activity and plasma activity during metabolic studies with ^{131}I-labelled γG-globulin. In order to compare results from all subjects the daily values (F_d) are expressed in percent of the average value (F_{av}) for the whole period for each subject. The vertical lines indicate the standard error of the mean.

protein will rapidly be taken up by the liver. If a denatured preparation has been used, plasma volume will be considerably greater determined with this, than with T-1824.

INTRAVASCULAR CATABOLISM

The metabolic parameters we are interested in are shown in Figure 2 : intravascular mass, extravascular mass, rate of synthesis and fractional catabolic rate. The γG-globulin seems to be catabolized in connection with the intravascular mass. In our opinion, therefore, the catabolic rate should be related to the intravascular mass. The fractional catabolic rate denotes that fraction of the intravascular mass, which is catabolized daily. This parameter indicates how fast the protein is broken down after production. The reciprocal value of the fractional catabolic rate is the same as the mean life time for a protein molecule in the intravascular mass.

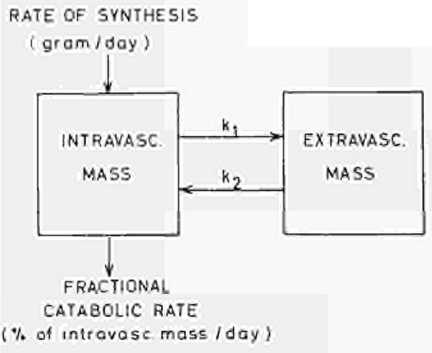

FIGURE 2. — Model of the parameters of γG-globulin metabolism. Exchange of protein between intravascular and extravascular compartment is symbolized by k_1 and k_2, which reflects the capillary permeability and lymphatic flow rate, respectively.

The γG-globulin has been considered to be broken down intravascularly because the activity excreted in the urine is a constant fraction of the plasma activity, even during the process of establishing equilibrium between extravascular and intravascular labelled protein. But this is not quite the case. As mentioned previously the U/P ratio of activity is usually low the first day or two of the study and relatively high the following days. Subsequently the fractions might be decreasing slightly. This pattern may be due either to extravascular degradation of the γG-globulin or to the effect of the iodide pool, if denaturation of the preparation can be excluded.

In order to clarify these points a mathematical model which takes the iodide pool into account was used (Figure 3). The plasma curve is composed of a sum of

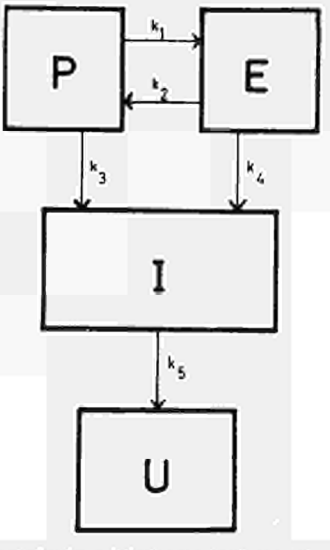

FIGURE 3. — Metabolism of γG-globulin : Mathematical model which includes the iodide pool : P = activity intravascularly. E = activity extravascularly. I = activity in iodide pool. U = activity excreted in the urine. k_5 = urinary excretion rate of iodide.

two or three exponentials. The form of the plasma curve is unaffected of the site of degradation. It is therefore possible to calculate the urinary excretion curve if 1) the protein is assumed to be broken down intravascularly only or 2) the protein is assumed to be broken down extravascularly only [4]. The excretion rate from the iodide pool, k_5, is about 1.5 days^{-1} [5].

These curves are shown in Figure 4 for normal subjects and in Figure 5 for patients with cirrhosis of the liver. It is apparent that the experimental data follow the theoretical curve for intravascular degradation very closely. It is seen that a slight fall in the U/P ratio is not greater than should be expected theoretically if the iodide pool is taken into account, indicating metabolic homogeneity of the labelled γG-globulin.

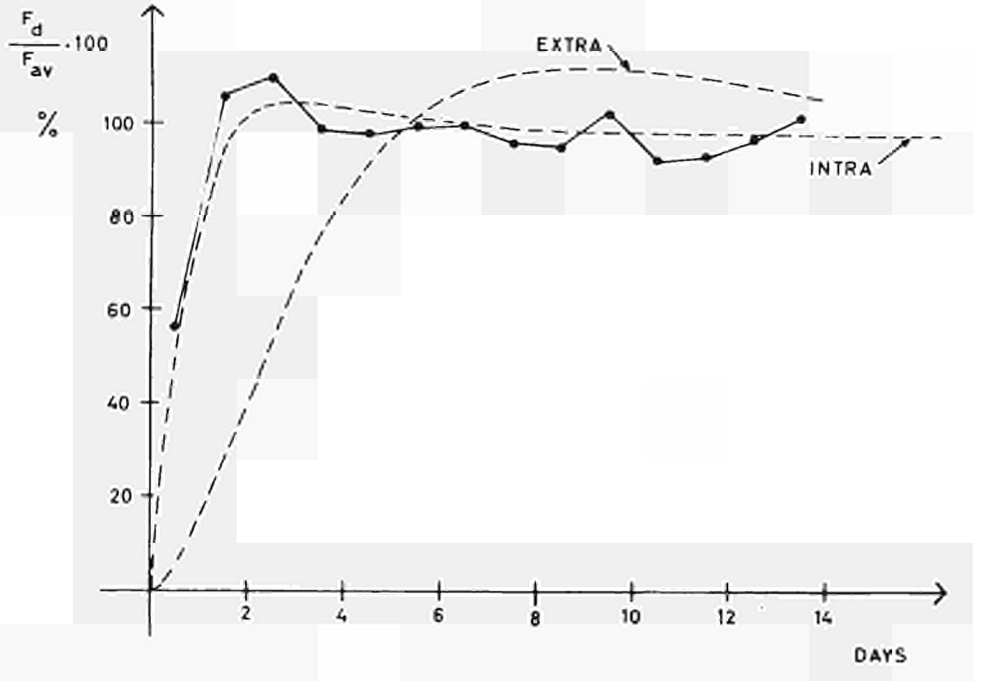

FIGURE 4. — Theoretical (- - - - - - - - - - -) and experimental (●——●) variations in urinary excretion of activity in metabolic studies with ^{131}I-γG-globulin. The experimental U/P ratios are calculated relatively (symbols as in Figure 1) from the results of 21 studies in normal subjects.

These findings, of course, do not mean that γG-globulin is catabolized in the blood stream. In fact we know that incubation with blood or plasma has no effect on labelled γG-globulin. But these findings suggest that it is broken down at extravascular sites in so rapid exchange with plasma that the specific activity there is identical with that of plasma.

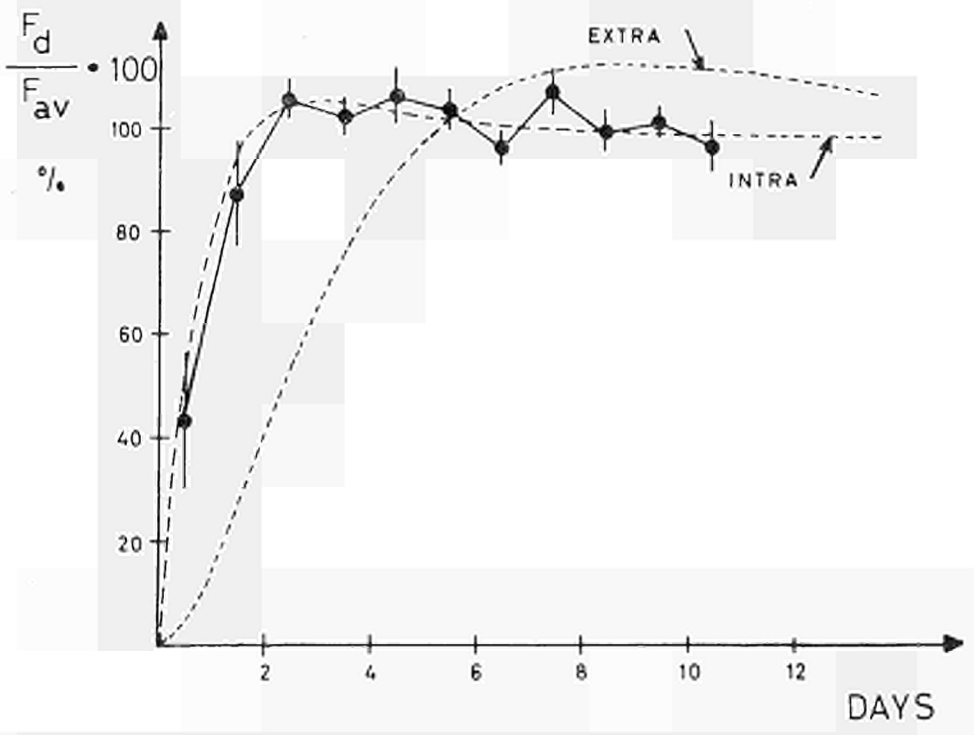

FIGURE 5. — Theoretical compared with experimental variations in U/P ratios in cirrhosis of the liver (Symbols as in Figure 4)

CATABOLISM

In recent years it has been shown that certain types of hypoproteinemia are caused by leakage of plasma proteins into the gastrointestinal tract and subsequent digestion by the intestinal enzymes. It has been suggested that the same mechanism may play a role for the catabolism of γG-globulin in normal subjects, because gammaglobulin has been found both in saliva and in gastric and intestinal juice.

A couple of years ago we made an experimental study of the transfer of proteinbound radioactivity from the plasma to the lumen of isolated intestinal segments in dogs [6]. We calculated that an average of 40 % of gammaglobulin was broken down in the intestinal lumen.

The intestinal segments were isolated between tape ligatures and then rinsed with saline through a thin Nelaton catheter. The question arises, of course, if the transfer of γG-globulin might be caused by the trauma from the surgical procedure. We have the following arguments in favour of the validity of our study :

1. When a segment was subdivided with one more tape ligature the transfer rate of protein to the lumen of the gut was not increased.

2. We found a significant correlation between the exchange rate (from plasma to extravascular compartments) and that fraction of the gammaglobulin which

was catabolized in the gut. These two parameters were determined independently in two different experiments. It is improbable, therefore, that the transfer we measured was artificial, because an artefact most likely would not be correlated to a physiological parameter.

3. In each experiment the rinsing was repeated from two to four times on each segment. The transfer rate was the same from time to time. It is unlikely that the rinsing or the surgical procedure should have damaged the mucosa. Otherwise the transfer rate would not have been constant.

However, experiments of the above mentioned type is open to criticism of obvious reasons. In addition, studies with ^{51}Cr-labelled albumin show insignificant excretion of activity in feces of normals [7]. The question of gastrointestinal degradation in normal subjects has not been finally settled. The crucial experiment remains to be planned and carried out.

Gammaglobulin might also be catabolized universally, for instance in connection with its function as an antibody. After combination with antigenic substances invading the body the antigen-antibody-complex should be catabolized after phagocytosis in the reticulo-endothelial system. The following observation suggests that this mechanism is of little quantitative significance.

In rabbits it is possible to produce high concentrations of gammaglobulin in serum by hyperimmunisation with a polyvalent pneumococci vaccine. The increase in gammaglobulin is due almost entirely to specific antibody. The synthetic rate of gammaglobulin increased from 0.5 to 4.3 grams/day while the fractional catabolic rate was unchanged, 35 per cent of the intravascular mass per day [10]. Under the assumption that the increase in the synthetic rate is antibody against the pneumococci, it is easy to calculate that each pneumococci must have stimulated the production of five thousand million antibody molecules.

Even if each pneumococci was able to bind several millions antibody molecules under the formation of an antigen-antibody-complex, the production of antibody would be several thousand times greater than necessary to neutralize the injected pneumococci.

Therefore, it is doubtful if gammaglobulin to any significant degree is catabolized through an antigen-antibody-reaction. The catabolic mechanism is probably an unspecific process the nature of which we know little of.

Metabolism of γG-globulin in normals

In addition to the conditions previously mentioned two more have to be fulfilled before results of turnover studies can be accepted : the subject must be in a metabolic steady state and a satisfactory mathematical model must be used for the calculations.

The mathematical analysis is based on the assumption that the metabolic parameters are constant during the study. At present no satisfactory mathematical model exists with which it is possible to calculate all parameters under unsteady state conditions. It is necessary, therefore, to ensure that the subject is in a steady

state. We use the following criteria : 1) the weight of the patient must be constant during the study, 2) the concentration of the protein must be constant, 3) the plasma curve and the whole body curve must be parallel when the final slope is obtained.

A satisfactory mathematical model should reflect the conditions in the body as close as possible. γG-globulin seems to be catabolized intravascularly. Several models take this into account [11, 12]. Alternatively can be used a model which is independent of the site of catabolism. The most general one has been developed by Nosslin [13]. Previous models assume that extravascular protein is localized in one or two compartments. Nosslin's model operates with an infinity of extravascular compartments which may be interconnected in any possible way. The protein may be catabolized from any one or more compartments. The only basic assumption is that the protein from the protein producing cells should be released primarily to the blood stream. Nosslin's calculations are very simple. The fractional catabolic rate is identical with the reciprocal value of the area under the plasma curve. The total body mass of the protein is related to the intravascular mass as the area under the whole body curve is related to the area under the plasma curve.

In table I are shown the normal values for γG-globulin metabolism. The fractional catabolic rate is about 7 %/day and the rate of synthesis is an average of

TABLE I. — Metabolism of γG-globulin in 21 normal subjects

	Mean	Range
Serum γ-globulin (g/l)	12.7	9.8-15.8
Plasma volume (l)	2.8	1.9-3.7
Plasma pool (g)	36	22-53
Total body pool (g)	69	42-104
Fractional catabolic rate (%/day)	6.9	4.3-9.3
Rate of synthesis (g/day)	2.5	1.4-4.0

2.5 grams/day. These values are higher than those reported from other laboratories. This may be due to actual differences in the metabolism of γG-globulin in different populations. But it may also be due to errors in the measurements. One of the most frequent ones is insufficient urine collections. This error is difficult to control — unless whole body counting is employed — and until new evidence has been presented I am reluctant to accept values for a European population much lower than those we have found in Copenhagen.

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DISCUSSION

M. A. ROTHSCHILD (*New York*) : Do you believe that the catabolism in the gastrointestinal tract is related to specific process or may it represent the simple transudation of serum ?

S. B. ANDERSEN : In experiments similar to ours other investigators have found transfer of ^{131}I-labeled albumin to the lumen of intestinal segments. Even if this is not conclusive evidence that gastrointestinal loss of plasma protein takes place, I think that this suggests a bulk loss of serum. The gastrointestinal catabolism therefore should not be a specific process.

G. MILHAUD (*Paris*) : How did you estimate the daily synthetic rate of gammaglobulin ?

S. B. ANDERSEN : The subject I studied was as far as I could judge in a metabolic steady state. So when I know the fractional catabolic rate and the intravascular mass then I know the synthesis rate also in g/day, because these two should be equal.

L. O. PLANTIN (*Stockholm*) : With respect to the completeness of urine collection I would like to show the results from two investigations where there can be no question about incomplete urine collection.

The first figure shows the turnover curves in a patient with cerebral hemorrhage. Urine was collected through a catheter "à demeure". We note that the fractional catabolic rate (U/P ratio) is constant throughout the investigation period of 60 days. The extravascular curve is far from parallel with the plasma curve and seems to be almost at a constant level and is still over 30 % of the given dose after 60 days.

The second figure shows the turnover in a healthy male, who was injected with ^{131}I-gammaglobulin and where in addition to urine collection whole body measurements were made for over 50 days. In this case we obtain parallel curves for extravascular and plasma activity. The retained dose curve, obtained in the usual way by subtracting urine excretion from the given dose, is very close to the whole body curve. The difference can be explained by loss of some iodide activity through the sweat. These two results suggest that there can and must be some other explanation to deviating plasma and extravascular curves than incomplete urine collection. I would also like to ask if Dr. Andersen's whole body curves were obtained by actual whole body measurements or by calculations from the urine excretion.

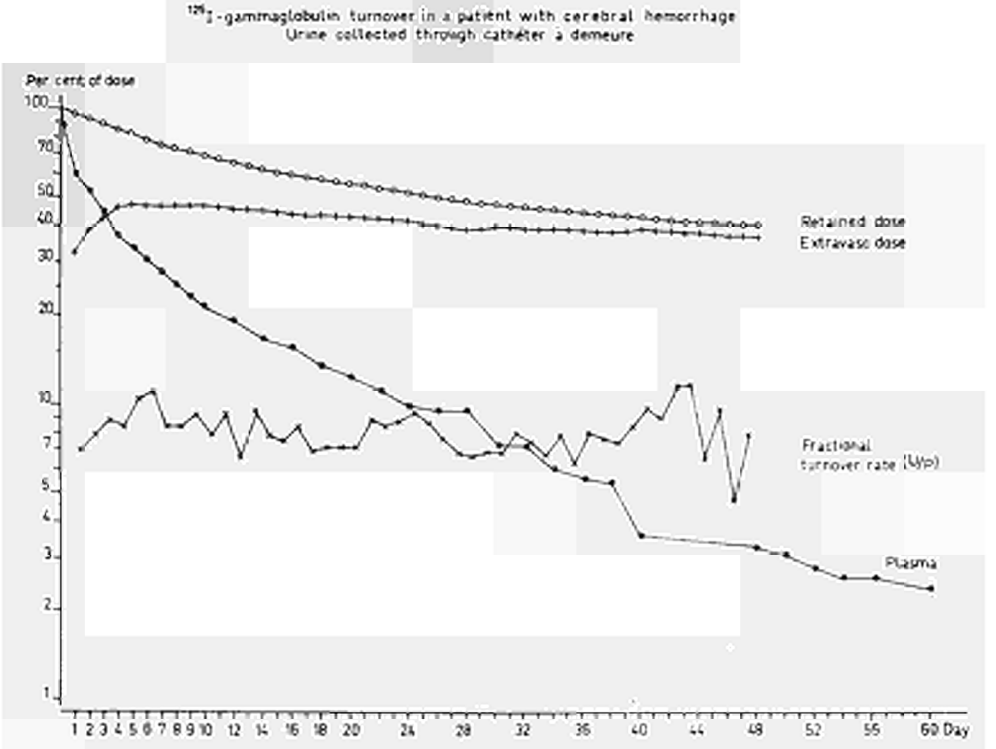

Figure 1

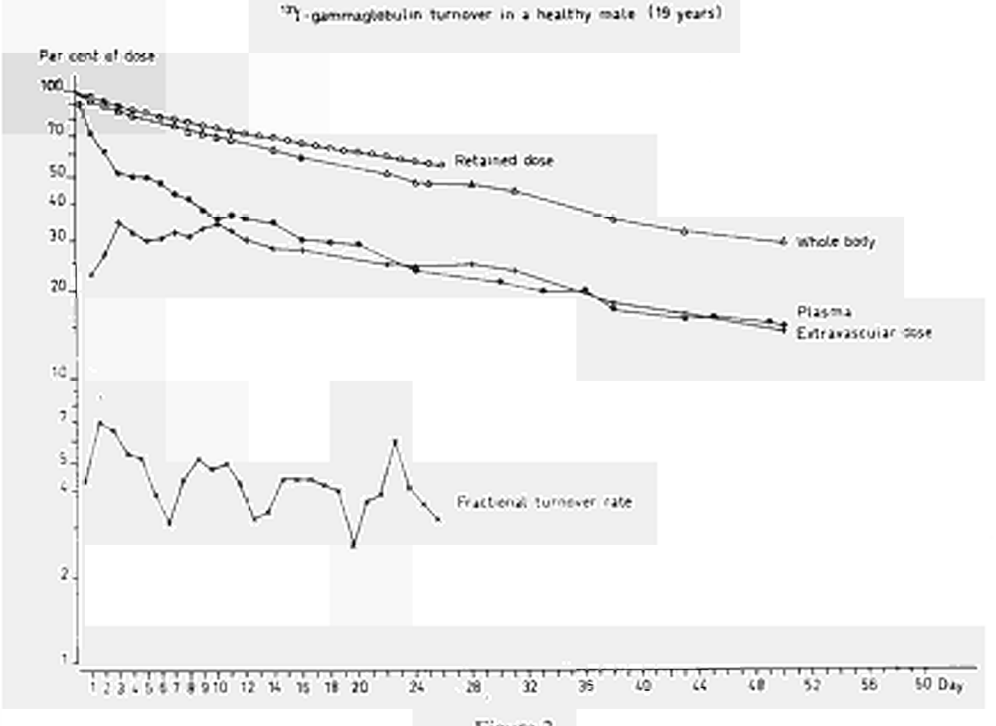

Figure 2

T. FREEMAN (*London*) : I have a comment in connection with this question. Measuring both total body counting and urine activities we quite frequently have found a discrepancy between these two. In some of these instances we think we have collected all the urine, so loss of activity must be by some other ways, but we could find no evidence of this in feces or sweat.

Concerning gammaglobulin catabolism in hypergammaglobulinemia, I have had different results from Dr. Andersen, in that those patients we have studied with a high gammaglobulin concentration have had increased gammaglobulin catabolism. Many of these were patients with myeloma, and this may be a special case but it was also found in hypergammaglobulinemia from other causes.

In relation to the gastrointestinal tract as a site for catabolism of gammaglobulin and other plasma proteins, I would like to point out that Waldman's work with chromium labeled albumin and copper labeled ceruloplasmin is against this. Both these proteins when given by mouth can be recovered quantitatively in the feces, however if they are given intravenously little or no activity can be found in the stool, in spite of the fact that they can survive in the blood stream for several days.

S. B. ANDERSEN : There may be two explanations for deviating retained curve and plasma curve if the curve of retained dose is calculated from the urine excretions of activity : 1) insufficient collections of excreted activity, 2) the subject is not in a steady state condition.

In the first case the insufficient collection of excreted activity may be due to incomplete urine collection or to loss of activity by other routes than urine (or feces). These other routes may be sweat. Even in a climate as cold as in Denmark this may account for significant amounts. I have studied the excretion of iodide in myself being sure that the urine collections were complete. I could only account for about 95 % of the injected dose. Nothing was retained in the body as controlled by total body counting. This shows that even if you have a complete urine collection you may very well have an insufficient collection of excreted activity. It is therefore important to control the results by total body counting.

The other possibility that Dr. Plantin's patient was in an unsteady state is not quite improbable considering the disease of the patient. Both possibilities might play a rôle for the finding of deviating curves. As answer to Dr. Plantin's last question I can inform that our results from the last two years have been controlled by total body counting.

As for Dr. Freeman's comment on hypergammaglobulinemia I do not quite agree. In 21 patients with cirrhosis of the liver the fractional catabolic rate was increased in five only, four of which were treated with 30-60 mg of prednisone daily. These findings show that the fractional catabolic rate in hypergammaglobulinemia due to liver cirrhosis most often is within normal limits. In patients with paraproteinemia fractional catabolic rate was increased in four out of fifteen. Three of these had myeloma in a very advanced stage. They died within 2 months of the

study. These observations show that the fractional catabolic rate in hypergammaglobulinemia due to paraproteinemia most often also is within normal limits.

Concerning Dr. Freeman's comment on the gastrointestinal tract as a site for catabolism of gammaglobulin and possibly other plasma proteins I fully agree with him that we have no conclusive evidence for this. On the other hand Dr. Waldman's finding with ^{51}Cr-labeled albumin may not reflect the true conditions. We know that ^{51}Cr-labeled albumin is denatured and that denatured proteins are taken up by reticulo-endothelial cells. The low fecal excretion of ^{51}Cr after intravenous injection might then be due to the fact that reticulo-endothelial cells in the gastrointestinal mucosa took up the molecules of ^{51}Cr-labeled albumin during the passage from the blood stream to the lumen of the gut. There is some evidence for this conception. Dr. Wetterfors has shown in irrigation experiments with dog intestines that ^{51}Cr-labeled albumin passes more slowly from the blood to the lumen of the gut than to ^{131}I-labeled albumin. Of course other explanations than phagocytosis by reticulo-endothelial cells might explain this finding.

Y. COHEN (*Paris*) : J'ai été très intéressé par la communication du Dr. Andersen et je voudrais lui poser la question suivante : A-t-il une idée de la localisation dans l'intestin de la zone de dégradation de la gammaglobuline, soit au niveau cellulaire (glandes de Liberkuhn, cellules de Paneth, cellules à plateau strié), soit dans les voies lymphatiques ou les plaques de Peyer, etc...

S. B. ANDERSEN : I imagine that the intestinal catabolism of gammaglobulin occurs in the lumen of the gut under the influence of intestinal proteolytic enzymes. After passage through the capillary wall the protein may pass between the epithelial cells of the mucosa. This has not been shown in normal subjects. But in patients suffering from protein losing gastroenteropathy electron microscopy has shown spaces between the epithelial cells suggesting that something is passing. If the same took place — on a smaller scale — in normal subjects we had an explanation of the route of transfer of gammaglobulin from the blood stream to the lumen of the gut.

P. DYKES (*Birmingham*) : Elevation of the fractional catabolic rate on the second and third days after injection might well be explained by catabolism not from a space in immediate juxtaposition with the plasma, but from a space which equilibrates with a transfer half time of about 24 hours. In kinetic studies such an early compartment exists and probably represents the liver and viscera.

S. B. ANDERSEN : I have never calculated the variations in fractional catabolic rate under the assumption that the gammaglobulin is catabolized in an extravascular compartment with a transfer half time of 24 hours. This would correspond to a transfer rate of about 0.7 day^{-1}, a value which is not negligible compared to a fractional catabolic rate of almost 0.1 day^{-1}. This means that the specific activity of the protein in this hypothetical compartment would definitely be different from

the specific activity of the protein in plasma. If I interpret my results correctly the catabolism of gammaglobulin should take place in a place in so rapid exchange with plasma that the specific activities are identical. Therefore I believe that the catabolism of gammaglobulin takes place in a much more rapidly mixing compartment than the one suggested by you.

THE USE OF ^{131}I-FIBRINOGEN FOR *IN VIVO* STUDIES

E. REGOECZI

National Institute for Medical Research,
Mill Hill, London, N. W. 7. Great Britain

ABSTRACT

Iodinated fibrinogen can be very useful in various *in vivo* fields of work, such as in measuring turnover rates in health and disease, in experimental coagulation research (to test the effect of various substances on the coagulability of the blood), and in diagnosis (for instance of malignant tumours).

In the present communication results are reviewed concerning the metabolism and distribution of fibrinogen in humans, rabbits and dogs. Various considerations are discussed which suggest that masses of fibrinogen catabolized daily per unit body wt. are proportional to fibrinogen concentration. Some possible mechanisms for this are discussed.

Several technical criteria for obtaining reliable results with iodine-labelled fibrinogen *in vivo* are outlined. The purification of the protein should be carried out quickly and by gentle means and it should be subsequently iodinated at very low iodine substitution values. An upper limit for substitution using the iodine monochloride method is set at a mean of 0.5 atoms iodine per molecule of fibrinogen. The significance of the body iodide parameters in interpreting *in vivo* results are also pointed out.

Of the various labelled plasma proteins which have been introduced in recent years into biological research, fibrinogen appears to be a particularly interesting one because of its specific rôle in blood coagulation.

Although ^{127}I-fibrinogen was produced and its physicochemical properties were investigated by Laki and Steiner [1] as early as 1952, several years elapsed before the first attempts were made to carry out *in vivo* studies with this protein labelled with radioactive iodine. Some of the pioneer work [2] seems nowadays to be of more or less historical value only, and the beginning of fundamental developments dates back not further than 1963, i.e. when McFarlane [3] described the criteria under which the iodine label can be introduced into fibrinogen without seriously altering its *in vivo* behaviour. The work done in this field is still relatively small compared with that done on other proteins (e.g. albumin or γ-globulin) and available results are difficult to interpret because of the variety of techniques used. In the general picture of labelled fibrinogen, which is presented below, emphasis has been given to basic technical considerations and numerous matters which still require clarification are discussed.

FIBRINOGEN TURNOVER IN NORMAL HUMANS.

Relevant data obtained by different workers using ^{131}I-fibrinogen are summarized in Table I.

TABLE I. — ^{131}I-Fibrinogen Metabolism in Normal Humans

Reference	No. of cases	Source of Fibrinogen	Iodine : protein ratios	Biol. half-life (days)	Intravascular fraction %	Fractional catabolic rate	Absolute catabolic rate
	A	B	C	D	E	F	G
Christensen [1] (1958)	8	commercial (Cohn Fr. I ?)	?	4.3 (4.0-4.7)	50	32	?
Adelson *et al.* [5]	3	labelled whole plasma	?	2.3 (1.5-3.0)	?	?	?
McFarlane *et al.* [6] (1964)	10	$(NH_4)_2SO_4$ ppt^e.	0.5	3.1 (2.1-3.8)	75 (60-90)	31 (25-37)	46 (31-63)
Amris and Amris [7] (1964)	3	commercial ("Kabi")	0.3-1.0	4.2 (4.2-4.3)	82 (75-90)	21 (19-23)	33 (17-62)
Zetterqvist *et al.* [8] (1964)	?	Bombäck's	1.5	4.4^f, 4.7^m (3.8-6.1)	?	?	?

C = Iodine atoms incorporated per fibrinogen molecule.
D = Average biological half-lives measured over single exponential sections of intravascular activity curves and the figures in brackets show the range of variation. Indices *f* and *m* in Zetterqvist's data denote mean values for the sexes.
E = Intravascular fibrinogen pool is expressed as fraction of total body fibrinogen.
F = Per cent intravascular pool broken down per day.
G = mg fibrinogen catabolised per day per kg body weight.

There is clearly a wide scatter in the results and in some cases important relevant information is not available. Until more results are forthcoming one should not be too discouraged even where, as in the data by Adelson and co-workers, biological half-lives were only one half of the values given by Zetterqvist *et al.* However, discrepancies of this order inevitably pose the question whether the different methods of preparing and iodinating fibrinogen for metabolic purposes are fully comparable and this is considered further below.

From McFarlane's and Amris' data in Column G of Table I it follows that normal subjects catabolize on an average 43 mg fibrinogen/day/kg body wt. This is based on altogether 13 observations, and because of the fluctuating levels of plasma fibrinogen it is important that the number of these investigations should be enlarged in order to facilitate statistical analysis of the physiological range of fibrinogen catabolism. A typical experiment from McFarlane's collection is shown in Figure 1.

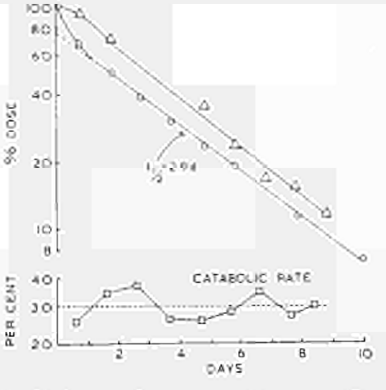

FIGURE 1. — Catabolism of ^{131}I-fibrinogen in a normal human. Catabolic rates (lower diagram) are urine radioactivities expressed as percentage of the mean protein-bound radioactivity in the plasma (○) for each collection period. Total body radioactivities (△) were calculated from the cumulative excretion of the dose.

FIBRINOGEN METABOLISM IN LABORATORY ANIMALS.

Two sets of experimental data each from normal dogs and rabbits are shown in Table II. They reflect satisfactory reproducibility within a species, and also a similar general pattern of fibrinogen turnover in the two species. Unfortunately, values for absolute catabolic rates in the dog experiments were not calculated. Since, however, the concentration of plasma fibrinogen in the dog [12] is similar to that in rabbits, it seems justified to assume that absolute catabolic rates in dogs and rabbits are essentially the same. Moreover, a comparison of corresponding values in Tables I and II suggests that, on a basis of body weight, the absolute amount of fibrinogen catabolized per kg of human is very similar in magnitude to that in adult rabbits and possibly in the dog as well. Incidentally, most of the data underlying the above deduction were obtained using salt-precipitated [$(NH_4)_2SO_4$] fibrinogen which appears to give more reproducible results.

TABLE II. — ^{131}I-Fibrinogen Turnover in Dogs (D) and Rabbits (R)

Reference	Species	No. of cases	Biol. half-life (days)	Intravascular fraction (%)	Fractional catabolic rate	Absolute catabolic rate
		A	D	E	F	G
Lewis *et al.* [9] (1961)	D	20	2.4 (1.8-3.1)	66	42[a]	?
Adelson *et al.* [5] (1961)	D	4	2.3 (1.7-3.0)	75[a]	40[a]	?
Regoeczi *et al.* [10] (1964)	R	25 (5*y*+20*o*)	2.7 (2.1-3.5)	79 (65-85)	34 (25-43)	41 (18-63)
Atencio and Reeve [11] (1965)	R	14 (7*y*+7*o*)	2.3*y*, 2.4*o* (2.0-2.7)	57*y*, 76*o* (48-85)	44*y* 37*o* (28-53)	50*y*, 38*o* (31-73)

For (D), (E), (F), and (G) see Table I. Indices *y* and *o* refer to values obtained in young and older animals respectively.

[a] These approximate figures are not contained in the original papers but were calculated from graphs or data given in their publications.

The half-life of ^{131}I-labelled fibrinogen in rats has been reported to be 1.2 days [13].

KINETIC CONSIDERATIONS.

Christensen [4] observed that whereas the prevailing plasma fibrinogen concentration in several healthy humans varied greatly nevertheless the biological half-life of ^{131}I-fibrinogen was remarkably constant. In view of a possible significance which this phenomenon might have in relation to the mechanism of fibrinogen metabolism, Regoeczi *et al.* [14] compared the behaviour of biologically screened [15] labelled fibrinogen in the same animals before and after artificially increasing the pool size by means of bacterial endotoxin injections. They found that whereas ^{131}I-specific activities of circulating fibrinogen fell sharply after the injection with increasing pool sizes yet the slopes of the protein-bound radioactivity per unit volume of plasma either remained unchanged or became only slightly accelerated. In addition it is a matter of common experience with fibrinogen that those considerable fluctuations of the pool size which occur under "physiological" conditions (possibly accentuated by minor injuries and excitement due to blood sampling and hand-

ling) do not coincide with any substantial changes in the radioactivity curves. Thus plots of specific activities on the one hand and protein-bound activities per ml of plasma on the other can be divergent in the case of fibrinogen in a way which is never observed with iodinated albumin.

These observations point to a considerable constancy in the fractional catabolic rate of fibrinogen, and this appears to hold over a wide range irrespective of the size of the fibrinogen pool. The available data indicate, therefore, that fibrinogen catabolism is a first-order kinetic process [9, 10], although the underlying mechanism by which this is achieved is not at all clear.

Relationships between concentration and catabolism of various plasma proteins have been established by numerous investigators and Freeman [16] has reviewed their data in addition to his own. He concludes that fractional catabolic rates can be related either directly or inversely to protein concentrations, e.g. albumin and γ_G-globulin are directly related and haptoglobin and transferrin inversely. Fibrinogen with its fractional catabolic rate showing no significant dependence on protein concentration does not fit into either category, and it must be assumed to have a different normal catabolic pathway.

Interstitial fibrinogen.

The presence of fibrinogen in the extravascular space has been known for some time [17], but until recently quantitative assessments were subject to controversy. As discussed further below, partial denaturation of the fibrinogen molecule in the course of purifying the protein or iodinating it greatly reduces the reliability of the labelled fibrinogen as an indicator of the distribution of the native protein [3]. Improved labelled proteins is the most likely explanation for the remarkable apparent shrinkage of extravascular fibrinogen pool values from 50-85 % of the total body fibrinogen a few years ago to 15-35 % in the most recent literature.

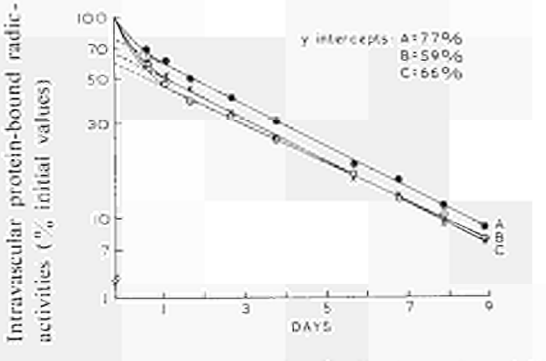

FIGURE 2. — Variations in the intra/extravascular distribution ratios of fibrinogen in three rabbits (2.79-3.04 kg body wt.) as indicated by the extrapolation value of the ^{131}I-fibrinogen curves. Each animal was injected with an aliquot of the same batch of labelled material.

Using ^{131}I-fibrinogen which was prepared under certain conditions (Cf. below) most of the intravascular radioactivity curves in adult humans and rabbits extrapolate at zero time to 70-80 % of the administered dose, but deviations in both directions are not uncommon (Cf. Figure 2). Results obtained by Atencio and Reeve [11] in rabbits of two different age groups suggest that the ratio of intra- to extravascular fibrinogen increases with the age of the animal (Cf. Table II).

Intravenously injected labelled fibrinogen equilibrates with the extravascular compartment in a noticeably shorter time than does albumin. The single exponential slope of intravascular fibrinogen is reached in humans [6, 18] within 2 days and in dogs [5, 9] and rabbits [10, 11] in 24-36 hours, in spite of the fact that considerably more albumin than fibrinogen is being transferred across the capillary wall. (The ratio of the two fluxes in the rabbit is about 2:1 calculated as fractions of the i.v. pool and 27:1 in terms of protein masses.) Calculations also showed that the mean transit time of 99 % of the fibrinogen molecules within the extravascular compartment is only half that of albumin [11, 19].

These results show that the fluid extravascular space which is accessible to fibrinogen is considerably smaller than to other plasma proteins, but is approximately of the same order as that presented to other non-protein macromolecules [20, 21]. Many other observations, for example the distribution of fibrinogen in tissues as revealed by means of fluorescent antibodies [17], and analyses of lymph from different regions [22], all support this conclusion. Furthermore, it is possible — and indeed some unpublished results by McFarlane strongly uphold this view — that the interstitial distribution of this protein is not uniform, muscle lymph in particular having little or no fibrinogen. Clearly a comprehensive and quantitative histo-anatomical description of the interstitial fibrinogen pool is still required.

Theories of the normal catabolism of fibrinogen.

Fibrin deposits are known to play an important rôle in the pathology of atherosclerosis [23], and in view of the rapid turnover of coagulation factors it has also been suggested that fibrin would be essential for the maintenance of the integrity of the vascular endothelium [24]. This interesting hypothesis was given a dynamic aspect in 1956 by Astrup [25] who claimed that continuous formation and lysis of fibrin films proceeds on the surface of vessels under physiological conditions. Since then a number of experiments which were designed to identify a possible mechanism for this in fact gave results which are not wholly in favour of its existence. The turnover of ^{131}I-fibrinogen is not affected by heparinisation [9] nor by experimental hypoprothrombinæmia produced by coumarin derivates [3, 5, 9]. Platelet survival is greatly altered in hypo- and hypercoagulable states, but without reference to the behaviour of fibrinogen [5]. In patients with haemophilia A [8, 26] (classical haemophilia, Factor VIII deficiency), or hypoproconvertinæmia [8] (Factor VII deficiency) or von Willebrand's disease [8] the fractional catabolic rate was essentially the same as in subjects with normal coagulation of blood. Similarly, antifibrinolytic treatment also does not seem to delay the *in vivo* breakdown of labelled fibri-

nogen [13, 27-29]. For these reasons, the widely shared view today is that even if some fibrinogen is steadily converted into fibrin in healthy subjects, the proportion which this represents of the total catabolized fibrinogen is insignificant.

In the author's opinion, the normal degradation of fibrinogen is most likely to take place during transcapillary transport of fibrinogen, so that breakdown occurs either entirely within the vascular endothelium or is at least initiated there. This concept emerges from a simple consideration, namely that fibrinogen while moving through the endothelial cells must face a unique exposure to enzymic action, partly because of the pronounced tendency of plasminogen to complex with fibrinogen [30] and partly because the activator of plasminogen is localized to the vascular endothelium [31-34]. Thus, the fibrinogen molecule as it leaves the capillary system can be visualized in a greatly simplified form as a substrate-proenzyme complex passing through a barrier of the activator. Unlike the situation in the kidney where electronmicroscopic evidence supports the existence of pores in the glomerular capillaries it is believed that in other organs transmitted substances must pass through the endothelial cytoplasm (cytopempsis) [35]. It is conceivable that under these favourable conditions, all three substances being brought together within the same cell, plasminogen activation with subsequent destruction of the "attached" fibrinogen occurs, and is responsible for the phenomenon of "catabolism".

This theory would provide an acceptable explanation for the first order kinetic nature of fibrinogen catabolism (Cf. above), but it could not explain the apparent ineffectiveness of epsilon aminocaproic acid on normal fibrinogen turnover.

Technical considerations

Fibrinogen in our experience is more susceptible to denaturation changes during iodination than is albumin and in this connection it would be helpful to know of the experiences of others. Some points which are regarded as important in our laboratory will be discussed briefly below.

Preparation and iodination of fibrinogen.

A fibrinogen preparation for metabolic purposes should be at least 90 % clottable in terms of protein-bound radioactivity. The use of partially purified materials, for example with clottabilities ranging from 50 to 70 %, has repeatedly been described and the disadvantages of this were thought to have been overcome by measuring only clot radioactivities in the samples. However, neither total body nor urinary activities can have any unequivocal meaning in such an experiment in which a contaminated similarly labelled protein, usually γ-globulin, is present. Moreover, unavoidable occlusion of other (radioactive) proteins [36, 37, 38] in the clot may give rise to additional errors.

Fibronogen better than 90 % pure can be prepared by a number of methods and the residual non-clottable materials may be classified as (*a*) non-specific con-

taminants, i.e. proteins not involved in coagulation, (*b*) specific contaminants, and (*c*) occasionally some denatured incoagulable fibrinogen. Since this large molecule is readily denatured, we prefer to prepare the protein immediately before iodination and to carry out the latter procedure as quickly as possible — for example avoiding prolonged dialysis. Contact with organic solvents should also be avoided but repeated precipitations using ammonium sulphate in decreasing (from 25 to 20 %) saturations [3] appears to cause no damage. Labelled fibrinogen prepared in this way and injected into rabbits showed no signs of denaturation and circulating protein-bound radioactivities were 94-98 % clottable with thrombin [39].

Fibrinogen prepared by almost any method contains plasminogen, and further investigations are required to decide whether this may have implications for turnover studies. The difficulties of obtaining a plasminogen-free fibrinogen which could be used for comparison are well known. Plasminogen can be removed by Mosesson's procedure [40] but judged by the low solubility of the final product the method appears to be somewhat drastic for subsequent *in vivo* purposes. The method proposed by Brakman [41] is milder but unfortunately is not completely satisfactory with human material.

Mosesson and Finlayson [42-44] have recently shown that purified fibrinogen can be subfractionated on DEAE-cellulose and this may prove to have metabolic implications. However, we feel — perhaps without a good experimental basis — that the use of fibrinogen prepared by adsorption procedures for subsequent iodination purposes is dangerous.

The crucial point in *iodinating* fibrinogen is that as shown by McFarlane [3] this protein cannot be associated with more than 0.5 atoms of iodine per molecule of fibrinogen without essentially altering its *in vivo* behaviour relatively to that of the biosynthetic carbon-labelled protein. Using ratios higher than this, certain fractions of the injected material are removed from the circulation by rapid initial catabolism, giving rise — as already mentioned — to overestimates of the size of the extravascular fibrinogen pool. The validity of this critical 0.5 level has fully been confirmed by other investigators [11, 19]. Preparations iodinated at even lower rates have been claimed by Atencio *et al.* [19] to behave *in vivo* indistinguishably from the 0.5-atom fibrinogen.

Iodination ratios must of course be visualized as mean values for the random distribution of the label in a population of protein molecules with a given number of reactive sites. Relevant calculations using Oncley's data [45] show that after iodinating at a mean value of 0.5 atoms, about 62 % of the protein is labelled with 1 atom, about 30 % with 2 and the remaining 8 % with 3 or 4 atoms of iodine. However, working at a mean value of 2.0 the proportion of the molecules with more than one atom becomes much higher, now only 13 % being tagged with 1, 28 % with 2, another 28 % with 3 and about 30 % with 4 to 7 atoms of iodine. Whereas when albumin is "multi-iodinated" within reasonable limits in this way, it still behaves normally *in vivo*, but fibrinogen is clearly "over-iodinated".

Finally, it seems worth mentioning that the recommanded upper iodination

ratio of 0.5 is in *sensu stricto* only 0.25, the fibrinogen molecule being most probably a dimer [46]. Since at a mean value of 0.25 atoms about 80 % of the labelling is with 1 atom iodine, 19 % with 2 and only 1 % with more than two, the biological conclusion seems to be that fibrinogen molecules having more than two atoms of iodine are recognizably different in the body from the native protein.

DETECTING DENATURED LABELLED MATERIAL.

Evidence for the presence of such material can be obtained by observing the behaviour of

a) intravascular non-precipitable radioactivities,

b) urinary excretion of the liberated label,

c) slope of the total body activity curves,

— especially by comparing the values measured over the first 48 hours with those obtained during the rest of the experiment [13, 47].

A low extrapolation value of the intravascular protein-bound radioactivity curve at zero time *alone* does not necessarily indicate a bad preparation because of the physiological variations in the pool ratios (Figure 2).

The question, whether the *in vivo* behaviour of an iodinated fibrinogen preparation can be predicted on the basis of comparative clottability measurements before and after iodination, has to be left open until evidence is obtained that losses in clottability and the extent of denaturation in biological sense are proportional.

To evaluate various methods of preparing fibrinogen for labelling, the preparations should be compared in the same animal at the same time using different labels. Figure 3 shows that minor alterations may otherwise remain undetectable.

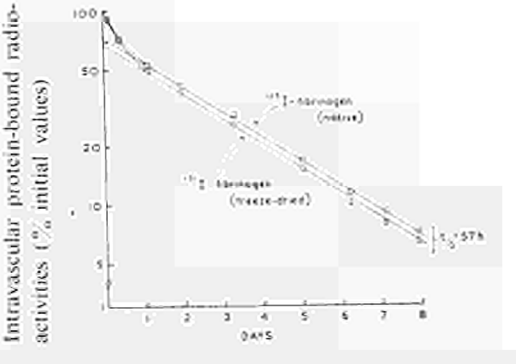

FIGURE 3. — The *in vivo* behaviour of labelled fibrinogen after freeze-drying. A preparation was divided into two and each labelled with a different iodine isotope and then one aliquot was freeze-dried. The curves obtained after injecting a mixture of both showed that about 6-7 % of the freezed-dried material has been removed by rapid initial catabolism. Thereafter the slopes were identical.

IODIDE PARAMETERS AND FIBRINOGEN TURNOVER.

For a given renal excretion rate of inactive iodide the amount of non-precipitable plasma radioactivity (measured conventionally as a fraction of the total radioactivity in the sample) is substantially higher during fibrinogen turnover than in studies with albumin, because of its faster catabolic rate. Therefore, e.g. "TCA-values" which in rabbits are usually below 1 % 24 hours after injecting albumin, are in the case of fibrinogen normally 1.5-2.5 %.

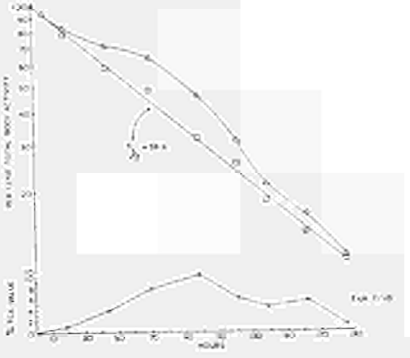

FIGURE 4. — Atypical ^{131}I-fibrinogen turnover : Fluctuating total body radioactivity in a rabbit (△) with intercurrent urinary infection (unsterile catheter). Non-precipitable radioactivities in the plasma (lower diagram) were also elevated and their absolute values multiplied by the ^{131}I-iodide space factor provided a means of obtaining a more satisfactory curve for the total body protein-bound radioactivity (□).

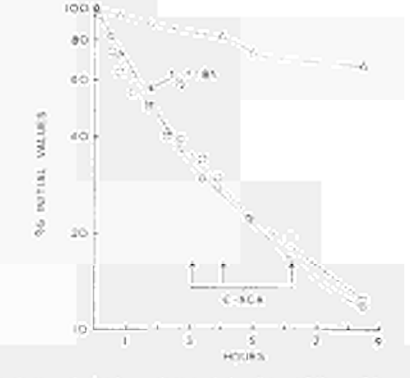

FIGURE 5. — Plasma protein-bound (○) and total body (△) radioactivities in a rabbit after i.v. injection of 2.5 mg ^{131}I-fibrin. This was separated from a spontaneously clotted labelled fibrinogen preparation and was solubilized in a minimal amount of urea for injection. The discrepancy between the disappearance rates of this material from the plasma and the body is obbious. The amounts of retained liberated label in the body were calculated as explained in Figure 4 and the values thus obtained (□) show that throughout the whole experiment practically all protein-bound radioactivity has been retained in the intravascular compartment. The arrows indicate injections of 1 g epsilon aminocaproic acid.

Indeed, situations not infrequently arise with fibrinogen in which rates of ^{131}I-liberation from the protein greatly exceed the rates of excretion, thus giving rise to underestimates of catabolic rates. Thus activity may accumulate because of a renal defect in iodide excretion or because of a greatly accelerated liberation, for example in any condition in which a significant portion of the labelled fibrinogen is converted to fibrin in the body (Figures 4 and 5).

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DISCUSSION

L. DONATO (*Pisa*) : After the work reported this morning by Dr. Rosa, it seems to me that we can safely state that at the same iodination degree quite different distributions of iodine within and between the molecules can take place. The validity of approximating this latter distribution on the basis of the Oncley's binomial equation is therefore questionable.

E. REGOECZI : McFarlane found by animal test using his ICl method that above a mean of 0.6 atom of iodine considerable proportions of denaturated fibrinogen were present. We have no means of ascertaining whether this arose from random or non-random distribution of the label. The experimental error in McFarlane's comparison between ^{131}I-and ^{14}C-labeled proteins also cannot be stated precisely but probably did not exceed $\pm$ 5 %.

I agree with Prof. Donato that Dr. Rosa's electrolytic method may in principle give more uniform distribution of the iodine and if it does then it may be possible to agree on a higher permissible mean level of iodine substitution. However, it seems to me that this must be demonstrated by *in vivo* methods.

USE OF LABELED PROTEINS FOR STUDYING THE REGULATORY PROCESSES OF PLASMA PROTEIN METABOLISM (*)

M. A. ROTHSCHILD, M. ORATZ and S. S. SCHREIBER

Radioisotope Service, V. A. Hospital
the Department of Medicine, New York University School of Medicine,
and the Department of Biochemistry, New York University College of Dentistry,
New York, N. Y., U.S.A.

ABSTRACT

The metabolism of albumin and γ-globulin has been studied using proteins labeled with ^{131}I. The balance between serum protein degradation and synthesis has been altered in an attempt to determine the reaction of each to various stimuli. Albumin degradation is increased following the administration of exogenous adrenocortical and thyroid hormones, and in the presence of an elevated albumin pool following albumin infusions. Albumin degradation is depressed when the serum albumin level and/or pool size are low. In contrast albumin synthesis is not sensitive to low levels of serum albumin *per se* but is capable of significant increments upon the administration of adrenocortical and thyroid hormones. Albumin synthesis is depressed in the presence of increased colloids other than albumin.

Gammaglobulin degradation is elevated when the pool size is increased during hyperimmunization but is unaltered during cortisone administration. Gammaglobulin synthesis is not stimulated by cortisone but is capable of marked increments following an appropriate antigenic stimulation.

Serum protein degradation appears to be related to the concentration of the protein, or to the pool size or both. *In vitro* incubation and perfusion studies indicated that while many tissues possess the potential for degradation of the serum proteins the accessibility to a degradative site may play a limiting rôle.

The synthetic mechanism is not related to pool size or concentration nor are degradation and synthesis interdependent. A colloid osmotic mechanism affecting control by regulating albumin synthesis is postulated.

Significant advances have been made in the field of protein metabolism through the use of labeled proteins to study endogenous protein turnover. This discussion shall be limited primarily to the use of the iodine label for this technique has a unique advantage based upon the fact that in the absence of thyroidal accumulation of iodide released from degraded protein there is quantitative excretion of these products of degradation in the urine [1,3]. Before reviewing some of the experimental data a closer view of the method is important.

The labeled protein must be metabolically indistinguishable from native protein [4] (Figure 1). Heavy iodination [5, 6], exposure to excessive radiation [7], and

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1. Labeled protein indistinguishable from native protein.
2. Label not reutilized.
3. "Iodide" release reflects degradation.
4. Excretion of "iodide" very rapid in comparison to degradation.
5. Protein distribution between intravascular and extravascular spaces rapid in comparison to degradation.
6. Excretion of "iodide" parallels intravascular distribution of labeled protein.

FIGURE 1. — Important factors in the use of ^{131}I labeled proteins

significant denaturation during separation must be avoided [8]. The label, once removed must not be reutilized or reincorporated and be quantitatively excreted [6]. As pointed out by Hughes [6], there is no evidence for exchange between cold ^{127}I-labeled protein and ^{131}I-iodide and it has been clearly shown that the turnover of ^{131}I-labeled albumin and globulin parallels the metabolism of ^{14}C endogenously labeled proteins [1]. Furthermore these studies add support to the concept that simple deiodination does not occur and *in vitro* studies have repeatedly failed to

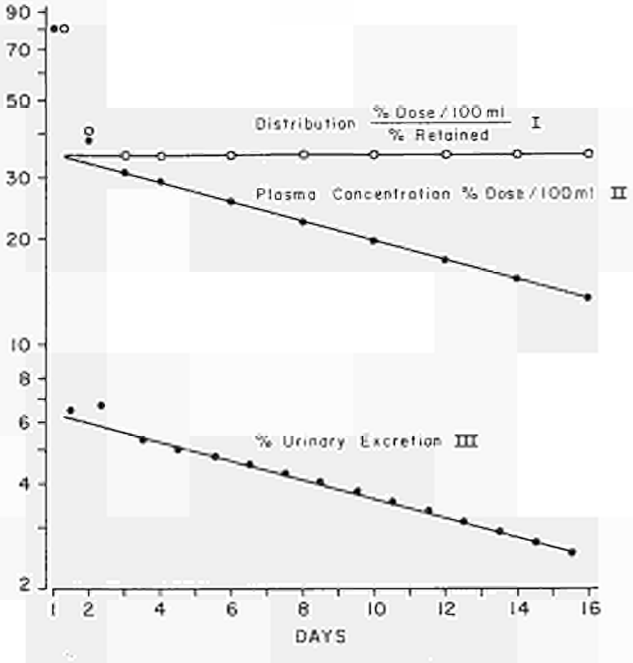

FIGURE 2. — ^{131}I-albumin metabolism
Sample set of curves for a typical rabbit experiment. Curve I. Distribution curve. Curve II. Plasma concentration. Curve III. Urinary excretion (See text) [5].

reveal any significant release of non precipitable material when incubated with tissue homogenates and thus while degradation did not occur neither did simple deiodination [9]. In other studies Beeken employing subcellular fractions has demonstrated equivalent rates of release of tyrosine or peptides and non precipitable ^{131}I [10].

So far we have mentioned only basic properties of the label. Now there are three main physiological principles which are also vital to the proper interpretation of turnover data employing the iodine tag. Berson, Yalow, Reeve, Lewellin, McFarlane, Matthews, Campbell and Freeman [11,19] among others have described detailed mathematical approaches for the evaluation of such data and these factors have been stressed. First the excretion of the products of degradation are rapid in comparison to degradation so that the latter becomes the rate limiting factor [2, 4, 5]. Second—distribution between intravascular and extravascular areas is also extremely rapid in comparison to degradation — the intravascular pool equilibrating with the extravascular pools at rates with half times of a few hours to a day whereas degradation is normally described with a half time of many days [2-4, 5-7, 11-19]. Third, the excretion of the label parallels the concentration in the plasma, and not that in extracellular sites [11].

A sample set of curves that would be obtained in an ideal metabolic study in a rabbit are shown in Figure 2 [5]. Curve II is the raw plasma data plotted as % dose/100 ml and Curve III represents the daily urinary excretion. If the plasma curve is corrected for the amount of tracer excreted per day then Curve I is derived. After a few days all change in this curve can be accounted for by excretion indicating that loss from the plasma due to continued expansion of the ^{131}I-albumin into a larger volume has been completed and thus if this curve remains horizontal then distribution equilibrium has been achieved. Employing this iodine label there are three individual methods for the calculation of protein degradation. One is to use the raw plasma data deriving the rate of metabolism from the slope of a plasma decay curve [3]. The second is to rely on the rate of urinary excretion of breakdown products [11], and the third is to relate, in some fashion, the daily urinary excretion to the mean plasma concentration for that day [13, 20]. This may be called the clearance procedure (Figure 3). The renal clearance of plasma iodide is determined from the quotient of the urinary excretion on any day and the mean plasma concentration for that day. The product of this value and the mean serum protein concentration results in a value for protein degradation. Such a procedure is independent

1. Renal clearance ml/day	=	$\frac{\text{Urinary excretion } \mu c/\text{day}}{\text{Plasma concentration } \mu c/\text{ml}}$
2. Metabolic clearance g/day	=	Renal clearance (ml/day) × Plasma albumin (g/ml)

FIGURE 3. — Methods for determining metabolic clearance [30]

of pool size measurements [17] and thus valid in non-steady states. Many modifications of the basic method of calculation have been proposed. Suffice it to say that in steady state conditions all methods agree and in the non-steady state all methods that relate the daily plasma activity to the urinary excretion also result in values for the amount of protein metabolized which are in good agreement with each other. In terms of measuring synthesis, a convenient procedure is to remeasure the protein pool size by means of a 2nd injection (Figure 4). Following the initial injection and an appropriate control period, experimental procedures are instituted

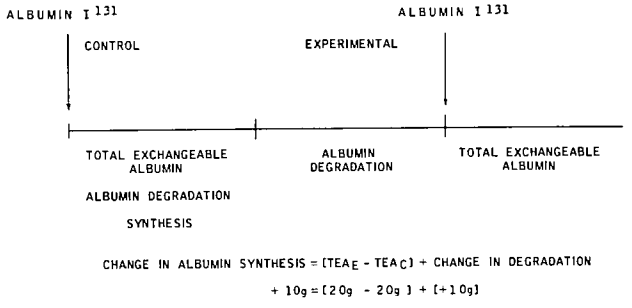

FIGURE 4. — Reinjection technique for the measurement of protein synthesis following a control and an experimental period

and degradation determined daily by means of a clearance method. A 2nd injection is then made and the pool size re-evaluated. By comparing changes in the pool size to the changes in degradation it is possible to determine if synthesis has kept pace with, lagged behind, or remained unaltered during the changes in degradation.

In regulating protein metabolism there must be a balance between protein production and degradation. In reviewing some of the factors affecting these various parameters I shall dwell mainly with serum albumin and γ-globulin.

While decreases in the serum albumin level are frequently seen in various disease states [21], hyperalbuminemia is not seen except perhaps in acute dehydration. Furthermore, while plasmapheresis experiments had shown that the loss of protein could be remade [22], such changes could have been mediated via alterations in degradation and not an increase in synthesis. Studies in patients with proteinuria seemed to support this concept [23]. The urinary excretion of radioactivity was divided between ^{131}I bound to and excreted with albumin and ^{131}I which was non precipitable and derived from degraded protein. Between one quarter and one-half of the excreted ^{131}I was still precipitable and thus albumin degradation was decreased. Since these subjects were in steady state conditions with regard to albumin metabolism, albumin synthesis was equal to the sum of the amount of albumin lost and degraded and albumin synthesis was not increased. It is interesting to speculate that the decrease in degradation may be an attempt to compensate for these renal losses for an increase in albumin synthesis would result in simply a greater urinary loss of protein.

In order to determine if albumin production was capable of increasing it was decided to use excess thyroid as a stimulus. The patients were injected with ^{131}I-albumin and control values for albumin distribution and degradation obtained [24]. During the control period serum albumin levels remained constant and there were no changes in albumin distribution. At this point desiccated thyroid in doses of 10-16 grains per day were administered and observations continued. As the thyroid was administered the amount of radioactivity excreted in the urine increased and the slope of the plasma decay curve increased. Calculations revealed that the amount of albumin degraded increased in every study. A 2nd dose of ^{131}I-albumin was injected and albumin distribution remeasured. In this group of patients albumin degradation increased by 30 % and 80 grams of albumin were degraded in excess of the control period. The total exchangeable albumin pool had fallen only slightly indicating that albumin synthesis must have increased to prevent the loss of exchangeable albumin in the face of the absolute increase in degradation (Figure 5).

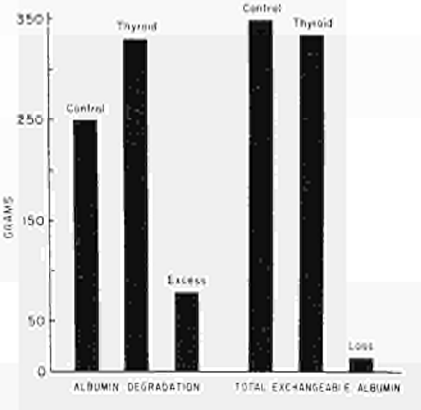

FIGURE 5. — Albumin metabolism and thyroid administration.
During the administration of thyroid 80 grams of albumin were degraded in excess of an equivalent control period. There was a loss of exchangeable albumin of 15 grams.

In another group of patients and in rabbits the effects of cortisone acetate were studied in order to determine if albumin synthesis would be altered in association with the expected increase in albumin degradation. In both groups studied there was a rapid increase in the amount of albumin degraded averaging 58 % in the rabbits and 30 % in the patients. However, albumin synthesis also rose and in response to this appropriate stimulus, albumin synthesis was capable of keeping pace with degradation and no loss of albumin occurred [25-27]. Cortisone was catabolic in terms of albumin but was not as had been felt antianabolic.

The metabolism of γ-globulin, however, is not affected in the same way by cortisone. Cortisone acetate 4 mg/kg/day was administered to 4 rabbits over a two week period and the studies conducted as described above (Figure 6). Following cortisone the γ-globulin pool fell 23 %. γ-globulin degradation was unaltered and

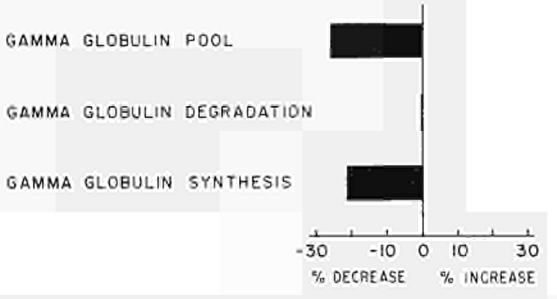

FIGURE 6. — Cortisone and γ-globulin metabolism.
The effects of cortisone acetate 4 mg/kg/day on the metabolism of γ-globulin.

γ-globulin synthesis fell 22 % from 259 mg/day to 201 mg/day [8]. While these changes are not marked, they clearly show that the fractional rate of γ-globulin degradation is increased but synthesis is not stimulated by cortisone as is the production of albumin. The administration of thyroid, however, has been reported to increase γ-globulin turnover [29, 30]. Thus thyroid hormone is capable of producing perhaps a rather non specific increase in protein turnover while the reaction of these same proteins to the adrenocortical hormones is very specific. The direction of change of the protein pool in the presence of these stimuli is dependent on the predominant effect of these hormones on degradation or synthesis.

Now that it was clear that both the synthetic and degradative systems were capable of being accelerated how could they be inhibited? It had long been observed that in many disease states there appeared to be a depression of the serum albumin level whenever the globulin fraction of the blood increased [31-36]. This possible dependent relationship suggested to Bjørneboe in 1945 that colloid content might somehow play a rôle in protein metabolism perhaps by altering protein distribution. A colloid osmotic regulatory system responsible for these reciprocal changes was suggested [35, 36]. In order to study this question rabbits were injected with ^{131}I-albumin and following control observations, high molecular weight dextran was infused [36]. After a new equilibrium state had been achieved, ^{131}I-albumin was re-injected and the pool size remeasured (Figure 7). Albumin degradation fell 22 % and the exchangeable albumin pool decreased 16 % indicating that at some point albumin synthesis must have fallen below albumin degradation. In the presence of another colloid, albumin levels fell and these low albumin levels *per se* did not result in an increment in albumin production.

This study was extended employing rabbits hyperimmunized with a polyvalent pneumococcal antigen according to the method of Bjørneboe [31]. During the rapid development of hypergammaglobulinemia the exact same sequence of events occurred as during the dextran infusions. Albumin levels fell as the γ-globulin concentration rose and a decrease in degradation occurred which was most marked when the albumin pool size had fallen to low levels. Finally a new steady state was achieved at which time the exchangeable albumin pool had fallen and albumin

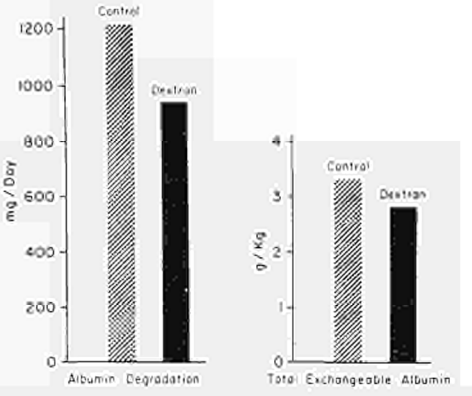

FIGURE 7. — Dextran and albumin metabolism.

The effect of dextran, average molecular wt. 188,000 in doses of 1.5 g/day on albumin metabolism. The exchangeable albumin pool decreased in the face of a fall in albumin degradation indicating that albumin synthesis must have decreased even further.

synthesis and degradation were at new but lower levels. Total protein concentration increased 52 %, albumin levels fell 38 %, exchangeable albumin decreased 30 % due mainly to a 23 % decrease in albumin synthesis from a mean of 273 mg/kg/day to 217 mg/kg/day (Figure 8) [37].

While this depression of albumin production was taking place, gamma globulin synthesis was markedly accelerated [33, 39] (Figure 9). Gamma globulin degradation rose rapidly as the γ-globulin levels rose and exceeded the control value by 450 % to a level of 330 mg/kg/day. A 2nd injection of ^{131}I-γ-globulin indicated a pool size nearly 6 times the initial value while γ-globulin synthesis increased 700 %.

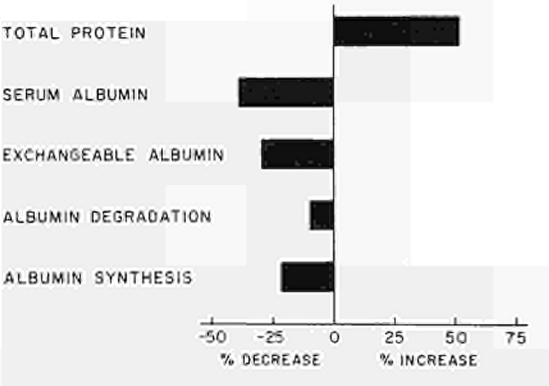

FIGURE 8. — Hyperimmunization and albumin metabolism.

The effects of rapid hyperimmunization on albumin metabolism. The total protein concentration rose 52 % while there was a decrease in the serum albumin level and exchangeable albumin pool due mainly to a decrease in albumin synthesis.

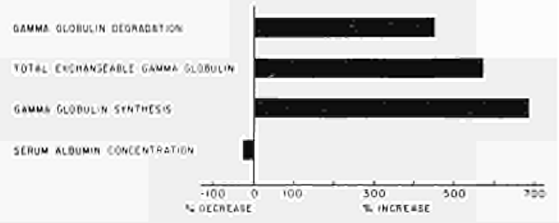

FIGURE 9. — Hyperimmunization and γ-globulin metabolism.
The effects of rapid hyperimmunization on γ-globulin metabolism. While the serum albumin level decreased, there were marked increases in the γ-globulin levels, pool size and synthesis.

Serum albumin levels decreased 27 % or about 1 g/100 ml. At peak production γ-globulin synthesis required about 1/3 of the average daily nitrogen intake; and, as been suggested in other diseases when the globulin levels are markedly elevated, it seemed possible that the rapid production of one protein might competitively interfere with the synthesis of others [33]. In order to investigate this possibility, two groups of studies were performed. The first involved the infusion of pooled γ-globulin into rabbits. The infusion of 7-10 g of γ-globulin over a 10-16 day period resulted in a depression of the albumin pool from 13.5-8.4 g due to a decrease in albumin synthesis from 1070 mg/day to 744 mg/day [40]. These studies confirmed the results during hyperimmunization and during dextran administration. During the infusion of γ-globulin there was no excess demand on the body's own amino acid pool. The second study was undertaken to see if the rapid hyperimmunization would in any way effect the production or destruction of a protein not involved in maintenance of colloid osmotic pressure. Hemoglobin seemed to fit this criterion and in 7 rabbits values for red cell mass, total circulating hemoglobin and red blood cell survival were obtained using ^{51}Cr labelled red cells. Values for serum protein, serum albumin and hemoglobin concentrations were obtained before, during and after the rapid hyperimmunization. Total protein rose 25 % associated with a 22 % fall in circulating albumin levels from 3.2 to 2.5 g/100 ml. There were no significant changes in red cell mass or hemoglobin and the red cell survival time of about 2 weeks was not altered. These studies indicate that during rapid hyperimmunization the production of hemoglobin is not affected and excessive colloid accumulation appears to be prevented by a specific decrease in albumin production [41], and probably not secondary to a nitrogen lack.

So far we have dealt mainly with some mechanisms controlling protein synthesis. Now for a moment let us turn to degradation. Albumin degradation is low in conditions where the serum albumin level is depressed [35, 36, 40, 47, 48]. Actually albumin degradation has been observed not to fall until the serum albumin levels had started to decrease during hyperimmunization [37], and at the end of the study the values for albumin degradation and synthesis agreed indicating equilibrium at a new steady state [37]. Also an absolute decrease in degradation has been seen in some patients with proteinuria and hypoalbuminemia [22]. It should be pointed

out that alterations in degradation may be missed if only the fractional rate of turnover is used [36, 37]. In hyperimmunized rabbits and following dextran infusions the fractional rate of loss of ^{131}I-albumin from the plasma is unaltered yet there are marked changes in the amount of albumin degraded per day. On the other hand elevations in the serum proteins are associated with changes in degradative rates; both the absolute amount degraded and the fractional rate. Bjørneboe, Andersen and others have demonstrated this point [38], and in rabbits as the γ-globulin levels rose, γ-globulin degradation increased from 8 to 12 % per day [39]. Fahey has shown that the fractional rate of degradation may increase until the γ-globulin levels reached 2-3 g/100 ml after that the fractional rate remained relatively stable—only the absolute amount of γ-globulin degraded increasing [49]. During albumin infusions the fractional turnover rate of ^{131}I-albumin increased 22 % [50].

While these data suggest that the plasma protein degradation is probably related to serum levels or pool size or both there are no conclusive data on the site of plasma protein degradation. It is well appreciated that the site of plasma protein degradation is probably in close approximation to the plasma [1, 11, 51] and not in distant extra plasma sites but the specific area is still a mystery. Tissue homogenates fail to release non precipitable iodide after incubation with labeled gammaglobulin or albumin. However, perfusion of the kidney and spleen with ^{131}I-labeled albumin and globulin demonstrated an increase in non-precipitable ^{131}I. During perfusion of the dog kidney with ^{131}I-albumin, non-precipitable activity increased at a rate of about 100 % per hour, but no increase occurred when ^{131}I-gammaglobulin was used. In splenic perfusions the rate of release of ^{131}I was 50 % per hour for ^{131}I-albumin and 25 % per hour when gammaglobulin was used as the substrate [52]. While the reasons for these differences are not clear, it is tempting to speculate that many tissues possess the capacity to degrade protein and the limiting factor is the accessibility of the degrading site for this protein.

Finally, let us turn to protein distribution. While most serum proteins rapidly achieve equilibrium between intravascular and extravascular sites with specific rates of equilibration, different tissues maintain different concentrations in their interstitial fluid. For example, the skin contains very large amounts of exchangeable protein in its interstitial fluid whereas the liver has only very limited amounts of protein so located [52, 53]. In terms of albumin it is interesting to return to the observation that albumin production is not stimulated by low plasma albumin levels *ver se* for it may be that if a colloid osmotic regulatory system does exist such a mechanism may be located in an extravascular site. In exploring this possibility hepatic albumin distribution was measured in control rabbits and in rabbits receiving 1.5 g dextran daily where the synthesis of albumin was depressed. ^{14}C-sucrose was used to measure extracellular fluid and ^{131}I-albumin to measure the plasma volume and the ^{14}C and ^{131}I activity per gram of liver compared to that contained in 1 ml of plasma. In addition, studies were performed in these two groups of rabbits employing ^{131}I-albumin to measure both albumin metabolism and equilibrium albumin distribution. Six to 10 min prior to sacrifice the rabbits were given ^{125}I-albu-

min, the livers removed and again the ^{131}I and ^{125}I per gram of tissue compared to that contained in one ml of plasma. In these groups of rabbits albumin metabolism was measured before and after dextran while the hepatic spaces were determined in separate sets of rabbits. The ^{131}I-albumin served to measure hepatic exchangeable albumin while ^{125}I-albumin, the hepatic plasma albumin, the difference being the albumin located in the interstitial space. While albumin degradation exchangeable albumin and albumin levels fell, the amount of albumin located in the hepatic intersitial space increased some 93 % from 27-52 mg/100 g wet liver (Figure 10). There was no significant change in hepatic interstitial volume. These results are in accord with the concept that extravascular protein distribution may play a rôle in regulating protein synthesis.

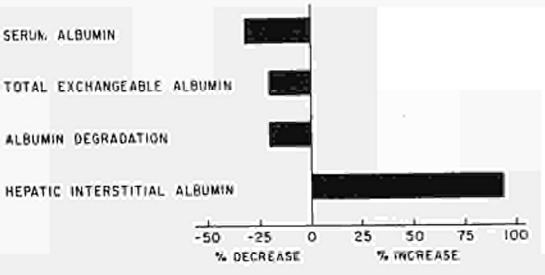

FIGURE 10. — Albumin metabolism and hepatic albumin distribution during dextran administration. While the serum albumin level, exchangeable albumin pool and albumin degradation decreased, hepatic interstitial albumin increased from 27 to 52 mg/100 g wet liver weight.

The experiments that I have briefly outlined above have given us some insight into the forces and counter forces influencing protein metabolism. Degradation seems to be related to concentration, can be stimulated by hormonal action and the site of degradation may well be a more general one than we believe. Synthesis seems capable of marked increments and while the most potent stimulus for γ-globulin is an appropriate antigenic exposure, albumin production may well be controlled by the effective colloid concentration. The mechanisms affecting protein degradation and synthesis are still unclear, and further work is necessary before the regulatory systems that keep protein metabolism in balance, the interplay of one protein with another become understood.

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THE EFFECT OF PLASMA CONCENTRATION ON THE CATABOLIC RATE OF HUMAN SERUM ALBUMIN

P. W. DYKES, J. W. L. DAVIES, C. R. RICKETTS and D. R. STANWORTH

Department of Experimental Pathology,

University of Birmingham, Great Britain

ABSTRACT

Catabolism of plasma proteins has been shown to be related to their concentration, but the nature of this relationship is to date unclear. A study was undertaken in human subjects, in whom hypoalbuminemia was the result of cirrhosis o the liver. The rate of catabolism was found to be unexpectedly low, and well below that anticipated from the plasma concentration. Albumin infusions resulted in the return to normal of both the serum concentration and the fractional rate of catabolism. The data appear to support the hypothesis that the rate of catabolism is dependent upon plasma concentration, by a square law relationship.

Study of catabolism of human γ-globulin in rabbits has shown that an S10 fraction, believed to represent a dimer form, has a plasma disappearance rate indistinguishable from that of the normal S7, suggesting that at least for this protein, the dimer does not represent the normal route of catabolism.

It has been demonstrated that the rate of catabolism of plasma albumin is related to its concentration, and most authors have concluded that this relationship is linear [1, 2]. The evidence for this conclusion is, however, meagre and largely confined to short term animal experiments [3, 4, 5]. In addition, much of the supporting evidence comes from work on other plasma proteins such as γ-globulin [6] and fibrinogen [7], and there is no reason to believe that different proteins are catabolised in the same way. Patients suffering from hypoalbuminemia due to congenital causes [8], to cirrhosis [9, 10], and to malnutrition [11], have all been shown to have depressed fractional rates of catabolism.

The present study describes observations in human patients suffering from cirrhosis of the liver, in whom the serum albumin concentration was raised to normal by infusions of human serum albumin. Observations are also described of rates of catabolism in rabbits of 7S and 10S human γ-globulin fractions; the 10S was believed to be a dimer, probably formed in the preparation and sterilisation of the γ-globulin.

METHODS

All subjects suffered from hypoalbuminemia, the result of cirrhosis of the liver in all except one. Liver biopsy in this patient was virtually normal, and hypoalbuminemia may here have been nutritional in origin. All patients received potassium iodide (200 mg/day) throughout the study, and were either at rest in bed or ambulant to a minor extent. Occasional measurements of creatinine content suggest-

ed satisfactorily complete urine collections; the estimations did not vary by more than 10 % from the mean value for each patient except in a few specimens which were discarded. All patients were on a normal diet apart from restriction of sodium intake where this was necessary.

Albumin and γ-globulin were prepared by an ether fractionation process at the Lister Institute of Preventive Medicine, and labelled with ^{131}I and ^{125}I by the iodine monochloride method of McFarlane [12]. The mean ratio of iodide bound to protein was less than one atom per molecule. Previous examination of the γ-globulin by ultracentrifugation revealed 81 % 7S, 0.5 % 19S macroglobulin, 9.5 % 10S, and 9 % 4.5S. Electrophoresis on cellulose acetate at pH 8.6 showed that 81 % of the radioactivity was associated with γ-globulin, 6 % with ß-globulins, 9 % with α-globulins, and 4 % with albumin [13].

The labelled albumin was injected intravenously, and the γ-globulin administered intramuscularly or intravenously. In the latter instance, it was mixed with an excess of unlabelled albumin and injected slowly over a ten minute period. Venous blood samples were taken ten minutes after albumin injection from which to estimate plasma volume, and further samples were taken daily or as necessary. The blood was taken into E. D. T. A. and the plasma separated. Unlabelled albumin, also obtained from the Lister Institute, was infused intravenously at the rate of fifty grammes per day. The infusions were given daily for several days, followed by an observational period, also of several days, during which time measurements were made of the fractional catabolic rate.

The total protein concentration was estimated by the biuret method [14], and the albumin and γ-globulin percentages by electrophoretic separation on filter paper [15]. The radioactivity of plasma and urine samples was measured by scintillation counting in a well type sodium iodide crystal.

Pure preparations of albumin and γ-globulin were obtained for specific activity measurements by cold ethanol fractionation of plasma [13].

Calculations were made of plasma volume, also of fractional and absolute rates of protein catabolism on the basis of urine and plasma radioactivity, and plasma protein concentrations. The value for absolute catabolic rate was obtained in two different ways by using not only urine : plasma ratios (U/P %) (Protein Method), but also plasma albumin and γ-globulin specific activity measurements related to total urine radioactivity (Specific Activity Method).

Results and discussion

Serial daily measurements of catabolic rate in a cirrhotic patient being infused with human albumin revealed a progressive rise in the fractional rate as the serum concentration increased. Hence, a scheme of step-wise rise in concentration was designed by short intermittent courses of infusion, leaving periods of a few days between each, when it was hoped the serum concentration would remain reasonably constant. Mean values for each period have been reported fully elsewhere [10], and are plotted in Figure 1. It is clear that these data do not fit a simple linear relation-

ship, but may be better described by a square law function, illustrated by the sketched dotted line. If both axes were plotted logarithmically, this curve would become a straight line, with a slope of two. This has been done (Figure 2*a* and *b*), and regression equations fitted. From the two equations of *y.x* and *x.y*, and using the ratio of the error variances on each axis [16], the regression line of the functional relationship

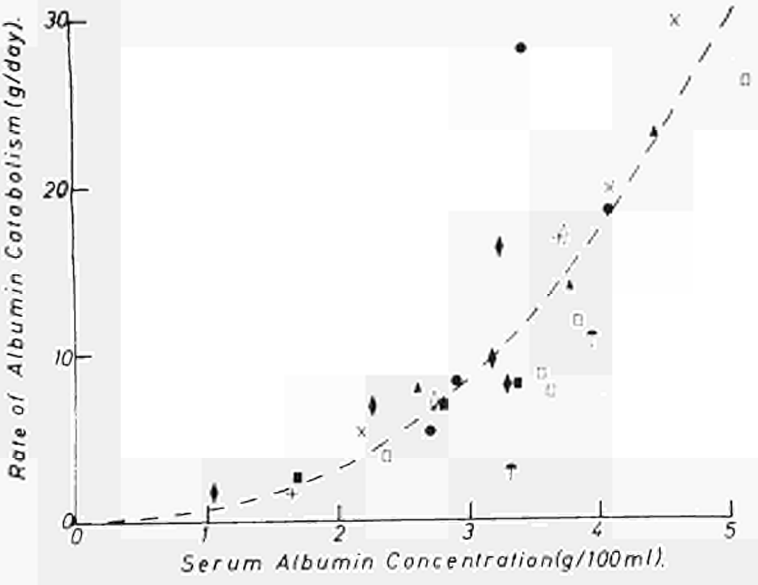

FIGURE 1. — The relationship between the rate of albumin catabolism (protein method) and serum concentration

(Symbols in this and succeeding figures refer to individual patients).

has been drawn, its calculated slope being 2.47. This value does not differ significantly from 2.0. When the abscissa is changed to total plasma albumin (Figure 2*b*), similar equations are obtained, with slopes of 1.9 and 2.4 for *y.x* and *x.y* respectively. The functional relationship cannot here be calculated, however, as the additional source of variance on the abscissa, plasma volume, is not easily definable. It is, however, probable that abscissal variance is hereby increased, and the equation for the functional relationship must have a slope of a little less than 2.4.

Unfortunately, both axes in these figures contain the measurement of plasma albumin concentration, and it was fortunate that a further comparison was possible which was not open to this objection. These values, obtained by the Specific Activity method, have been plotted in Figure 3*a* and *b*, and again the slopes of the calculated relationship do not differ significantly from 2.0. The slope of the functional relationship for Figure 3*a* is 3.30, and the two slopes drawn in Figure 3*b* are 1.4 and 4.0.

Nonetheless, it was possible that catabolic rate was grossly affected by the sudden changes in blood volume and nitrogen balance induced by albumin infusions, and so measurements were made in three patients several months later during the

maintenance period and at differing albumin concentrations. These values are plotted separately (Figure 4*a* and *b*) and again the values for the slope do not differ significantly from two (2.15 for the functional relationship illustrated in Figure 4*a*, and 1.83 and 2.01 for Figure 4*b*). A further test of the effects which rapid infusion might have on protein catabolism was made by using labelled γ-globulin. This was injected at the same time as the labelled albumin, and after five days, catabolic rate was measured during the whole observational period. No consistent change occurred either in plasma concentration or in catabolic rate, thus adding further support to the specificity of the albumin changes. It is further concluded that in man, albumin and γ-globulin catabolism are entirely separate metabolic processes.

Although a second order reaction had not previously been suggested relating the rate of catabolism and plasma protein concentration, a number of studies in the literature contain data bearing upon such an hypothesis. Most of the authors have not considered this relationship in any detail, but their data can be used to test the present hypothesis. Six reports contain sufficient information to enable this comparison to be made. Two were published soon after the introduction of isotope labelled proteins into clinical research [9, 17, 18], the others more recently

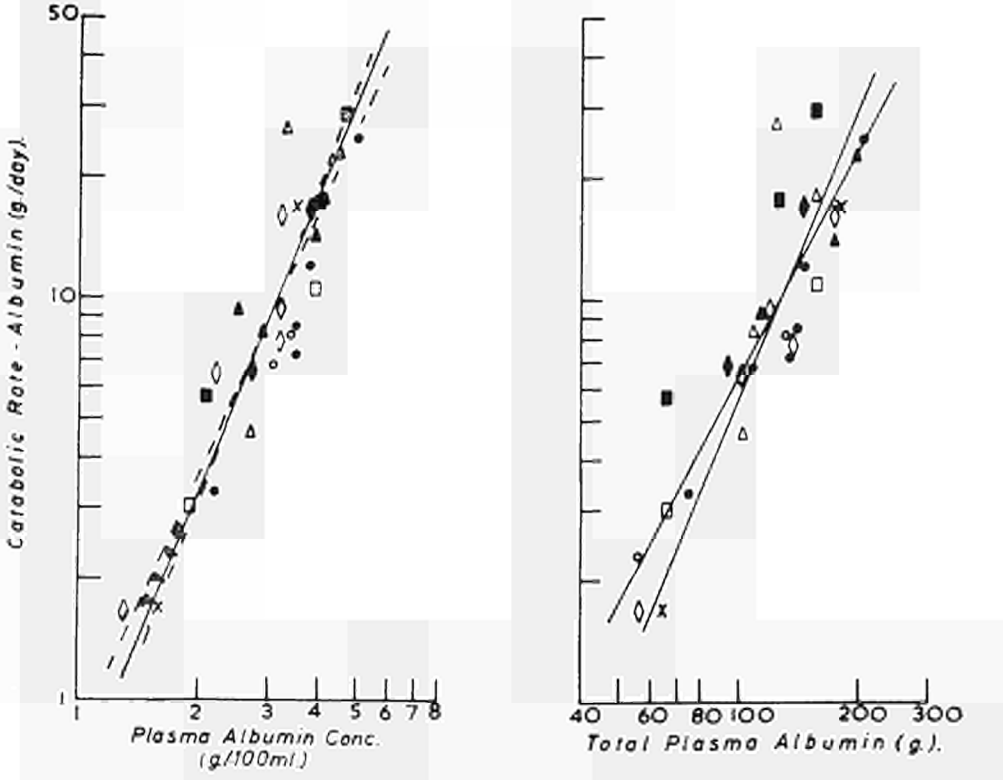

FIGURE 2. — The relationship, albumin catabolic rate (protein method) against plasma concentration (*a*), and against total plasma albumin (*b*)

The relationship between serum albumin concentration and its rate of catabolism

Source	b(y.x)	1 / b(x.y)	Difference of slopes	
			from 1	from 2
Starling (1951)	0.9	1.4	N.S.	< 0.01
Berson and Yalow (1953) . . .	1.55	3.2	< 0.05	N.S.
Jeejeebhoy (Pers. Commun.) . .	1.8	2.4	< 0.001	N.S.
Jeejeebhoy (1962)	2.0	4.7	< 0.02	N.S.
Wilkinson and Mendenhall (1963)	2.15	4.1	< 0.05	N.S.
Cohen and Hansen (1962) . . .	1.8	2.8	< 0.01	N.S.
Picou and Waterlow (1962) . . .	1.05	1.45	N.S.	N.S.

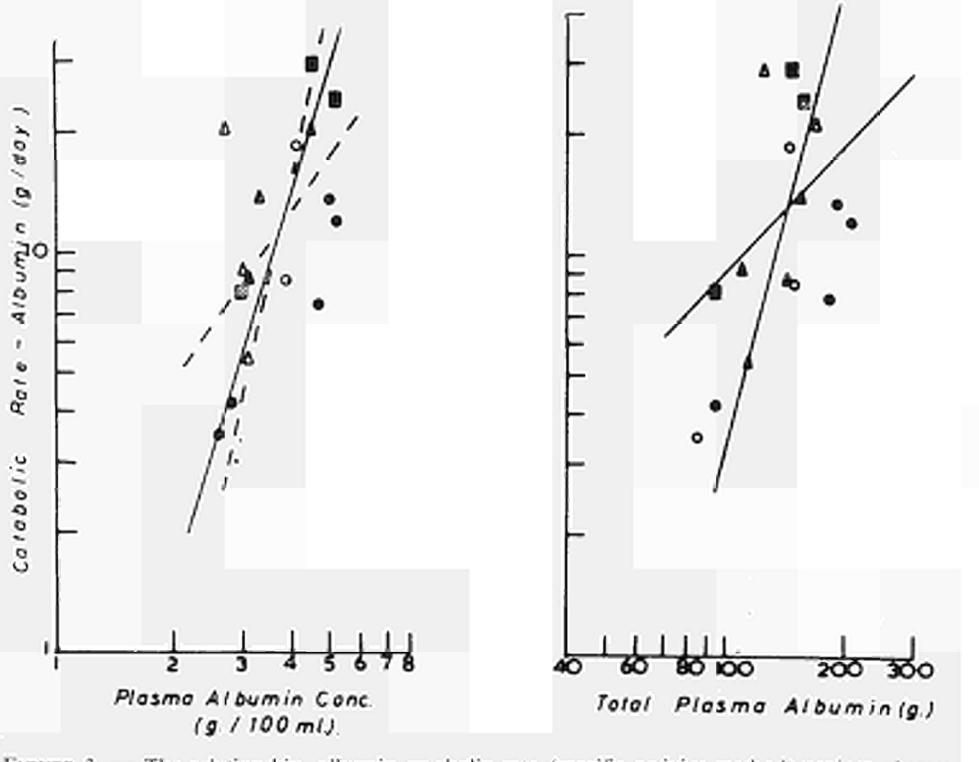

FIGURE 3. — The relationship, albumin catabolic rate (specific activity method) against plasma concentration (*a*), and against total plasma albumin (*b*)

[11, 19, 20, 21]. There is, in addition, a useful report by Bennhold and Kallee [8] of a patient with idiopathic hypoproteinemia in whom the abnormality is apparently due to defective synthesis. Patients in the other series suffered from cirrhosis of the liver, kwashiorkor, and protein losing enteropathy. Data are not always available in exactly the same form as that used here, but within the limits of the information provided, it has been converted to values as closely comparable as possible. Regression cœfficients [$b(y.x)$ and $1/b(x.y)$] are listed in the table, where it is apparent that with the exception of data from Sterling and from Picou and Waterlow, all studies are consistent with a square law relationship. It is probable that the protein used by Sterling, showing very high rates of catabolism, was not of satisfactory biological standard, thus leaving only one important study not in agreement with the present hypothesis.

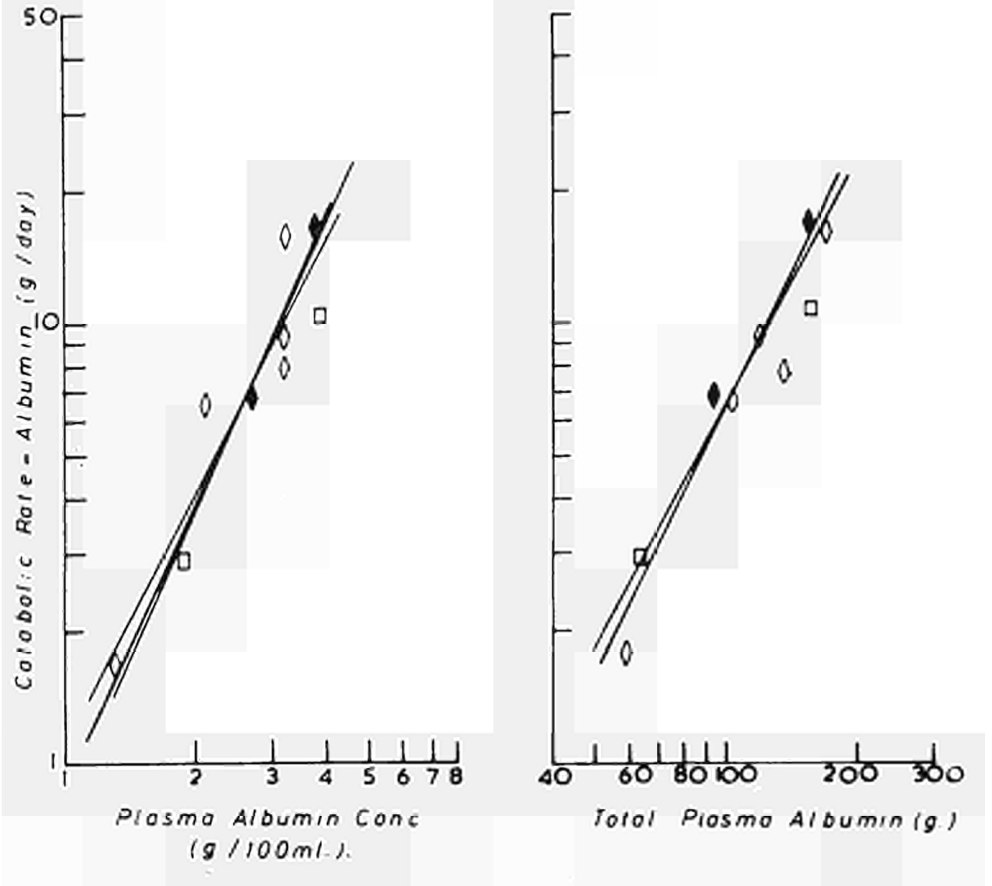

FIGURE 4. — The relationship, albumin catabolic rate (protein method) against plasma concentration (*a*), and against total circulating albumin (*b*) for observations which are unaffected by the period of rapid infusions.

Further, Cohen and Hansen [11] also published data on rates of γ-globulin catabolism which appeared to support a square law relationship for this protein, and Sell [22] has also reported rising fractional rates of catabolism in mice.

Thus, it appears that the rate of catabolism of human albumin is not related linearly to its serum concentration or intravascular pool size, but by a more complex function which probably follows the kinetics of a second order reaction. The underlying physiological reasons for this are unclear, but one of the more probable is that the route of catabolism is via a dimer formed within the distribution of albumin and rapidly broken down at catabolic sites. This possibility has the advantage of explaining simply the site of albumin catabolism, i.e. the blood or tissues in close association where protein concentration is high.

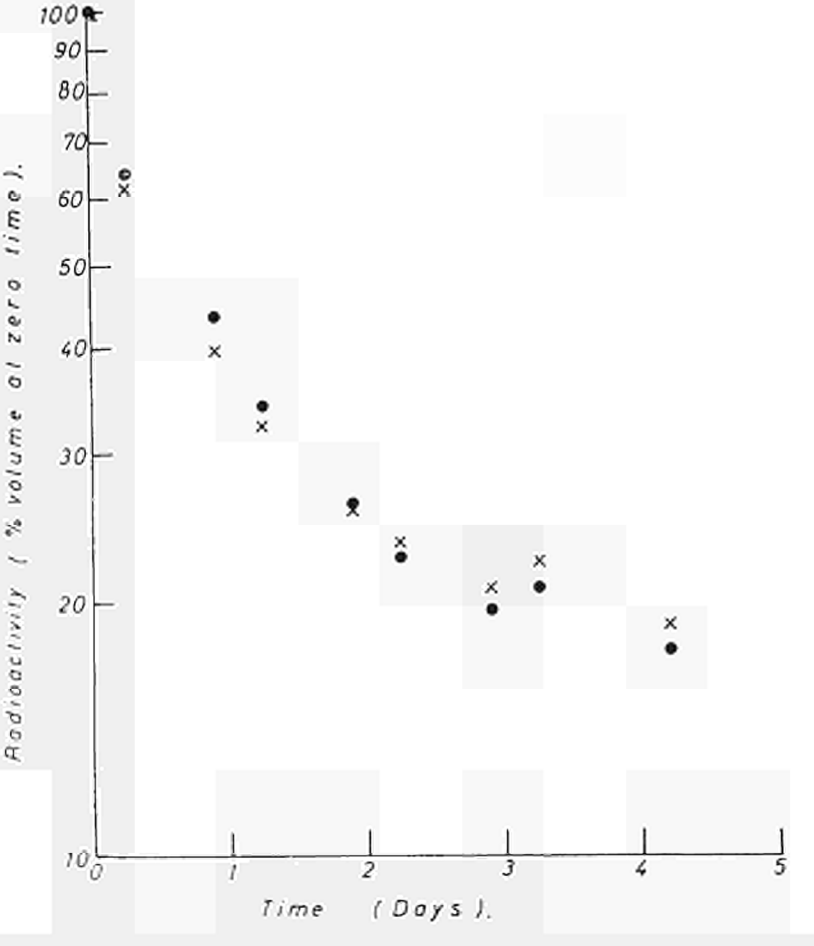

FIGURE 5. — The decline of plasma radioactivity, after the injection into a normal rabbit of two fractions of human γ-globulin, one predominantly 7S (X), the other predominantly 10S (●).

In searching for a readily available, undenatured dimer, a fractionation of American Red Cross γ-globulin on Sephadex G. 200 was found to concentrate a 10S fraction. A sample thus prepared and containing 60 % 10S was injected into normal rabbits together with 7S γ-globulin prepared from the same source, and labelled with a different isotope of iodine. This procedure has been carried out in six rabbits, and in each instance, the rate of distribution and catabolism of the two fractions was found to be identical (Figure 4). Twenty-four hours after intravenous injection of these fractions, a plasma sample was examined ultracentrifugally, and preliminary observations suggest that the isotopes were distributed in the same proportion as in the injected solution. Thus, this particular dimer does not appear to be the route of catabolism in the species tested, and further studies must be carried out both to test the validity of the present hypothesis, and to explore possible underlying reasons.

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DISCUSSION

C. M. E. MATTHEWS (*London*) : I would like to ask Dr. Rothschild how he decides when a new steady state has been reached ? A constant protein concentration in the plasma is not sufficient necessarily to indicate a steady state. I think it is dangerous to equate synthesis with catabolism under such circumstances, and the measurement of total exchangeable albumin may not be valid.

Secondly I think albumin synthesis rate can increase as a result of a reduced albumin concentration, as I found in plasmapheresis experiments in rabbits, which are mentioned in my paper (Effects of plasmapheresis on albumin pools in rabbits C. M. E. Matthews, *J. Clin. Invest.*, **40** : 603, 1961).

Finally Dr. Rothschild seemed to imply that a constant ratio of intravascular to total retained radioactivity indicated equilibrium between extra- and intravascular pools with equal specific activities, but this only means that radioactivities in the different pools are decreasing in parallel.

M. A. ROTHSCHILD : A new steady state was determined to be present with respect to albumin in exactly the same way as during the original control period. Not only were the serum albumin levels constant but also the plasma decay curve was stable and the distribution curve determined from the second dose of ^{131}I-albumin remained at a horizontal asymptote indicating equilibrium of this dose and thus the experimental pool could be determined.

In the conditions mentioned, namely, during proteinuria, and during the infusion of dextran and gammaglobulin and associated with the rapid development of hypergammaglobulinemia, low serum albumin levels *per se* did not result in a change or stimulate albumin synthesis to increase. Plasmapheresis removes all the serum proteins and probably results in changes in distribution as well as alterations in degradation and synthesis as you have shown. This is a different state from one in which one serum colloid or protein is increased and another appears to decrease. It is interesting to consider that when the additional colloid is albumin excessive colloidal accumulation is prevented by an increase in the fractional and absolute rates of albumin degradation. Albumin synthesis remains unaltered. Synthesis and degradation are thus not interdependent.

It is well to point out again that the specific activity in the various body compartments are slightly different but as long as distribution is rapid in comparison to degradation then changes in specific activity in one area will rapidly be reflected in another and the measurements of pool size will be valid. Actually any technique under these states will result in valid determinations.

A. H. GORDON (*London*) : Dr. Rothschild's sytem in which gammaglobulin is injected daily into rabbits may well yield important results because by this mean colloid osmotic pressure can be altered without the introduction of any substance

not normally present in the blood. However as he has himself pointed out, the much greater molecular size of gammaglobulin as compared with albumin must mean that plasma colloid osmotic pressure is reduced as a result of the gammaglobulin injection. If as he suggests colloid osmotic pressure is a controlling factor for the rate of synthesis of albumin, it would seem to follow that this rate should be increased and not decreased as he has in fact found to occur in these conditions. Since the colloid osmotic pressure of the interstitial fluid, which he believes may be responsible in the control of the albumin synthesis rate, must presumably follow changes in the plasma colloid osmotic pressure, it would seem to be more useful to look for other quite different factors controlling this rate.

An example of control of the rate of synthesis of a group of plasma glycoproteins by a factor in rat blood may be mentioned. Blood taken from rats two days after injection of an irritant substance has been found to increase synthesis of certain globulins by the isolated perfused rat liver. In view of this observation of the effect of a factor in blood on the rate of synthesis of certain plasma proteins, increased attention to the possible existence of messenger substances responsible for the control of plasma protein synthesis rates may be more rewarding than further investigation of factors such as colloid osmotic pressure.

M. A. ROTHSCHILD : Following the infusion of gammaglobulin the loss in the serum albumin was not compensated by the increase in gammaglobulin levels in terms of colloid osmotic pressure and a similar observation was made after dextran infusions. However the site of albumin production is in contact with extravascular fluid and in the liver, at least, changes in interstitial colloid content do not necessarily parallel those of the plasma. Also the existence of such a colloid osmotic regulatory system was proposed because there are many other experimental and clinical observations where reciprocal depressions of serum albumin occur in the face of significant gammaglobulin elevations. Finally since albumin is the most important serum protein for maintaining colloid osmotic pressure, alterations in a regulatory system aimed at maintaining a steady colloid state would not necessarily influence the metabolism of other proteins. Their production would probably be related to other specific functions. While many experimental data seem to favor the existence of a colloid osmotic system, I agree that the proof of the existence and the importance of this mechanism must await further evidence. I did not mean to exclude other stimuli which alter protein metabolism, such as the hormone, action nitrogen intake or health and environment. All of these alter protein turnover, perhaps by other independent methods.

W. MULLIGAN (*Glasgow*) : The apparent reciprocal relationship between gammaglobulin and albumin concentrations has often been attributed to a homeostatic adjustment in colloid osmotic pressure. An interesting example occurs in the young calf which is born without gammaglobulin but where the level raises markedly during the first 24 hours of life associated with the absorption of colostral globulins.

Pierce at Cambridge some years ago noted a drop in albumin levels associated with the increase in gammaglobulin and suggested that this might be due to the maintenance of colloid osmotic pressure. We have looked for the same phenomenon in the baby pig during the first few hours of life but failed to find it. In fact albumin and gammaglobulin levels rose at the same time.

In considering whether colloid osmotic pressure is really involved in these adjustments in concentration it would appear to be important that workers should actually measure colloid osmotic pressure in these various situations.

E. REGOECZI (*London*) : In discussing regulatory mechanisms for controlling plasma protein levels, I feel fibrinogen deserves at least brief mention. Dr. Rothschild has shown results obtained with ^{131}I-albumin from which he concluded that albumin synthesis was not related to the concentration or to the pool size of the protein. This may not be true for fibrinogen. So e.g. Miller *et al.* found in liver perfusion experiments that net fibrinogen synthesis was a function of the fibrinogen concentration in the perfusing blood, although unpublished data by Gordon are not fully in favour of Miller's observations. Moreover, Mutschler and I have observed using livers from acutely defibrinated rats (i.e. injecting Malayan viper venom) that significantly increased quantities of fibrinogen were synthesized even when the organ was perfused with blood from normal rats. Evidently fibrinogen has a different mechanism for the regulation of its synthesis than the one described for albumin.

LABELLED PLASMA PROTEINS FOR METABOLIC STUDIES

President : J. COURSAGET
Département de Biologie du C. E. A., C. E. N. Saclay, France

REMARQUES PRÉLIMINAIRES

C'est pour moi un grand honneur d'avoir été appelé à présider cette séance et je crains fort de vous décevoir en succédant à de distingués collègues beaucoup mieux qualifiés. S'il m'est, en effet, arrivé voici quelque quinze années de préparer des protéines plasmatiques marquées avec divers isotopes stables afin d'en étudier le comportement biologique, je n'ai guère participé aux intéressants développements intervenus depuis lors dans ce domaine.

Les communications qui nous ont déjà été présentées nous ont montré que, grâce principalement aux isotopes radioactifs 131 et 125 de l'iode, d'importants progrès ont été réalisés depuis l'époque où nous ne disposions que du carbone-13 et de l'azote-15. Cependant la relative facilité de marquage des protéines par iodation soulève le sérieux problème de l'identité de comportement des protéines chargées d'atomes d'iode et des protéines naturelles correspondantes. Nous payons ainsi d'une inquiétude nouvelle une plus grande aisance expérimentale.

Et je crois que l'on n'insistera jamais assez sur l'importance des preuves de validité, appropriées à chaque recherche particulière, du marquage par addition d'atomes lourds à une molécule organique, surtout si l'on désire explorer ses propiétés spécifiques, enzymologiques ou immunologiques.

En ce qui concerne les études cinétiques, il est tentant certes d'admettre l'hypothèse que les processus en cause sont du premier ordre et les relations exponentielles obtenues sont aisées à manier. Cependant, les modèles proposés sur cette base comportent des espaces, des compartiments dont la signification physiologique n'est pas toujours claire. La réalité biologique ne saurait certainement, dans nombre de cas, être schématisée de manière aussi brutale. Nous devons espérer que les calculatrices électroniques permettront dans l'avenir de saisir cette réalité avec une plus grande finesse.

Mais ce sont sans doute les mécanismes intervenant dans les processus de la biosynthèse et de la dégradation métabolique des protéines plasmatiques qui demeurent les points les plus obscurs. En contraste avec les importants progrès réalisés durant ces récentes années en matière de régulation de la biosynthèse des protéines cellulaires, nous demeurons très ignorants des facteurs fondamentaux qui assurent l'homéostasie des protéines dans le plasma. Cette situation est particulièrement gênante pour l'interprétation des résultats obtenus au cours d'états pathologiques. Sans doute quelques suggestions ont-elles été présentées, mais elles demeurent hautement hypothétiques et il faut espérer que des progrès significatifs pourront être réalisés au cours des prochaines années.

DISTRIBUTION OF INJECTED ^{131}I OR ^{125}I LABELLED HOMOLOGOUS PLASMA PROTEINS AMONG SUBCELLULAR PARTICLES OF RAT LIVER

A. H. GORDON and P. JACQUES (*)

National Institute for Medical Research, Mill Hill, London, Great Britain

ABSTRACT

^{125}I labelled rat albumin and ^{131}I rat transferrin were screened separately and injected into rats.

After periods from 5 min to 6 h the livers were perfused with saline and fractionated by a method similar to that of de Duve *et al.* (1955).

As has been previously observed with heterologous proteins the specific activities of the light mitochondrial (lysosome rich) fractions (L.) derived from both ^{125}I-albumin and from ^{131}I-transferrin were greater than those from any of the other fractions.

In contrast however, to results with heterologous proteins no systematic change in the distribution pattern with time after dosage was seen. Another difference was that much more activity appeared in the 1×10^5 g. min supernatant fraction (S.). As the transferrin to albumin ratio in this fraction was much higher than that in the plasma of the liver donor this high value of the S fraction cannot be explained only by residual plasma. The ratio of the specific activities of the L to S fraction was found to be more than twice as high for transferrin as for albumin. The distribution pattern of non protein radioactivity was in general rather similar to that found for the proteins, thus suggesting proteolysis of homologous proteins inside the granules.

Previous work has indicated that catabolism of varying proportions of some plasma proteins takes place in the liver (Gordon, 1963). Thus in the isolated perfused rat liver larger proportions of both transferrin and γ-globulin (Cohen, Gordon and Matthews, 1962) are catabolised than of albumin (Cohen and Gordon, 1958). However, in experiments of this kind with plasma proteins which circulated beforehand for 48 hours in another rat, the rate of catabolism in the perfused liver never exceeded 30 % of that known to occur in the whole animal. The aim of the present experiments was to obtain some information about the cellular sites of that catabolism. For this purpose, two homologous proteins, albumin and transferrin labelled with ^{131}I or ^{125}I and screened, were injected together into rats. After appropriate intervals, the livers were perfused briefly with cold saline, homogenized in 0.25 M sucrose and fractionated by differential centrifugation; the scheme adopted was essentially that described by de Duve *et al.* (1955) except that the microsomal and supernatant fractions were separated after 1×10^6 *g. min* only.

(*) Chercheur Qualifié of the Fonds National Belge de la Recherche Scientifique.

Results

The amounts of the two injected isotopes retained in the liver after perfusion with saline at various periods after the original injection are shown in Figure 1. The observed radioactivities per g wet liver have been expressed as the volumes

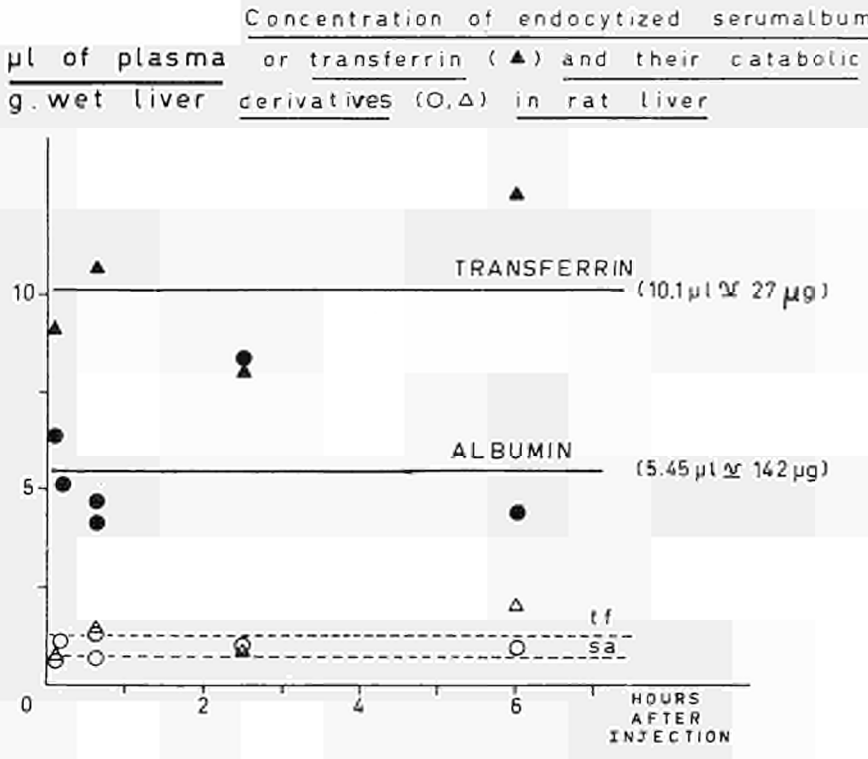

Figure 1

of plasma occupied by each labelled protein before entry into the liver. Evidently, on average, transferrin has been concentrated by a factor of 2 compared with albumin. Treatment with TCA indicated that approximately 12 % of each isotope in the liver was present in combination with products of catabolism as compared with 0.6 % of each in the blood.

Figure 2 illustrates the distribution of the radioactive proteins and their split products in five experiments; that of lysosomal acid phosphatase is also given for comparison. On average 35 % of albumin and 65 of transferrin can be sedimented at 10^6 *g. min.* At shorter times after injection, the specific activities of both albumin and transferrin were found to be highest in the L fraction where lysosomes and microbodies were mostly concentrated; later, higher values, especially of albumin, were found in the supernatant. Considering only the particulate fractions, the L fraction from both proteins always showed highest specific activity at all times

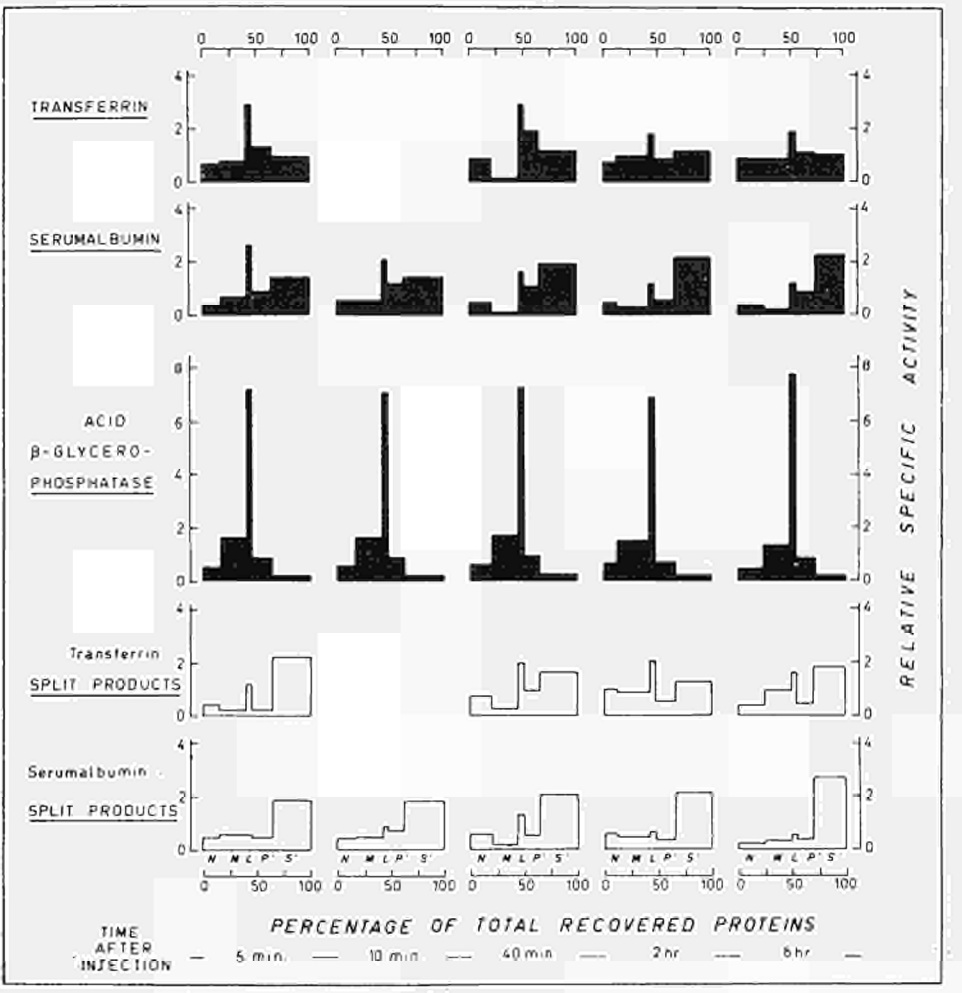

Figure 2

investigated. This was also true for the catabolic products of the injected proteins; their distribution paralleled that of the corresponding protein except for a short period after injection when they were more concentrated in the supernatant. To investigate the possible importance of adsorption to cell particles, plasma containing both proteins was added to the liver homogenate of an uninjected rat; 97 % of albumin was recovered in the supernatant and the 20 % of sedimented transferrin was found distributed evenly between the particulate fractions.

Discussion

An appreciable fraction of both proteins after having been taken up by the liver became bound in cytoplasmic granules. The lysosomal nature of these granules,

already suggested by the peak of specific activity in fraction L, has been established with albumin in an experiment in which the albumin was injected together with Triton WR-1339 six hours before fractionation (Jacques and Wattiaux, 1964). As with endocytized peroxidase the equilibrium density of lysosomes and the granules bearing both ^{125}I-albumin and its split products was considerably lowered in a gradient of hypertonic sucrose whereas that of mitochondria and microbodies remained unchanged. Although cytoplasmic particles (phagosomes) have not yet been proved to be the only conveyors of ingested proteins from the plasma to the lysosomes, endocytosis remains the most likely mechanism of uptake (Novikoff, 1961; de Duve and Wattiaux, 1966). If in fact evidence can be provided that little redistribution of the catabolic products takes place during isolation, then the similarity of distribution of these substances and that of the original proteins in sucrose gradients and after fractionations such as that shown in Figure 2 indicates the importance of the acid cathepsins as the agents causing the breakdown of native plasma proteins inside the lysosomes. A mechanism of this type has been postulated for ^{131}I-insulin (Jacques and Wattiaux- De Coninck, unpublished) and human ^{131}I-serum albumin (Mego and McQueen, 1965) after pinocytosis by rat liver.

As plasma proteins do not accumulate in the liver, there must be an exact balance, *in vivo*, between their rate of entry and their rate of disappearance. Minimum rates of entry of albumin and transferrin are known because the rates of catabolism of both these proteins have been measured in the perfused rat liver (Cohen and Gordon, 1958; Gordon, 1963). As already mentioned, equilibrium had apparently been established in the present experiments as early as 5 min after injection of the albumin and transferrin. To our surprise, at this time the amounts of albumin and transferrin actually observed in the liver, after careful perfusion with saline. were both already more than 10 times the amounts which would have entered the

TABLE I

	Albumin	Transferrin
Amounts of each protein found in liver at 5 min after injection (μg/g wet wt)	142	27
Amount of each protein which would have entered liver in 5 min if rate of entry were equal to rate of catabolism (μg/g wet wt)	12	2.1
The same as % of amount actually present at 5 min after injection	8.5	7.8
Time that would be required for entry of the amount of protein found after 5 min if it has entered only at the rate of catabolism (hours)	1.0	1.08

liver if the rate of entry had been no more than the expected rate of catabolism (Table I). The simplest explanation of the large excess of labelled albumin and transferrin over the expected amounts would, of course, be the retention in the liver of trapped plasma. Although the retention of traces of plasma as a result of inefficient perfusion with saline may well have occurred another mechanism also seems likely. This conclusion follows from the observation that the ratio of transferrin to albumin in the liver homogenate is approximately twice that in the plasma (Figure 1). Evidently concentration of transferrin relative to albumin has occurred during the process of absorption into the cells.

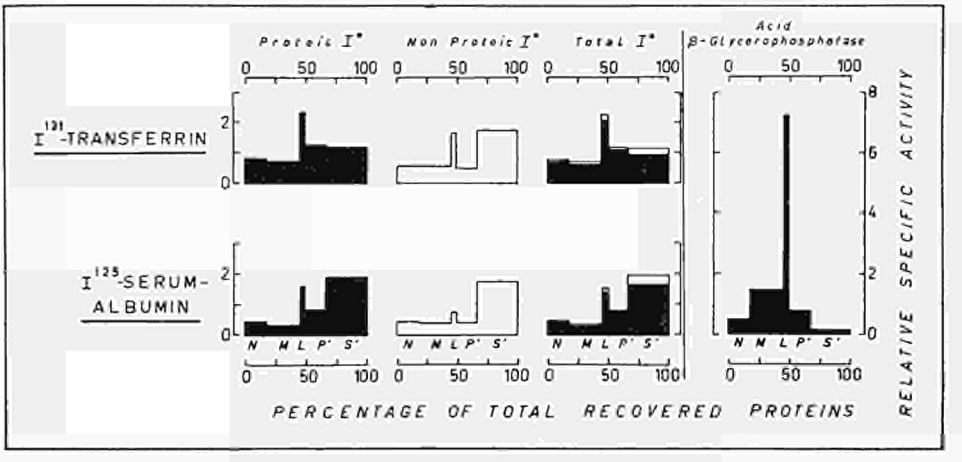

Figure 3. — Average distribution patterns.

An important consequence of the observation of relatively large amounts of albumin and transferrin in the liver cells is that if these proteins are not present as trapped plasma and have entered the cells at a rate much higher than that needed for catabolism then the excess of each protein which has not been catabolised must return to the vascular system either directly to the plasma by some kind of regurgitation or indirectly via lymph.

Although the transport of substances conveyed through cells by cytoplasmic granules (diacytosis) with little if any enzymic breakdown, has been reported for some epithelia (Odor, 1956; Farquhar and Palade, 1960; Straus, 1961; Brambell *et al.*, 1964), the concept of transit of plasma proteins through liver cells is, of course, still hypothetical. However, if the existence of such a process can be firmly established, a possible explanation would be available for the catabolism of plasma proteins in many other organs. Thus, protein molecules slightly altered during passage through the liver or the kidney tubules would be picked up by cells of the R. E. system wherever these might be found.

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DISCUSSION

M. A. ROTHSCHILD (*New York*) : What fraction of the total radioactivity per gram of tissues could be recovered in the subcellular fractions ?

P. JACQUES : Only a few per cent of the injected labeled proteins were recovered in the total liver but as much as seventy per cent of the labeled transferrin present in the liver or fifty per cent of the albumin could be sedimented with the cytoplasmic particles; these are minimal values since the 10^6 g min supernatant is likely to contain micropinocytic vacuoles.

HEPATIC ALBUMIN DISTRIBUTION DURING DEXTRAN AND DEXTRAN AND CORTISONE ADMINISTRATION(*)

M. A. ROTHSCHILD, M. ORATZ and S. S. SCHREIBER

Radioisotope Service, V. A. Hospital, the Department of Medicine, New York University School of Medicine, and the Department of Biochemistry, New York University College of Dentistry, New York, N. Y. U.S.A.

ABSTRACT

Hypoalbuminemia *per se* does not stimulate albumin production and dextran and gammaglobulin infusions depress albumin synthesis suggesting a colloid osmotic regulatory mechanism controlling albumin production. Since the plasma colloid osmotic pressure was not reduced, this mechanism was postulated to reside in hepatic extravascular fluid. This report describes the measurement of hepatic extracellular space (^{14}C-sucrose), plasma albumin (^{125}I-albumin), and total exchangeable albumin (^{131}I-albumin) in control rabbits, rabbits treated with dextran, and dextran and cortisone. Following distribution equilibrium of ^{131}I-albumin, ^{125}I-albumin and ^{14}C-sucrose were injected, livers and lung removed and tissue radioactivity compared with that in the plasma. Hepatic interstitial volume averaged 12 % in all groups. During dextran administration total exchangeable albumin fell 20 %, albumin degradation fell from 273 to 217 mg/kg/day, hepatic interstitial albumin rose from 27 to 52 mg/100 g liver weight. Cortisone reversed these findings. Exchangeable albumin remained stable and albumin degradation rose from 245 to 320 mg/kg/day. Hepatic interstitial albumin decreased to 11 mg/100 g wet liver weight. The available hepatic interstitial space for albumin was limited to 10 % of the sucrose space. In the lung the pulmonary albumin space approximated 50 % of that for sucrose. Albumin is excluded from most of the hepatic interstitial fluid and this volume appears to provide a sensitive system for monitoring changes in colloid concentration. These results support the concept that albumin synthesis may be regulated by interstitial albumin or colloid within the liver.

In previous studies [1] it was observed that the administration of dextran to rabbits resulted in a decrease in the serum albumin level and a loss of total exchangeable albumin. Similarly, the production of experimental hypergammaglobulinemia has been shown to cause hypoalbuminemia and a lowered albumin pool [2, 4]. In both situations the decrease in albumin pool size was due to a diminished rate of albumin synthesis [1, 4]. These observations indicated that albumin synthesis was not stimulated by low plasma albumin levels *per se* and suggested that the mechanism responsible for albumin production might respond to changes in colloid osmotic pressure. Although the calculated osmotic pressure [5, 6] in the plasma was not elevated in either circumstance when albumin synthesis was lowered, the possibility that such a colloid osmotic regulatory system was located within an extravascular site, perhaps within the liver itself, could not be excluded [1].

(*) Supported in part by United States Public Health Service Grant AM-02489.

The present report describes the measurement of albumin metabolism and hepatic albumin distribution in rabbits during the control state, in the presence of dextran and during dextran and cortisone administration.

METHODS

Extracellular space was measured with ^{14}C-sucrose. Following the intravenous injection of 20-30 microcuries of ^{14}C-sucrose, bloods were obtained at 20 minute intervals for 1 hour then 1-2 microcuries of ^{131}I-albumin were injected in 15 control rabbits, 10 rabbits treated with 1.5 g of dextran daily and 10 rabbits treated with cortisone acetate 3 mg/kg/day in addition to the dextran for 10-18 days. The animals were anesthetized 5 minutes after the injection of ^{131}I-albumin and heparanized blood obtained and the livers removed. The concentration of each isotope in liver tissue was compared to that in blood giving a simultaneous measurement of hepatic plasma volume and hepatic extracellular volume. The difference between these two values represents the hepatic extracellular-extravascular volume or interstitial volume (Table I). In 9 other rabbits ^{131}I-albumin was injected and metabolic studies

TABLE I

A. 1) Tissue extracellular volume ml/g	=	Equilibrium ^{14}C-sucrose space
2) Tissue plasma volume ml/g	=	6-10 minute ^{131}I-albumin space
B. 1) Tissue exchangeable albumin g/100 g wet tissue	=	Equilibrium ^{131}I-albumin space X serum albumin concentration
2) Tissue plasma albumin g/100 wet tissue	=	6-10 minute ^{125}I-albumin space X serum albumin concentration

conducted during a control period following which the dextran infusions of 1.5 grams/day, were instituted for 2 weeks, and albumin metabolism remeasured with a 2nd injection of ^{131}I-albumin. After distribution equilibrium this 2nd dose of ^{131}I-albumin and following the re-measurement of albumin metabolism, ^{125}I-albumin was injected, the animals anesthetized and livers removed as described above. Thus in these animals albumin metabolism was determined before and during dextran treatment as well as simultaneous measurements of total available space for albumin distribution in the liver determined with ^{131}I-albumin, and hepatic plasma volume determined with ^{125}I-albumin. The product of these spaces and the serum albumin concentration represents the hepatic exchangeable albumin and plasma albumin. The difference between these quantities is the albumin located in the hepatic interstitial space (Table I). Similar studies were done in 6 rabbits injected with both dextran and cortisone, and the hepatic interstitial albumin in

both groups was compared to values obtained in 7 control rabbits. Total protein was determined by a biuret reaction [7] and protein partition by means of a Kern microelectrophoresis unit [1]. Dextran was determined by the method of Roe [8].

RESULTS

Following the administration of dextran the hepatic extracellular volume increased (Table II). This increase was primarily due to an increased hepatic plasma volume. Since the total liver water remained unaltered, these changes were at the

TABLE II. — Hepatic spaces

	Control	Dextran	Dextran + Cortisone
Extracellular volume (ml/100 g)	21.4 ± 0.9	24.9 ± 1.1	19.8 ± 1.0
% change		+ 14	— 20
Plasma volume (ml/100 g)	9.3 ± 0.6	11.5 ± 0.4	10.2 ± 0.7
% change		+ 24	— 11
Interstitial volume (ml/100 g)	12.5 ± 1.1	13.4 ± 1.2	9.6 ± 1.2
% change		+ 7	— 28
Hepatic water (ml/100 g)	74.1 ± 1.2	74.1 ± 1.3	70.5 ± 0.7

TABLE III. — Albumin metabolism

	Serum albumin g/100 ml		T. E. A. g/kg		Albumin degradation mg/kg/day		Hepatic interstitial albumin mg/100 g	
	C	E	C	E	C	E	Control*	Dextran
Mean	3.6	2.6	3.3	2.6	273	217	27	52
± SE	0.1	0.1	0.2	0.1	17	15	6	6
% change		−33		−21		−21		+93
P value		<0.001		<0.001		<0.05		<0.02

C = Control.
E = Experimental.
* = Mean of 7 control rabbits.

expense of cellular water. Upon the addition of cortisone the extracellular space, plasma volume and interstitial volume decreased and there was a fall in total hepatic water. The effects of dextran on albumin metabolism and distribution are summarized in Table III. Serum albumin fell 33 %, exchangeable albumin pool 21 % and albumin degradation fell 21 %. In the face of this decrease in the serum albumin levels and the exchangeable albumin pool: hepatic interstitial albumin rose from 27 to 52 mg/100 gms of wet liver weight .The presence of any dextran in the interstitial fluid of the liver would have resulted in an even larger increment in colloid concentration than that due to albumin alone. The results of the studies in rabbits treated with dextran and cortisone are shown in Table IV. In contrast to the dextran rabbits the serum albumin concentration decreased only 18 % and there was no change in the exchangeable albumin pool albumin degradation increased by 35 % and this change was accompanied by a decrease in hepatic interstitial albumin. The addition of cortisone was accompanied by an increase in body plasma volume without a decrease in the dextran level in the plasma indicating that extravascular dextran was probably shifted into the plasma. This shift of dextran would probably result in a greater decrease in interstitial colloid osmotic pressure than the change in albumin would indicate.

TABLE IV. — Albumin metabolism

	Serum albumin g/100 ml		T. E. A. g/kg		Albumin degradation mg/kg/day		Hepatic interstitial albumin mg/100 g	
	C	E	C	E	C	E	Control*	Dextran Cortisone
Mean	3.4	2.8	3.0	2.9	245	320	27	11
± SE	0.1	0.1	0.1	0.1	10	26	6	4
% change . . .		—18		—3		+ 35		—60
P value		<0.01				<0.05		<0.05

* Mean of 7 control rabbits.

DISCUSSION

These studies demonstrate again that albumin synthesis does not increase in relation to low levels of serum albumin and that the extracellular distribution of albumin does not necessarily change in the same direction as the plasma albumin concentration.

Upon the administration of dextran there were changes in the hepatic plasma volume and extracellular space which did not exactly parallel the body as a whole.

The hepatic extracellular volume did increase but this was primarily due to an increase in plasma volume. This change in volume was at the expense of cellular water since total liver water remained unaltered. However, in the presence of a fall in plasma albumin levels during dextran infusions, the hepatic interstitial albumin concentration rose. The presence of any dextran in the interstitial fluid of the liver would have resulted in an even large increment in colloid concentration than that due to albumin alone. A lowered albumin synthesis was associated with an increased interstitial colloid concentration.

Following the addition of cortisone, the changes within the liver produced by dextran were reversed and the extravascular albumin content of the liver decreased. The addition of cortisone was also accompanied by an increase in body plasma volume without a decrease in the dextran level in the plasma as has been noted before [1] indicating that extravascular dextran was probably shifted into the plasma resulting in a decrease in interstitial colloid concentration probably more marked than the change in albumin level would indicate.

Previous studies have shown that in the presence of excess adrenocortical hormones the rate of albumin synthesis increases [12] particularly in rabbits pre-treated with dextran. In the latter group a significant shift in extravascular albumin back into the plasma occurred and this sift was again observed. Similar shifts associated with increased protein synthesis have been seen with thyroid administration and in Kwashiorkor. In the latter, the diminished albumin pool is due largely to a loss of extravascular albumin which is replenished by an increase in albumin synthesis upon the administration of an adequate diet [13].

While it is recognized that an unequivocal statement concerning the relationship between albumin synthesis and hepatic interstitial albumin cannot be made the concept proposed seems to fit the data. The results of this study show that the hepatic interstitial albumin exchangeable with plasma albumin is low and albumin synthesis seems to vary inversely with the levels of interstitial albumin or colloid within the liver.

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DISCUSSION

T. FREEMAN (*London*) : Your measurement of extravascular liver albumin depends on the difference between liver plasma space measured with ^{125}I-albumin at 5-10 minutes and liver total extravascular albumin measured with ^{131}I-albumin after equilibrium. If the former is erroneously high due to escape of labeled albumin prior to sampling the estimate of the extravascular albumin would be erroneously low. Could the changes you observe in apparent extravascular albumin after dextran and dextran-cortisone be due to changes in permeability ? Can you exclude this ?

M. A. ROTHSCHILD : While we have not excluded the possibility of specific alterations in sinusoidal permeability, such changes do not seem to explain our results. Firstly, in the case of dextran-treated rabbits both the plasma volume and total exchangeable albumin space within the liver were elevated above normal. If anything the plasma volume increase actually minimized the degree of expansion of the available total extravascular space for albumin distribution. In the rabbits treated with cortisone, on the other hand, the hepatic plasma pool/100 g wet liver weight was small and did not parallel the marked increase in the plasma volume for the body as a whole. Thus the data do not seem to indicate a leak at least within the liver itself. It is well known that there is extremely rapid equilibrium between the plasma and hepatic lymph. It has also been shown that there is no specific removal of ^{131}I-albumin into the liver in one passage through the portal circulation. Also, in conditions with marked edema and congestive heart failure the ratio of ^{32}P labeled red cells to ^{131}I-albumin in whole blood remains constant after the first circulations indicate again no significant specific loss of albumin during these early time periods. Thus while it is still possible that the hepatic plasma volume may have been overestimated the leak must have been small and furthermore if present would have tended to minimize the results.

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ETUDE DE LA DEMI-VIE DE L'α_2-MACROGLOBULINE

C. REUGE, C. BLATRIX, J. P. BREVET et M. STEINBUCH

Centre National de Transfusion Sanguine, Paris
et Centre Hospitalier de Créteil, Créteil, France
CEA Saclay

Abstract

The labelling of a pure protein, α_2-macroglobulin, was effected by two different techniques using chloroform or chloramine T.

This protein was isolated from human plasma by a technique based on the use of rivanol and adsorption of the impurities on bentonite and DEAE cellulose. The purity of this preparation of α_2-macroglobulin was checked by several techniques — electrophoretic and immuno-electrophoretic — and was shown to be homogeneous by ultracentrifugation study.

The half-life of various preparations of this protein was studied in human subjects. The results obtained by various labelling techniques are also reported.

Resume

Le marquage d'une protéine pure, l'α_2-macroglobuline, a été réalisé par deux techniques différentes au chloroforme et à la chloramine T.

Cette protéine a été isolée pure à partir du plasma humain par une technique basée sur l'emploi du rivanol et l'adsorption d'impureté sur bentonite et DEAE cellulose. La pureté de cette préparation d'α_2-macroglobuline a été vérifiée par différentes techniques électrophorétique, immuno-électrophorétique et s'est montrée homogène à l'ultracentrifugation.

La demi-vie de différentes préparations de cette protéine a été étudiée chez l'Homme ainsi que les résultats des différents moyens de marquage.

Technique de preparation de l'α_2-macroglobuline

L'α_2-macroglobuline a été préparée à partir du plasma par une technique faisant intervenir successivement plusieurs précipitations par le rivanol, l'adsorption du plasminogène contaminant par la bentonite et celle des dernières impuretés sur DEAE-cellulose, l'α_2-M restant non-adsorbée dans les conditions choisies. La protéine purifiée peut être ensuite concentrée par les moyens habituels [1].

L'α_2-M s'est révélée homogène à l'ultracentrifugation, en électrophorèse en gel d'amidon et en immuno-électrophorèse (Figure 1).

Techniques de marquage

Le marquage de l'α_2-M par l'iode-131 a été effectué par deux techniques différentes. L'une est basée sur l'emploi de la chloramine T comme oxydant [2, 2bis, 3], l'autre sur l'emploi de IO_3K, en présence de IK et de SO_4H_2, le chloroforme étant utilisé comme véhicule [4]. Le temps de marquage pour la technique à la chloramine T est

d'une minute. En ce qui concerne la deuxième méthode, on prépare le réactif radioactif par extraction au chloroforme de l'iode-131, cette préparation est ensuite mise en contact avec la solution protéique en assurant une agitation lente et continue pendant plusieurs heures.

Le chloroforme étant très dénaturant pour de nombreuses protéines, il n'est pas étonnant que la première technique nous ait donné de meilleurs résultats et c'est pourquoi nous l'avons retenue pour des études ultérieures.

Quel que soit le procédé de marquage on a éliminé l'iode libre par absorption sur IRA-400 puis par dialyse contre du citrate trisodique 0,1 M. La teneur de nos préparations en iode libre était inférieure à 1 % dans tous les cas.

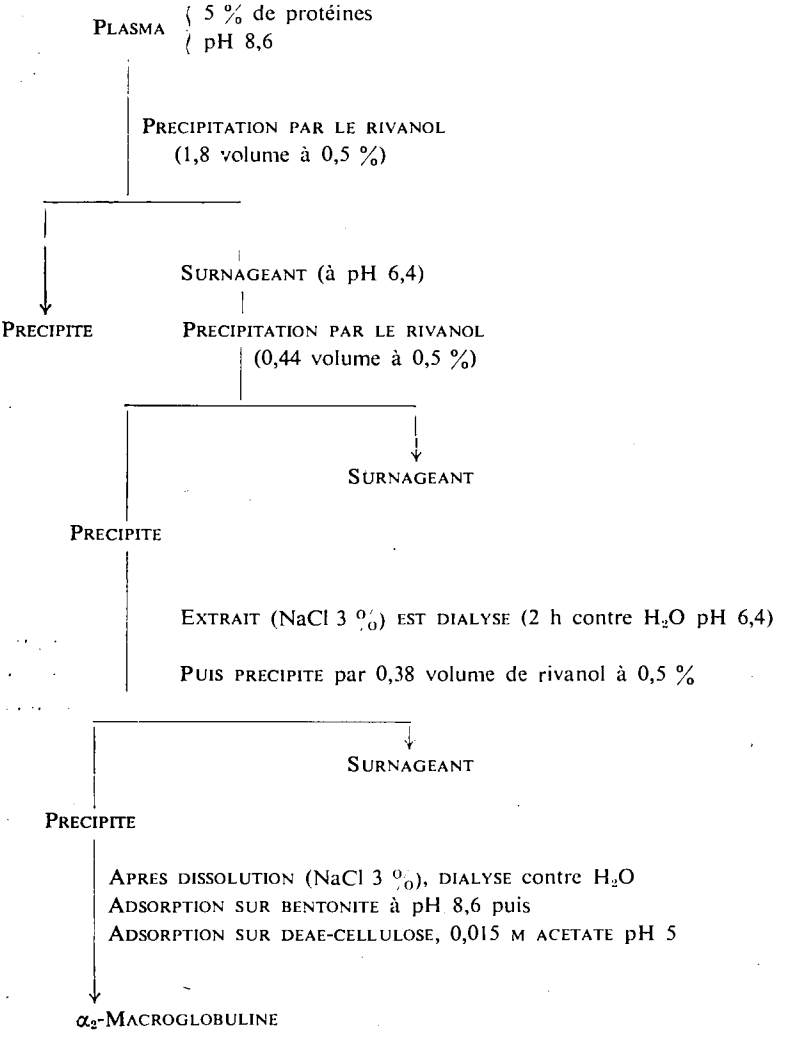

FIGURE 1. — Technique d'isolement de l'α_2-macroglobuline

L'absence de dénaturation de l'α_2-M marquée a été vérifiée par électrophorèse en gel d'amidon et immuno-électrophorèse. En plus nous avons vérifié si les propriétés biologiques de l'α_2-M se trouvaient modifiées après marquage et nous avons choisi son activité comme antithrombine progressive pour apprécier l'intégrité de notre protéine [5]. En effet l'α_2-M se comporte comme l'antithrombine III et les tests ont démontré que la chloramine T était moins dénaturante que le chloroforme ce qui n'est pas étonnant car on sait que le chloroforme détruit l'activité de l'antithrombine progressive du plasma (tableau I).

Après ces vérifications *in vitro* nous avons procédé à l'étude in *vivo*.

TABLEAU I. — Etude de l'activité de l'α_2-M marquée comme antithrombine progressive
Système coagulant : 0,05 ml thrombine 40 u/ml + 0,05 ml de solution à tester + 0,4 ml fibrinogène à 1,75 g/L de protéines coagulables

	Temps d'incubation	Temps de coagulation
Témoin : Thrombine Roche 40 u/ml + Tampon citrate	0 mn 5 mn 10 mn	15 s 16 s 17 s
α_2-M non marquée (Thrombine Roche 40 u/ml + α_2-M 1 %)	0 mn 5 mn 10 mn	17/18 s 125 s 256 s
α_2-M marquée chloroforme (Thrombine Roche 40 u/ml + α_2-M marquée 1 %)	0 mn 5 mn 10 mn	18 s 70 s 150 s
α_2-M marquée chloramine T (Thrombine Roche 40 u/ml + α_2-M marquée 1 %)	0 mn 5 mn 10 mn	18 s 105 s 195 s

INJECTION DES PROTEINES CHEZ L'HOMME

L'α_2-M a été injectée à des malades chroniques, ne présentant cliniquement ni signe d'insuffisance cardiaque, ni œdèmes ou épanchements dans les séreuses, et biologiquement ne présentant ni anémie, ni troubles métaboliques, la vitesse de sédimentation et les diagrammes électrophorétiques étant toujours normaux. Le premier prélèvement a été fait 15 minutes après l'injection pour le dosage de la radioactivité de départ; puis toutes les 6 heures pendant 24 heures et enfin tous les deux

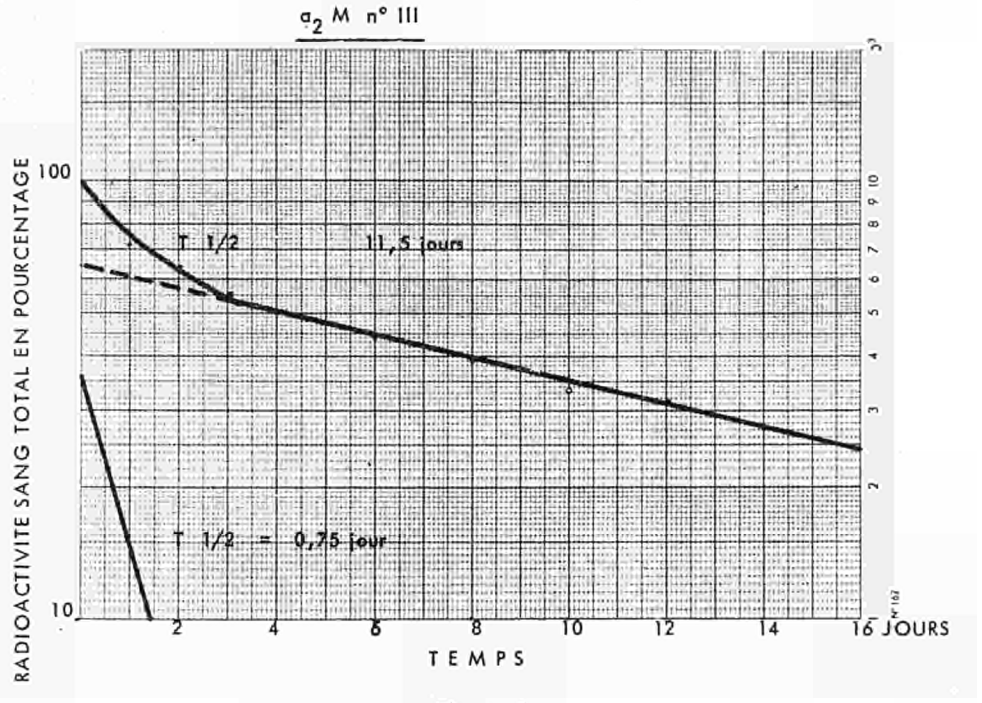

Figure 2

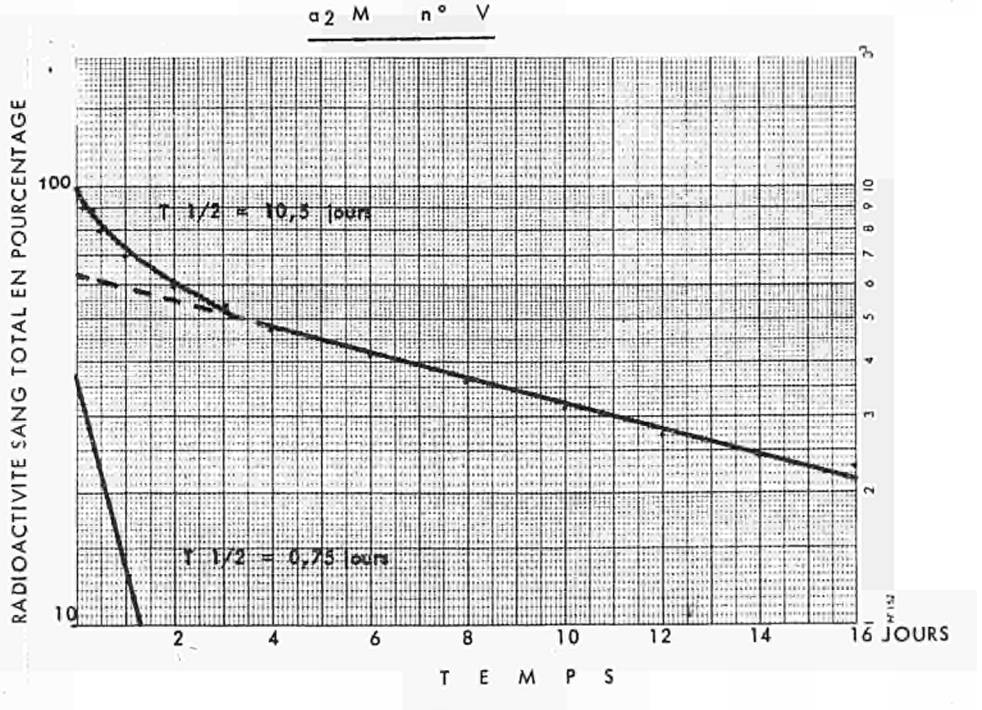

Figure 3

jours pendant 16 jours. En ce qui concerne les comptages, ils ont été pratiqués dans un compteur à scintillation automatique pendant 10 minutes pour chaque prélèvement. Le calcul de la demi-vie et le taux de dégradation ont été faits selon la technique proposée par Matthews [6].

Resultats et discussion

Les meilleurs résultats ont été obtenus en utilisant un produit de départ non lyophilisé et marqué en présence de chloramine. De cette façon on a pu obtenir des courbes de décroissance de radio-activité plasmatique superposables notamment chez 4 témoins, à partir desquelles on a pu définir d'une part une exponentielle lente correspondant à la dégradation de la protéine dans le secteur intravasculaire, et d'autre part une exponentielle rapide correspondant à l'équilibre de diffusion entre le secteur vasculaire et le liquide interstitiel (Figures 2 et 3). Si le marquage est effectué sur un produit préalablement lyophilisé ou sur un produit liquide mais

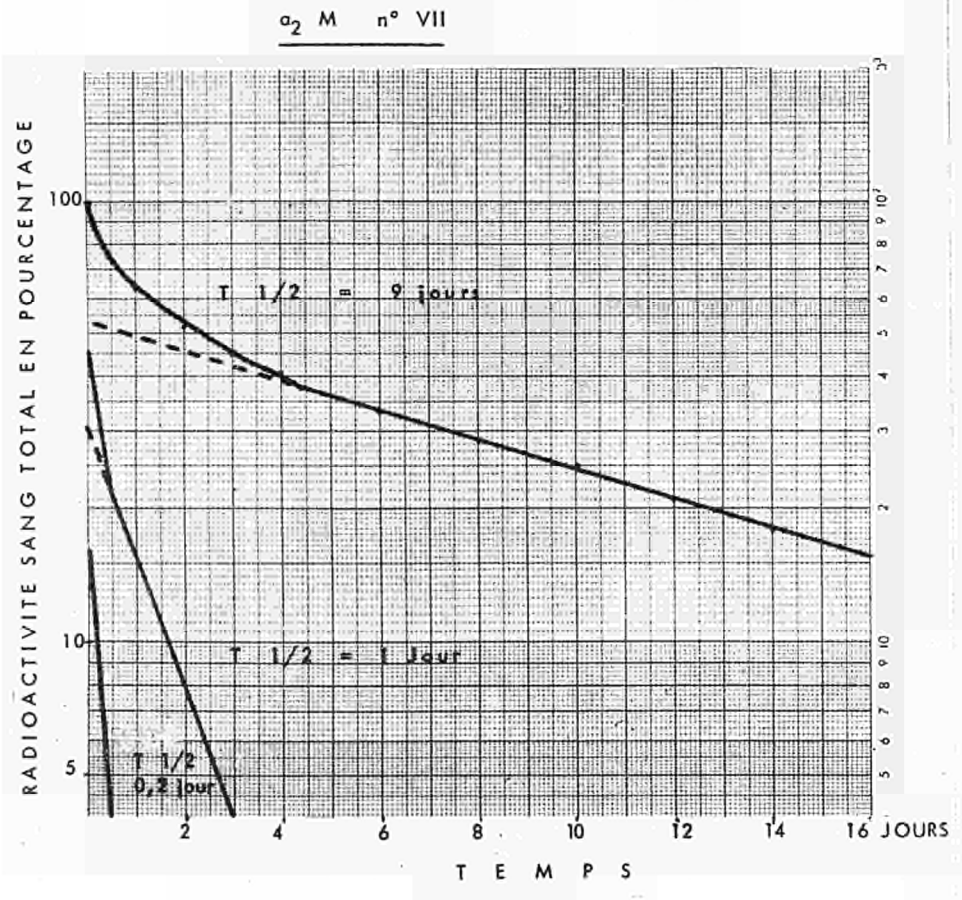

Figure 4

en présence de chloroforme, la courbe obtenue débute par une chute rapide de la radio-activité dans les deux premières heures, ce qui permet de définir une troisième exponentielle, très rapide, et qui indique une certaine dénaturation de la protéine injectée (Figure 4). L'ensemble des résultats figure sur le tableau II. Comme mesure de la demi-vie on obtient pour l'exponentielle lente un chiffre moyen de 10,5 jours et pour l'exponentielle rapide un chiffre de 17 h 30 et pour le pourcentage de dégradation quotidien un chiffre moyen de 10,1 % (K 12), établi pour les courbes VI et VII en tenant compte de la radio-activité correspondant à la protéine dénaturée soit pour la courbe VI 16 % de la radio-activité de départ, et pour la courbe VII 14 %. Sur le tableau figure également le K 13 (% de protéines intravasculaires passant par jour dans le pool extra-vasculaire) et le K 31 (% de protéines extravasculaires passant par jour dans le pool intravasculaire).

TABLEAU II. — Résumé des résultats concernant l'α_2-macroglobuline

	Etat du produit avant marquage	Technique marquage	Nombre d'atomes Iode fixé par molécule PM. 900.000	Activité spécifique µc/mg	Protéines injectées en mg	$T_{1/2}$ en jour	Taux de dégradation/jour % $K_{1\cdot2}$	K_{13}	K_{31}
I[a]	Lyophilisé	Chloramine	1	1,5	70	8,5	/		
II	Liquide	Chloramine	3	6	15	9	11,7	27	60,4
III	Liquide	Chloramine	3	6	15	11,5	9	27	62,2
IV	Liquide	Chloramine	3	6	15	10	9,7	27	73,1
V	Liquide	Chloramine	3	6	15	10,5	10,1	35	59,4
VI[a]	Liquide	IK + 10_3K + SO_4H_2 chloroforme	0,6	1,9	50	13	10,3		
VII[a]	Liquide	IK + 10_3K + SO_4H_2 chloroforme	0,6	1,9	50	9	13,1		
					Moyenne	10,5	10,6		

[a] Echantillons présentant une troisième exponentielle d'élimination très rapide correspondant à une dénaturation partielle de la protéine. Les résultats de II à VII correspondent au même lot de α_2-M

En conclusion on peut dire que la demi-vie de l'α_2-M (10,5 jours) est nettement plus longue que la demi-vie de l'autre macroglobuline du plasma, la γM-globuline, qui est d'environ 5 jours chez les sujets normaux [7].

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DISCUSSION

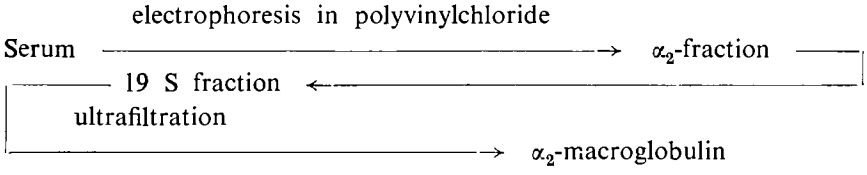
V. Bocci (*Sienna*) : May I ask what amounts of Chloramine T and α_2-macroglobulin you have used ?

C. Reuge : Le taux de Chloramine est toujours le même; il représente 1 % du poids de la protéine.

G. Milhaud (*Paris*) : Je voudrais vous demander si le marquage en présence du chloroforme n'a pas un effet sur les lipides que peut contenir votre protéine ?

C. Reuge : L'α_2-macroglobuline ne contient pas de lipide.

R. Norberg-Stenbeck (*Stockholm*) : I want to point out that we have prepared α_2-macroglobulin by combining gel filtration on Sephadex G 200 and zone electrophoresis in polyvinylchloride according to the following scheme :



The method is quite simple and gives a pure undenaturated fraction.

EXTRAVASCULAR DEGRADATION OF ALBUMIN IN HUMANS

K. HØEDT-RASMUSSEN, E. HASCH and S. JARNUM

Medical Department B, Bispebjerg Hospital
Department of Clinical Chemistry, Bispebjerg Hospital
Medical Department P, Rigshospitalet
Copenhagen, Denmark

Abstract

The commonly accepted site of albumin degradation is the intravascular pool. The methods for calculating the catabolic rate assuming intravascular degradation are based upon the activity in urine/activity in plasma ratio. The same result can be obtained by analysis of the plasma curve. Theoretically, these calculations would give the same results after both intravenous and subcutaneous administration of iodized albumin.

In 10 cases, ^{131}I-albumin was injected subcutaneously and the radioactivity was measured in urine and in blood samples for three weeks. In 6 of the cases, ^{125}I-albumin was simultaneously injected intravenously.

The urine excretion of activity was almost identical after subcutaneous and intravenous administration of albumin, while the plasma activity after subcutaneous injection first reached its maximum value after about 5 days. Calculation of the intravascular degradation of albumin in accordance with various commonly accepted principles leads to the conclusion that 10-20 % of the subcutaneously injected albumin is catabolized extravascularly in normal subjects. In patients suffering from protein-losing diseases, there was found to be a smaller percentage of extravascular albumin catabolism than in normal subjects.

The finding is discussed with particular reference to protein denaturation and lymphatic flow.

When radioiodinated albumin is injected intravenously, the labelled protein is broken down and the liberated radioiodide is excreted in the urine. The decline of the plasma concentration and the renal excretion of radioiodide run a parallel course, which has led to the conclusion that albumin is broken down within or close to the intravascular compartment. For the same reason the catabolic rate can be calculated from the plasma curve alone or as the renal clearance of labelled protein in plasma (U/P ratio).

In the present investigations the degradation of labelled albumin was measured after subcutaneous injection of the protein with the specific purpose to demonstrate a possible extravascular site of catabolism.

Methods

Two labelled preparations of human albumin (Behringswerke, Marburg, Germany) were applied, one labelled with ^{125}I, one with ^{131}I. More than 99.5 per cent of the label was proteinbound. For each study about 20 μCi of either preparation

were used. To prevent thyroid uptake of the label, 150 mg of stable KI was give orally each day throughout the study beginning 2 days before the injection. A patients received simultaneous injections of ^{125}I-albumin intravenously and ^{131} albumin subcutaneously or into the peritoneal cavity. The dose was given in 1 ml isotonic saline.

Daily determinations were performed of plasma activity, renal radioiodic excretion and, by external counting, of the radioactivity at the site of subcutaneo injection. The two isotopes were measured separately by means of single chann analyzers.

CALCULATIONS

ANALYSIS OF PLASMA CURVE.

A simple way of calculation was given by Nosslin [1]. The fractional catabol rate (that fraction of the Intravascular pool which is broken per day) is obtain from the reciprocal of the area under the plasma curve (Eq. 1). With a slight tran cription the equation is identical to that given by Matthews [2].

$$\text{Fractional catabolic rate} = \frac{100}{\int_0^\infty C_P \, dt} \text{ per cent/day} \qquad (1)$$

where C_P, is the plasma concentration.

If the degradation occurs only in the intravascular compartment, the ar $\left(\int_0^\infty C_P \, dt\right)$ will be the same no matter whether the injection is given "momentarily (i.e. intravenously) or not. Following subcutaneous injection one may visuali the input function from subcutaneous tissue to plasma pool as a protracted intr venous injection, and the area under the ensuing plasma curve should be identic to that following a true intravenous injection of the protein provided the plasn concentration is given in the same unit. A relative value is applied as unit : observ concentration as fraction of the theoretical concentration obtained, if the tot injected dose were present in the plasma pool. The latter is known from the simu taneous intravenous injection of ^{125}I-albumin. Following subcutaneous injection t area was determined by a combination of weighing and calculation. The area the observed part of the curve (until the last plasma sample) was determined b weighing. The rest was calculated, since it had to be extrapolated to infinite. Th was possible, because the plasma curve almost invariably assumed a final monoexp nential decline. Hence

$$A_{\text{extrapol.}} = \frac{T\ 1/2}{0.693} C_{Pi} \qquad (2)$$

where

$A_{\text{extrapol.}}$ = area under extrapolated part of the plasma curve
C_{Pi} = relative plasma concentration in the last plasma sample
T 1/2 = halftime (days) of the final exponential decline.

Now, the extravascular degradation ($D_{extravasc.}$) was calculated from

$$D_{extravasc.} = \frac{A_{iv}-A_{sc}}{A_{iv}} \times 100 \text{ per cent of } D_{intravasc.} \quad (3)$$

where

A_{iv} = area under plasma curve of ^{125}I-albumin after intravenous injection
A_{sc} = area under plasma curve of ^{131}I-albumin after subcutaneous injection.

METABOLIC CLEARANCE.

The fractional catabolic rate was also calculated as the average of daily U/P ratio determinations expressed as fraction of the plasma volume [3].

$$\text{Fractional catabolic rate} : \frac{U/P}{PV} \text{ day}^{-1} \quad (4)$$

CASE MATERIAL

GROUP 1.

Six patients from 31 to 81 years of age received *subcutaneous* and intravenous injections. None of them had abnormal gastrointestinal protein loss, oedema or ascites. One (No. 3) had a slight proteinuria due to chronic pyelonephritis. The diagnoses are listed in table I.

TABLE I. — Extravascular degradation and subcutaneous elimination rate of labelled albumin in 6 patients

Patient	Age	Sex	Diagnosis	Serum albumin g/100 ml	Synthetic rate of albumin g/kg/day	Extravascular albumin degradation (Eq. 3, see text), %	Disappearance rate from subcut. inject. site, 0.693/T 1/2
1	78	F	Left hemiparesis	3.65	0.146	29	0.20
2	79	F	Senile dementia	4.86	0.162	19	0.22
3	81	F	Diabetes Chronic pyelonephritis	2.12	0.266	8	0.26
4	43	M	Disc degeneration	4.00	2.205	1	0.53
5	46	F	Cyclic œdema	4.68	0.185	14	0.77
6	31	F	Traumatic œdema of left leg	4.70	0.231	8	1.16
Mean				4	0.206	13	0.52

GROUP 2.

Three patients with cirrhosis of the liver and ascites had *intraperitoneal* and intravenous injections (table II).

RESULTS

GROUP 1 (subcutaneous injection).

In group 1 the albumin synthesis was within normal range in everyone (table I). An extravascular degradation was demonstrated in all except one (No. 4). On an average it amounted to 13 per cent of the simultaneous "intravascular" degradation of ^{125}I-albumin (given intravenously).

The elimination of ^{131}I-albumin from the subcutaneous injection site was rather slow in the 3 first patients, which may be due to the fact that they were elderly bedridden patients (table I, last column).

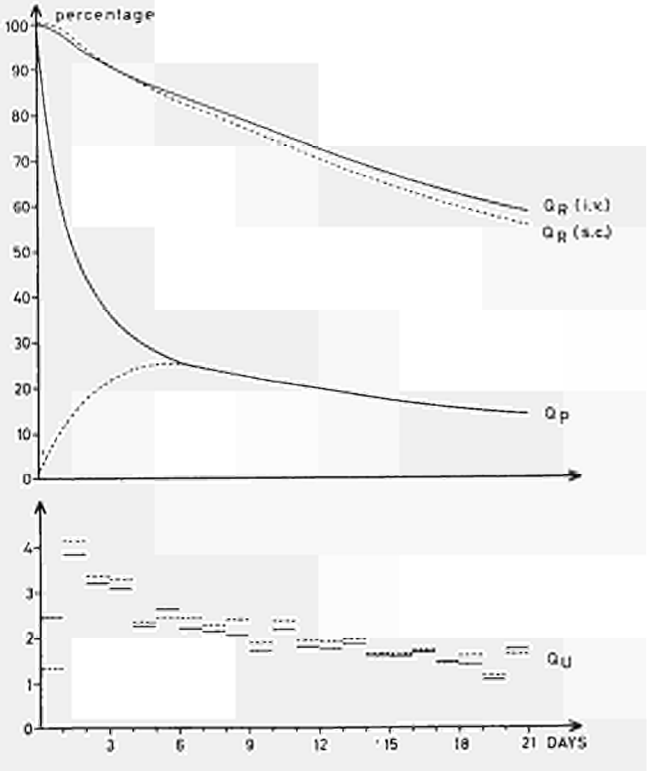


FIGURE 1. — Plasma concentration of labelled albumin (top) and urinary radioiodide excretion (bottom) following subcutaneous (^{131}I-albumin) and intravenous (^{125}I-albumin) injection. The values are relative (per cent of injected dose), and each one represents the average of six studies (group 1).

(- - - - - -) : ^{131}I-albumin or ^{131}I (subc.).

(————) : ^{125}I-albumin or ^{125}I (i.v.).

The average *renal radioiodide excretion and plasma concentration* of all six patients are graphically depicted in Figure 1. It is seen that the radioiodide excretion was almost identical after subcutaneous and intravenous injection.

The fractional catabolic rate calculated from daily U/P-ratios is shown in Figure 2. The high catabolic rate of ^{131}I-albumin (given subcutaneously) is obvious.

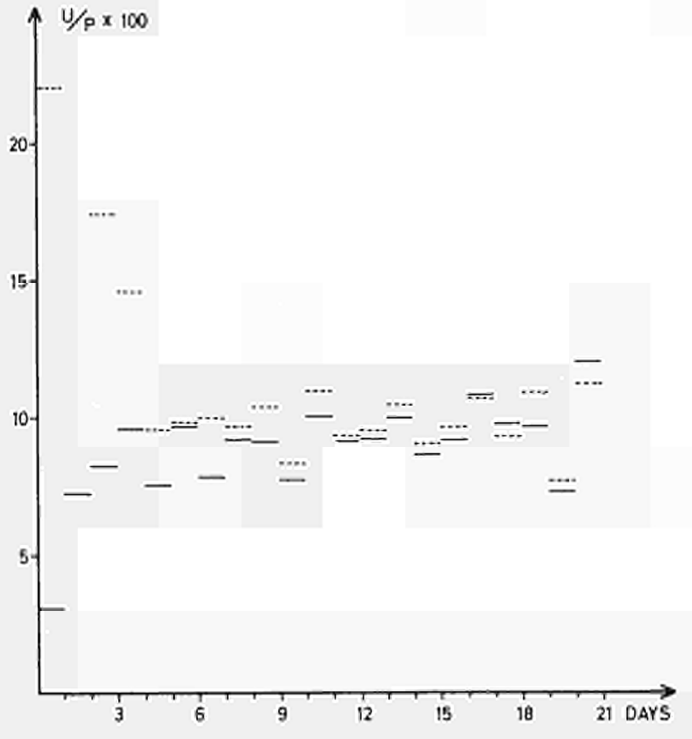


FIGURE 2. — Daily fractional catabolic rates calculated as U/P — ratio following subcutaneous ^{131}I-albumin injection (- - - - - - -) and intravenous ^{125}I-albumin injection (————). Each curve represents the average of six studies.

TABLE II. — Extravascular degradation of labelled albumin injected intraperitoneally in 3 patients with cirrhosis and ascites

Patient	Age	Sex	Serum albumin, g/100 ml	Synthetic rate of albumin, g/kg/day	Extravascular albumin degradation (Eq. 3), %
1	50	F	2.30	0.148	0
2	78	F	3.00	0.103	11
3	52	F	2.58	0.088	13

GROUP 2 (intraperitoneal injection).

The findings in group 2, who received intraperitoneal ^{131}I-albumin and intravenous ^{125}I-albumin injections, were similar to those of group 1 (table II). An extravascular degradation was evident in 2 patients.

DISCUSSION

The fact that the metabolic clearance (U/P ratio) remains constant from day to day after intravenous injection of iodine-labelled albumin has been considered a proof that albumin breakdown takes place in the intravascular compartment. In the present investigations an extraordinary high U/P ratio was found to be present during the first days following subcutaneous injection of labelled albumin. Later, when the plasma curve followed an exponential decline, the U/P ratio did not differ from that observed for labelled albumin given intravenously.

An undue high initial excretion of radioiodide in the kidneys usually indicates denaturation of the labelled protein. However, denaturation could hardly account for the observed high U/P ratio. First, the amount of radioiodide excreted during the first days was of the same order of magnitude (as fraction of the injected dose) after both intravenous and subcutaneous injection. Furthermore, when the ^{131}I-labelled albumin preparations used for subcutaneous injection, were given intravenously to other patients, no undue high renal radioiodide excretion was observed within the first days. As a final check another ^{131}I-albumin preparation was given to normal persons. Two had a subcutaneous injection, and 2 an intravenous injection. The excretion pattern of liberated ^{131}I did not differ from that of the previous studies. Consequently, the phenomenon could not be ascribed to the use of denatured protein. Still, denaturation might occur somewhere on the absorption route from subcutaneous tissue to blood, but this would equal a degradation.

We therefore conclude that an appreciable part of ^{131}I-labelled albumin injected subcutaneously is broken down in the extravascular compartment before it reaches the blood.

Now the question arises whether this is incompatible with the common methods of calculating albumin degradation rate. Many of these methods assume — or are claimed to assume — an "intravascular" degradation. However, for the mathematical correctness of the various formulas it is insignificant whether degradation occurs intra- or extravascularly, if only the transfer rate of the protein from blood to degradation site is much larger than the degradation rate. In fact, Nosslin has shown that the formula using the area under the plasma curve (Eq. 1) is valid no matter where in the body protein degradation takes place (cited by Andersen 1964) [1].

When radioiodinated albumin is given intravenously, a period of 3 to 6 days will elapse before an approximate equilibrium is present between specific activity in the intra- and the extravascular compartment. However, since the transfer rate of albumin from blood to extravascular space is about 150 per cent of the plasma pool per day [4], an extravascular degradation site is easily conceivable. Our finding

that renal radioiodide excretion during the first days was nearly the same after intravenous and subcutaneous injection, actually suggests that the extravascular degradation may take place at any "site" of the extravascular compartment, since the subcutaneous tissue of the calf is almost as remote from the blood as it can be.

Following subcutaneous injection an average of 13 % of the total degradation took place extravascularly. This is a minimum figure, since we have no way to assess the fraction of extravascular degradation once the labelled protein has reached the blood. For the same reason it is not amazing that persons with a high absorption rate seemingly had a smaller extravascular degradation (table I).

As to the actual site of extravascular degradation, the present investigations may offer some clue. It is close at hand to consider the lymphatic system a possible degradation site, since a similar "extravascular" degradation was found after subcutaneous injection and after injection into ascitic fluid. The lymphatic system represents the only common absorption route from the 2 injection sites. It is in keeping with our findings in two patients with abstructive lymphedema of the legs. In these patients the absorption of ^{131}I-albumin injected subcutaneously was very slow and the renal ^{131}I-excretion extremely low.

Conclusion

Following subcutaneous and intraperitoneal injection of radioiodine-labelled albumin a high fractional catabolic rate (U/P ratio) was found during the first days after the injection. It seems to reflect extravascular degradation of the protein. The phenomenon is not incompatible with the common models used for calculation of degradation rate, when labelled albumin is given intravenously, but the formulas derived from these models can only be applied when the labelled protein is injected intravenously.

The figures given for extravascular degradation are minimum values. The lymphatic system is believed to represent an important extravascular degradation site.

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DISCUSSION

P. DYKES (*Birmingham*) : In studying albumin transfer rates between plasma and ascitic fluid in humans, I injected ^{131}I-albumin intraperitoneally and measured also urinary excretion of ^{131}I. Figures obtained for fractional catabolic rate were never high enough to suggest a significant amount of extravascular catabolism.

K. HØEDT-RASMUSSEN : We have also injected ^{131}I-albumin in the ascitic fluid of patient suffering of cirrhosis and our results are the same as yours but you did not say what your plasma concentration was at the time you measured the urine excretion. The point is that you have an urine excretion in spite of a very low plasma concentration.

M. A. ROTHSCHILD (*New York*) : It is hard to reconcile this view of an extravascular degradative site particularly in patients with ascites, with Berson and Yalow previous studies (Berson S. A., Yalow T. S., *J. Clin. Invest.*, 33, 322, 1954). These authors have clearly shown that following the injection of iodinated human serum albumin into ascitic fluid the rate of degradation paralleled the intravascular concentration not that in the ascitic fluid. It is possible that a slight degree of denaturation of a small fraction of the tracer protein could account for the high excretion observed in the first few days following subcutaneous injection.

METABOLISME DE LA ^{131}I-SERUMALBUMINE CHEZ LE RAT

G. MILHAUD et P. GUILLIEN

Laboratoire des Isotopes, Institut Pasteur, Paris

ABSTRACT

Rat serumalbumin was prepared and labeled with ^{131}I. After I. V. injection, the changes of the specific radioactivity, R_s, with time can be expressed by the relation :

$$R_s = A_1 e^{-a1t} + A_2 e^{-a2t}$$

Serumalbumin metabolism can be represented by a two compartments model. In the normal rat as well as in the rat with tied ductus lymphaticus we have measured : the masses of the compartments, the rate of exchange between the compartments, the rate of total way out from the pool, the fecal excretion of serumalbumin.

RÉSUMÉ

La sérumalbumine de rat a été préparée et marquée à l'iode 131. Après une injection I. V., les changements de la radioactivité spécifique, R_s, dans le temps peuvent s'exprimer selon l'équation :

$$R_s = A_1 e^{-a1t} + A_2 e^{-a2t}$$

On peut représenter le métabolisme de la sérumalbumine au moyen d'un modèle à deux compartiments. Nous avons mesuré, chez le rat normal et chez le rat à canal lymphatique lié, la masse des compartiments, la vitesse d'échange entre les compartiments, la vitesse de sortie hors du fonds commun, l'élimination fécale de sérumalbumine.

Nous nous sommes proposé d'étudier l'influence de la ligature du canal thoracique sur le métabolisme de la sérumalbumine chez le rat.

L'albumine de rat est précipitée, à partir du sérum, par relargage répété au sulfate d'ammoniaque en milieu acide. Le précipité est repris dans du tampon phosphate 0,10 M de pH 7,4 et dialysé contre une solution de chlorure de sodium à 9 ‰. L'analyse électrophorétique sur papier montre la présence d'un seul composé migrant comme l'albumine (Figure 1). L'albumine est iodée à pH 8, en utilisant le persulfate d'ammonium en présence de carbonate de guanidine pour oxyder l'iodure-131. La quantité d'iodure utilisée est telle que l'albumine contient environ 1 atome d'iode par molécule. Après dialyse, la pureté de la sérumalbumine marquée est vérifiée par électrophorèse en milieu gélosé suivie de radioautographie à l'aide de film Kodak no-screen. Toute la radioactivité est présente dans la tache correspondant à la sérumalbumine. Le rendement en radioactivité varie entre 35 et 45 %.

FIGURE 1. — Analyse électrophorétique. A : Sérum de rat; B : Albumine de rat

Nous avons utilisé des rats mâles WISTAR CF, pesant de 130 à 140 g, répartis en trois groupes :

1) *Le groupe L* comprend 5 rats chez lesquels le canal thoracique est ligaturé pour provoquer une stase lymphatique dans le réseau intestinal. Après anesthésie à l'éther, la voie d'abord est postéro-latérale gauche, sous la dernière côte; le canal thoracique est satellite de l'aorte. Son repérage est facilité par l'administration par gavage d'un ml d'huile d'olive, 90 mn avant l'intervention : le canal lymphatique apparaît sous forme d'un cordon blanchâtre. Il est ligaturé à l'aide d'un fil de nylon.

2) *Le groupe G* comprend 3 rats, chez lesquels la veine cave supérieure gauche est ligaturée pour essayer de provoquer une hyperpression dans le système cave supérieur gauche, où se déverse le canal thoracique. L'ouverture du thorax se fait par exérèse de la partie antéro-latérale de la deuxième côte. Le pneumothorax est réduit, après suture serrée de la brèche, par aspiration de l'air restant dans l'hémithorax à l'aide d'une aiguille montée sur une seringue étanche.

3) *Le groupe D* comprend 4 rats, chez lesquels la veine cave supérieure droite est liée, comme il a été décrit pour le groupe précédent (il existe deux systèmes veineux caves supérieurs chez le rat [11]) : ce groupe sert de groupe témoin.

Entre le 14e et le 16e jour après l'intervention chirurgicale, on injecte la sérum-albumine de rat marquée par voie intra-veineuse, puis on prélève du sang par ponction dans le sinus rétroorbitaire aux temps 0,25; 22; 43; 91 et 163 h. On mesure la radioactivité plasmatique à l'aide d'un compteur à scintillation. Les selles sont recueillies entre 0 et 48 h après l'injection d'albumine; on administre du carmin par gavage, pour délimiter la fin de la période expérimentale. Les selles sont broyées au mixer dans de l'eau distillée et comptées, puis la radioactivité liée aux protéines est mesurée en précipitant les protéines par l'acide trichloracétique à 10 %.

RESULTATS

La variation de la radioactivité spécifique de la sérumalbumine, R_s, en fonction du temps peut être exprimée par la relation :

$$R^s = A_1 e^{-a1t} + A_2 e^{-a2t}$$

Un modèle très simple, à deux compartiments, peut représenter le métabolisme de la sérumalbumine (Figure 2) :

— le compartiment plasmatique P, sur lequel on fait porter l'entrée et la sortie de l'albumine.

— le compartiment extravasculaire E.

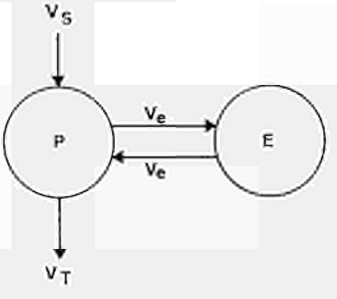

FIGURE 2. — Représentation schématique du métabolisme de la sérumalbumine chez le rat. P et E sont les deux compartiments du fonds commun, V_e, la vitesse d'échange entre P et E; V_s désigne la vitesse de synthèse de la sérumalbumine et V_T, la vitesse de sortie irréversible.

Le Tableau I rapporte les valeurs moyennes de P, E et de trois paramètres :

— la demi-vie de la sérumalbumine, définie comme le temps nécessaire au renouvellement de la moitié de la masse totale de l'albumine T ½.

— la vitesse totale de sortie hors du fonds commun, V_T.

— l'excrétion de la sérumalbumine dans les selles, V_f.

TABLEAU I. — Valeur moyenne et erreur-type de la moyenne des masses des compartiments P et E, des vitesses V_T et V_f et de la demi-vie $T_{1/2}$ de la sérumalbumine dans les différents groupes.

Groupes	Nombre de rats	P (mg)	E (mg)	V_T (mg/j)	V_f (mg/j)	$T_{1/2\ (h)}$
D	4	420,40±25,225	516,94±18,575	656,37±34,877	50,19± 5,487	17,31±0,448
G	3	423,85±13,513	439,72±48,863	675,25±15,401	50,19±6,568	15,41±1,309
L	5	448,73±17,889	410,98±13,513	758,90±32,303	99,09±15,946	13,60±0,615

On n'observe aucune différence statistiquement significative, pour ces trois paramètres, entre les groupes D et G (Tableau II). Par contre, L diffère de façon significative de D : différence hautement significative pour la demi-vie et la vitesse totale de sortie, V_T; différence significative pour l'excrétion de radioactivité dans les selles, V_f. Enfin, L diffère de G de façon hautement significative pour la demi-vie et de façon significative pour les deux autres paramètres, V_T et V_f.

TABLEAU II. — Comparaison des valeurs des vitesses V_T et V_f et de la demi-vie $T_{1/2}$ de la sérum-albumine dans les différents groupes.

Groupes	V_T	V_f	$T_{1/2}$
D et G	—	—	—
D et L	+ +	+	+ +
G et L	+	+	+ +

— pas de différence significative.
+ différence significative $P > 0{,}01$.
++ différence hautement significative $P \leqslant 0{,}01$.

DISCUSSION

Les résultats obtenus lors de la ligature du canal thoracique montrent que l'on peut reproduire expérimentalement une entéropathie avec déperdition de sérum-albumine, comportant une diminution de la demi-vie et une augmentation de l'excrétion fécale de protéines. Il est, par contre, surprenant que la ligature du système cave supérieur gauche n'ait pas provoqué des perturbations analogues, en gênant l'afflux de lymphe provenant du canal thoracique. Ceci peut toutefois s'expliquer par l'existence d'anastomoses veineuses et lymphatiques entre les systèmes caves supérieurs droit et gauche chez le rat, [1], qui pourraient permettre l'établissement d'une circulation collatérale importante, 15 jours après l'intervention chirurgicale. Chez l'homme, l'atrésie et la compression du canal thoracique ont été constatées, notamment dans sa portion sous-diaphragmatique [2]. On observe alors, comme chez le rat, une entéropathie avec déperdition d'albumine. Enfin, chez l'homme, l'hyperpression dans la veine cave supérieure ne suffit pas à provoquer une déperdition de protéines, si des anomalies de la circulation lymphatique ne sont pas associées [3].

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DISCUSSION

P. VESIN (*Paris*) : Je voudrais insister sur l'intérêt que représente le modèle expérimental que le Dr. Milhaud a réalisé chez le rat pour ceux qui étudient chez l'homme les entéropathies avec perte de protéines à l'aide de protéines marquées. En effet, il a réalisé non seulement une entéropathie exsudative avec perte de protéines, mais le tableau réalisé est absolument semblable, tant du point de vue biologique que du point de vue clinique, au tableau que l'on observe chez les malades présentant des affections lymphatiques localisées à la partie inférieure du canal thoracique.

Le grand intérêt de son travail est que ces malades sont extrêmement rares en clinique humaine, et qu'il est par conséquent difficile de faire des études sur ce sujet. Or, non seulement par le modèle que le Dr. Milhaud a mis au point et qui je pense est assez facilement reproductible du point de vue de la physiopathologie expérimentale, il sera possible d'étudier le sort de l'albumine marquée, mais également d'autres protéines plasmatiques qui sont perturbées dans les entéropathies avec perte de protéines, en particulier les immunoglobulines du système Gamma : Gamma A, Gamma M et Gamma G.

PREPARATION OF ^{3}H SERUM PROTEINS AND THEIR USE IN STUDIES ON CATABOLISM OF PROTEINS (*)

G. B. GERBER, A. REUTER, F. KENNES and J. REMY-DEFRAIGNE

Euratom and Centre d'Etude de l'Energie Nucléaire, Mol, Belgium

ABSTRACT

The concentration of several serum protein fractions decreases after whole-body irradiation, an effect which could be due to a decreased synthesis or an increased intra- or extrahepatic catabolism.

Studies on incorporation of phenylalanine and tryptophan demonstrated that synthesis of the fractions involved increases rather than decreases. The aromatic aminoacids were chosen since they occur in a relatively large concentration in the fractions most affected after irradiation.

Hepatic catabolism was then investigated. To this end, serum proteins labeled with ^{3}H-phenylalanine were biosynthetically prepared by a rat liver perfusion. Labeled serum proteins were then added to livers perfused at different time periods after irradiation and the decrease of total activity and specific activity were followed over periods of perfusion up to 12 hours. Formation of volatile radioactivity due to degradation of liberated phenylalanine was also measured. These data do not suggest a difference in hepatic catabolism after irradiation. However, the differences in activity, even during a 12 hour period of perfusion, were rather small. Next, a mixture of serum proteins labeled with ^{131}I and of serum proteins biosynthetically prepared with ^{3}H-phenylalanine was injected into rats and the ^{131}I and ^{3}H radioactivity in serum proteins, as well as in feces and in urine, was followed in normal and X-irradiated rats. These data suggest that extrahepatic loss of proteins is responsible for the changes in concentration of serum proteins after irradiation.

It is well known [1-3] that the concentration of several serum protein fractions is altered in X-irradiated animals, but the interpretation of these changes remains uncertain. The changes most notable in rats after total body exposure in the lethal dose range are : a decrease in prealbumin, albumin and γ-globulins and an increase in $\alpha 1$, $\alpha 2$ and β globulins. However, this pattern is not always consistent and depends, at least in part, upon the development of the radiation syndrome. A decrease in concentration of a protein fraction in the serum such as prealbumin for example, could be the result of either a decrease in synthesis, an increase in hepatic or extrahepatic catabolism, a change in distribution between vascular and extravascular space or of a combination of these factors.

In our present investigation we have studied some of these factors in the intact rat and in the isolated perfused liver. The technique of liver perfusion is very suitable for such studies since the isolated liver is capable of synthesizing many serum

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proteins with exception of the γ globulins [4-6]. Furthermore, the isolated liver also can degrade many serum proteins, although it is not yet clear which importance this hepatic catabolism possesses for the intact animal.

Methods

Perfusion of the isolated liver was carried out by a modification of the method of Miller (Figure 1) [4, 7]. If one works under sterile conditions the liver remains viable for up to 12 hours.

A mixture of ^{131}I serum proteins was prepared by iodination at pH 8. About 1-2 molecules of iodine were bound pro molecule of protein under these conditions. Unreacted iodine was removed by Amberlite and the proteins were screened from denatured material by injecting them into a rat for three days.

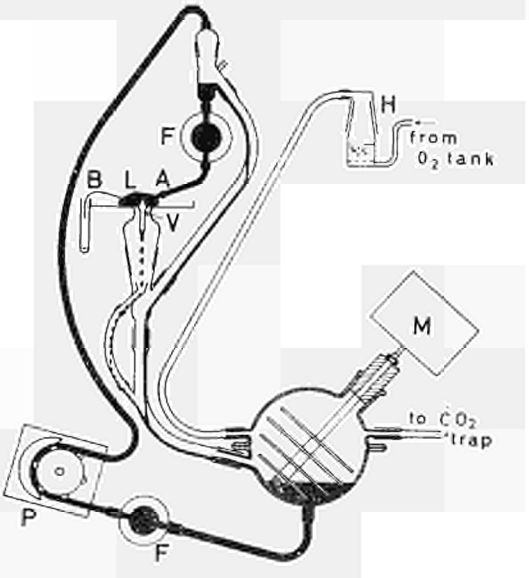

Diagram of the apparatus used for liver perfusion

A inflow cannula.
B bile cannula
F filter
H humidifier
L liver
M motor
P pump
V outflow cann.

Figure 1. — Schema of the perfusion of the liver

Serum proteins labeled with ^{3}H-phenylalanine (specific activity 0.8 Ci/mM) were prepared biosynthetically in the perfused liver. In a typical experiment, ^{3}H-phenylalanine was added to a liver perfusion (volume of perfusate 30 ml) in three doses of 12 mCi at 2 hour intervals. Two hours after the last addition, from 20 to 30 % of the radioactivity had been incorporated into proteins, the rest had been degraded,

	Activity (% of total activity)					
	Prealbumin	Albumin	α1 globulins	α2 globulins	β globulins	γ globulins
^{131}I	15.6	59.9	3.1	1.3	6.8	12.3
Phenylalanine ^{3}H	15.8	40.8	16.0	15.0	11.9	≤ 1
Tryptophan ^{3}H	25.7	14.6	12.3	24.7	20.7	≤ 2.0

FIGURE 2. — Distribution of label in serum proteins prepared by iodination or biosynthetically from ^{3}H-phenylalanine or ^{3}H-tryptophan in the perfused liver

mostly to tritiumwater. The distribution of the ^{3}H and ^{131}I label in the different fractions, as determined by micro agargel electrophoresis, is shown in Figure 2. It can be seen that phenylalanine is incorporated preferentially into prealbumin and γ globulins, whereas albumin contains relatively little radioactivity. This high incorporation into prealbumin and into α and β globulins was the reason why we have chosen labeling with phenylalanine or tryptophan for these experiments.

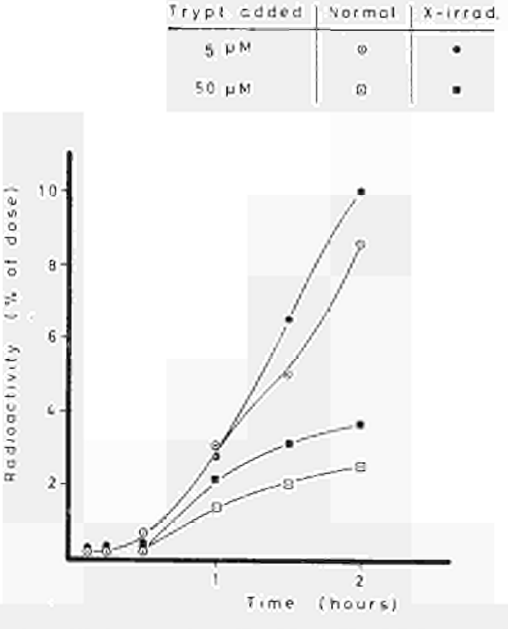

FIGURE 3. — Incorporation of ^{3}H-tryptophan (5 µM or 50 µM) into total serum proteins during perfusion of normal and X-irradiated liver (24 hours after 1000 r)

Results

Synthesis of serum proteins.

Earlier experiments [8] on the perfused rat liver using ^{3}H-phenylalanine, showed that 7 days after a dose of 950 r, synthesis of most serum proteins does not decrease; if anything, it rather increases in case of α1 and α2 globulins. Other studies [9] have confirmed the increased incorporation of ^{3}H phenylalanine into several proteins of the intact mouse from the 3rd tot the 5th day after exposure. Maximal incorporation occurs on the 4th day after irradiation. Recently, we have carried out an investigation into metabolism of tryptophan 24 hours after exposure to a dose of 1,000 *r* [10]. In these studies we also followed incorporation of ^{3}H-tryptophan (at

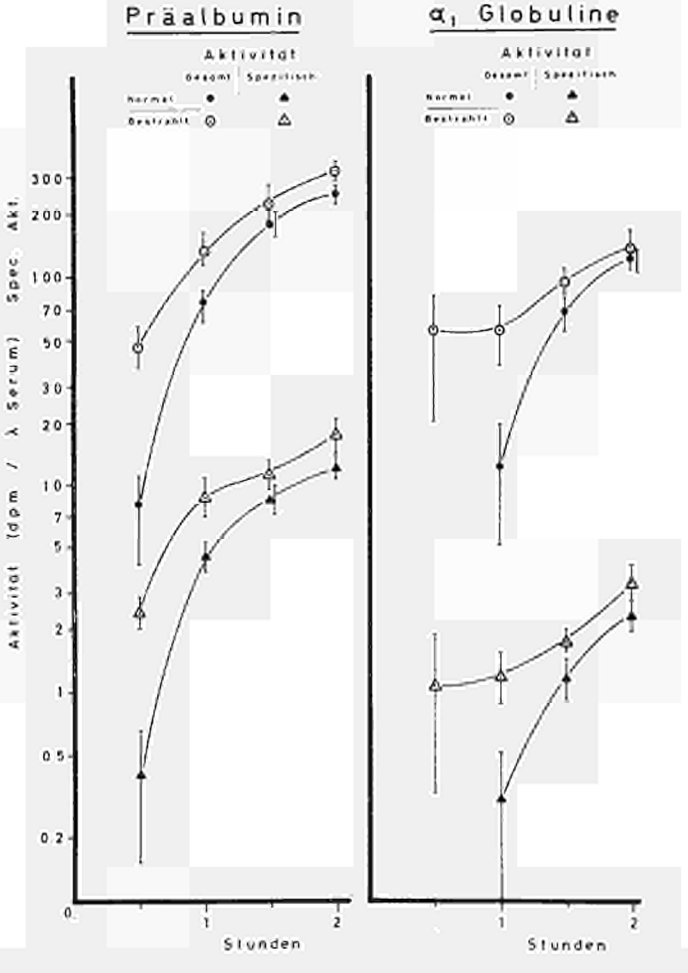

Figure 4. — Incorporation of ^{3}H-tryptophan (5μM) into prealbumin and α_1 globulins by normal and X-irradiated perfused liver

a dose level of 5 μM) into serum proteins [11]. The data (Figure 3) show that incorporation of tryptophan into total proteins by the irradiated liver is increased slightly. The same finding pertains also to some isolated serum protein fractions. The incorporation into prealbumin is shown as an example (Figure 4). It appears, however, that the rate of incorporation is changed more markedly than the final level of incorporation. The increase in incorporation of tryptophan may in part be due to the slight reduction in tryptophan catabolism of X-irradiated animals. Nevertheless, these experiments confirm that a decrease in synthesis is not the responsible factor for the radiation induced decrease in serum proteins.

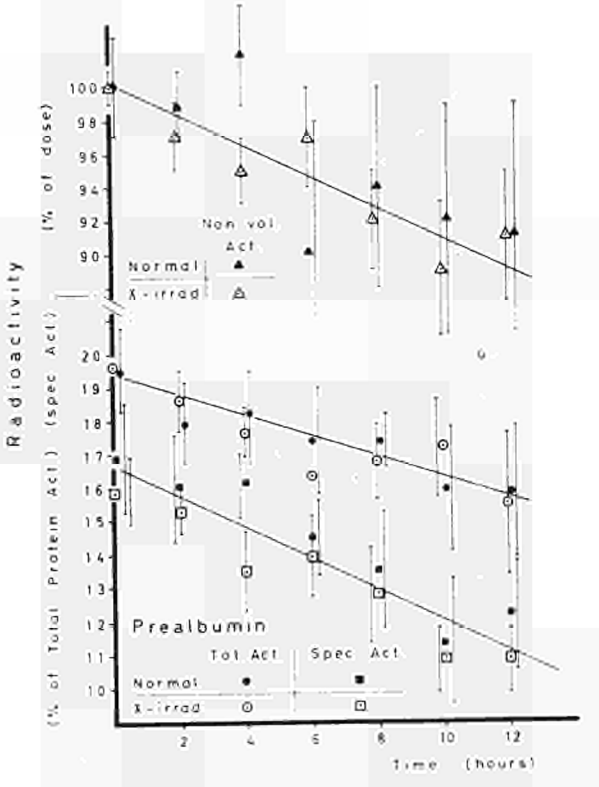

FIGURE 5. — Degradation of prealbumin in a mixture of serum proteins labeled with ^{3}H-phenylalanine by the perfused normal and X-irradiated liver (3 to 5 days after 950 r). Presented are total radioactivity in % of total proteins and specific activity in dpm/μg protein concentration. The upper part of the curve presents the decrease in nonvolatile radioactivity.

HEPATIC CATABOLISM.

Next hepatic catabolism of serum proteins labeled with ^{3}H-phenylalanine was investigated. Seven livers from X-irradiated rats 3 to 5 days after exposure to 850 r and 4 livers from normal rats were perfused with a mixture of serum proteins labeled with ^{3}H-phenylalanine for time periods up to 12 hours. Samples were taken

at two hours intervals and specific and total activity of the serum proteins as well as formation of volatile radioactivity (derived from degradation of liberated phenylalanine) was determined. We could not expect that serum proteins of relatively slow turnover, such as albumin, would show a noticeable catabolism during the 12 hour period of perfusion. However, fractions of more rapid turnover could have presented significant changes if hepatic catabolism was important. Furthermore, if the radiation induced decrease in concentration (up to 20 % per day) of a fraction like prealbumin were the result only of an accelerated hepatic catabolism, the alte-

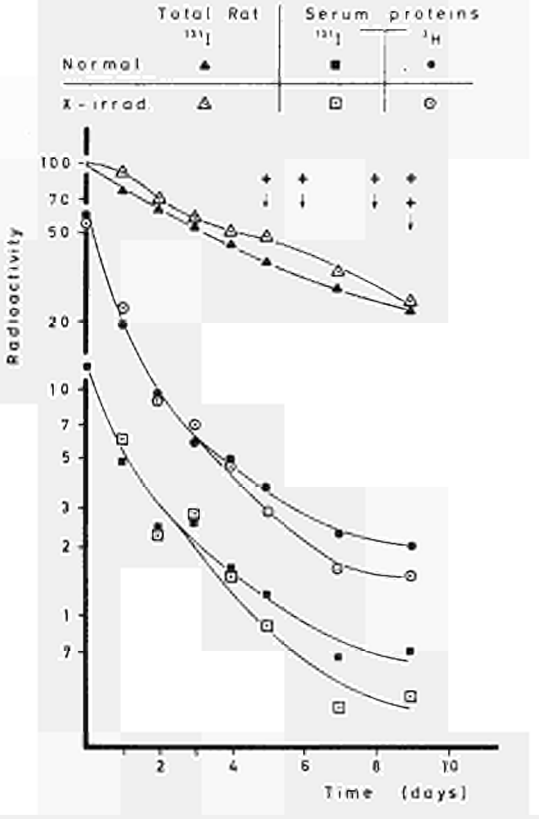

FIGURE 6. — ^{131}I activity in total rats and ^{131}I and ^{3}H-phenylalanine in serum proteins after an injection of a mixture of proteins labeled with ^{131}I and ^{3}H-phenylalanine. One group of rats received 950 r whole-body irradiation at time zero.

ration should have been recognizable. As the following Figure 5 shows, specific activity and total activity of prealbumin decrease significantly during the perfusion. The catabolism and turnover of prealbumin is in the order of 20 mg/day for a pool of 74 mg in the perfused system, but this calculation is naturally subjected to a large error (about 50 %). No significant difference exists between livers from normal and X-irradiated rats. On the same figure data on nonvolatile radioactivity

are presented. Formation of volatile activity due to degradation of liberated phenylalanine is in the order of 10 % per 24 hours. It is known from our previous experiments, that reutilisation of phenylalanine amounts to about 20 tot 30 %.

CATABOLISM OF SERUM PROTEINS IN THE TOTAL RAT.

In the following experiments we studied the catabolism of a mixture of ^{131}I and ^{3}H-phenylalanine labeled serum proteins (Figure 2) in the intact rat. Five rats were exposed to 800 r whole-body X-irradiation and injected intravenously with

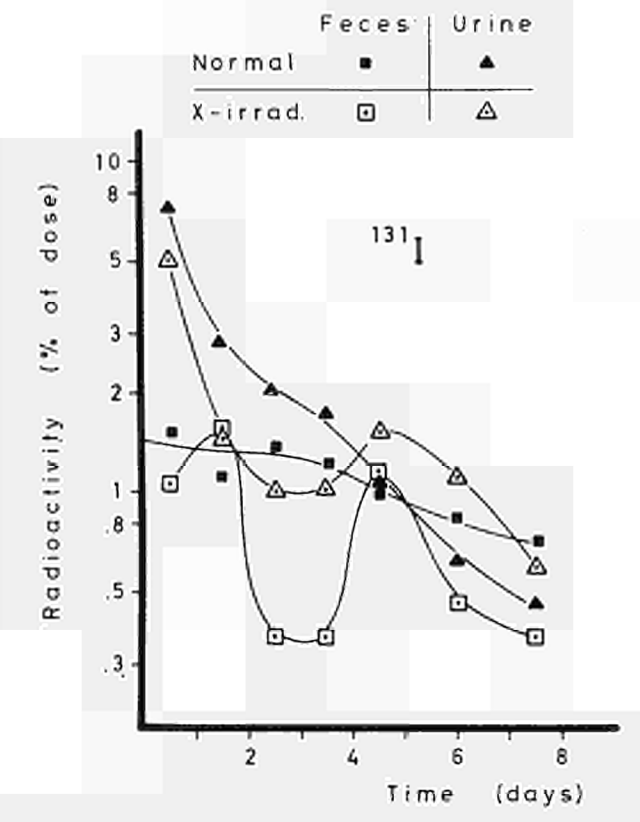

FIGURE 7. — Excretion into feces and urine of ^{131}I radioactivity. The experimental groups are the same as in Figure 6

3 ml of the labeled proteins immediately thereafter. Five other rats served as controls. Blood samples from the tail were taken 1 hour, 1, 2, 3, 4, 5, 7 and 9 days after injection. Urine and feces were collected separately; however, some mixing of urine and feces was unavoidable, especially during the period of diarrhoea of the irradiated rats.

Data on ^{131}I radioactivity of the total rat as well as of ^{3}H and ^{131}I activity of total serum proteins are shown in Figure 6. Radioactivity is retained slightly by the X-irradiated rats on the 2nd to 3rd day and again on the 4th-6th day after exposure. This retention is also reflected in the excretion pattern (Figure 7). No difference between normal and irradiated rats exists with respect to the ^{3}H of ^{131}I radioactivity of circulating total serum proteins up to the 4th day after exposure (Figure 6). Later, as the radiation disease enters its lethal stage, radioactivity in proteins of X-irradiated rats decreases more rapidly than in those of the controls.

A similar pattern is seen in the individual protein fractions. Prealbumin is shown as an example in Figure 8. The activity time curves for ^{3}H and ^{131}I-prealbumin are parallel and decrease after the initial period of mixing. At later times, catabolism becomes slower. The radioactivity of prealbumin from the X-irradiated animals drops suddenly on the 4th day after exposure but afterwards decreases with the same slope as in normal rats.

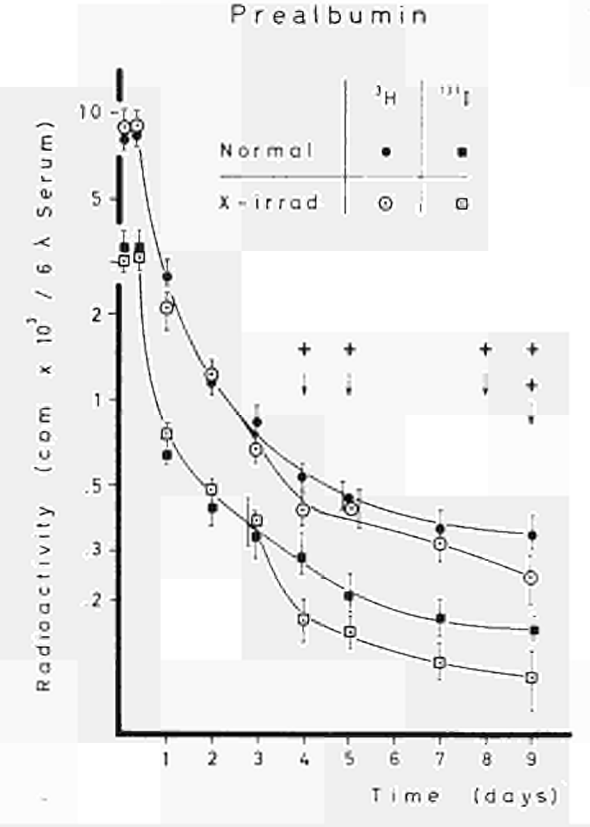

FIGURE 8. — ^{131}I and ^{3}H radioactivity of prealbumin in normal and X-irradiated rat. The experimental groups are the same as in Figure 6

CONCLUSIONS

Whereas our data are not yet complete and do not allow definite conclusions, they suggest that the decrease in certain serum proteins is due to a change in the intra- and extravascular distribution and not to a loss into urine or intestine. Such an explanation has been proposed already by Shaber [11] in the case of fibrinogen. It is conceivable that the different behaviour of the various protein fractions has its cause either in a differential behaviour during transit into the extravascular space or in a difference in adjustment of the *de novo* synthesis to the altered conditions.

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DISCUSSION

A. S. MACFARLANE (*London*) : On ground of expense tritiated proteins are likely to replace ^{14}C ones for tracer experiments in the future but using a variety of tritiated amino acids many people have not had very happy experiences. For instance, glycine labeled in the methyl group was poorly used for plasma protein synthesis and almost not at all for porphyrin synthesis. Even phenylalanine labeled with tritium in the benzene ring in our hands was not well used although I think that Gerber *et al.* have had most success with this amino end. A new specifically labeled lysine from Amersham which we have lately used gave the same incorporation into plasma protein as its ^{14}C counterpart and at one twentieth the cost. Dr. Gerber and his collaborators are to be congratulated on having prepared their proteins at 20 % efficiency with the help of the perfused liver whereas we get only 4-5 % incorporation into total plasma proteins in the intact animal. I think these proteins will find increasing use especially as comparison ones for iodine labeled proteins.

G. GERBER : In our experiments we have compared the turnover of the ^{131}I-labeled proteins with that of the ^{3}H-phenylalanine labeled proteins. In all protein fractions we found that the decrease of radioactivity was parallel in both cases. It should however be pointed out that due to the fact that a mixture of proteins was used which had to be separated by agar electrophoresis before counting, the accuracy of this determination is not very large.

J. COURSAGET (*Chairman*) : I would like to ask Dr. Gerber what was the irradiation dose in his experiment ?

G. GERBER : We studied incorporation of ^{3}H-tryptophan one day after 1000 r, degradation of phenylalanine labeled proteins in the perfused liver 3-5 days after exposure to 850 r and the catabolism of ^{131}I and ^{3}H-phenylalanine labeled proteins in the intact rat after 850 r.

J. COURSAGET : In experiments we made 15 years ago on irradiated rabbits, we noticed that for doses above 400 r there was a marked decrease in the speed of synthesis of antibodies labeled with ^{14}C-valine. Have you observed an effect of that sort ?

G. GERBER : Dr. Sassen and Dr. Reuter have studied incorporation of ^{3}H-phenylalanine into the globulins after exposure to 700 or 1000 r. They found that total radioactivity was slightly increased 4 days after exposure. However, due to the fact that the concentration of the globulins had decreased at this time, specific activity was considerably increased in comparison with normal mice.

STUDIES ON THE COMPOSITION OF CHROMIUM-51-LABELLED HUMAN SERUM ALBUMIN

R. HÖFER, H. SCHATZ and N. THUMB

2nd Medical University Clinic, Vienna, Austria

Abstract

Starting from observations on differences in metabolic behaviour of ^{131}I-labelled human serum albumin and ^{51}Cr-labelled human serum albumin or ^{131}I-labelled polyvinylpyrrolidone (PVP) the composition of ^{51}Cr-labelled human serum albumin was studied.

Gel filtration yielded three ultraviolet (uv) absorbing peaks and three radioactivity peaks. Radioactivity peaks I and II and uv.-absorption peaks I and II were located in identical fractions and were identified immunologically as albumin.

The findings suggest formation of (high specific activity) aggregates as a consequence of the labelling process.

The possibility is discussed that radioactivity peak III consists of complex Cr salts. Excretion and turnover patterns of ^{51}Cr-labelled human serum albumin are discussed in the light of these findings.

Introduction

Using ^{51}Cr-labelled HSA for the study of protein loosing enteropathia we made in accordance with other authors [1, 6, 9, 10] a number of observations typical for the metabolic behaviour of this tracer substance :

1) Fecal excretion values of ^{51}Cr-labelled HSA in normals were lower than those obtained with ^{131}I-labelled PVP.
2) Fecal excretion values of ^{51}Cr-labelled HSA in patients with gastrointestinal protein loss were higher than those obtained with ^{131}I-labelled PVP.
3) During the first 24 hours a relatively high percentage of the injected activity was excreted with the urine — the amount varied from preparation to preparation.
4) Using whole-body counting techniques the half-life of the dose retained is extremely long — 40 days and more,
5) The half-life of ^{51}Cr-labelled HSA in the plasma is shorter than that observed for iodinated albumin. It amounts to 8 days and less and varies with the preparation used.

Material and Methods

^{51}Cr-labelled HSA was obtained from the Radiochemical Division of "Farbwerke Hoechst", Frankfurt/Main, Germany. Commercial preparations as well as special preparations (varying specific activity with and without stabilizer) were investigated.

In addition to paper- and microagargelelectrophoresis [11] immunoelectrophoresis [7] followed by autoradiography, and agar gel precipitation [4], a number of gel filtration experiments were performed :

Sephadex G 25, G 150 and G 200 columns of varying size up to 750 × 30 mm were used and eluated with Trisbuffer pH 7.3, ionic strength 0.1 or 0.2. Fractions of 3 ml were collected and protein concentration was measured at 280 mμ using a Beckman DB-spectrophotometer. Radioactivity of the fractions was measured in a well type scintillation counter.

For analytical ultracentifugation a Spinco Model E at a speed of 59780 RPM was used.

Results

In paper- and microagargelelectrophoresis the ^{51}Cr-labelled HSA migrated in a single band in the albumin region. Using strip counting and autoradiography radioactivity could be detected in this band only. Immunoelectrophoresis (Figure 1) with antihuman serum of the preparation (upper well) gave a single precipitation line in the typical albumin region. There was, however, a slight but constant deformation of the line suggesting that the line consists of two confluing bows. ^{51}Cr human serum albumin incubated with normal human serum was placed in the lower well. Autoradiography showed that radioactivity was exclusively bound to albumin (Figure 2).

Analytical ultracentrifugation of the native preparation showed a main peak with a sedimentation rate of 4.3 S and a small 6.1 S peak (85 % and 15 % respec-

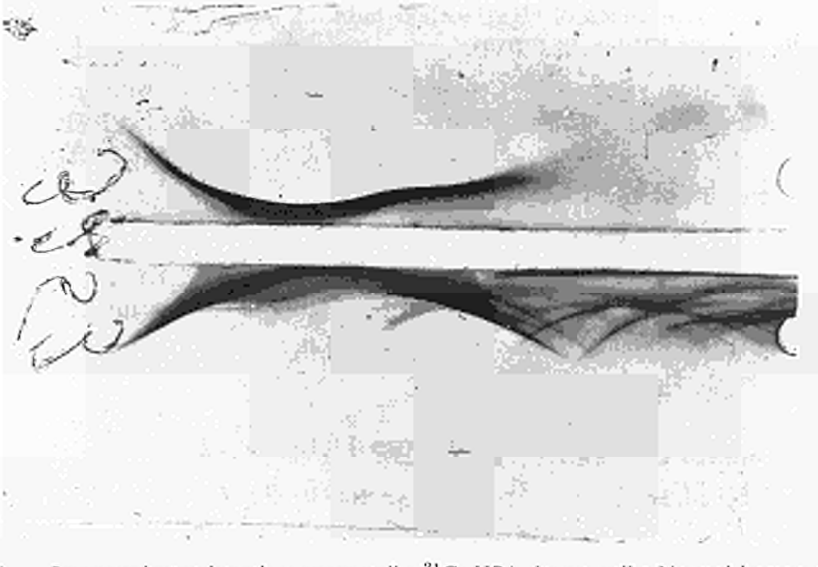

FIGURE 1. — Immunoelectrophoresis : upper well : ^{31}Cr-HSA, lower well : Normal human serum incubated with ^{51}Cr-HSA for 30 min at 37° C. Antihuman serum was used.

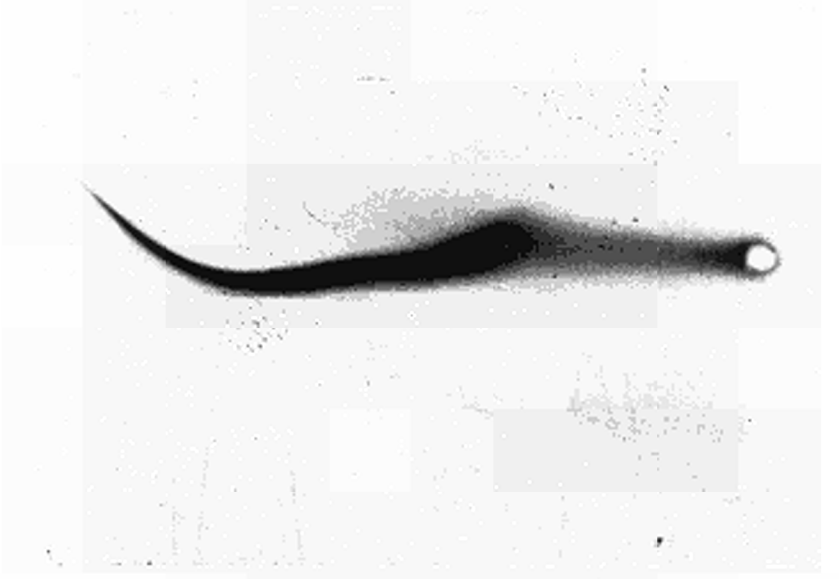

FIGURE 2. — Autoradiography of figure 1.

tively). Fractionation on Sephadex G 150 and G 200 yielded three protein peaks and three activity peaks (Figures 3, 4). The concentration-fraction curves for radioactivity and for uv.-absorption constantly showed peak I and peak II in corresponding positions. The relative concentration, however, of peak I : peak II was different for radioactivity and uv.-absorption,

The ratio of peak I to peak II was 1 : 5 for uv.-absorption and 1 : 2 for radioactivity. The third radioactivity peak appeared in earlier fractions than the third uv.-absorption peak. The third uv.-absorption peak corresponds to the PHB-ester which is added to the commercial preparation after the labelling procedure as could be shown by fractionation of PHB-free preparations under identical conditions. The third radioactivity peak appears during fractionation of PHB containing preparations as well as in PHB free preparations. Radioactivity peak III is absent when peak I and/or II are refractionated. It is, therefore, not due to an unspecific elution effect of the Sephadex column.

In the radioactivity peak III fractions of PHB free material no uv.-absorbing material can be detected. Furthermore, ninhydrin reaction was negative with these fractions, even when all fractions containing radioactivity peak III were concentrated up to 50 fold. Thus radioactivity peak III is neither labelled PHB nor labelled polypeptides nor labelled aminoacids. Further fractionation on Sephadex G 25 showed that radioactivity peak III is also not composed of simple chromium salts like chromate, since it appears in much earlier fractions than chromate. The individual protein peaks (uv.-absorption peaks I and II) were concentrated by vacuum dialysis and identified immunologically and by analytical ultracentrifugation. Peaks I and II were immunologically albumin and showed reaction of complete identity

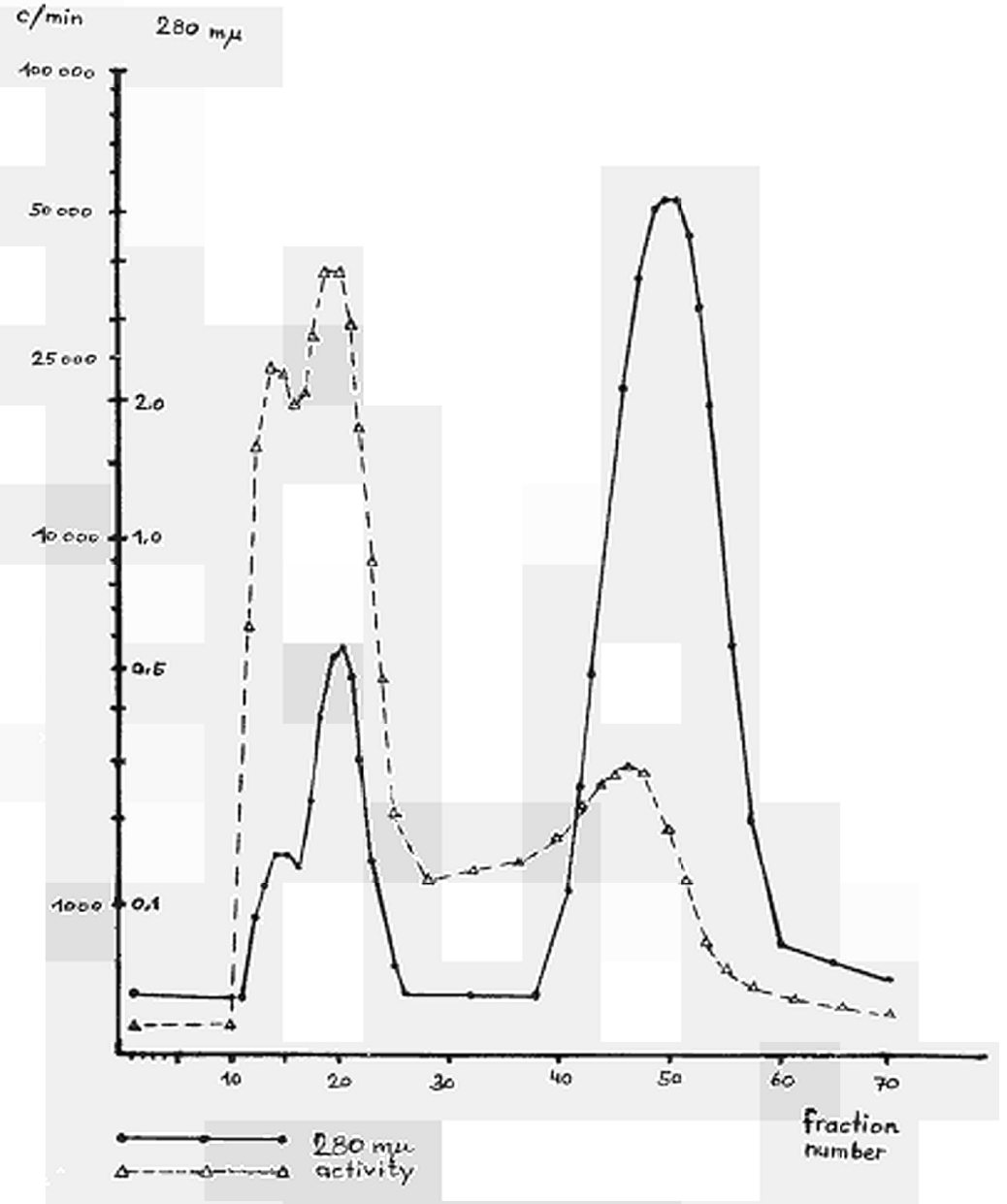

FIGURE 3. — Fractionation of ^{51}Cr-albumin on Sephadex G 150 column 450 × 25 mm, Trisbuffer pH 7.3; μ = 0.1; 3 ml fractions.

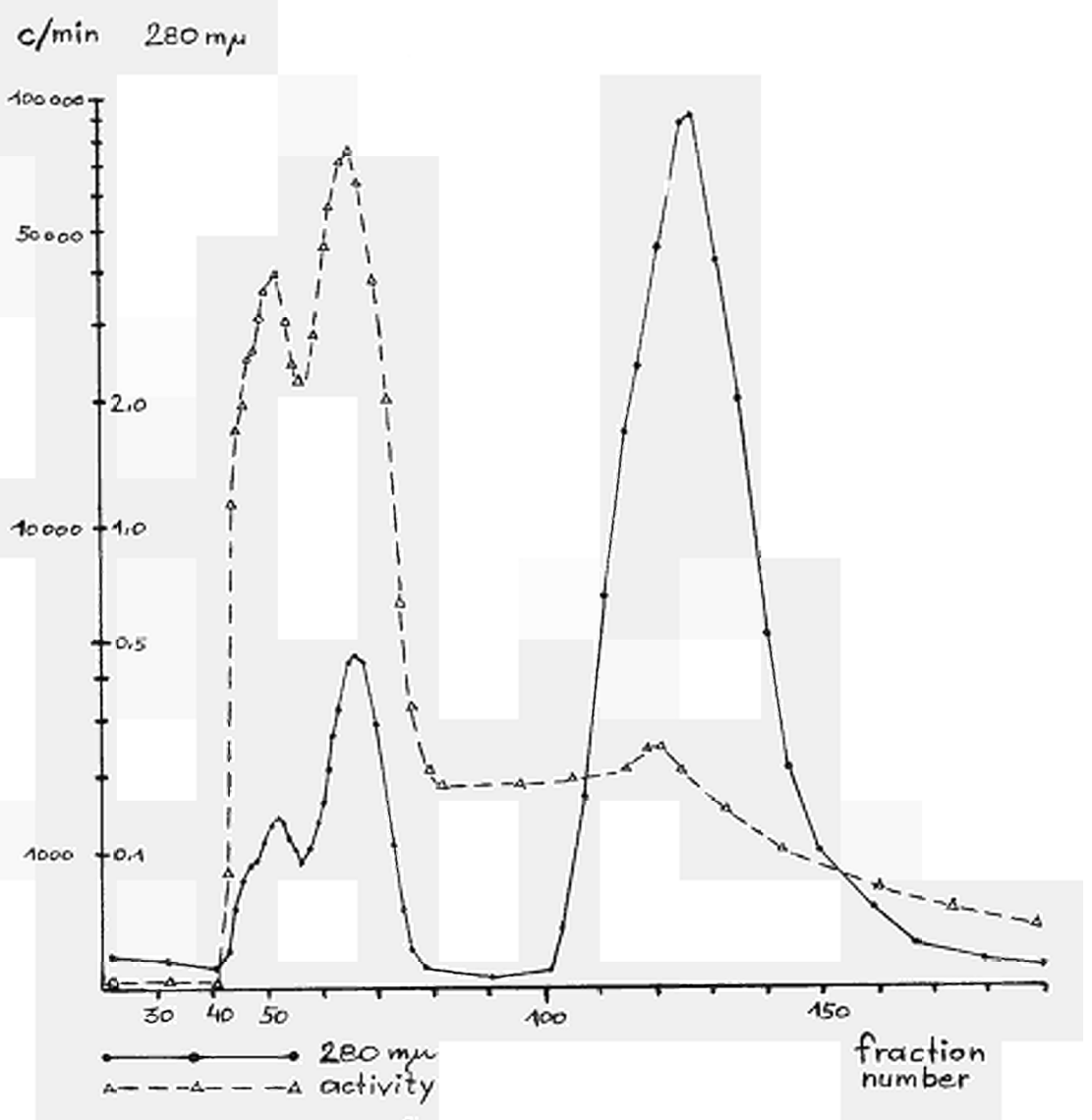

FIGURE 4. — Fractionation of ^{51}Cr-albumin on Sephadex G 200 column 750 × 30 mm, Trisbuffer pH 7.3; μ = 0.1; 3 ml fractions.

in the Ouchterlony plate. Anti human serum and specific antiserum against albumin were used. In analytical ultracentrifugation peak I showed two components, one with a sedimentation rate of 6.2 S and a second with 4.1 S. Peak II was 4.0 S material only.

Radioactivity in urine (Figure 5) was not precipitable with all conventional protein precipitants.

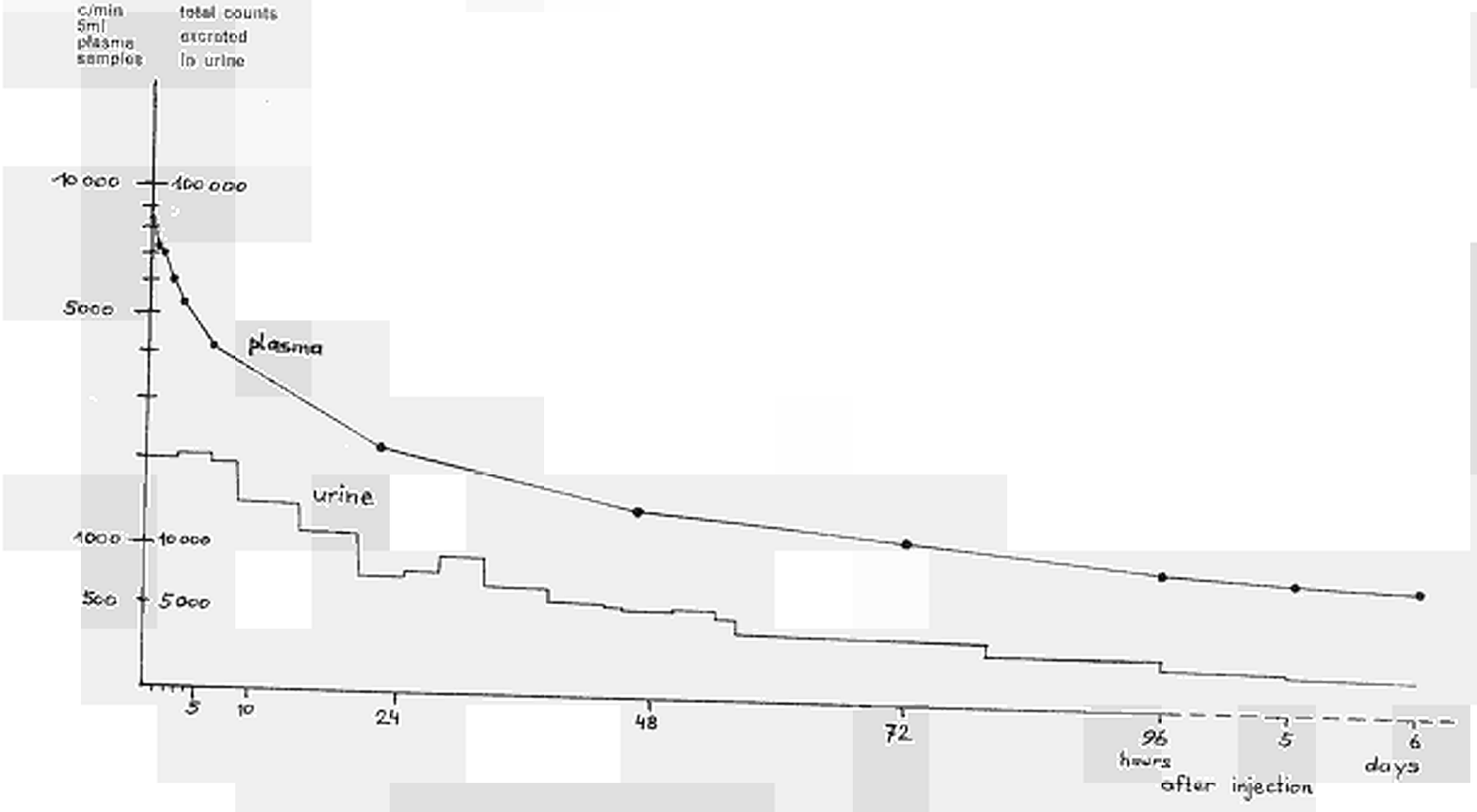

FIGURE 5. — Plasma radioactivity and urinary excretion of radioactivity in a healthy subject after intravenous injection of ^{51}Cr-HSA.

Discussion

It is relatively easy to explain the differences in fecal excretion values between ^{51}Cr-labelled HSA and ^{131}I-labelled PVP. The lower normal values of ^{51}Cr-labelled HSA are certainly due to the fact that the albumin molecules are much larger than the PVP molecules.

The higher fecal excretion values on the other hand found for ^{51}Cr-labelled albumin in pathological cases may sufficiently be explained by the longer half-life of chromium labelled HSA in the circulation as compared with radioiodine labelled PVP.

Schultze and Hughes [8] pointed out that the chromium label of ^{51}Cr-HSA is readily transferred to other proteins of tissue cells at the site of albumin break-down and they demonstrated this fact by autoradiographic studies. Such a label transfer must result in a prolonged apparent half-life of ^{51}Cr-labelled HSA when *whole body counting* techniques are used and when results are compared with those obtained with radioiodine labelled HSA where the label is readily excreted after break-down of the albumin molecule. The shorter half-life of ^{51}Cr-HSA in the *plasma* again in comparison with radioiodine labelled HSA must be attributed to some kind of denaturation of the albumin molecule which occurs in the course of the labelling procedure.

Studies we carried out so far show in accordance with other reports [3,5] that chromium labelling leads to formation of albumin aggregates with higher specific activity than the monomere [8]. We would suggest that these "polymere" fractions are more readily removed from circulation, thereby causing the observed shortening of *plasma half-life* of ^{51}Cr-HSA. The removal of the high specific activity polymere component from plasma would be followed by break-down and label transfer — this could explain the observation by Schultze and Hughes that chromated albumin *polymeres* show a longer half-life (*in the whole-body*) than monomeres.

The high urinary excretion of radioactivity during the first 24 hours following intravenous injection of chromated HSA remains to be explained. It is very improbable that it is due to a break-down of some parts of the chromated albumin since short period collections of urine after intravenous injection of ^{51}Cr-HSA show that the excretion of radioactivity starts and is highest within the first hour after injection (Figure 5). Furthermore, as pointed out above, break-down of part of the chromated substance should lead to an increased label transfer rather than to an increased urinary label excretion.

It can be seen from our fractionation experiments on Sephadex columns that the only non albumin fraction of our preparation is radioactivity-peak III which is, as already pointed out, not labelled protein, not labelled polypeptides, not labelled aminoacids and not a simple chromium salt and we have not yet been able to identify this unknown chromium compound. We would suggest, however, that this unknown compound is the one that is excreted during the first 24 hours in the urine since the percentage of radioactivity found in peak III is of the same order

of magnitude than the percentage of radioactivity excreted within the first 24 hours after injection and it is interesting to point out here, that Cohen [2] found complex chromium salts in the urine after intravenous injection of chromium labelled albumin and it could well be that our radioactivity peak III is composed of such complex chromium salts.

In summary, we would like to say that ^{51}Cr-HSA cannot be used for albumin turnover studies because of the formation of highly labelled aggregates, and because of label transfer after albumin break-down. On the other hand ^{51}Cr-HSA is a very valuable tracer substance for the study of gastrointestinal protein loss since it simulates much better physiological conditions than ^{131}I-labelled PVP. With the preparation on hand it is, however, not possible to obtain quantitative results since large aggregates of high specific activity are formed and since a portion of the tracer substance is present in an unknown form which is probably lost into the urine. Further *in vivo* experiments with the separated pure monomer fraction of chromated HSA going on now could possibly increase considerabely the value of studies of gastrointestinal protein loss with chromium labelled HSA.

ACKNOWLEDGEMENTS

We would like to thank Dr. E. H. Belcher from the International Atomic Energy Agency in Vienna for performing the whole body measurements with the whole body counter of the Agency's laboratory.

We would like to thank further the Radiochemical Division of Farbwerke Hoechst Frankfurt/Main for supplying us with the preparation of chromium labelled human serum albumin for our experiments.

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DISCUSSION

E. NIEMANN (*Frankfurt*) : In Ergänzung zu Dr. Höfers Ausführungen ist folgendes zu sagen :

1. Die in der Chromalbumin-Lösung enthaltene als Stabilisator bezeichnete Substanz dient nicht der Stabilisierung der Chromalbumins, sondern ist lediglich ein Bakteriostatikum.
2. Es ist sehr unwahrscheinlich, dass es sich bei der über Sephadex abgetrennten niedermolekularen Verbindung um eine Chromaminosäure handelt.
3. Versuche an Hunden haben gezeigt, dass ein geringer Prozentsatz der im Urin ausgeschiedenen ^{51}Cr-markierten Substanz Chromat ist.

Y. COHEN (*Paris*) : Le Dr. Höfer pourrait-il préciser la nature du stabilisateur qu'il emploie dans les préparations d'albumine humaine dites marquées au chrome-51 ?

E. NIEMANN : Es handelt sich um ein Gemisch von Methyl- und Propylester der p-Hydroxydbenzoesäure.

THE ROLE OF PLASMA TRANSFERRIN IN IRON METABOLISM AS STUDIED WITH ^{131}I-LABELLED TRANSFERRIN

S. VENTURA, U. SENIN, R. PALUMBO, A. DEL FAVERO and L. COLI

Istituto di Clinica medica generale e Terapia medica dell'Università di Perugia, Italy

ABSTRACT

The authors have investigated the rôle of plasma transferrin in iron metabolism by studying the intraorganic movements of ^{59}Fe-transferrin, ^{131}I-transferrin and ^{59}Fe-transferrin-^{131}I in normal persons and in patients with iron deficiency anemia and aplastic anemia.

The results of the investigations allow the authors to conclude that transferrin molecule does not show any attitude to direct the iron molecule in its intraorganic movements.

Up to now we derived our knowledge on the behaviour of iron metabolism in normal and pathological conditions, by injecting intravenously radioactive iron bound to transferrin; only if linked to transferrin, the iron follows its normal intraorganic pathway.

Iron kinetic studies have shown that the quota of the metal released from the daily hemoglobin breakdown and carried to the marrow erythroblastic tissue is the main physiological path of iron metabolism. Secondary pathways are represented by the mineral storage and intestinal absorption.

In all these intraorganic movements, the transferrin molecule plays a very important rôle, as iron can move inside the body and reach the cells where it is utilized, only if linked with the transferrin molecule.

This property of the transferrin depends upon its iron-binding capacity.

As 80 % of plasma iron transferrin is carried, in normal conditions, to the erythropoietic marrow, the problem arises if such affinity is conditioned by a peculiar property of transferrin to direct iron toward this tissue.

Our researches try to reply to such a question.

Crystalline transferrin was prepared from Cohn fraction IV-4 by the method of Inman and co-workers; physio-chemical analysis indicated that this protein was pure at a high degree (at least 95 %). Transferrin was labelled with radioactive iodine.

Radioactive transferrin, either free of iron or saturated with radioactive iron, was injected intravenously to normal and anemic subjects.

A first study gave evidence, according to previous results of Katz and Away and Brown, that iodination does not produce any detectable alteration on the physiological behaviour of the transferrin molecule.

In fact in our experiments, ^{59}Fe bound to iodinated transferrin and injected intravenously to normal subjects leaves the plasma, reaches the bone marrow and

then comes back to the blood as radioactive hemoglobin, in the same manner as does ^{59}Fe bound to normal transferrin. The same result may be obtained in iron deficient patients, in which ^{59}Fe linked to iodinated transferrin shows the same behaviour than ^{59}Fe bound to normal transferrin. In conclusion, iodination does not modify the biological properties of the transferrin molecule.

In a new series of experiments we investigated the transferrin metabolism in normal and anemic patients, examining its plasmatic behaviour, its intraorganic movements and, finally, its urinary excretion. This metabolic study of the transferrin molecule was always carried out together with an analysis of the behaviour of ^{59}Fe bound to the iodinated transferrin. In the normal subject (Figure 1), the intravenous injection of transferrin labelled with ^{59}Fe and ^{131}I is followed, during the first hours, by a linear disappearance of the radioactivity due to ^{59}Fe and to iodinated transferrin. The initial exponential rate of decrease of radioactivity is much higher for iron (T/2 = 1.30 hours) than for transferrin (T/2 = 15.30 hours).

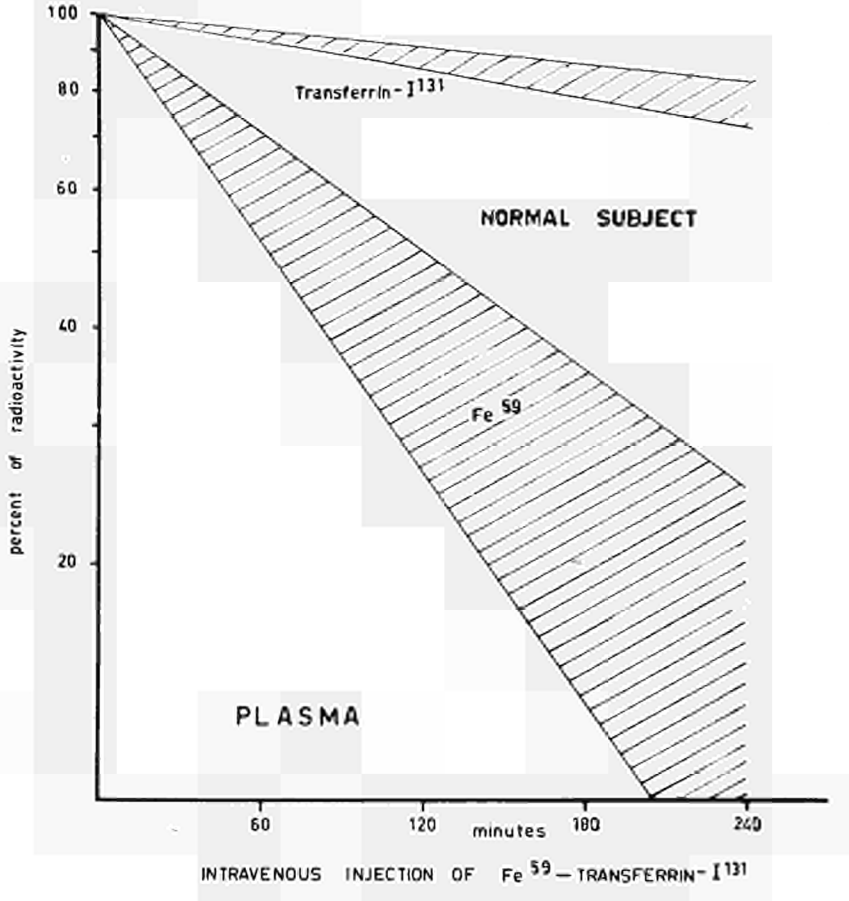

Figure 1

In the following days (Figure 2), the exponential rate of disappearance of radioactivity undergoes a modification. The first exponential rate of ^{59}Fe, which is related to the passage of plasma iron to the erythropoietic tissue, is followed by a second one, much slower, which, according to the iron-kinetic mathematical model of Pollycove and Mortimer, depends upon the re-entry of iron into plasma from the erythropoietic labile pool iron.

On the other hand radioactive transferrin decreases in a curvilinear manner during the first days and such a behaviour may be related to equilibration of transferrin in various body compartments. Plasma disappearance rate of transferrin assumes, during the following days, a much slower and linear behaviour; this exponential component of iodinated transferrin disappearance has a half-time of 8.7 days and is probably related to the daily catabolic rate of transferrin. The urinary excretion of radioactive iodine, which is released from transferrin molecule and

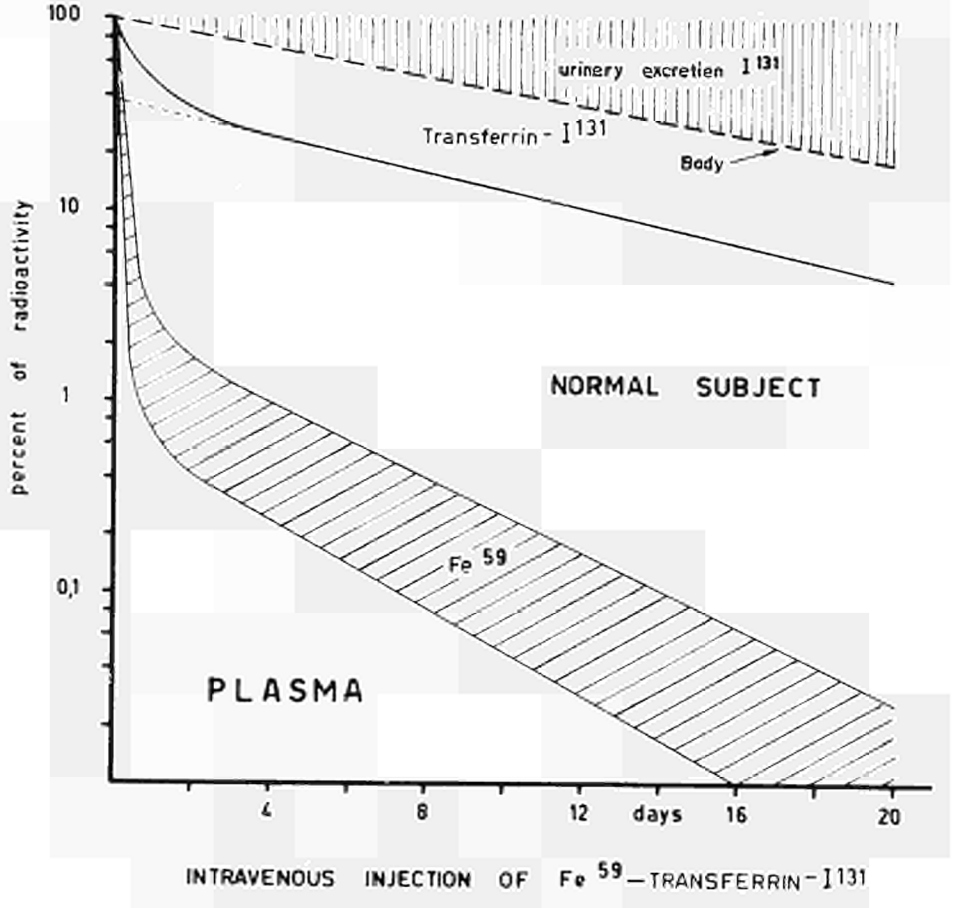

Figure 2

excreted almost entirely in the urines, if uptake by the thyroid gland is blocked, has to be considered as an expression of the transferrin degradation. From such a behaviour it derives a proportionality between plasma and tissue transferrin.

In iron deficient patients (Figure 3), the intravenous injection of ^{59}Fe bound to iodinated transferrin, shows the following behaviour : ^{59}Fe leaves the plasma more rapidly than normal, as it usually does in such an iron disease. Transferrin molecule, on the contrary, does not show any significant modification from its behaviour in normal subjects.

In the opposite iron pathological condition : namely the aplastic anemia, ^{59}Fe is retained in the plasma, while iodinated transferrin follows its normal plasmatic behaviour (Figure 4). We may so arrive to the conclusion that plasma disappearance rate of iodinated transferrin is not affected by the state of iron metabolism.

It is possible to get the same conclusion by studying the intraorganic movements of radioactive transferrin; this study is performed by detecting the gamma-ray

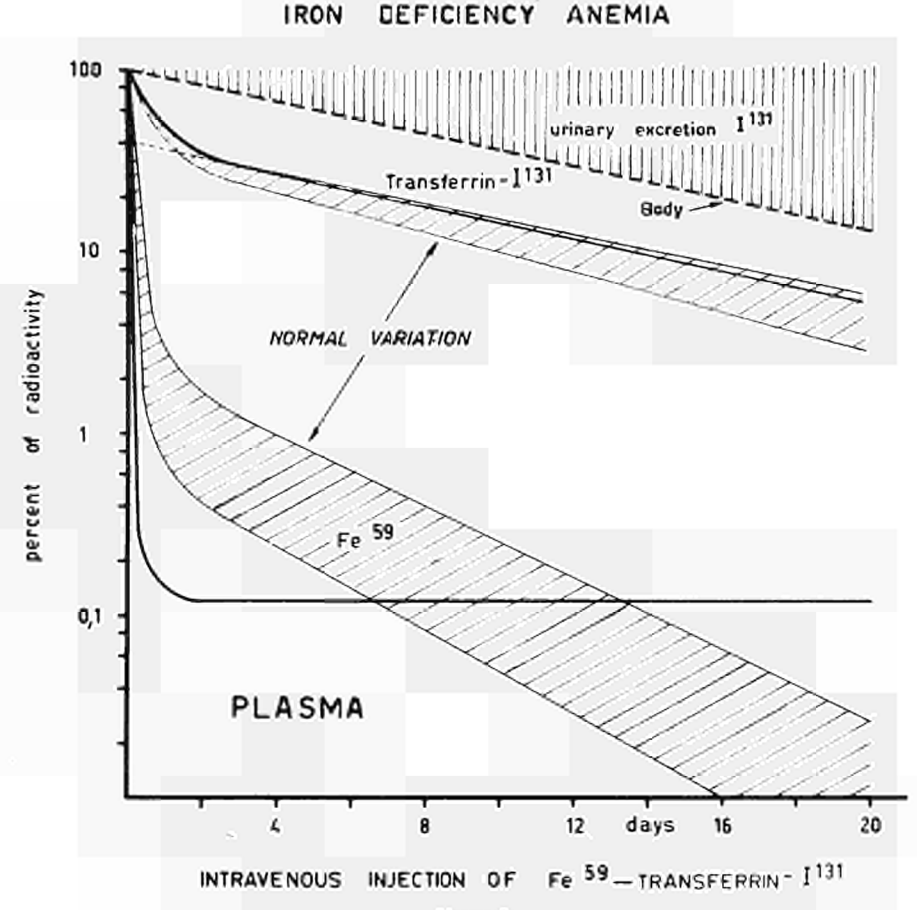

Figure 3

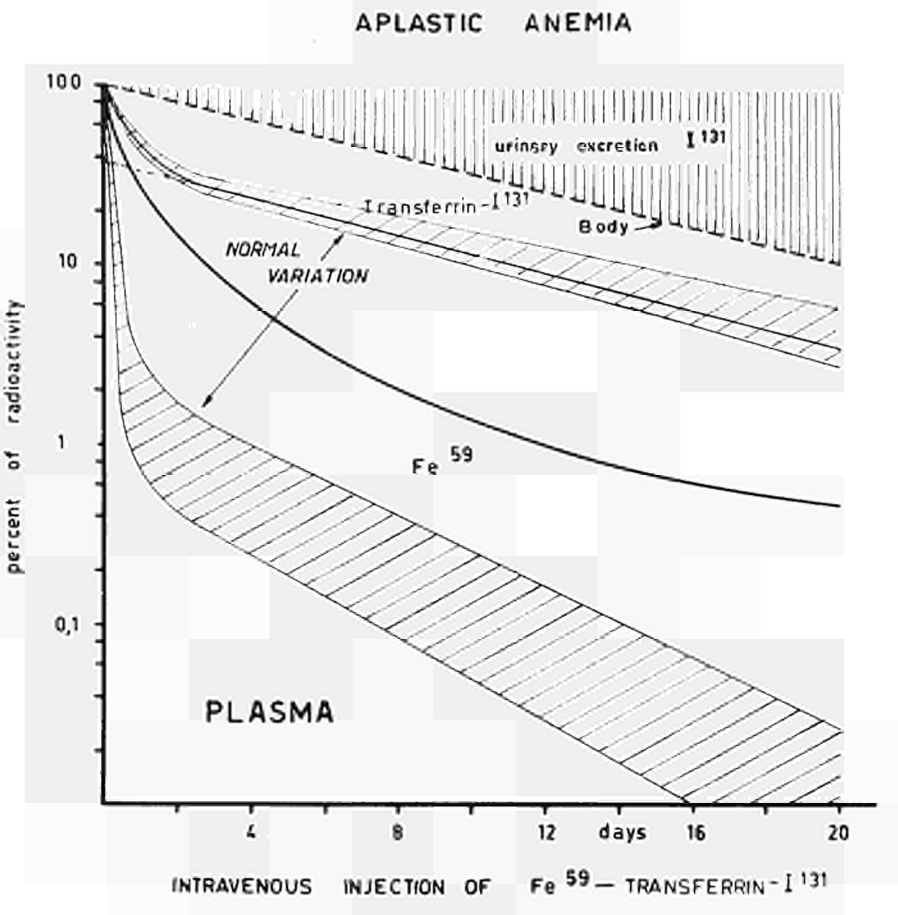

Figure 4

emission by body surface measurements over some organs directly related to iron metabolism : the sacrum (bone marrow), the liver (iron stores) and the spleen. Here we deal only with the radioactivity behaviour over the sacrum. Such a tissue, normally rich in bone marrow and erythroblasts, usually shows a selective accumulation of iron which leaves the plasma. If we now take into consideration the sacrum behaviour of the radioactivity after intravenous injection in normal subjects of ^{59}Fe bound to ^{131}I-transferrin (Figure 5), we can see that ^{59}Fe undergoes its normal selective accumulation followed in the subsequent days by a release into the blood as radioactive hemoglobin. On the contrary iodinated transferrin does not show any specific accumulation, but its initial radioactivity decreases gradually during the following days.

In iron deficiency anemia (Figure 6), the hyperplasia of erythropoietic tissue induces a rapid accumulation of radio-iron in the marrow, but does not affect the transferrin behaviour.

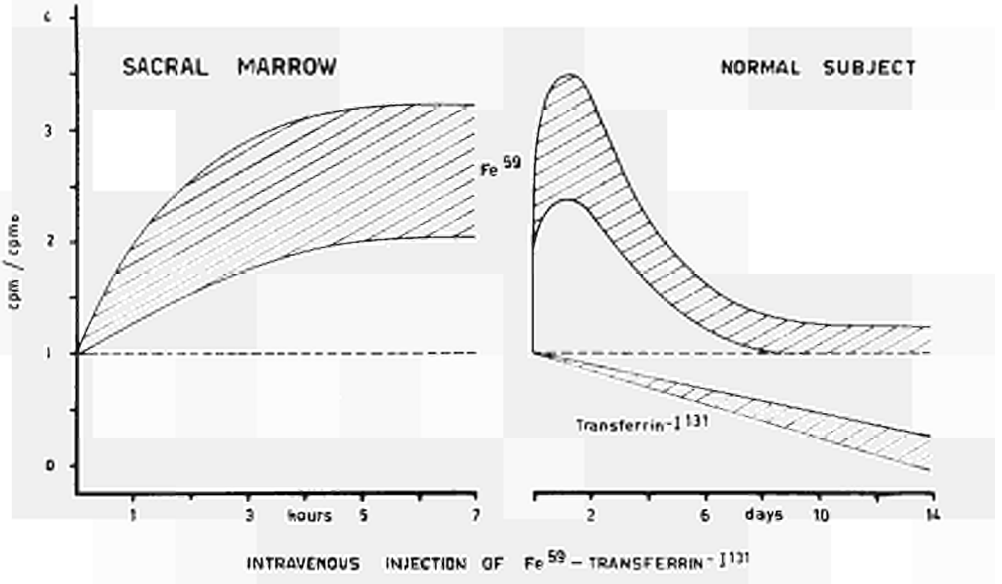

Figure 5

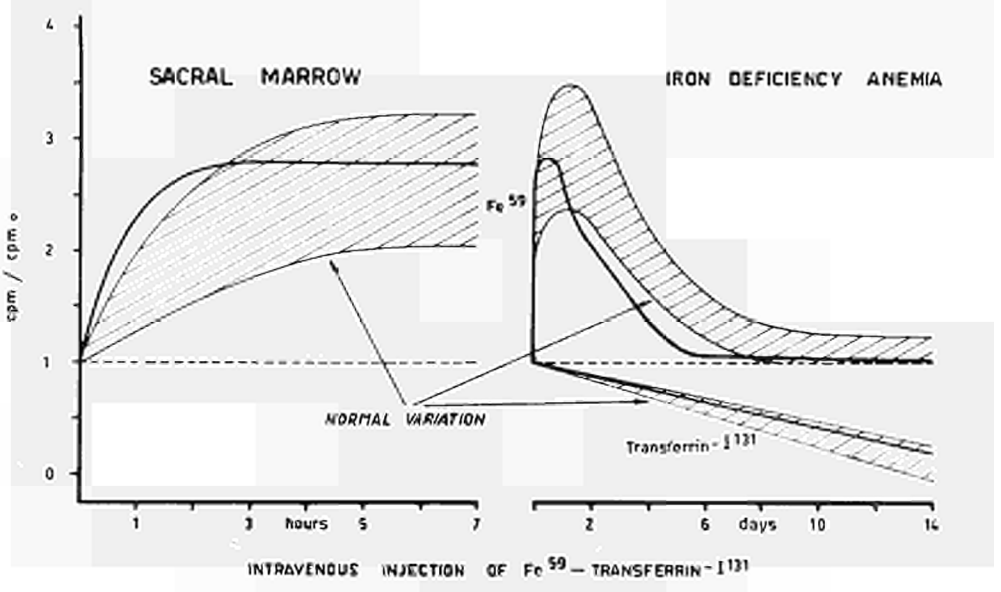

Figure 6

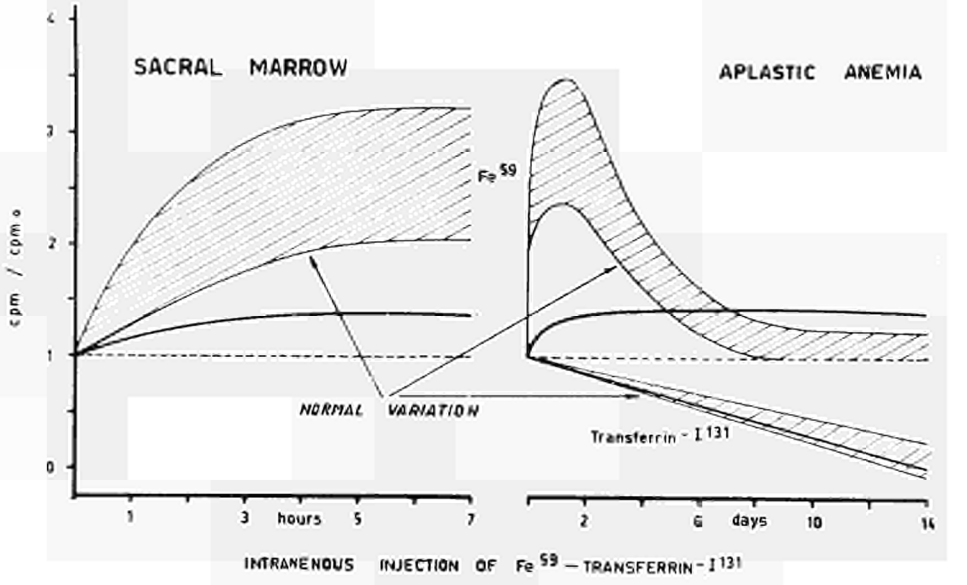

Figure 7

In aplastic anemia the lack of erythropoietic tissue in the bone marrow gives reason of the very low passage of iron from plasma to sacrum while the iodinated transferrin behaves as in normal (Figure 7).

The final conclusion is that the transferrin molecule does not show any sensibility towards the intraorganic state of iron metabolism. This plasmatic protein behaves in a constant manner whatever may be the erythropoietic activity of the subject. So the transferrin molecule seems to possess only its iron binding capacity and our experiments have failed to show any attitude to direct the iron molecule in its intraorganic movements.

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ANALOG COMPUTER STUDY OF THE KINETICS OF EXTRAVASCULAR DISTRIBUTION OF ^{131}I LABELLED PLASMA PROTEINS IN NORMAL AND TUMORAL TISSUES (*)

N. RICCIONI, M. F. BECCHINI, F. VITEK and L. DONATO

Clinica Medica Generale dell'Universita' di Pisa

Centro di Medicina Nucleare, Pisa, Italy

ABSTRACT

An attempt has been made to interpret the time course of radioactivity in extravascular space following injection of human serum albumin (RIHSA) and fibrinogen (RIHF), both labelled with ^{131}I, in normal tissues, inflammatory lesions and tumors.

An analog computer has been used to simulate the observed behaviour, on the basis of compartment models. Extravascular activity curves observed in any case with RIHSA and in normal and inflammatory tissues with RIHF can be simulated on the basis of compartmental model with constant exchange rates. On the contrary the extravascular activity curves observed with RIHF in tumors cannot be accounted for by such a model with all constant rates of exchange. The compatibility of simulated curves with those in tumors has been achieved by decreasing feedback from extravascular spaces to plasma for a certain time after which it retained constant lower value. The relative implications are discussed.

In recent years, using ^{131}I-labelled preparation of plasma Cohn fraction I, containing about 65 % of radioactivity in fibrinogen and 35 % in albumin, and measuring the time course of the radioactivity over inflammatory lesions tumors, Monasterio *et al.* have shown that the pattern of the curves representing the extravascular radioactivity in these two types of lesions present significant differences, which have been proved to be of diagnostic value [1, 2, 3]. An example is shown in Figure 1.

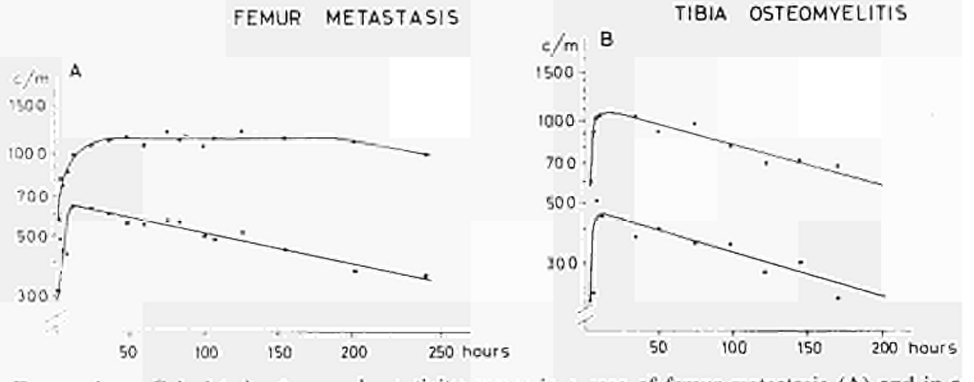

FIGURE 1. — Calculated extravascular activity curves in a case of femur metastasis (A) and in a case of tibia osteomyelitis (B) after administration of ^{131}I-labelled Cohn fraction 1; upper curves — lesion, lower curves — normal opposite side.

(*) Work performed as part of the program of the Association Contract Euratom-ULB-Università di Pisa No. 026-4-63 BIAC.

All the measurements are performed by external counting under standardized conditions, and the curves over a healthy symmetrical region are regularly recorded for purpose of comparison : in the inflammatory process the EV radioactivity rises rapidly to a peak value higher than that over the healthy reference region and it starts then to decrease regularly with a pattern that simply resembles a magnification of the normal behaviour. On the other hand, over the tumors, after a similar ascending part, the counting rate tends to level off for variable length of time, after which it starts to drop regularly. This pattern seems to be due to the fibrinogen moiety of the preparation, since it has been shown to be prevented by heparin treatment [4].

The aim of this communication is that of reporting on the attempts that we have made to interpret the extravascular activity curves in inflammatory processes and tumors using an analog computer for analysis and simulation of the curves obtained by successively injecting into the same subjects the Cohn fraction 1, high purity albumin, or high purity fibrinogen, all labelled with ^{131}I.

Curves Examined

The material for analysis and simulation was represented by the curves obtained in 5 subjects, four with bone tumors of various types and one with tarsus osteitis.

The Cohn fraction 1 curves were obtained in each case and they all showed the typical pattern. In three tumor cases and in the case with the inflammatory process, ^{131}I-HSA labelled with the same technique was subsequently injected, and curves were recorded over the lesion and symmetrical healthy region. In the fourth tumor case, a high purity preparation of fibrinogen (95 % pure) labelled with ^{131}I was injected.

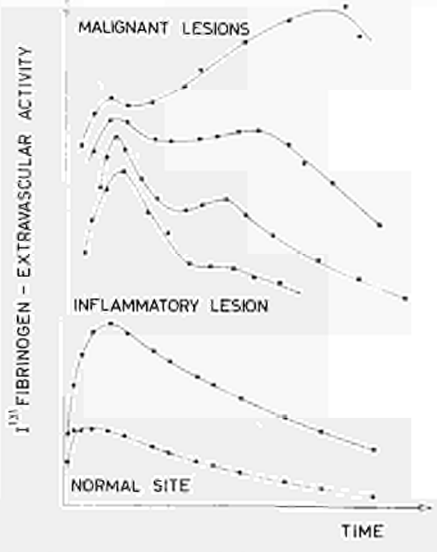

Figure 2. — Calculated extravascular activity curves after administration of RIHF.

The curves obtained with ^{131}I-HSA were used to correct by subtraction the Cohn fraction 1 curves after suitable normalization for injected activity, and knowing the percentage of albumin in the preparation.

Pure fibrinogen curves were thus obtained, and attempts were made to simulate them with an analog computer.

Figure 2 shows the curves obtained : they have a peculiar pattern, particularly in tumors. All four fibrinogen curves show an obvious inversion in their trend after the peak concentration is reached, and decrease is started; the new rise varying from minimal to a new peak even overcoming the first one.

In the examined case of inflammatory process this pattern is absent, but it is obvious that the radioactivity over the healthy side drops more rapidly.

Computer Simulation

Intravascular activity curves, available in all cases, were submitted to multi-exponential analysis so that they could be simulated on the computer : a sum of two exponentials was sufficient in every case. Intravascular activity was assumed to be the specific precursor of EV activity, and not to be influenced by feedback of activity from the lesion.

The models with which the simulation attempts were made are shown in the next figures.

The simplest model is that in which K_1 and K_2 are the fractional rates at which the activity is transferred from the intra- to an extravascular compartment and vice-versa (see Figure 3). This simple model offered no difficulties in fitting the fibrinogen

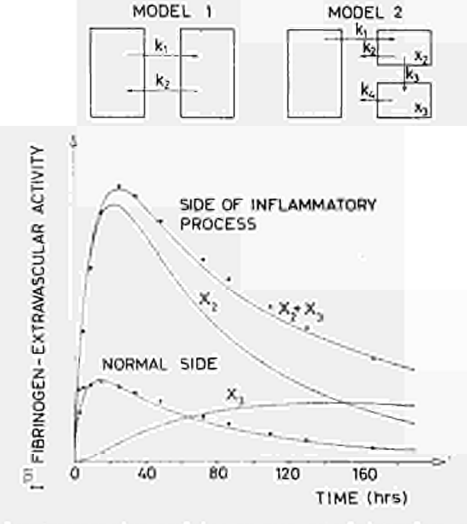

Figure 3. — Calculated extravascular activity curves administration of RIHF in inflammatory process. Points : experimental values; continuous line : fitting by an analog computer. Simulation of normal side is based on model 1; simulation of inflammatory process on model 2.

curves over healthy region. It was not possible to simulate the curves of the inflammatory process, neither (obviously) those of the tumors, by this simple model.

In the next step it was assumed that from the first EV compartment a fraction of activity K_3 were transferred to a second EV compartment, and that K_4 were the rate at which activity from this latter compartment was fed back to blood.

This model proved adequate to describe the time course of activity over inflammatory process, but not in tumors.

To simulate the curves in tumors, one had to device a model such that, at a time at which feedback from EV to blood already exceedes activity transfer in the opposite direction, an inversion of this trend could occur, resulting in a new rise, of more or less long duration and extent.

In presence of a progressively decreasing plasma activity, one of the possible interpretations is that the new rise be due to a reduction of the activity fed back to blood from EV. On the other hand, this interpretation has to be in keeping with the assumption of steady state conditions.

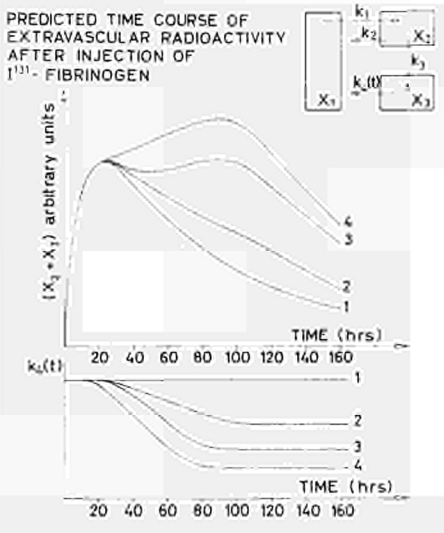

FIGURE 4. — The effect of the value of the coefficient $K_4(t)$ on the shape of simulated curves.

After various attempts it was shown that, with reference to the two EV compartments model, the possibility of fitting the tumor curves was that in which K_4 was progressively reduced from its value at 0 time to a new lower value to be reached and maintained after a variable time.

The effect of varying the extent of the reduction of K_4, and its duration, is shown in the Figure 4. As one can see all the various patterns observed in tumors can be easily reproduced, and that in the inflammatory process as well.

An example of the agreement of predicted with experimental curve is shown in the Figure 5.

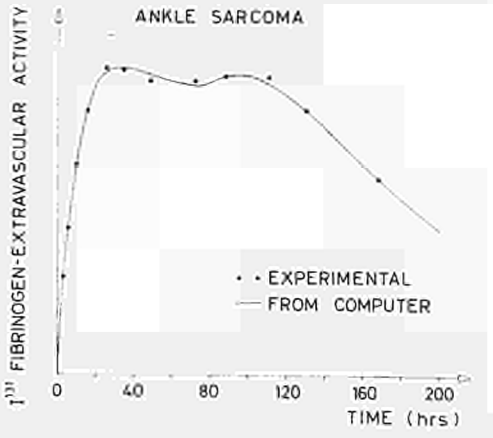

FIGURE 5. — Comparison of measured RIHF — extravascular activity in Ankle sarcoma with the curve obtained from an analog computer.

DISCUSSION

The problem is now that of examining the possible physical justification of such a model, and its compatibility with steady state assumptions.

An interpretation can be offered if one takes in mind that : *a*) more or less important amounts of fibrin are present in tumors tissue; *b*) heparin treatment prevents the occurrence of the typical tumor pattern after injection of the labelled protein.

The model could be thought to result from a physical situation in which the second EV compartment is actually made up of fibrinogen and fibrin, and in which the gelification of fibrinogen into fibrin is not an instantaneous process, attaining equilibrium from 0 time. This could result both from the type of the reaction involved, or from the structures of the extravascular spaces in tumors, in which the contact between diffused fibrinogen and thrombin like enzymes may not be immediate. This latter assumption could be accounted for by uneven distribution of poor mixing of the indicator in this compartment.

This hypothesis offers no difficulties in explaining the curves. Since the type of measurement performed here, the shape of the EV curve is determined by the balance between total input and total output to and from EV space, the time course of the activity in tumors could be explained assuming that activity can return from EV space to blood by true feedback of fibrinogen or lysis of fibrin. If this latter process is slower in rate than the first one, which is quite acceptable, and the gelification of fibrinogen is not instantaneous, the overall rate of feedback of activity will initially be that due to exchange of fibrinogen, but it will then progressively decrease, as more and more ^{131}I-fibrinogen is converted into fibrin, to attain finally an equilibrium state in which the rate of feedback becomes constant because the indicator has attained equilibrium of distribution; the value of this final rate will actually and obviously

depend on the fraction of fibrinogen in fibrin form in the compartment two and on the relative rates of feedback of fibrinogen and fibrinolysis.

In this type of model the subdivision of the extravascular system into two compartments is unnecessary since exactly the same result with different constant could be obtained with a single EV compartment.

At the light of this hypothesis which can explain the time course of EV activity in tumors and inflammatory tissues, the difference between the two types of process could be accounted for by :

a) smaller amount of fibrin in inflammatory tissues in respect to tumors.

b) rate of fibrinolysis in inflammatory tissues greater that in tumors, and therefore closer to the rate of feedback to blood of ungelified fibrinogen.

c) faster diffusion of fibrinogen in the EV space of inflammatory tissues in comparison to tumors, or better mixing conditions in the former in comparison with the latter.

It is obvious that any combination of the above three conditions may be responsible for the observed differences.

The validity of common model capable to justify the behaviour observed both in tumors and inflammatory process is supported by some recent findings. The use of antifibrinolytic agents in inflammatory process tends to produce the typical tumor pattern [5].

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DISCUSSION

C. M. E. MATTHEWS (*London*) : Have you worked out the details of a model for the unlabeled fibrinogen in a steady state, which would lead to this kind of effect of variation of rate constant with time, or are you just saying qualitatively that a delay in this pool would give rise to such an effect ?

F. VITEK : We do not study the model of fibrinogen kinetics under normal conditions. The model described enables only qualitative description of the system. Of course, if it would be possible to separate extravascular impulse-rate curve in two parts, one of which could describe the pure fibrinogen only, another mathematical approach could be used and, for example, the determination of the life time of a new substance formed should be possible. But the separation of the impulse-rate curve measured above the tumor region into these two parts is not possible.

J. COURSAGET (*Chairman*) : If you allow me a last remark, I would like to say that one of the major conclusions we may draw from this session is that the fate of labeled plasma proteins is of a major interest to a great variety of scientists which belong to disciplines as different as chemistry, physiology, immunology, pathology, even radiobiology. It seems that further progresses will be largely dependent on the degree of cooperation which can be achieved between those who are preparing very pure reliable labeled plasma proteins and those who are using them for biological or chemical investigations.

LABELED PROTEIC HORMONES FOR METABOLIC STUDIES

Chairman : E. F. PFEIFFER

Abteilung für Klinische Endokrinologie, I. Medizinische Universitätsklinik, Frankfurt/Main, Deutschland

PRELIMINARY REMARKS BY THE CHAIRMAN ON

Lack of correlation between immunologic neutralization of biologic action and antigenic reactivity of certain proteic hormones (insulin, ACTH)

I take it as a great honour and privilege to chair this symposium on « Labeled proteic hormones for metabolic studies" in the course of which so many eminent scientists from different countries will give their views on problems, of our mutual interest and concern. The first four papers should deal critically with the use of labeled proteic hormones in radioimmunoassays. Evidently, the general technical precepts as uniform binding to the protein without changing its physical, chemical, immunologic or biologic properties should be even better preserved in the labeling of hormones than with regard to other proteins. Many examples are known where alterations of a proteic hormone were ensued by diminishing and even abolishing its biologic activity without changing its immunologic reactivity and vice versa. Handling of ACTH containing solutions for more than 20 minutes already suffices

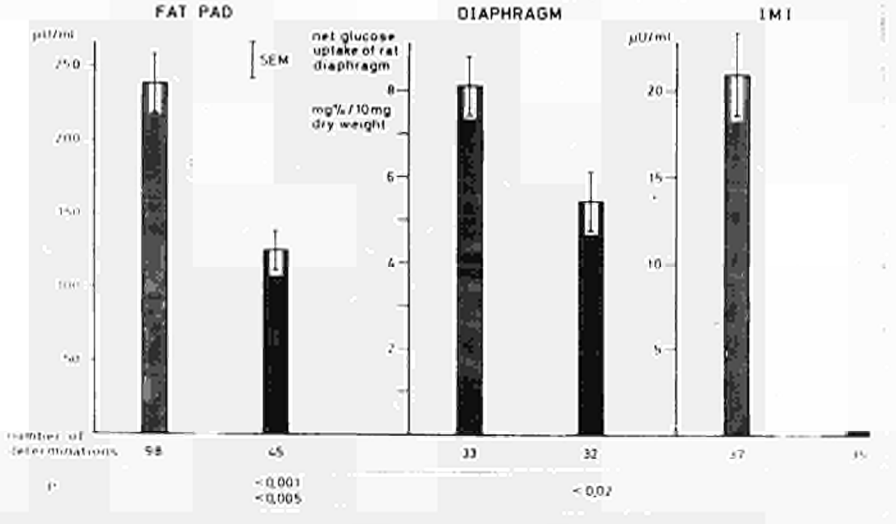

Figure 1. — Effect of sera of normal and Houssay dogs on isolated fat pads and diaphragm in comparison with immunologically measurable insulin (IMI).

for destroying its steroidogenic capacity. How sure can we be that denaturated or biologically inactive hormones are not included in immunoassay values? It is one of the purposes of the first and even more of the second part of our symposium to determine, as good as possible, the reasons for the discrepancies existing between bioassay and immunoassay values for certain hormones. I hope that speakers and discussors will come back on that point once and again. Fortunately, our main concern will be the 3 polypeptide hormones of small molecular size, ACTH, glucagon, and insulin, leading the more critical problems connected with the HGH and the parathormone to a special round table conference.

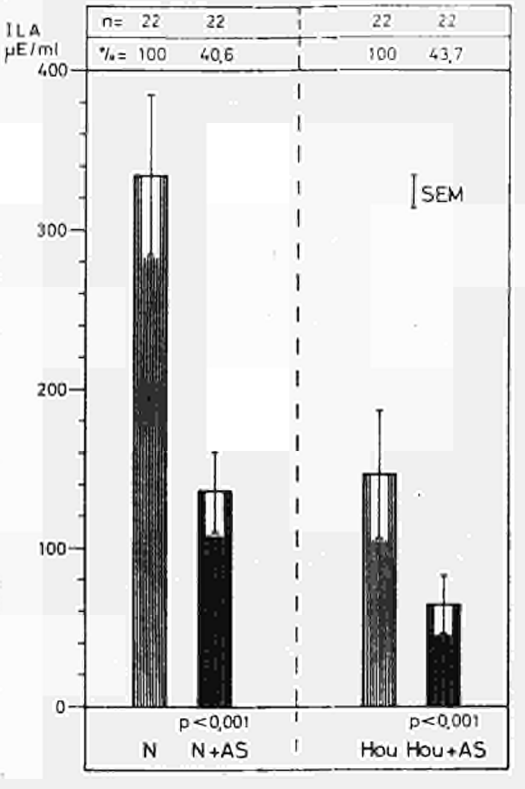

FIGURE 2. — Inhibition of ILA (fat pad) of sera from 22 normal and Houssay-dogs by guinea-pig anti-insulin serum

You will permit me to briefly pick out one, I feel, hot point, i.e. the lack of correlation between immunologic neutralization of biologic action and antigenetic reactivity of certain polypeptide hormones.

We were first confronted with that problem when studying serum insulin in Houssay-dogs in which both the pituitary and the pancreas were removed for providing survival without substitution with exogenous insulin. The studies were begun

in collaboration with Dr. and Mrs. Sirek from Toronto [10, 11, 13], but were continued in our laboratory upon return of our associate Dr. Schöffling to Frankfurt [2, 12]. I shall refer to these newer findings.

In Figure 1 is shown that the two operations induced the diminishment but not the disappearance of plasma ILA on both fat pad and diaphragm until the end of the experiment, i.e. for more than 100 days, whereas the Immunologically Measurable Insulin (IMI) values promptly fell to zero upon removal of the pancreas.

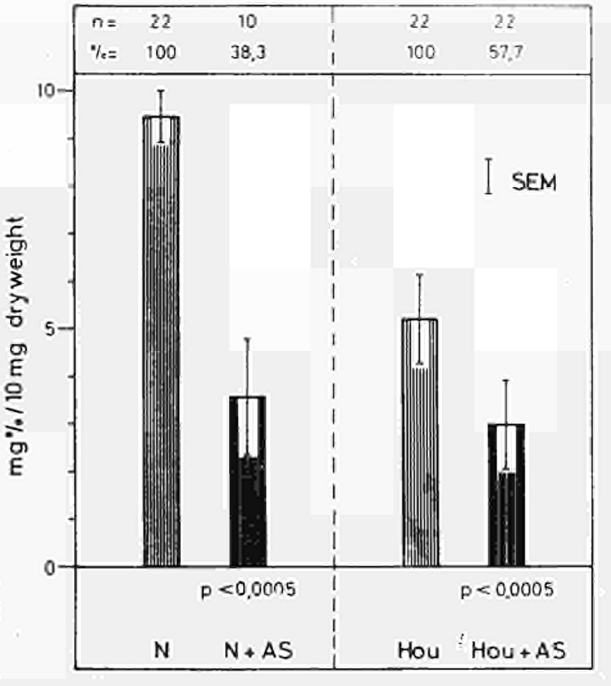

FIGURE 3. — Inhibition of glucose uptake of rat diaphragm by guinea-pig anti-insulin serum in sera of normal and Houssay-dogs

The persisting ILA still was immunologically suppressible on both the fat pad (Figure 2) and the diaphragm (Figure 3), and the same observation was made when the ILA was extracted from the serum. However the intravenous injection of the same antiserum in Houssay-dogs did not alter the blood glucose concentration, the hyperglycemia being confined to the controls (Figure 4).

Hence, the biologic ILA of serum and serum extracts of Houssay-dogs were inhibited by an antiserum *in vitro*, whereas the same antiserum neither did neutralize ILA *in vitro* nor demonstrate any IMI *in vitro*.

I shall desist from listing other examples of the said discrepancy, as amply provided by insulins from primitive species acting on mammalian tissues [1, 3] or

insulins produced by malignant tissues [10] for discribing an analogous observation concerning ACTH.

In contrast to insulin, ACTH values determined by immunoassay, in the standards as well as in normal serum or plasma, are higher than the ACTH activities measured on the basis of the steroidogenic capacity in hypophysectomized rats [4, 9]. The same observation is made, in general, also in Cushing's Syndrome and Addison's disease where elevated values are measured. Therefore, it was a surprise

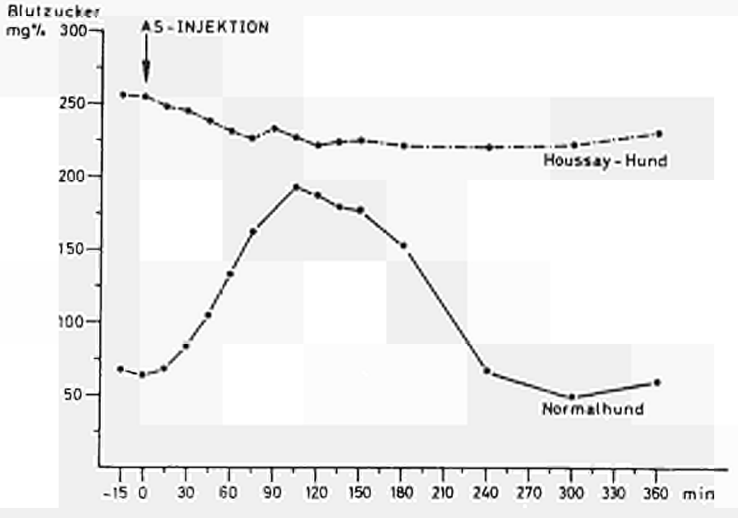

FIGURE 4. — Verhalten des Blutzuckers nach I.V.-Injektion von Meerschweinchen-anti-Insulin-Serum (3 ml/kg) beim normalen und hypophysektomiert pankreatektomierten (Houssay) Hund.

when in a case of Cushing's disease due to the so-called "ectopic ACTH-Syndrome", i.e. a carcinoma producing ACTH like susbstances [7], the elevated bioassay values in the serum constantly exceeded the immuno-ACTH concentrations which were found to be even lower than in normal subjects.

We try to interpret these findings, in conformity with a recent remark of Felber (1965) with the pituitary inhibition due to secondary adenocortical overactivity; this inhibition should be reflected more precisely by the immuno-ACTH values, measuring mainly the endogenous pituitary ACTH in plasma, than by the biologically determined ACTH activity, perhaps being mostly of tumourous origin. If so, the ACTH extracted from the carcinoma or its metastases, must show high activity in the bioassay but react only weakly in the immunoassay. As shown in Table I, this assumption was confirmed by the experimental data, demonstrating the high steroidogenic and low immunologic activity of the ACTH which Dr. Stewart extracted from large amounts of metastatic tissue secured *post mortem*, pituitary ACTH being reduced to 1/4 to 1/10 of the normal content.

TABLE I. — Biological and immunological ACTH quantities in a patient with a metastatic carcinoma of the lung associated with Cushing's syndrom

	methods of determination	
	biological	immunological
Plasma (mu/100 ml)	2.0	0.2
Pituitary (mu/mg)	11.6	0.1
Metastases (mu/g)	10.0	0.11

However, this high steroidogenic activity was nearly completely neutralized by the simultaneous injection of the antiserum into the hypophysectomized rats, the same antiserum which, *in vitro*, did measure only a small quantity in the radioimmunoassay (Table II).

These results shed some doubts on recent speculations, derived from studies on synthetic ACTH and ACTH fragments, localizing the steroidogenic site of ACTH activity in the N-terminal portion and the immunologic site in the C-terminal portion [6].

TABLE II. — Neutralization of ACTH-like activity with specific ACTH antiserum in metastases of a carcinoma of the lung associated with Cushing's syndrom

	ACTH content	ACTH content + antiserum
Metastases (mu/g)	10.0	> 0.03
ACTH standard (0.5 mu/inj.)	0.45	> 0.02
Liver extract control (mu/g)	> 0.03	

These hypotheses of a clear-cut molecular topography of ACTH activities are also difficult to reconcile with the immunologic neutralization of other biologic actions residing in the N-terminal portion, as the skin darkening or lipolytic effect [8, 14]. Either the antibodies produced against the antigenic property residing in the C-terminal portion do also affect the N-terminal component, or the folding occuring in the course of the formation of the antigen-antibody-complexes does prevent the biologic active site to fix to its cell receptors, etc.

Anybody seriously interested in this problem should profitably consult the work of the biochemists concerning the neutralization of enzyme action by specific antisera. The heterogeneity and multiplicity of the mechanisms by which antibodies inhibit, do not inhibit, and even increase enzyme activities is impressive. The most recent data are given in a small volume by title "Immunchemie" which has been published by Springer in 1965.

For the time being, we better defer from equating immunologic inhibition of hormone action with its immunologic reactivity. It would be gratefully acknowledged if anybody willing to contribute to that point would participate, in the appropriate place, in the discussions of this morning's session.

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BASIC PRINCIPLES IN THE USE OF LABELED PEPTIDE HORMONES WITH PARTICULAR REFERENCE TO RADIOIMMUNOASSAY

R. S. YALOW and S. A. BERSON

Veterans Administration Hospital, Bronx, New York, N. Y. U.S.A.

ABSTRACT

Two primary considerations in the use of labeled proteins in biomedical investigations are : I. — a labeled protein is a valid tracer for its parent unlabeled protein in a particular system, S, if, and only if, the labeled and unlabeled proteins exhibit identical behaviour in S, similarities or dissimilarities of behaviour in all other systems being irrelevant and II. — a labeled protein may be used other than as a tracer for the parent unlabeled protein, in which case identity of behaviour of labeled and unlabeled proteins may be irrelevant to the validity of application of the labeled protein.

Radioimmunoassay is an illustration of II. Factors are considered in detail which affect the sensitivity and precision of immunoassay, namely, 1) selection of a suitable antiserum 2) labeling of hormone at high specific activity 3) purification from " preparation damage " 4) minimization of "incubation damage" 5) distinction between bound and free labeled hormone. The significance of iodinating at more than 1 atom per molecule protein in affecting the suitability of a tracer and a method for evaluating the extent of iodination of iodoinsulin are described.

We have earlier [1] emphasized two considerations for the use of labeled proteins in biomedical investigations which may be stated in the form of theorems.

Theorem I : a labeled protein is a valid tracer for its parent unlabeled protein in a particular system, S, if, and only if, the labeled and unlabeled proteins exhibit identical behaviour in S, similarities or dissimilarities of behaviour in all other systems being irrelevant.

Theorem II : a labeled protein may be used other than as a tracer for the parent unlabeled protein, in which case identity of behaviour of labeled and unlabeled proteins may be irrelevant to the validity of application of the labeled protein.

We present here a few obvious examples of the applicability of these theorems. Much of the early work on labeled proteins involved studies of the metabolic turnover of ^{131}I-labeled serum albumin in man [2, 3]. It was observed that some preparations of ^{131}I-labeled albumin that are subject to an abnormally rapid metabolic degradation *in vivo* exhibit no abnormalities on ultracentrifugation [3]. On the other hand, a detectable difference in electrophoretic mobilities of labeled and unlabeled albumin [4] (which might simply be the consequence of the presence of a tyrosyl iodine) certainly does not vitiate the use of ^{131}I-labeled albumin in turnover

studies since some labeled preparations show the same turnover rate as unlabeled albumin [2, 5]. Similarly, for testing the validity of ^{131}I-labeled hormone in turnover studies it is important to establish only that labeled and unlabeled hormones are turned over at the same rates and not that the labeled hormone maintains its hormonal potency. In general it has been difficult to ascertain the hormonal potency of a labeled preparation that is completely satisfactory for metabolic or other studies. Lightly iodinated insulin, for example, is turned over at the same rate as unlabeled insulin in the rabbit [6] but assay of the hormonal potency of a preparation containing 1 iodine atom per 20-40 insulin molecules hardly tests the iodinated component [6]. In order to test the biologic activity of iodoinsulin it is necessary that most of the insulin molecules be iodinated. Since Harington [7] has shown that highly iodinated insulin is virtually without hormonal activity, the question is reduced essentially to the activity of monoiodoinsulin. However, at an average of 1 1 atom/molecule insulin, a significant fraction of the insulin molecules have 2 iodine atoms, some have 3 and a small percentage even more [8, 9]. Thus, even by choosing a stoichiometric ratio of iodine to insulin we are left with a heterogenous mixture of variously iodinated insulins. We shall describe later a method for selecting more homogeneous components from these mixtures.

^{131}I-labeled insulin can provide further examples of both theorems. In the study of the immunochemical reaction between insulin and insulin antibody, crystalline insulins from several different mammalian species were labeled and used as tracers for the unlabeled hormones [10, 11]. It was therefore necessary to establish that the labeled and unlabeled hormones reacted identically with the antibodies. This was found to be the case not only for insulin [9] but also for the reaction of ^{131}I-labeled growth hormone with growth hormone antibodies [12]. It cannot be too strongly emphasized that where Theorem I is to be applied, the condition of identity of behaviour must be specifically tested for the system S, not only in general but in particular. For example, an ^{131}I-labeled hormone may react exactly as does the unlabeled hormone in a certain antiserum but quite differently in another, perhaps because the antibodies in the latter are directed principally against a site of the hormone near the substituted iodine atom. For this reason the behavior of one antiserum with labeled hormone, unlabeled hormone and hormone fragments cannot be transferred unreservedly to another antiserum. Moreover, a monoiodinated hormone may exhibit unimpaired immunochemical reactivity whereas the multiply iodinated hormone may react poorly. For instance, we have found that singly and doubly iodinated insulins show unaltered reactions with a guinea-pig antiinsulin serum but at 3 and more iodine atoms/insulin monomer progressive diminution in immunoreactivity becomes evident [9].

The last comments bring us to an illustration of Theorem II. In the radioimmunoassay of peptide hormones, labeled hormones are exploited in such a way that the validity of the method does not depend on the identity of reaction of labeled and unlabeled hormones in any system [13]. The theoretical basis of the assay is expressed in the set of competing immunochemical reactions.

$$\underset{(F)}{\text{Free } {}^{131}\text{I-Hormone}} + \text{Hormone Antibody} \rightleftarrows \underset{(B)}{\text{Antibody-bound } {}^{131}\text{I-Hormone}}$$

$$+ \text{ Unlabeled hormone}$$

$$\uparrow\downarrow$$

$$\text{Antibody-bound unlabeled hormone}$$

The labeled hormone must be able to bind to antibody but there is no essential requirement that its binding be identical to the binding of unlabeled hormone. It is only necessary that endogenous plasma hormone and unlabeled hormone exhibit the same competitive inhibition of binding of the labeled hormone. Then, the concentration of hormone in an unknown plasma is determined by comparing the inhi-

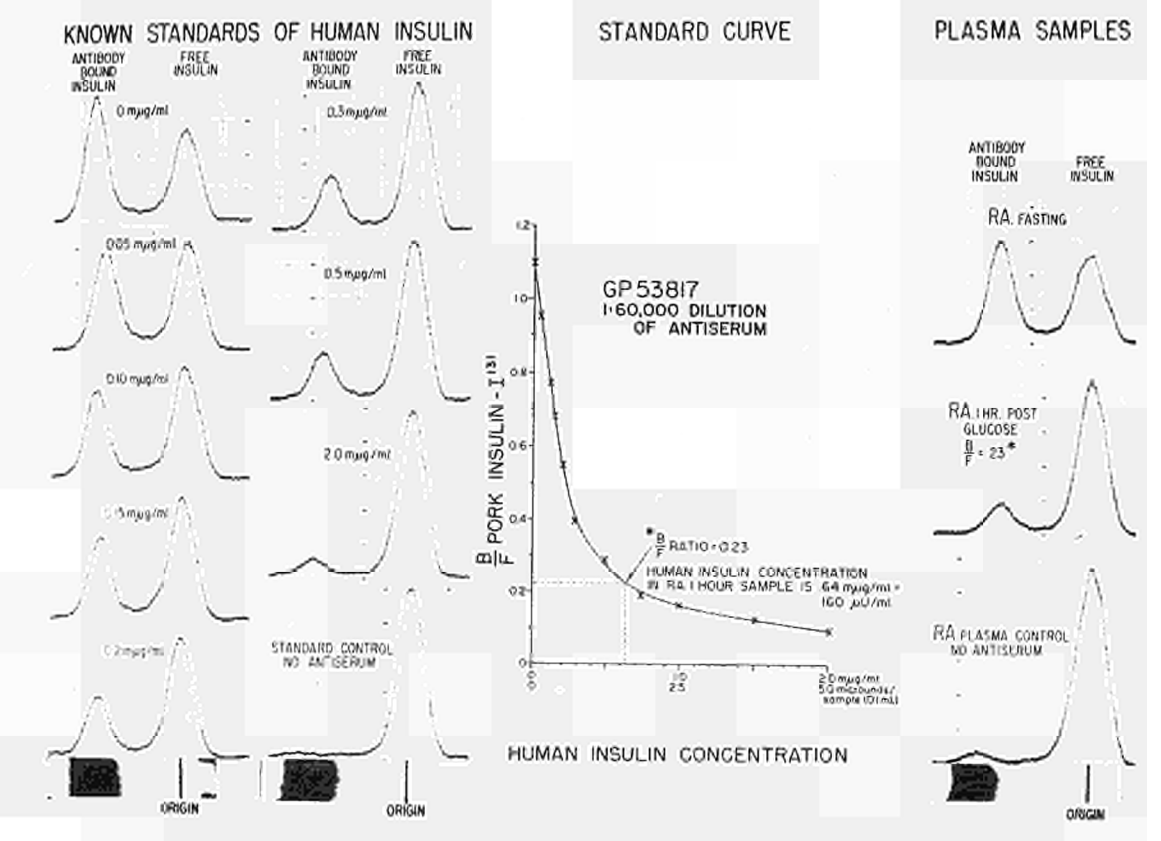

FIGURE 1. — Chromatoelectrophoresis of ^{131}I-insulin-antiserum mixtures containing known concentrations of human insulin (left) and unknown plasma (right). At the bottom of each set is a control mixture to which no antiserum had been added; migrating activity in control tubes represents damaged fractions. The standard curve (middle) is obtained from measurement of areas under each of the two peaks in the complete series of chromatograms of which 8 are shown. The insulin concentration in the 1 hour post-glucose specimen of patient RA is calculated as shown. (Reproduced from Ref. 22.)

bition it produces with that produced by the standards. The most direct way of doing this is to plot the ratio (B/F) of antibody-bound (B) to free (F) labeled hormone as a function of hormone concentration in known standards and to match the B/F ratio in unknown samples to the standard curve (Figure 1).

Having emphasized the application of Theorem II and the nonessentiality of establishing identity of reaction of labeled and unlabeled hormones in radioimmunoassay, we wish to devote the remainder of this presentation to a consideration of factors determining sensitivity and precision of the assay. Five principal factors merit discussion : 1) selection of a suitable antiserum. 2) labeling of hormone at high specific activity. 3) purification of labeled hormone from labeled components damaged during preparation (« preparation damage »). 4) minimization of and correction for damage during incubation with plasma (« incubation damage »). 5) separation of bound and free labeled hormone.

1. — Selection of antiserum

The slope of the standard curve is dependent primarily on the interaction energy between antigen and antibody [10, 14, 15]. It is a matter of experience that antisera obtained from different animals of the same species immunized on the same schedule vary in their energy of reaction with antigen [12, 16]. Proper choice of antisera

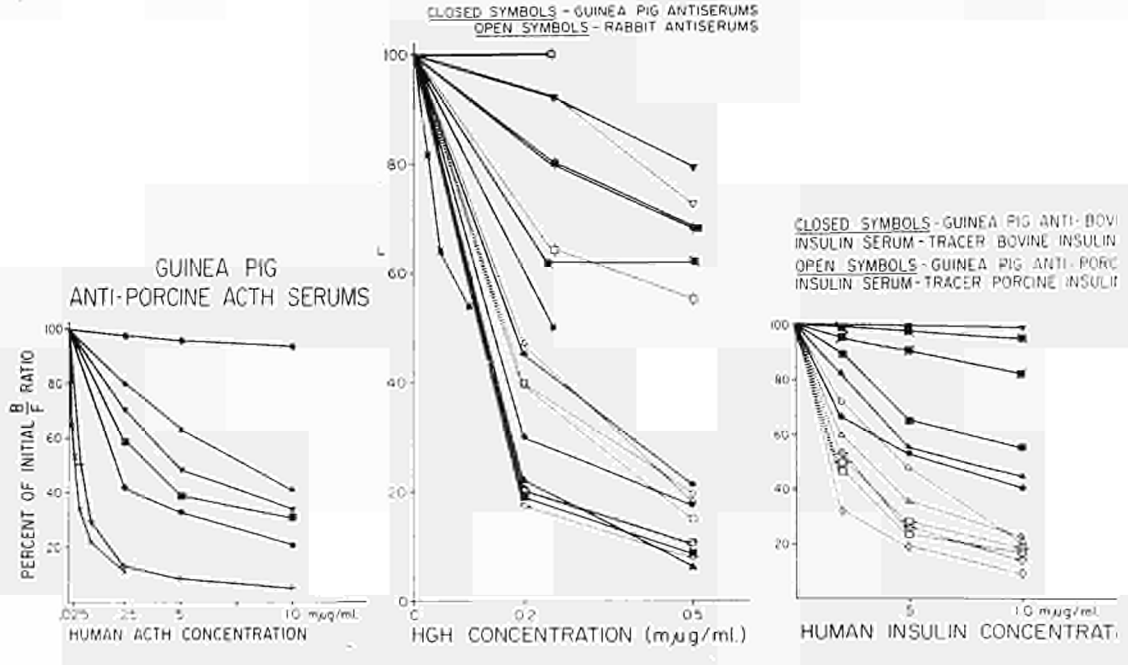

Figure 2. — Percent reduction in ratio (B/F) of bound (B) to free (F) ^{131}I-labeled hormone as a function of the concentration of unlabeled hormone. Initial B/F ratios 0.6 : 1 to 3 : 1; antiserum dilutions 1 : 1000 — 1 : 2,000,000.

can result in a hundred fold or greater increase in sensitivity for detection of hormone (Figure 2). Insulin and growth hormone commonly give excellent antisera, at least in guinea-pigs. Furthermore, these hormones are present at sufficiently high levels in human plasma that sensitivity is usually not a serious problem. Nevertheless, for reasons to be given, it is desirable to assay plasma at a dilution of 1 : 10 to 1 : 20 or greater and therefore to aim for as great a sensitivity as can be obtained. For certain other hormones, parathyroid hormone and ACTH, for example, the plasma concentrations may be so low that the sensitivity must be more than 10 fold greater than that which would be adequate for insulin and growth hormone. Since the yield of such good antisera is relatively low, successful assays for some hormones may require an extensive program of immunization and testing of antisera.

2. — REQUIREMENTS FOR SPECIFIC ACTIVITY OF THE LABELED HORMONE

From a brief consideration of the immunochemical reactions shown previously, it is evident that if the antibody is present in marked excess over the hormone to be assayed, the binding of the labeled hormone cannot be significantly inhibited by unlabeled hormone. Therefore, in practice, the antiserum is diluted sufficiently to reduce the concentration of antibody-combining sites to the same order of magnitude as the concentration of plasma hormone. Similarly, the concentration of labeled hormone should be kept within the same range. Indeed, more precise determinations are obtained when the concentration of labeled hormone (« tracer ») is small compared to the concentration of hormone in plasma. The value of decreasing the concentration of tracer depends on the antisera available. With an insensitive antiserum little improvement is gained by decreasing the tracer beyond a certain level (Figure 3). The more sensitive the antiserum the greater is the reduction in B/F obtained with a given increment in hormone concentration (Figure 2). Since this holds true also for the tracer, the initial slope of the standard curve is sharper and the assay more sensitive, the lower the concentration of tracer[12]. For a given antiserum, therefore, the ability to measure low concentrations of hormone will be increased by increases in the efficiency of the counting equipment, in the volume of the incubation mixture, or in the concentration of plasma contained therein or by a decrease in the concentration of the tracer.

A decrease in the concentration of tracer with maintenance of suitable counting rates can be effected by an increase in specific activity of the labeled hormone. This certainly has a limit when both positions on all the tyrosyl residues have been occupied; indeed for most hormones, the effective limit may be considerably lower since, as already noted for insulin, multiply iodinated hormone may lose immunochemical reactivity. It seems generally desirable to keep the average iodination level to not more than 1 I atom/molecule although it may be useful to exceed this value for some hormones. In the case of ACTH, for example, the plasma concentration in normal fasting subjects is frequently less than 20 μμg/ml. If we are to assay at 1 : 2 dilution of plasma in a volume of 0.5 ml and would accept a total of 3000 counts/minute (after a 6 day incubation period) in a system with an efficiency

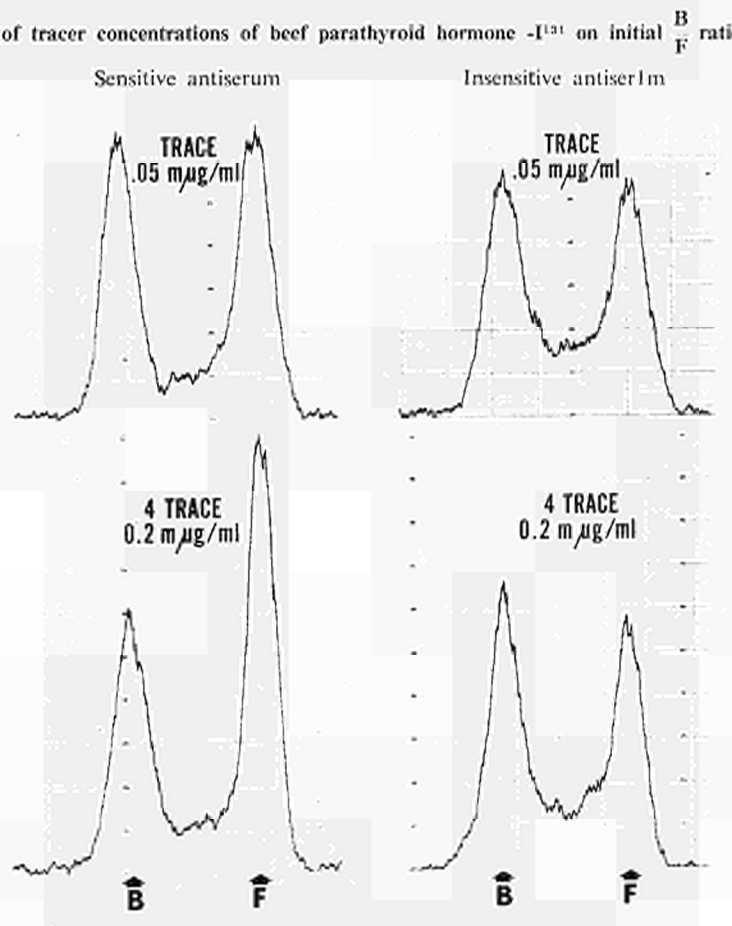

FIGURE 3. — Self-explanatory. (Reproduced from Ref. 19.)

of 10^6 c/m/μCi^{131}I, we must have added ^{131}I-ACTH in a concentration of 0.01 μCi/ml initially. For this tracer not to exceed 10 μμg/ml in the 1 : 2 plasma, the specific activity of the ^{131}I-ACTH must be 1 curie ^{131}I/mg protein. For ^{131}I contaminated with other isotopes to the extent of 70 % (the most nearly carrier-free material we have obtained) this means an average of about 1 I atom/molecule ACTH (MW = 4500). We have labeled at significantly higher levels, but a diminished immunochemical reactivity seems to offset the advantage of the higher specific activity.

As indicated earlier, the specific activity requirements of the labeled hormones are less stringent for hormones present at significantly higher concentrations than ACTH. Yet, since it is preferable to assay at plasma dilutions of 1:10 or greater in order to minimize "incubation damage" and since concentrations of hormone even lower than normal fasting levels may be of interest, it will often be desirable to exceed the minimum satisfactory specific activity.

A brief consideration of other radionuclides as potential labels for hormones in radioimmunoassay is pertinent. Under conditions for maximal sensitivity for measurement of plasma hormones, the concentrations of antigen and antibody are so low that the optimum time of incubation is generally about 3-5 days. Isotopes of iodine with shorter half-lives than ^{131}I would offer no great advantage. The use of longer-lived isotopes such as ^{125}I would result in a lower specific activity of the labeled hormone. When such lowering of the specific activity is acceptable and if the labeled hormone has a suitably long shelf-life, labeling with ^{125}I may be useful. Even were multiple substitutions with ^{14}C or ^{3}H possible, it does not appear likely that hormones labeled with these nuclides are potentially useful for radioimmunoassay in view of the very much lower specific activities attainable.

The method of iodination that we have used most extensively for the preparation of ^{131}I-labeled hormones at very high specific activities is that of Hunter and Greenwood [17], which uses chloramine T as an oxidant to effect virtually instantaneous iodination with yields generally about 90 %. Immediately after completion of iodination, the reaction mixture always contains, in addition to the unreacted iodide, a variable fraction of damaged ^{131}I-hormone which migrates with plasma proteins on paper chromatoelectrophoresis [18].

3. — Purification of labeled hormones

To obtain labeled hormone free of damaged components and iodide, we have used a technique that exploits the ability of undamaged hormone to adsorb to cellulose [13]. Immediately after iodination the reaction mixture is added to 10-20 μl plasma and placed on a small column packed with .2-.4 ml cellulose powder. Undamaged hormone is adsorbed to the column; damaged fractions bound to plasma proteins and unreacted iodide ^{131}I pass through with the plasma proteins. The column is then washed several times with buffer. For purification of labeled ACTH, parathyroid hormone and insulin, the adsorbed undamaged hormone is eluted rapidly with whole plasma into several test tubes containing several milliliters of an appropriate buffer. For HGH, since elution with plasma does not produce a satisfactory preparation, elution with 20 % acetone in veronal buffer has been used [19].

^{131}I-Insulin and ^{131}I-ACTH, when labeled at specific activities of 1000 mCi ^{131}I/mg, generally contain less than 3 % migrating components after purification. The bovine parathyroid hormone preparations we have employed (Aurbach, Rasmussen) have seldom been purified to less than 5 % migrating components even when labeled at low specific activity. The migrating components with various HGH preparations also average approximately 5 % (Figure 4). Since certain hormone preparations may contain significant amounts of contaminating proteins or altered hormone molecules that adsorb to cellulose and still are immunochemically unreactive, it is often desirable to test the labeled preparation for ability to bind completely to antibody by incubating of a small amount of labeled hormone with a strong antiserum for an hour or so at room temperature and analyzing the incubation

mixture by chromatoelectrophoresis (Figure 4). However, it should be noted that ability to bind completely does not necessarily indicate immunochemical reactivity identical to that of unlabeled hormone.

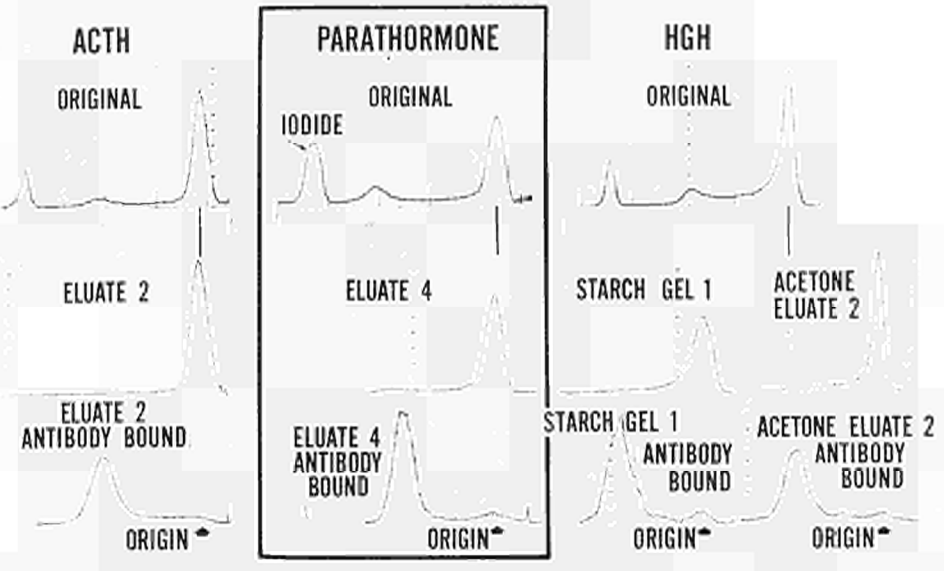

FIGURE 4. — Scans of paper chromatoelectrophoretograms of labeled hormone preparations added to control (non-immune) plasma (Rows 1 and 2) and specific antisera (Row 3). Paper strips, showing the origin (vertical line at right) and stained migrating serum albumin, are shown below the scans of the original preparations prior to purification. In Row 2 are shown plasma eluates of labeled ACTH and parathyroid hormone purified by adsorption chromatography on cellulose columns and extract from starch gel and acetone eluate from cellulose column purification of ^{131}I-HGH. (Reproduced from Ref. 19.)

In earlier studies we regularly purified HGH using preparative starch gel electrophoresis [20]. Immediately following iodination the reaction mixture is added to an equal volume of non-immune plasma and subjected to starch gel electrophoresis according to the method of Smithies [21]. After 10 hours at 150V, the gel is removed, radioautographs are obtained and used as a guide for the sectioning of the intense radioactive bands. The latter are cut out and separately frozen for 3-4 hours. When these sections are thawed, their fluid contents are readily extracted and placed in separate tubes. A sample from each tube is tested for damage and ability to bind to antibody. ^{131}I-HGH prepared from several highly purified preparations of Wilhelmi (e.g. HS502B2, HS372B, etc.) migrates almost completely in the pre-albumin zone (Figure 5); several thin cuts from this region will show virtually complete binding to antibody (Figure 4). Different results have been obtained [19] with a new highly purified preparation of Wilhelmi (HS612A), which is to be distributed as an inter-

national standard for radioimmunoassay. When a freshly prepared solution is labeled with ^{131}I, the radioactivity is concentrated principally in and just behind the albumin region (Figure 5). However, after repeated freezing and thawing of the same solution of HS612A, newly labeled preparations exhibit a more significant pre-albumin band of radioactivity and a loss of the more slowly migrating components (Figure 5). Labeled HGH612A from all cuts, including the post albumin zones, binds well to antibody.

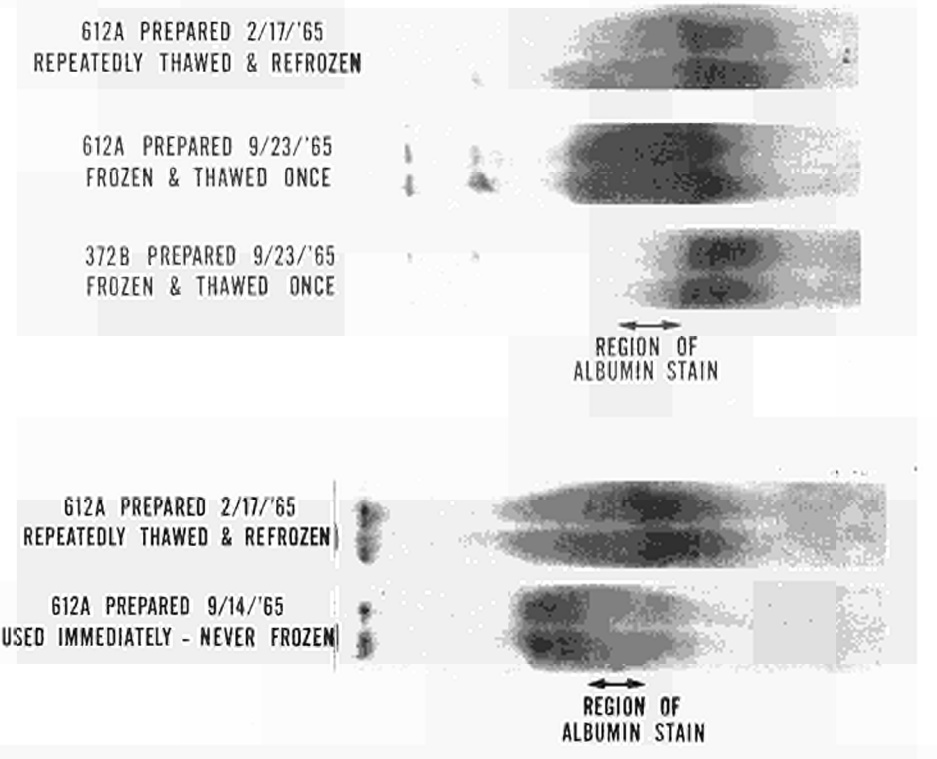

FIGURE 5. — All preparations of ^{131}I-HS372B as well as several other highly purified Wilhelmi human growth hormone preparations have shown virtually all radioactivity in the pre-albumin region. In contrast, freshly prepared ^{131}I-HS612A has reproducibly shown a major fraction with slower migration. After repeated thawing and refreezing of the unlabeled material, a significant fraction of freshly iodinated material migrates in the pre-albumin region. (Reproduced from Ref. 19.)

When ^{131}I-insulin of high specific activity is added to plasma and subjected to starch gel electrophoresis, the labeled insulin is found in a series of discrete spots [8, 9]. In order to determine whether the multiple components represented inhomogeneity of the starting material or were produced during labeling, comparisons were

made between iodinated preparations with a ten-fold difference in specific activities. The most anodal components were absent in the low specific activity preparations (Figure 6). Studies with labeled insulin preparations of the same specific activities but with varying amounts of carrier ^{127}I indicated that the individual bands corresponded to insulin molecules with different numbers of iodine atoms [8, 9] (Figure 7). These observations have been exploited to estimate the specific activity of commercially available ^{131}I preparations [9]. The estimation is made by comparing the starch gel electrophoresis patterns of insulins labeled with varying quantities of ^{131}I (Figure 8) with those obtained by labeling with small amounts of ^{131}I and graded known amounts of ^{127}I (Figure 7).

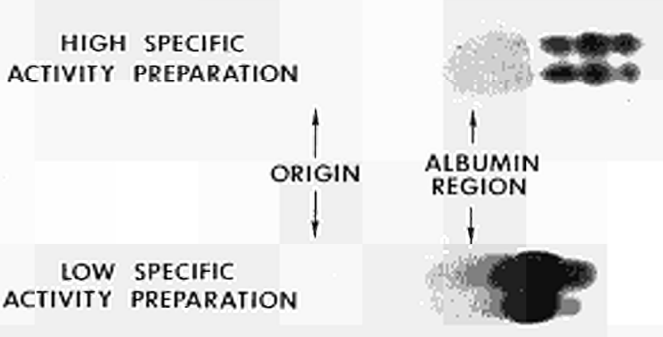

FIGURE 6. — Beef insulin (Lilly lot#719106) was labeled with ^{131}I and added to an equal volume of plasma for electrophoresis on starch gel. After electrophoresis the gel was removed from the mold, covered with Parafilm and placed in contact with x-ray film for radioautography. Bromphenol blue was added to the plasma prior to electrophoresis for ready identification of the serum albumin. High specific activity, ~520 mCi ^{131}I/mg insulin. Low specific activity ~50 mCi ^{131}I/mg insulin. (Reproduced from Ref. 8.)

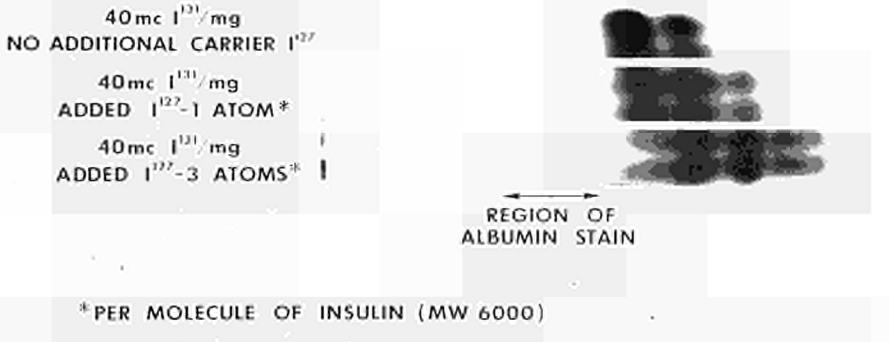

FIGURE 7. — Same experimental conditions as in Figure 5 except that the low specific activity preparation was 40 mCi ^{131}I/mg protein and ^{127}I was added to ^{131}I as indicated for 2 lower preparations. In all three preparations, iodination was approximately 95 % complete so that final labeled products contained essentially the initial iodine : protein ratio. (Reproduced from Ref. 8.)

FIGURE 8. — Same experimental conditions as in Figures 6 and 7 except that another lot of ^{131}I was used to prepare ^{131}I -insulin at the indicated specific activities

Since the ^{131}I supplied for clinical purposes is often of low specific activity and is protected from oxidation by reducing agents, only ^{131}I designated "for iodination" by isotope suppliers is suitable for the present purposes. However, uniform production of satisfactory labeled hormones is not always possible even with these special preparations. On occasion, lots of ^{131}I are unsatisfactory for iodination of hormones either because they result in low iodination yields or because the iodinated hormone fails to purify well. The identification of the noxious substance has not been made, but our studies with several such lots suggest that the difficulty does not always arise from low specific activity of the ^{131}I.

4. — MINIMIZATION OF INCUBATION DAMAGE

After the initial purification of labeled hormone, increasing amounts of labeled products which migrate with or are bound to serum proteins appear during incubation of the labeled hormones in plasma [18]. Such "incubation damage" occurs with all ^{131}I-labeled hormones but the factors responsible may differ with the individual hormones. However, virtually all plasmas become more damaging when stored for extended periods of time at room temperature or above, and minimally damaging plasmas are obtained when blood samples are taken with a heparinized syringe, immediately centrifuged and stored in a deep freeze.

^{131}I-Insulin is protected from damage in plasma with 0.5 % iodoacetamide for 20-30 min at 37° C [22]. Whereas we earlier considered that this effect might be due to the blocking of -SH groups present in plasma (and thus reducing the potential for reductive splitting of disulfide groups), the possibility exists that iodoacetamide may act by inhibiting sulfhydryl-dependent proteolytic enzymes, especially since free ^{131}I appears not infrequently to accompany incubation damage. Whereas -SH compounds, under appropriate conditions, damage insulin, mercaptoethanol in concentrations of 0.25-0.5 % has a strong protective effect on ^{131}I-ACTH. Although mercaptoethanol protection of ACTH against biological inactivation has been attributed to prevention of oxidation of the methionine group, we are not certain that this is the sole mechanism for its protection of ^{131}I-ACTH since mercaptoethanol is not helpful in protecting against damage to ^{131}I-parathyroid hormone,

which also is biologically inactivated by oxidation of the methionine group. It may well be that the protection of ^{131}I-ACTH by mercaptoethanol can be attributed to inactivation of disulfide-dependent proteolytic enzymes. Evidence for the rôle of plasma proteolytic enzymes in incubation damage arises from the observed effectiveness of trypsin inhibitors in protecting labeled hormones from damage in plasma [19].

5. — SEPARATION OF BOUND AND FREE LABELED HORMONE

Since free iodide ^{131} is not a part of the bound or free labeled hormone, it obviously must not be counted with either. Separation by paper chromatoelectrophoresis permits recognition of iodide ^{131} as a separate peak not included in the protein fractions. Test tube separations of bound and free hormones are generally blind to the appearance of free iodide ^{131} in the incubation mixtures and erroneous concentrations for immunoassayable hormone may be attributable to significant but variable deiodination in the incubation mixtures.

For all separation systems, proper controls must be set up for detection of and correction for the presence of damaged hormone that is bound non-specifically to serum protein and thus prevented from participating in the antigen-antibody reaction. The appropriate control for paper chromatoelectrophoretic separation is an incubation mixture prepared identically with standard or unknown solutions but without antibody; the migrating material is identifiable as damage. When cumulative damage due to preparation or incubation is small, adequate correction can be made but when it is greater than 20-25 % the determination of hormone concentration is subject to great error.

In the case of double antibody techniques for separation of bound and free hormone, damaged fractions bound to human plasma proteins are not available for binding to antibody and are therefore not precipitable with the second antibody. Proper controls in this procedure require a second precipitation with anti-whole human plasma for determination of protein-bound damaged fractions (as previously recommended [22]). To our knowledge this has not been done by workers relying on the double antibody separation.

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DISCUSSION

K. BRUNFELDT (*Gentofte*) : With the modern counting systems ^{125}I should not cause any difficulties, at least, when the double antibody method is used. If a method based on a scanning of paper strips or agar plates is used, ^{131}I may be preferable. Regarding the separation of iodinated insulin preparations, I should like to ask whether you have been able to obtain this with all your insulin preparations, and under which conditions the electrophoresis was carried out ? I should also like to direct your attention to the fact that it is possible to separate crystallized insulin into two fractions by continuous paper electrophoresis, the fast migrating one containing the major part of the Zn^{++} content of the original sample.

R. S. YALOW : Starch gel electrophoresis was carried out according to the method : Smithies, O. (*Adv. Protein Chem.* **14** : 65, 1959). Freshly dissolved crystalline beef and pork insulins from Lilly, and crystalline pork insulin from Novo behave identically in this system when iodinated. Prolonged storage at —15° C of these insulins in acid solutions (0.001M HCl) results in bands with increased anodal mobility for both labeled and unlabeled preparations, probably because of loss of amide groups.

F. C. GREENWOOD (*London*) : Concerning the intensive immunization schedule you mentioned, it would be useful to know exactly how many animals for example you require to immunize, to get a very good ACTH antiserum ? The other technical point is, how routine is the determination of specific activity of isotope samples ? I would like to congratulate yourself and Dr. Berson on this procedure since I understand the producers of carrier free ^{131}I have spent a long time trying to develop routine methods for specific activity.

R. S. YALOW : The best ACTH antiserum we have is capable of detecting 5 μμg/ml human ACTH. The animal was one of 18 immunized. In this group there was another animal whose antiserum had a sensitivity of 25-50 μμg/ml and a number of others had antibody detectable with antiserum dilutions of $1 : 10^4$ or greater. It should be emphasized that a very small tracer must be used for testing these very sensitive antiserums or the tracer alone would occupy all the antibody-binding sites.

With respect to the second question, we have been performing the specific activity determinations for almost each new lot of Isoserve iodine for the last month or two. We plan on doing it at monthly intervals for a while to intercompare Oak Ridge, Isoserve and Amersham iodines.

R. P. EKINS (*London*) : We have shown that the optimum amount of tracer to be employed in an immunoassay is 4/K (Mol/l) and the optimum amount of

antibody is 3/K (Mol/l) where K is equal to the reaction constant (1/Mol) (Ekins, 1963; Ekins and O'Riordan, 1966). The minimum detectable amount of hormone is given by $4\sqrt{2}/\sqrt{KVST}$ where V = volume (in liters) of incubation mixture subjected to the separation procedure, S = specific activity of tracer (c.p.m./Mol) and T = the total counting time per free/bound pair (min.). These theoretical considerations depend on the assumption that the error in the determination of the free to bound ratio comprises the counting error only, which I realise is invalid; however in certain assay procedure (I am thinking particularly of the assay of vitamin B_{12} using the analogous saturation assay technique (Barakat and Ekins, 1961) the total error in the determination of $R_{f/b}$ (or $R_{b/f}$) is indeed very close to the counting error (at the 1 % level). Under these circumstances, it may well be that better sensitivity is not obtained by reduction of the amount of tracer as Dr. Yalow has recommended without qualification.

In the assay of vitamin B_{12}, we have used activated charcoal to separate free and bound B_{12} and have found it a very useful reagent yielding an extremely simple technique. Dr. Herbert (Herbert *et al.*, 1965) has recently employed activated charcoal to separate free and bound insulin and has suggested its use in the assay of HGH. I wonder if Dr. Yalow has any comments to make on the use of this reagent. Regarding the point raised by Dr. Greenwood, Dr. Reith in our laboratory (Reith and Bown, 1964) has routinely measured the specific activity of ^{131}I supplied by the R. C. C. Amersham by iodinating ^{14}C-labeled tyrosine. Following chromatographic separation of the unreacted tyrosine it is a simple matter to calculate the specific activity on the ^{131}I supplied by measuring the ratio of the ^{131}I and ^{14}C activities in the iodotyrosines. Using this method, the proportions of ^{131}I in the material supplied was shown to be as low as approximatively 5 % though in recent preparations this proportion has somewhat improved.

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R. S. Yalow : A number of techniques have been used to separate bound (B) and free (F) labeled hormones. If charcoal absorbs 95-100 % of free hormone and less than 5 % of antibody-bound hormone under the conditions employed, it should be satisfactory as another method for separation of B and F. However, for each hormone and for each separation system the optimal conditions for separation must be evaluated.

I have seen the publication by Reith and Brown (*Nature*, **201** : 621 [1964]) on the experimental estimation of the specific activities of ^{131}I-iodotyrosines using

^{14}C-tyrosines in the iodination procedure. In this paper they made no reference to the specific activity of ^{131}I-iodide. Perhaps you could supply the exact reference for the study you have quoted.

A. E. FREEDLENDER (*Boston*) : Using the Soeldner modification of the method of Morgan and Lazarow we have assayed sera at dilution between 1 : 2 and 1 : 20. When results are corrected for dilution, equivalent values for insulin were obtained, indicating an absence of degradation in this system even at low dilutions of sera.

These results are in confirmation of the data recently published by Soeldner and Slone.

R. S. YALOW : Degradation of labeled insulin by plasma is quite variable and dependent on the plasma and on the individual lot of ^{131}I-insulin. On occasion ^{131}I-insulin added to plasma and stored for several weeks at 4° C has shown virtually no detectable increase in damage. An occasional check, therefore, is not definitive evidence for absence of damage with other lots of insulin and other plasmas. Using chromatoelectrophoresis we have evaluated each lot of ^{131}I-insulin and each patient's plasma for damage. By this time, evidence has accumulated that at 1 : 10 or 1 : 20 dilutions only occasional plasmas are significantly damaged, but in undiluted plasma or plasmas at low dilution, significant damage occurs quite frequently.

CORRELATION OF CHEMICAL CHANGES DUE TO IODINATION WITH INSULIN BIOLOGICAL ACTIVITY (*)

U. ROSA (°), A. MASSAGLIA (°), G. F. PENNISI (°), C. A. ROSSI (•) and I. COZZANI (•)

(°) *SORIN, Nuclear Research Center, Saluggia, Italy.*

(•) *Institute of Biological Chemistry, University of Pisa, Italy.*

ABSTRACT

The effect of progressive iodination on the biological activity of labelled insulin has been investigated. Iodination up to 8 iodine atoms per insulin molecule has been performed under standardized and controlled conditions. Preparations were tested for their biological activity using the epididymal fat pad method.

The distribution of iodine among the four tyrosine residues has been measured and tentatively correlated with the drop of biological activity occurring above a certain level of iodination, on one side, and the reduction in SS bridges reactivity towards sulphite on the other.

Insulin iodination beyond a certain substitution degree has been reported by several authors to cause the inactivation of the hormone [1-3]. Since the only occurring reaction is the conversion of tyrosine residues in the mono- and di-iodinated derivatives, inactivation must necessarily be correlated to this process.

In Figure 1, the insulin activity is plotted versus the average iodination degree for a series of iodo-insulin preparations. Biological activity was measured by the epididymal fat pad assay (Table I). Insulin iodination was carried out at pH 7.6 using the previsouly described electrolytic technique, under standard conditions. The Table I shows that hormonal activity is apparently unchanged up to a substitution degree of the order of 2 iodine atoms per molecule and becomes negligible at a substitution degree of the order of 5 iodine atoms per molecule.

At a first sight, these results would seem to indicate that below an iodination degree of 2 iodine atoms per mole, labelled ^{131}I-or ^{125}I-insulin could be considered an adequate biological tracer. In fact, such a clearcut conclusion is not warranted on the basis of these data. The internal variability of the bioassay, the contribution of the unlabelled insulin to the measured biological activity and the probably not uniform distribution of the iodine atoms among the insulin molecules, only permit to identify the iodination degree of 2, as the level at which the amount of inactivated insulin is large enough to induce a detectable loss of hormonal activity.

The question of the actual iodination level at which ^{131}I-or ^{125}I-labelled insulin can be considered an adequate biological tracer, is then still open.

(*) Work performed under EURATOM contracts No. 053-63-10 RISI and No. 048-64-6 BIOI.

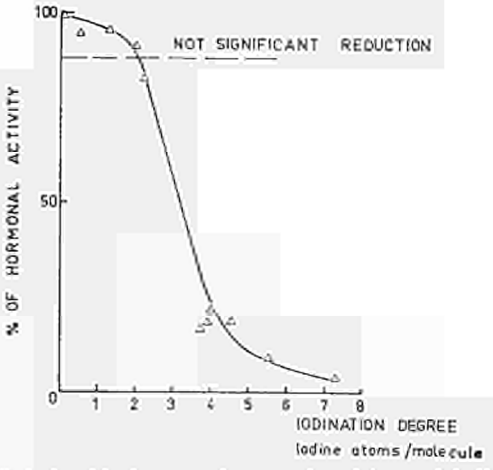


FIGURE 1. — Relationship between hormonal activity and iodination degree.

Some experiments reported by Fraenkel-Conrat suggest a way to interpret our results [1].

The inhibition of the hormonal activity depends on the attainment of a critical intramolecular structure, which is probably related to the distribution of iodine among the tyrosine residues.

A further step in the understanding of the inactivation process obviously requires a full knowledge of the relationship between the location of iodine in a iodinated insulin molecule and its hormonal activity.

Following this line, we have undertaken, on insulins iodinated at increasing degrees, the study of the distribution of iodine among the four tyrosine residues, and its correlation with the biological activity.

TABLE 1. — Relationship between degree of iodination and biological activity of iodoinsulins.

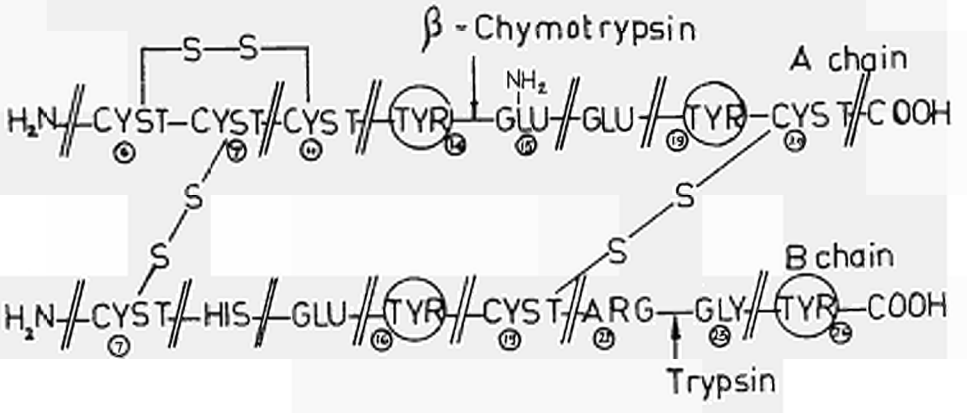
Iodination degree (I atoms per insulin molecule of M.W. 5734)	Hormonal activity in epididymal fat pad assay (% of activity of native insulin)	Observations
0.5	94.4 ± 4.14	Not significant
2.0	90.1 ± 1.13	Not significant
2.2	82.1 ± 6.60	—
3.7	16.5 ± 1.70	—
3.9	19.9 ± 2.60	—
4.0	22.0 ± 2.60	—
4.6	21.9 ± 4.01	—
5.5	7.0 ± 1.20	—
7.3	5 ± 2.90	—

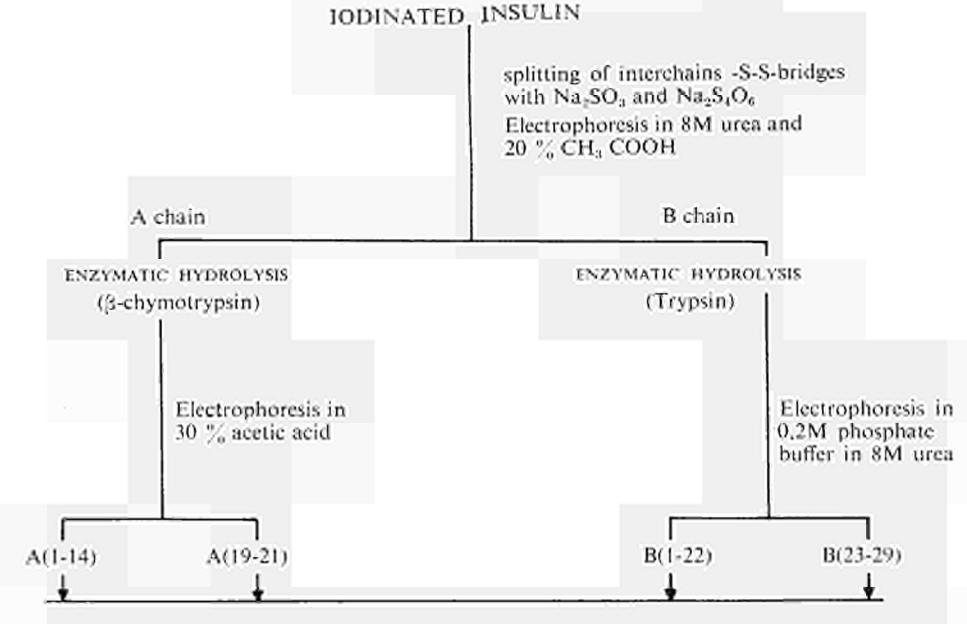
The procedure of fragmentation, which allowed the average iodine distribution to be evaluated, was the following (Figure 2) :

— A and B chains were separated by splitting the interchains -S-S- bonds, according to the procedure described by Bailey [4].

— the chains were separated by paper electrophoresis under the experimental conditions given by De Zoeten [5, 6] and eluted in the pure form from the paper strip.

— subsequent fragmentation of the chains was performed by enzymatic hydrolysis at a pH 8.4 with β-chymotrypsin or trypsin for A and B chains respectively.





MIT and DIT residues are chromatographically determined on each sample after total hydrolysis with pancreatin followed by chromatographic separation.

FIGURE 2. — Schematic representation of the fragmentation procedure.

— fragments separation was carried out by paper electrophoresis followed by selective elution with an appropriate solvent.
— each group of fragments was submitted to total hydrolysis with pancreatin. Mono- and di-iodinated tyrosine residues were separated by descending paper chromatography as MIT and DIT, and counted for ^{125}I activity.

As result of such a fragmentation procedure, a set of experimental data was obtained for each preparation of iodinated insulin.

These data concerned :

a) the distribution of iodine between the two chains, expressed in percentage of the total iodine bound to the insulin.
b) the distribution of the iodine bound to each chain between the two tyrosyl residues, expressed in percentage of the total iodine bound to the chain.
c) the amount of iodine bound to the single tyrosine residues.

The procedure employed for the calculations concerning the point *c*) requires a brief commentary. As a result of the fragmentation, the tyrosyl residues of a certain type (for example A 14 or A 19) are found, part as a mono-iodinated form, part as a di-iodinated form. By difference, the uniodinated tyrosines may be calculated. The results are expressed as percentage of the total tyrosines of that peculiar type.

The iodine distribution between the two chains is represented in Figure 3. In agreement with the results of De Zoeten [5, 6], the A chain shows a higher reactivity

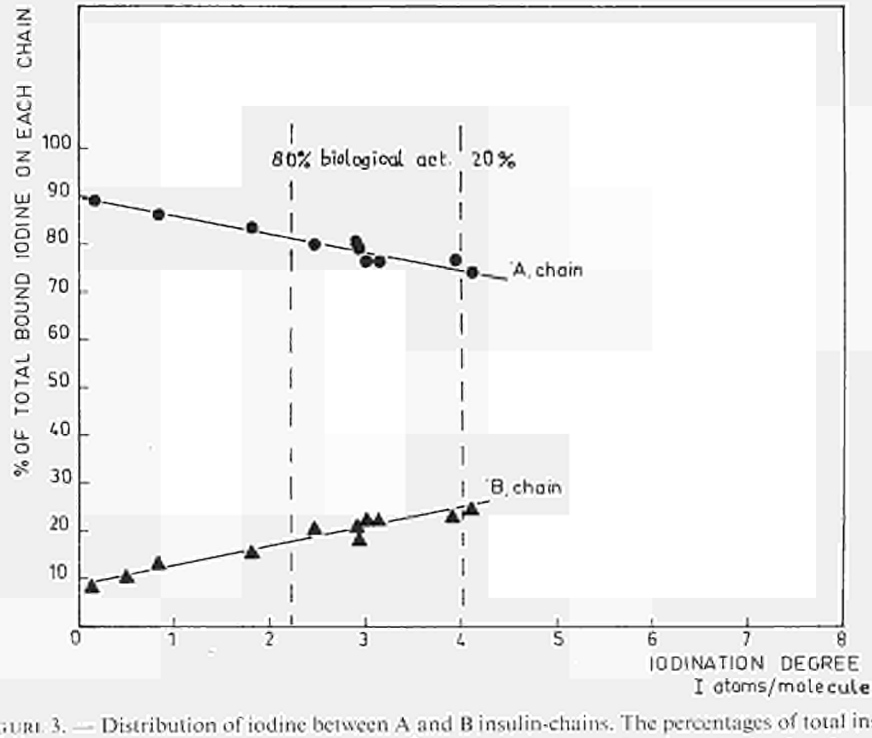


FIGURE 3. — Distribution of iodine between A and B insulin-chains. The percentages of total insulin-bound iodine, found in A chain (●) and in B chain (▲) are plotted vs. iodination degree.

towards iodine. Up to an iodination degree of 4 iodine atoms per mole, about the 70 % of the reacted iodine, is carried by the tyrosine residues of the A chain. As a consequence of this uneven distribution, the number of free tyrosine groups in the B chain is still 70 % of the initial value, even for an iodination degree of 4 (Figure 4). The dotted lines, on the Figures 3 and 4, define the range of iodination degree, within which the hormonal activity falls from 80 % to about 20 %. The distribution of iodine between B-16 and B-26 positions was experimentally found of the same order. Consequently, about 65-70 % of the tyrosine residues of each type were found to be not substituted, when the insulin was already highly inactivated.

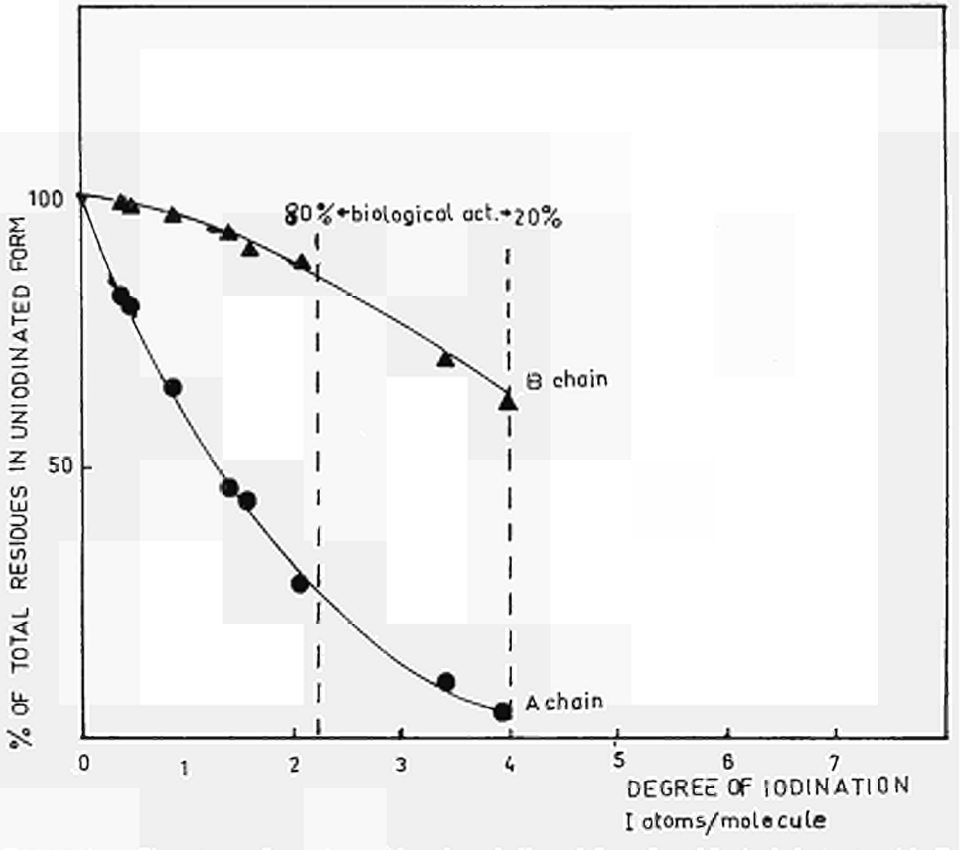


FIGURE 4. — Percentage of tyrosine residues in uniodinated form found in A chain (▲) and in B chain (●) at different values of the iodination degree.

Statistical considerations on these findings indicate that a critical rôle of the B chain in the inactivation process is highly unlikely.

In Figure 5 the number of uniodinated tyrosine residues in A 14 and A 19 positions is plotted versus the degree of iodination. At an average iodination degree of 4, all the A 19 and about 75 % of A 14 tyrosines have been substituted.

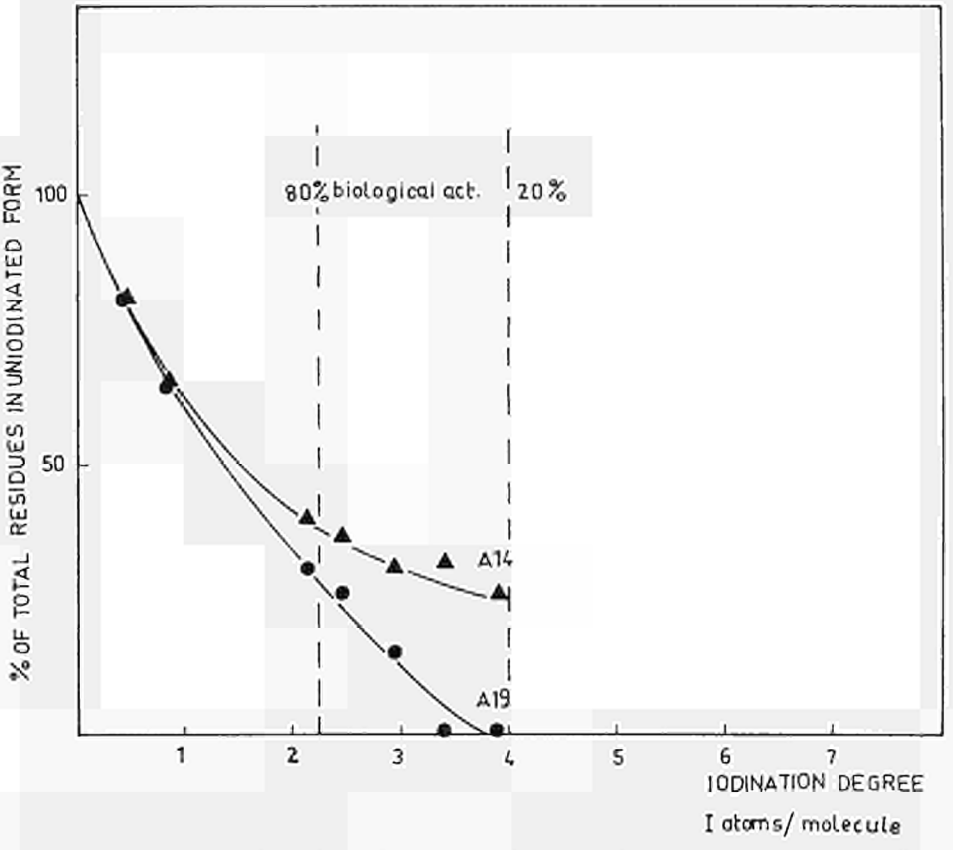


FIGURE 5. — Percentage of tyrosine residues A 14(▲)and A 19(●)in uniodinated form at different values of the iodination degree.

We have used these results in the attempt to identify the critical intramolecular combinations responsible for insulin inactivation. These analyses were performed with the aid of an IBM 7090 digital computer. Due to the limited number of available experimental data, the analysis was performed disregarding the B chain. This restriction, on a first approach, may be considered of limited importance, following our previous considerations. When A chain only is considered, 9 combinations are possible, as shown in Table II.

The probability for each combination was obtained as the product of the relative measured frequencies of its components; the fluctuation around the most probable distribution was disregarded in this first approach.

The hypothesis was made that every intramolecular combination could be either inhibitory or not inhibitory on the biological activity, thus obtaining a series of 2^9 (512) hypothetical structures. The computer was programmed to test the efficiency of every hypothesis in predicting the biological activity of each preparation for which distribution data were available.

TABLE II. — Possible intramolecular combinations (I.C.), deriving from the distribution of the iodine between Tyrosine residues A 14 and A 19

I.C.	Position A 14	Position A 19	Code (1)	
1	TYR	TYR	0	0
2	TYR	MIT	0	1
3	TYR	DIT	0	2
4	MIT	TYR	1	0
5	MIT	MIT	1	1
6	MIT	DIT	1	2
7	DIT	TYR	2	0
8	DIT	MIT	2	1
9	DIT	DIT	2	2

TYR = Uniodinated Tyrosine residue.
MIT = Mono-iodinated Tyrosine residue.
DIT = Di-iodinated Tyrosine residue.
(1) Code symbol identifying each Combination, for the Analysis at Digital computer.

The results of the analysis indicate that the hypothesis giving the best fit of our observations, about the correlation between biological activity and iodination degree, is that : inhibition is due to the intramolecular combinations 6, 8 and 9.

In other words, the inhibition effect due to the progressive iodination starts when one of the tyrosine residues of A chain has been mono-substituted and the other residue di-substituted. The confidence limits of these results will be well defined when the study, now in progress, will be completed.

We are fully aware of the limited significance of these data, due both to the various assumptions and restrictions introduced and to the limited number of experimental data.

However, from the practical point of view, it seems possible to conclude that in absence of di-iodinated tyrosines in the A chain, a preparation of iodinated insulin certainly retains its biological activity. In our preparative conditions, this requirement is met when insulin is iodinated below 0.5 iodine atoms per molecule.

In which way the inhibitory structures affect the biological activity is purely a matter of speculation.

However, it cannot be concluded without further investigations that one or more tyrosyl groups are directly involved in the hormonal activity.

One of the possible hypotheses is that tyrosine groups iodination creates a steric hindrance in the reaction on which hormonal effect is based. Before ending this communication, I would like to report briefly the results of an experiment that may suggest some possible interpretations of this phenomenon.

As it is well known, insulin contains three disulphide bridges per "monomer" unit, two connecting the A and B chains, the third one forming a ring in the A chain.

Cecil [7] has found that, when insulin reacts with sodium sulphite at a pH of about 7, only the two interchain -S-S- bridges are splitted.

When the Cecil's procedure was applied by us to study the reactivity of the sulphur bridges in a highly iodinated insulin, we found that only one -S-S- bond per insulin molecule was splitted.

Measurements carried out on iodoinsulin preparations, having an iodination degree ranging from 0.5 to 6 iodine atoms per molecule, gave the results reported in Figure 6.

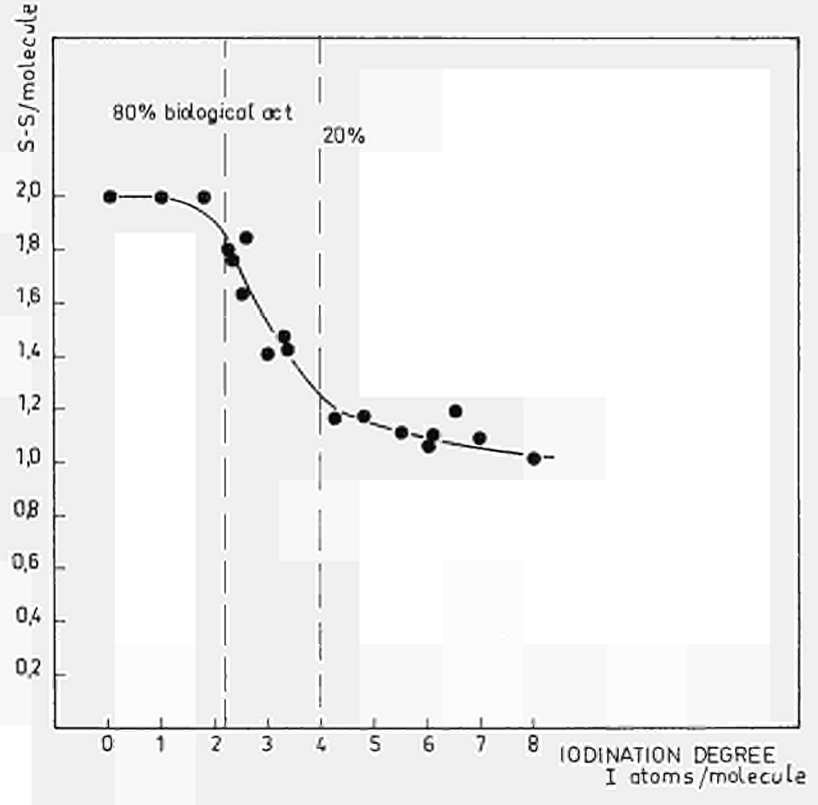


FIGURE 6. — Titratable S-S-/mole found in iodoinsulin preparations, having an iodination degree ranging from 1 to 8 iodine atoms per insulin molecule.

As it can be seen, iodination of insulin up to a substitution degree of about 2 iodine atoms per molecule, does not affect significantly the reactivity of the -S-S- bonds towards the sulphite, since the correct value of 2 -S-S- bonds per mole was measured. Iodination beyond 3 iodine atoms per molecule causes a progressive decrease in the value of the titratable -S-S- bonds. Only one -S-S- bond per molecule is splitted when the iodination degree is 4-5 iodine atoms per molecule, or more.

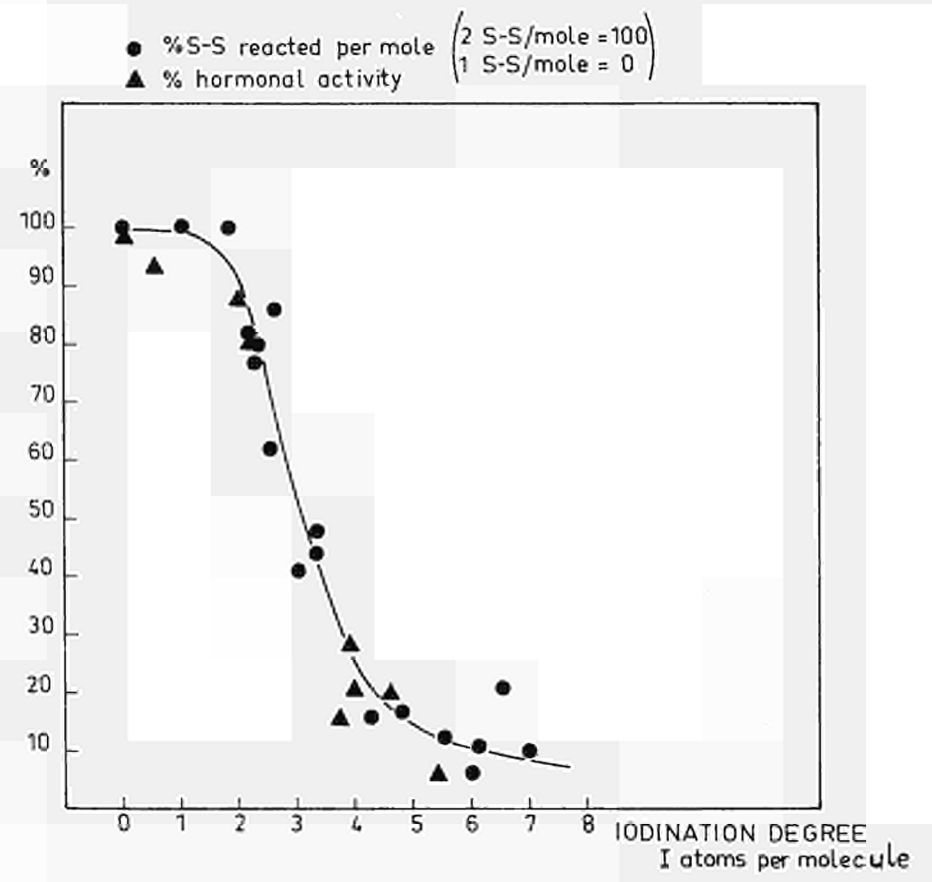


FIGURE 7. — Biological activity (▲) and -S-S- reacted/mole (●) versus iodination degree. Titration results are expressed in %, taking as 100 % the value of 2 -S-S-/mole found in native-insulin, and 0 % the value of 1 -S-S-/mole found in fully iodinated insulin.

This result appears to be an interesting example of how a substitution process, localized in the tyrosine groups, may interfere with the chemical behaviour of the other groups.

When the biological activity and the number of -S-S- reacted per insulin molecule, are represented on the same graph, as a function of the degree of iodination, both phenomena show a very similar trend (Figure 7). This was confirmed, when the computer program was used to correlate iodine distribution with the behaviour of the -S-S- bonds.

The same intramolecular distributions of iodine were found to account for both processes.

An attractive working hypothesis could be that splitting of interchains -S-S- bonds is an essential step in the insulin action.

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DISCUSSION

K. BRUNFELDT (*Gentofte*) : I should like to stress that by intravenous injection in rabbits we have found in our laboratory nearly same degree of inactivation as a function of the degree of iodination as has been demonstrated by Dr. Rosa.

E. SAMOLS (*London*) : It would seem to be worthwhile to clarify what is meant by biological activity of hormones. Dr. Rosa has shown a decrease in biological activity of insulin with increasing iodination. This measurement of native insulin activity is different from the determination of biological activity of "insulin" in biological fluids and extracts. For example Prof. Pfeiffer described a decrease of insulin like activity on addition of anti-insulin serum to the serum of Houssay dogs but no changes in blood glucose when these dogs were infused with the same anti-serum.

The possibility that the antiserum is acting primarily on the diaphragm or epidydimal fat pad rather than neutralizing insulin like activity must be considered. It should also be appreciated that bio-assay of low concentrations of insulin is beset by problems, and some knowledge about the index of precision of the bio-assays used would be relevant to the assessment of Dr. Rosa's results.

U. ROSA : Our results must be interpreted in the sense that they give a relationship between a localized substitution process on the insulin molecule and the hormonal activity measured under reproducible conditions in a well defined system. When the native insulin and the substituted one are compared by means of a bio-assay, I think that any difference we find must of course be attributed to the substitution process. On my opinion the example reported by Dr. Samols has nothing to do with this kind of experiments, although I quite agree that employing other kinds of bio-assays, one can possibly find a different relationship between iodination degree and biological activity.

As far as the precision of our bio-assay procedure is concerned, it was evaluated, on the basis of standard experiments, to be of the order of 15-20 %.

INFLUENCE OF IODINATION ON THE IMMUNOLOGIC PROPERTIES OF INSULIN (*)

H. A. OOMS and E. R. ARQUILLA

Laboratoire de Médecine Expérimentale et de Médecine Nucléaire
Université libre de Bruxelles, Belgium
and Department of Pathology, University of California, Los Angeles, U. S. A.

ABSTRACT

A double step, double tracer radioimmunoassay is presented which allowed us to study the immunologic behaviour of iodoinsulin. Iodination induces an impairment of the immunologic properties of insulin, increasing with the degree of iodination. This effect, slowly progressive is different from the biologic one.

In practice, this means that the degree of iodination should remain as low as possible.

A non saturation of the antibody sites by iodinated insulin has been found which has possible inference in the interpretation of the radioimmunoassay. Therefore every labelling methods should be evaluated, in order to use the best one.

The same problem probably exists for the other labelled proteinic hormones.

It has long been recognized that iodinated insulin preparations contain immunologic "damaged" hormone. Therefore methods have been developped to purify these preparations. Procedures are described for minimizing damage to the labelled hormone during its preparation, purification and incubation with plasma [24]. But the immunologic identity of these "purified" labelled insulin and non-labelled insulin has been much less questionned than the biological one, notwithstanding a lack of convincing experimental data. Only one indirect experimental argument is presented by two authors [4, 21]; they found "at any particular insulin concentration, the same degree of binding of ^{131}I-insulin whether the insulin present was derived entirely from the preparation of ^{131}I-insulin or was primarily non-iodinated insulin with only a tracer amount of iodoinsulin". It was assumed consequently that "the immunologic reactivity of insulin is not altered by the labelling procedure employed" [4].

The first purpose of the work was to set up a device allowing us to compare properly the immunologic identity of labelled and unlabelled insulins when both are put simultaneously in competition, as is the case in the radioimmunoassay. The comparison to be valid, requires both forms of insulin to be measured in the same experiment, by two different methods : one based on the distribution of the *radioactive molecules* (the radioimmunoassay itself); the second based on the direct evaluation of the *unlabelled molecules*, by transfer in a second system.

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In a second part the influence of various degrees of iodination is investigated. On the immunologic aspect of this problem, few data are found in the literature : Berson and Yalow [24] recommand the use of low iodination as probably advisable : "over-iodination appearing to have untoward effect on the stability and immunologic reactivity of insulin". On the contrary Izzo *et al.* [13] conclude that varying degree of iodination (1 to 5 at. I/mol) has no demonstrable effect on the binding of radioactive insulin to the insulin antibodies.

Comparison of labelled and non-labelled insulin

In the first part, we investigated the immunologic identity of labelled and non-labelled insulin.

Beef insulin has been used throughout the whole experiment to eliminate all problems of species specifity. ^{131}I- and ^{125}I-insulin were prepared by two different methods : Iodine monochloride [16] and elementary iodine [17]. The iodination degree was evaluated to 1 atom of iodine per molecule of insulin (M. W. 6,000).

Guinea-pig gammaglobulin was isolated from guinea-pig insulin antisera or normal serum by DEAE column chromatography [23]. The purity of this preparation was demonstrated by immunoelectrophoresis : it induced a single precipitating arc against rabbit anti-whole guinea-pig serum. This material was used for the production of rabbit antiserum against guinea-pig gammaglobulin. *The antisera* against beef insulin in the guinea-pig and against guinea-pig gammaglobulin in the rabbit were prepared according to Arquilla [1]. The insulin immune hemolysis inhibition assay (H. L.) has been set up by Arquilla. The λ, index of precision (S. D. of points about the regression line divided by the slope of the regression line) was 0.08. As radioimmunoassay (R. I. A.) : the double antibody method of Hales and Randle [10] was utilized slightly modified. The calculated λ was 0.05. Insulin biological assay : the rat epididymal fat pad method of Ball and Merril [2] was used to test the neutralizing biological capacity of the anti-insulin sera. The experimental design is summarized in Figure 1.

Guinea-pigs immunized with crystallin insulin produce anti-insulin serum. Rabbits injected with pure guinea-pig gammaglobulin produce precipitating antibodies directed against the guinea-pig gammaglobulin. The guinea-pig anti-insulin serum is mixed with the rabbit precipitating antiserum in adequate proportion to assure optimal precipitation; from the guinea-pig anti-serum, only the gammaglobulin, containing the anti-insulin antibodies are precipitated and isolated. To this precipitate is added a mixture of radioactive insulin (always the same tracer amount) and 10 times crystallized non-labelled insulin, in increasing amount.

Before use, the radioactive insulin has been purified on a Sephadex G. 75 or on cellulose column : 94 % of the radioactivity or more was able to bind to antibodies. After incubation, the precipitate containing the antibody-bound insulin is separated from the supernatant containing the free insulin. The duration of all incubations were controlled to allow completion of the antibody-antigen reaction.

In the first method used, the radioimmunoassay (R. I. A.), the results are based on the repartition of the radioactivity between the precipitate (or so-called antibody-bound insulin), and the supernatant (or so-called free insulin). The free insulin present in the supernatant is on the other hand directly measured by transfer in two different assays :

a) immune hemolysis assay (H. L. assay) or

b) another radioimmunoassay (2nd R. I. A.) using a different isotope of iodine as tracer for insulin (double tracer method) : in this second series of experiments different batchs of labelled insulin, other antisera, at other dilutions, were utilized. Also in one experiment, instead of mixing first the anti-insulin serum to the precipitating antibody to obtain a preformed precipitate (method B of Hales and Randle) the anti-insulin serum was first incubated with the insulins (labelled + unlabelled) and precipitation was carried out afterwards (method A of Hales and Randle). There was no difference in the result with pre- or post-formed precipitate.

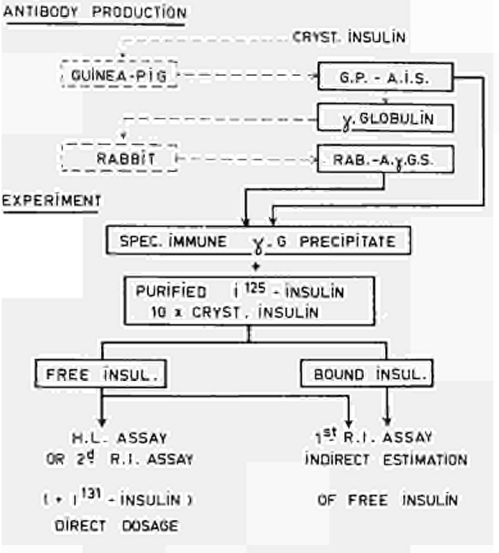

FIGURE 1. — Experimental design

If antibodies combined with both iodoinsulins and crystallin insulin to the same degree, then the free (or bound) insulin assayed *radioimmunologically* should have equalled the free (or bound) unlabelled insulin assayed directly by transfer in the second system.

Table I gives the result. At every concentration, in each experiment, less insulin is found free by the direct dosage, than is expected from the radioactive distribution.

The differences are statistically highly significant ($p < 0.01$). These results indicate that, when in competition with unlabelled insulin, iodinated insulin is preferentially displaced from the antibody complex and so demonstrates a significant impairment to combine with insulin antibodies.

TABLE I. — Comparison of the amount of free unlabelled insulin (remaining in the supernatant) as deduced

1° *indirectly* from the repartition of the radioactive insulin (1st R. I. A.).

2° *directly* measured by transfer in either an insulin immune hemolysis assay (H. L.) or a second radioimmunoassay (2nd R. I. A.).

Cryst. insulin added (μg)	Free insulin by	
	1st R. I. A. (in μg)	H. L. Assay (in μg)
0.5	0.11	0.03
1	0.39	0.016
2	1.17	0.55
4	2.89	1.9
6	4.8	2.9
(μU)	1st R. I. A. (in μU)	2nd R. I. A. (in μU)
250	33	0
500	186	86
1000	668	393

But from these experiments, another characteristic must be pointed out, which is illustrated in Figure 2.

Figure 2a represents the mean of 3 experiments obtained with free insulin dosed in the 2nd R. I. A. When 250 μU of insulin is added, no free insulin can be detected. This fits remarkably well the biological neutralizing capacity of the antiserum used; this concordance is an indirect proof of the validity of our results : in both conditions the titer of the antiserum was 1.5 U/ml. But, when no detectable insulin is found free, already 14 % of the radioactivity is displaced from the antibody into the supernatant.

Figure 2b represents the mean of 3 experiments obtained with the free insulin measured in the immune hemolysis system. Same results : when 12.5m U crystallin

insulin is added, almost no free insulin is found : again in very good correlation with the biological neutralizing capacity : but again, at this insulin level, 20 % of the radioactivity is displaced from the antibody.

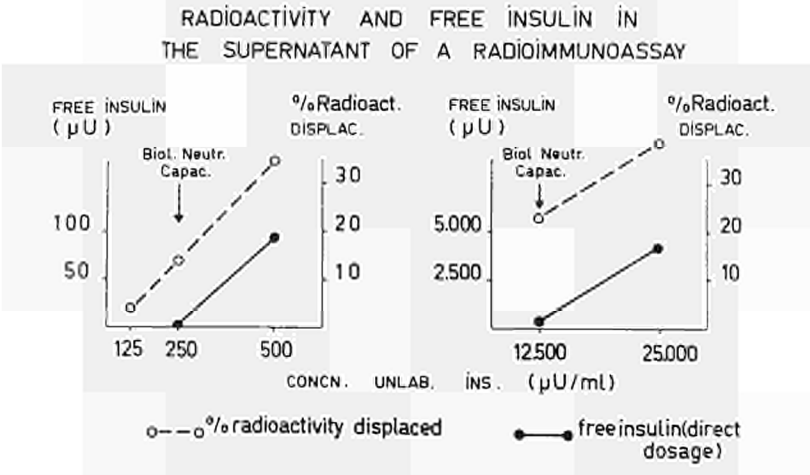

FIGURE 2. — Evolution of the % radioactivity displaced (0- - -0) and the free insulin directly dosed (●—●) in the supernatant of a radioimmunoassay
a) left figure : free insulin measured in the 2nd R. I. A.
b) right figure : free insulin dosed by H. L. Assay.

How can this be explained ? At any level below 250 μU (Figure 2a) or 12.5 mU (Figure 2 b), addition of crystallin insulin begins to displace fraction of the labelled molecules from the antibody complex into the supernatant. But at these levels as indicated by the further binding of higher amount of crystallin insulin, there are still antibody sites free, for unlabelled insulin, but not for the labelled molecules which remain unbound in the supernatant. So, iodoinsulin (or part of it) appears unable to saturate all the antibodies present in an anti-serum.

INFLUENCE OF THE DEGREE OF IODINATION ON THE IMMUNOLOGICAL PROPERTIES OF INSULIN

Recently we have received from Dr. Rosa radioactive insulin iodinated to varying degrees with ^{131}I or ^{125}I by the same method [16]. This allowed us to study the influence of the degree of iodination on the immunological properties of insulin. In each experiment two different iodoinsulins, one labelled with ^{131}I, the other with ^{125}I ,were mixed in tracer amount and incorporated in a radioimmunoassay. Figure 3 gives the curves obtained in experiments performed simultaneously with 3 different iodoinsulins; the more insulin is iodinated, the more it is preferentially displaced by equal amount of unlabelled insulin.

Figure 4 gives the results obtained with the different iodoinsulins prepared by Dr. Rosa's method in comparative R. I. A. performed on different days, these last 2 months. As reference, one single point of the R. I. A. curve was choosen to represent the radioactivity displaced.

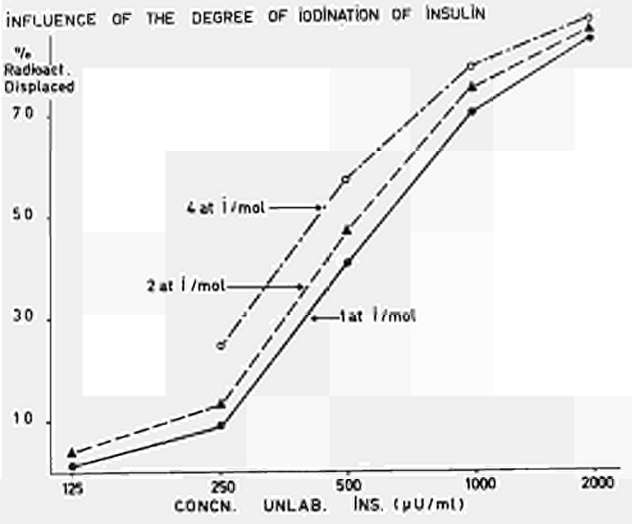

FIGURE 3. — Influence of the degree of iodination of insulin on its displacement by non-iodinated insulin

R. I. A. performed the same day with the same reagents: in one set of experiments, iodoinsulins with 1 and 2 at. I/mol were mixed; in the second, iodoinsulins with 1 and 4 at. I/mol. were mixed. In abcissa, % radioactivity displaced; in ordinate log. of crystall. insulin added.

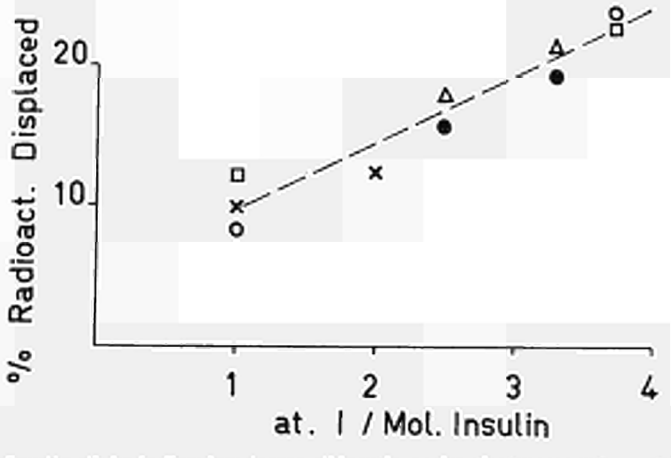

FIGURE 4. — Influence of the iodination degree of insulin on its displacement by unlabelled insulin. Experiments with various iodoinsulin batchs performed during a period of two months. As reference (for the % radioactivity displaced by unlabelled insulin): the level 250 μU unlabelled insulin added was choosen.

It is quite obvious that the displacement of radioactive insulin by cristallin insulin from the antibody is influenced by the degree of iodination. This effect appears different from the biological one. The influence here, is progressive, without sudden break in the curve, as is the case with the biological activity [20].

But these results are in contradiction with Izzo's results [13].

Therefore an experiment was set up, similar to that of Izzo. The binding of equal amount of two iodoinsulins ^{131}I and ^{125}I (1.1 and 3.8 at. I/mol insulin) is compared in presence of increasing amount of guinea-pig anti-insulin serum (Figure 5). Izzo *et al.* did not find any differences in their work. We find a significant one. This contradiction can be due to the different methods of iodination used : Rosa's method is probably more gentle and progressive, the oxidation method used by Izzo more explosive ?

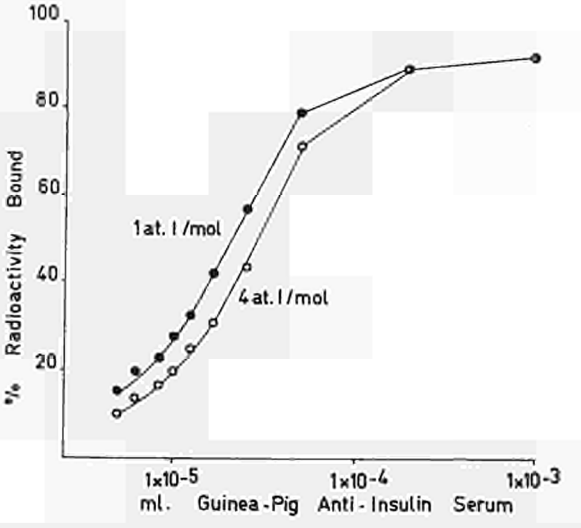

FIGURE 5. — Influence of the iodination degree of insulin on its ability to bind to increasing amount of anti-insulin antibodies

CONCLUSIONS

What conclusions to draw from these experiments :

First point : iodinated insulins were tested, prepared by three different methods : monochloride iodine, elementary iodine and electrolysis. At least with these preparations and the various antibodies used, these experiments demonstrate that iodination of insulin induces an impairment of its immunologic properties. This is consistent with different other data of the literature : indeed it has been shown that iodine binds preferentially to the tyrosine of the A chain [6, 7] ; tyrosines are important antigenic determinant [11]. Iodination of tyrosine is an important modification of a protein, as an antigen [11, 14]. Finally A chain appears, following the work of Wilson *et al.* [22] more critical than B chain in the neutralizing capacity of anti-insulin seras.

Second point : the impairment appears proportional to the degree of iodination (from 1 up to 4 at. I/mol). So increasing degree of iodination influences both the biologic and immunologic properties of insulin, but as shown, differently. This difference of the biologic and immunologic behaviour is in agreement with number of previous data. The results also confirm that the degree of iodination of insulin should remain as low as possible for R. I. A. use.

Third point : the non saturation of the antibody sites by iodoinsulins can have some inference in the interpretation of the radioimmunoassay. Indeed there are increasing doubts concerning the immunologic identity of extracted (crystallin) and the endogenous hormone [15, 18]. Endogenous hormone, if antigenically different, perhaps does not react with all the antibody sites directed against *crystallin* insulin. We show that iodoinsulin probably does not react with all the antibody sites. Both points together are a possible cause of error in the radioimmunoassay.

The problem raised up by the iodination of insulin should be probably extended to the labelling of other proteinic hormones. Less is known of these hormones than of insulin. What is known appears or the same or less favorable [5, 9, 12]. And yet, as far as we know, in the works dedicated to the R. I. A., we are not aware of any proper experimental device investigating the immunologic identity of the iodinated hormones.

Acknowlegments

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DISCUSSION

R. S. YALOW (*New York*) : 1) Do you believe that lose of immunologic integrity of the labeled hormone affects the validity of its use as tracer in immunoassay ? We have pointed out of course, that it is not.

2) While your experiments may prove that the iodinated proteins you prepare do not retain full immunologic activity, it is not safe for you to generalize concerning the immunologic behaviour of other preparations of iodinated hormone with other antisera. Obviously where labeled hormone is used as tracer for unlabeled hormone, the investigator is responsible for demonstrating that in his system labeled and unlabeled hormones react identically with antibody.

H. A. OOMS : I do not know if the question of immunological identity between the labeled and non-labeled hormone can yet be answered. Three insulins are present in a radioimmunoassay : endogenous insulin, extracted insulin and labeled extracted insulin. Should endogenous insulin be immunologically identical to the extracted hormone, then it would not be necessary for the radioactive hormone to be "identical" to the unlabeled one. But, as already told, if endogenous insulin is immunologically not identical to the extracted insulin, as suggested by recent works, then the use of a third form of hormone, also different, could lead to imprevisible error.

E. F. PFEIFFER (*Chairman*) : I should like to know Dr. Yalow's opinion about the many evidences (Fenton *et al.*, 1963; Renold *et al.*, 1963; Deckert, 1964) indicating antibody formation to species identical, homologous pancreas-insulin. Does not that mean that, in the course of extraction, insulin must be somewhat denaturated ?

R. S. YALOW : I am certain that the antigenicity of extracted hormone in homologous species implies that at least some fraction of the administrated hormone was not identical with endogenous plasma hormone. We might extend Dr. Pfeiffer's remarks to include the evidence from our laboratory and from other groups that human growth hormone is antigenic in man and we might even refer to the earlier work of Milgrom and Witebsky (*J.A.M.A.*, **174** : 56, 1960) who showed that precipitated and frozen *autologous* rabbit γ-globulin is antigenic in the rabbit. Thus, subtle changes may occur which can account for the antigenicity of homologous or even autologous serum proteins and hormones. I might call attention to a less subtle change that raises some concern about the interpretation of immunization experiments with supposedly "pure" beef or pork insulins. We have presented evidence (S. A. Berson and R. S. Yalow, *Am. J. Med.*, to be published in 1966) that Lilly pork insulin H 499667 contained a small, but significant contaminant of beef insulin. Dr. Otto Behrens of Lilly Laboratories several months ago confirmed that at the

time this lot of insulin was prepared (about 10 years ago), the facilities for preparation of beef and pork insulins were not completely separated and that contamination of pork insulin with beef and vice versa was not unexpected.

However, I am certain that Dr. Pfeiffer's question is not concerned with whether, because of accidental contamination or minor alteration extracted hormone appears to be antigenic in the homologous species, but rather that it is meant to imply that this antigenicity suggests that extracted insulin does not behave identically in the radioimmunoassay system with the endogenous insulin for which it serves as standard. We have in the past documented in detail our experimental evidence for the validity of radioimmunoassay when properly performed. The fact is that this is the only assay system in which dilution of plasma shows a linear response with respect to measured insulin concentration (R. S. Yalow and S. A. Berson, *J. Clin. Invest.*, 1960) so that in this critical respect at least plasma insulin does behave as pancreatic insulin.

SOURCES OF ERROR IN RADIOIMMUNOASSAY

L. G. HEDING

Novo Research Institute, Copenhagen, Denmark

ABSTRACT

Errors may stem from faults and irregularities in the analytical performance of the assay or they may be of a more specific nature related to the use of immunochemical reactions and labelled proteins. Some sources of error of the first kind are : adsorption of proteins to glassware, contamination of reagents with the compound to be assayed for, and lack of control with reaction times, temperature, pH, and salt concentration.

Sources of error in the second category include : (1) *species difference.* The standard antigen can be from another species than the antigen assayed, and then it should be carefully checked whether the reaction of the antigens from the two species towards the antibody is identical; (2) *structural difference.* The structure and thereby the specific immunological activity of the antigen may be changed in the extraction process used for the preparation; (3) *incomplete precipitation.* One major problem is the separation of free and antibody-bound antigen, and several different methods have been developed for this purpose. In the double antibody method, which includes the use of an anti-guinea pig γ-globulin rabbit serum, errors may occur due to cross reactions between human γ-globulin and the second antibody; (4) *presence of antibody in the sample.* Falsely high or low results, depending on the method of separation chosen, may occur if the samples (e. g. serum) already contain antibodies capable of reacting with the labelled antigen; (5) *inactivation of immunological activity.* Degradation of antigen or antibody, e.g. by enzymes, may give rise to falsely high values.

All the antigen in a sample may not be detectable in the assay, but may be present in an immunologically inactive state. Thus still another kind of error is possible, which may be important in the biological research.

The principle of the radioimmunoassay is simple. In constant volumes a known amount of antibody is mixed with a sample containing unlabelled antigen and a known amount of radioactively labelled antigen, and after a certain reaction time an equilibrium mixture of labelled and unlabelled antigen-antibody complexes has been formed. The amount of labelled complex formed will then be a function of the concentration of unlabelled antigen. A high concentration of unlabelled antigen will give a relatively low amount of labelled complex and vice versa.

As the amount of added labelled antigen is constant it is only necessary to determine the amount of either free or bound radioactivity, and a separation of these two components is the next step. The radioimmunoassay has first of all been used for determining hormones and unfortunately antibodies to hormones are in most cases incomplete, which means that they do not form a precipitate with their homologous antigen. Therefore, the separation of free and antibody-bound antigen

has to be done using special methods. Five different methods of separation have been used in the immunoassay :

1) Separation by chromatography or electrophoreses [1, 2].
2) Precipitation of the antibody-complex with an anti-γ-globulin antibody [3, 4, 5].
3) Precipitation of the antibody-complex with sodium sulfite [6] or ethanol [7].
4) Binding of the free antigen to an anion exchange resin [8, 9], or dextran-coated charcoal [10].
5) Separation by gel filtration on Sephadex G 75 [11].

Before mentioning some of the more specific sources of error related to the use of immunochemical reactions and labelled proteins I should like to discuss some errors, which may occur in the analytical techniques. Any of these errors may completely spoil the analysis and give wrong results.

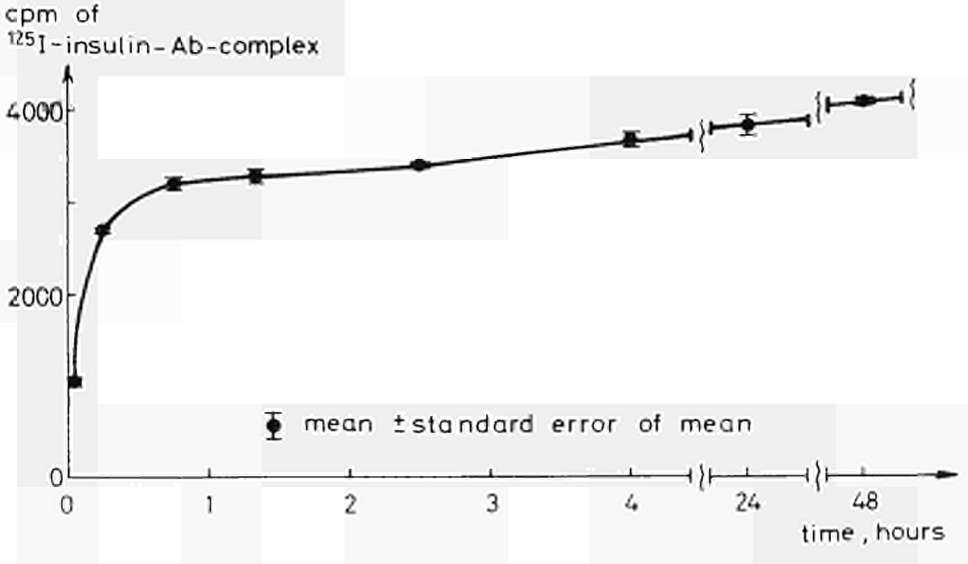

FIGURE 1. — Formation of insulin-antibody complex at different times

It is well known that proteins adsorb to the surface of glassware. At concentrations of protein hormones as low as normally used in radioimmunoassay a high percent of adsorption will occur if the hormone is not protected against adsorption by addition of other proteins, e.g. albumin. A concentration of 0.1 percent albumin is sufficient to prevent adsorption of insulin.

Another important source of error is contamination with the compound to be assayed for. When it is remembered that 1 mg of insulin is 25.000.000 μU of insulin and the amount of insulin in the sample may be 10 μU it can be understood that weighing and working with such or higher amounts in the laboratory presents a risk for contamination by air and glassware or other utensils.

When antigen-antibody-reactions take place a certain reaction time is needed before equilibrium is reached. Figure 1 shows a curve where the amount of formed antibody-bound insulin is plotted against varying reaction times. It takes several days to reach equilibrium in this system, and fortunately the analysis can be finished before this has occurred, but one has to choose a reaction time, which is so long that unavoidable accidental and systematic variations have no influence on the results. Variations in reaction time are not so easily controlled when the chromatographic or electrophoretic methods are employed, whereas an ethanol precipitation of 100 samples can be done within a few minutes.

The purity and the storage conditions of the antigen and the antiserum are very important. Radiation damage increases with the concentration of labelled antigen, which therefore must not be stored in too concentrated solutions. Normally

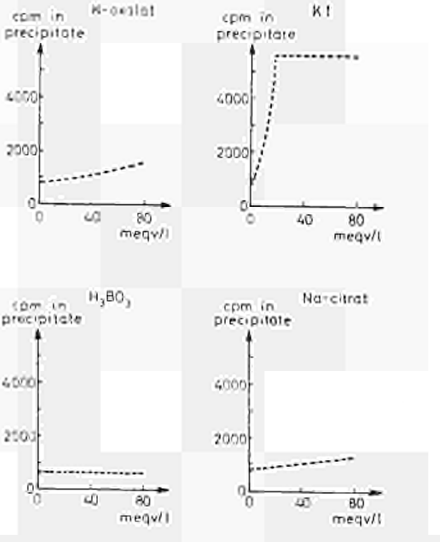

FIGURE 2. — Influence of different salts on the insulin-antibody reaction

an I-isotope is used for labelling the antigen and the ^{125}I-isotope is to be preferred to the ^{131}I, since it has a much longer half life time. ^{131}I has a half life of approximately 8 days which means that if a series of samples is counted over 20 hours the last sample will have lost 7 percent of the activity during the counting time, and the values must be corrected for this. ^{125}I has a half life of 60 days, and it can be mentioned that if a ^{125}I-insulin preparation with a specific activity of 10 mC/mg is stored at —30° C in the dilution used in the immunoassay, e.g. 300 μU/ml, the same solution can be used for more than six months without apparent loss of immunological activity. This can be checked by adding a surplus of antibody to the labelled antigen and determining how much becomes bound to the antibody.

A loss of potency of the antiserum can be observed if the sera are frozen and thawed several times, and if antisera are exposed to evaporation even at —30° C

a loss of immunological activity can also be shown. As ordinary rubber stoppers shrink at low temperature it can often be observed that the stoppers become too small after a storage at —30° C, and therefore a tight fitting cap should be used.

In the antigen-antibody-reaction pH must be under control. Morgan and Lazarow [5] have shown that the optimum pH is close to 7 for the insulin-antibody-reaction, and that a deviation of more than one pH-unit to either side causes formation of a smaller amount of antigen-antibody-complex. Variations of the pH will therefore give variations in the analytical estimates. Besides the pH the concentration of certain ions is of great importance to the reaction. Figures 2 and 3 show the influence of a number of different ions on the reaction between insulin and antibody : to a mixture of ^{125}I-pork insulin and insulin antiserum increasing amounts of different salts are added. It can be shown that only the monovalent anions influence the reaction and that the presence of one of these ions in a concentration above 20 mmol/l is necessary to ensure a complete reaction.

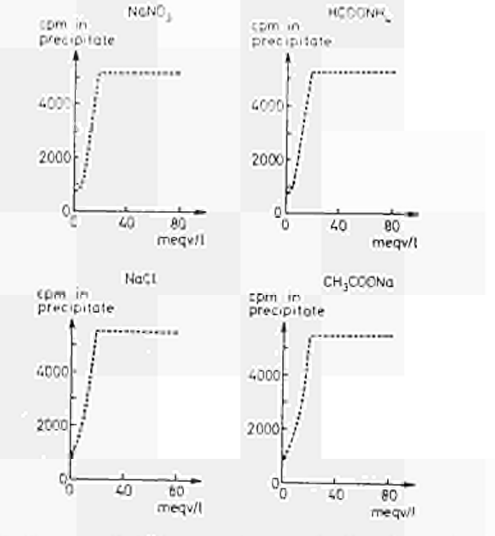

FIGURE 3. — Influence of different salts on the insulin-antibody reaction

If the concentration of necessary ions is too low or if the pH is wrong, too high estimates will result, and in general it can be said that most of the errors from the analytical techniques lead to falsely high values.

Errors due to wrong pH and salt concentration will naturally not occur when serum or plasma — not diluted too much — is analysed, but if extracts of serum are assayed, pH must be checked and the buffer used for dilution must contain enough active ions.

It is very important that the concentrations of antigen and antibody are adjusted such that the solution contains an excess of free antigen, when the first antibody-reaction is finished. This must not erroneously be interpreted as if the antibody

is saturated with antigen, since it does not bind all the antigen available. Figure 4 shows the amount of insulin bound at varying concentrations of free insulin; the antibody concentration is the same in all the determinations. When 180 μU is added to 1 ml of antiserum only 140 μU is bound, or 80 percent, but if 6,000 μU is added to the same amount of antibody 3,000 μU, or 50 percent, becomes bound to antibody, and if more insulin is added even more becomes bound.

Insulin binding of serum from a diabetic patient

I added insulin μU/ml	B bound ins. μU/ml	F free insulin μU/ml
180	144	36
370	244	126
1,570	930	640
1,490	865	625
5,900	2,950	2,950
12,900	5,160	7,740
25,000	8,760	16,240
113,400	15,850	113,400
238,000	16,700	221,300

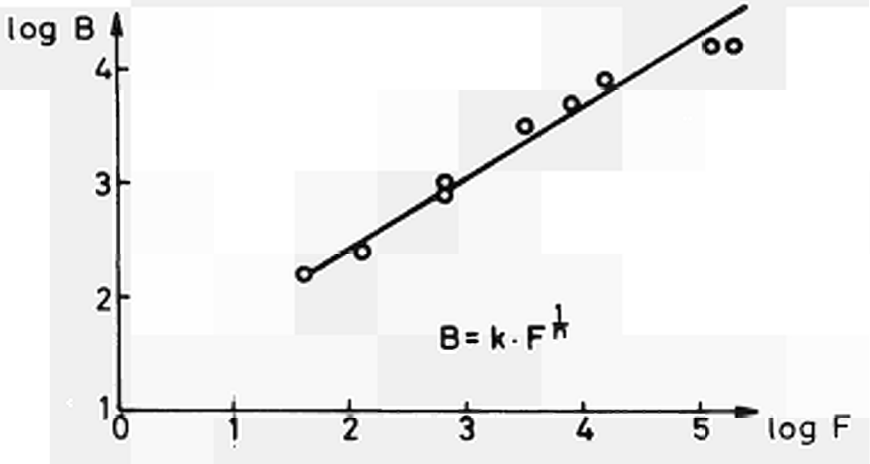

FIGURE 4. — Binding of insulin to antibody at different concentrations of insulin

A number of important trivial errors has been mentioned, and furthermore a few sources of errors more specially related to labelled proteins and immunoassay shall briefly be discussed.

Errors due to species differences of the antigen may occur because antibodies only react with the substance used to produce them and more or less with substances showing close chemical and structural relationship with the homologous antigen.

Apart from insulin it is very difficult to obtain pure preparations of the human hormones, and therefore one often has to use hormones from animals both for immunizing and as a standard for a human hormone. It is therefore very important to check that the hormones from the two species react identically with the antibody. Berson and Yalow[12] have shown that human and pork insulin could give different standard curves with several different antisera although the two hormones only differ in one amino acid. It is possible that this difference is dependent on other causes than the small variation in the chain sequence. However, in most cases insulin antisera react similary with pork and human insulin, whereas beef insulin often reacts differently.

It has been stated that it is necessary that the labelled and unlabelled antigen should have the same binding affinity to the antibody, but this is not the case, when the conditions are the same in the standard which is made at every run and in the assay system. Of course it is of no advantage if the labelled antigen has a much greater affinity to the antibody, since this will decrease the sensitivity of the assay. It is incidentally a very difficult task to determine whether the specific affinity of the labelled and the unlabelled antigen to the antibody is different.

It must be remembered that all the hormone preparations used to-day as standard have been extracted from animal tissue by a procedure which may involve treatment at low pH, extraction with an organic solvent, salting out, and so on. It cannot be excluded that these treatments may alter the hormones, e.g. in such a way that the chain conformation becomes different from the native structure, and hereby the immunological specificity can be changed. To-day one does not know whether the circulating hormones, measured with the immunoassay, are identical with the extracted hormones.

Furthermore it can not excluded that some of the natural hormones in blood are bound and threrefore do not react with antibody, but retain the activity in biological assays. This would explain differences between biological assays and immunoassays.

It is well known that persons treated with insulin form antibodies against insulin, and blood samples from persons containing antibodies against the antigen to be assayed therefore cannot be analysed directly. The assay will give either too high or too low estimates depending on the method of separation and the antiserum used.

One of the major problems in the radioimmunoassay is the separation of free and antibody-bound antigen, and one of the methods most commonly used is the double antibody method, where the antigen-antibody-complex is separated by an immunoprecipitation with an anti-guinea-pig γ-globulin antibody :

1) Ag-Ab (soluble) + anti-γ-globulin (soluble) $\rightarrow$ Ag-Ab-anti-γ-globulin (insoluble precipitate)

2) Ab (soluble) + anti-γ-globulin (soluble) $\rightarrow$ Ab-anti-γ-globulin (insoluble precipitate)

Unfortunately the anti-guinea-pig γ-globulin is able to cross react with human γ-globulin to a certain degree thereby causing a decrease in the amount of antibody capable of precipitating the antigen-antibody-complex [13]. In some instances this may cause an incomplete precipitation, which gives falsely high levels. It is generally accepted that this error can be avoided if a co-precipitation of the two antibodies is used as shown. The first antibody does not lose its ability to bind the antigen after the precipitation has taken place. If one still prefers the first modification for technical reasons a surplus of anti-γ-globulin must be used, and it must be checked that the incomplete precipitation does not occur.

The last source of error to be mentioned is inactivation of immunological activity due to degradation or denaturation of antigen and antibody. It is obvious that an enzymatic degradation of either antigen or antibody will disturb the results. Degradation or denaturation of the antibody in the samples will always give rise to erroneously high results, because the antibody will bind a smaller amount of labelled antigen. Degradation of both labelled and unlabelled antigen may give either too high or too low results (in most cases too high).

Degradation of the antigen does normally only occur to a slight degree in serum and plasma samples, but after a fractionation of e.g. serum by electrophoresis it has been shown by Orskov [14] that the very high insulin values found in some of the fractions were false and due to degradation of the labelled insulin. Fortunately it is easy to control whether the antigen is degraded by using the chromatographic technique, whereas antibody degradation is more difficult to observe.

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DISCUSSION

H. A. OOMS (*Bruxelles*) : In the double antibody method, one of the causes of error reported by Dr. Heding consists in incomplete precipitation of the complex "antigen-first antibody" by the second antibody (anti-guinea-pig gammaglobulin antiserum). This is in several papers generally only attributed to cross-reaction between human gammaglobulin and guinea-pig globulin. I do not know if demonstration of this cross-reaction, by immunoelectrophoresis has yet been published. But even if this is or can be the case, another cause we think more important in practice has to be taken into account : Morgan and Lazarow (*Diabetes* 13, 579, 1964) have demonstrated the interference of complement in the inhibition of the first antibody-second antibody reaction. Another cause of error as pointed out by Dr. Yalow is to neglect to evaluate the incubation "damage". This can be easily performed in the double antibody method. After the normal incubation period of the antigen -first antibody reaction, an excess of the first antibody is added and after a second incubation period the complex antigen-excess first antibody is precipitated by the second antibody.

C. N. HALES (*Cambridge*) : Concerning the evidence of a cross-reaction between human gammaglobulin and an antiserum to guinea-pig gammaglobulin prepared in rabbits, Prof. Randle and I have published such evidence (Hales C. N. and Randle P. J. (1963) *Biochem, J.*, **88**, 137) and a photography of the precipitation reaction quoted is contained in my Thesis (Hales C. N., Ph. D. Thesis, Cambridge University).

RADIOIMMUNOLOGISCHE BESTIMMUNG VON ACTH IM SERUM

F. MELANI

I. Medizinische Universitätsklinik
Abteilung für Klinische Endokrinologie, Johann Wolfgang Goethe-Universität, Frankfurt/Main, Deutschland

ABSTRACT

A radioimmunoassay of serum ACTH is presented. ^{131}I-ACTH was prepared of specific activities of 300-500 mC/mg by the method of Hunter and Greenwood and purified free of components damaged by Sephadex G-50 column after incubation with normal serum. ^{131}I-ACTH degradation products after two purifications are less than 5 % of total radioactivity. To separate antibody bound ^{131}I-ACTH we used an anion-exchange resin (Amberlite CG 400 I), already employed for serum insulin assay. For the preparation of standard curves we used an anti-ACTH-rabbit-serum diluted 1:40,000, 1:50,000. The sensitivity of the method is at level of 0.05-0.1 m μg/ml. The values of serum ACTH in normal subjects was found to range from 0-0.4 m μg/ml. Elevated ACTH-levels were also demonstrated in an Addison-patient and in two Cushing patients after adrenalectomy.

ZUSAMMENFASSUNG

Es wird über eine radioimmunologische Bestimmung von ACTH-Serum berichtet. Nach der Methode von Hunter und Greenwood wurde ^{131}J-ACTH mit spezifischer Aktivität von 300-500 mCi/mg hergestellt und nach Inkubation mit normalem Serum über eine Sephadex-G-50-Säule von beschädigten Bestandteilen gereinigt. Nach zweimaliger Reinigung machen die ^{131}J-ACTH Abbauprodukte weniger als 5 % der Gesamtradioaktivität aus. Zur Isolierung von antikörpergebundenem ^{131}J-ACTH verwendeten wir ein Anionenaustauschharz (Amberlit CG 400 I), das bereits zur Insulin-Serum-Bestimmung verwendet wurde. Zur Aufstellung der Standardkurven verwendenten wir ein Anti-ACTH-Kaninchen-Serum in einer Verdünnung von 1:40.000 und 1:50.000. Die Empfindlichkeit der Methode liegt im Bereich von 0,05-0,1 m μg/ml. Es wurde festgestellt daß bei Gesunden die Werte für ACTH-Serum zwischen 0 und 0,4 m μg/ml liegen. Hohe ACTH-Werte wurden bei einem Patienten mit Addisonscher Krankheit und bei zwei Patienten mit Cushingscher Krankheit nach Adrenalektomie nachgewiesen.

Die Messung von ACTH im Blut mit der radioimmunologischen Technik wurde dadurch möglich, daß einerseits hochgereinigte ACTH-Präparate zur Verfügung gestellt, andererseits Antikörper gegen ACTH bei Tieren erzeugt wurden. Untersuchungen von McGarry und Mitarb. [6], Yalow und Mitarb. [12], Himura und Mitarb. [4] über die biologischen und immunologischen Eigenschaften von natürlichen und synthetischen ACTH-Präparaten haben gezeigt :

1. Die biologische Aktivität des ACTH liegt bei den ersten 20-26 Aminosäuren des Moleküls. Die vollsynthetischen Peptide, die die ersten 24 (α1-24) oder 26

(α1-26) Aminosäuren des adrenocorticotropen Hormons darstellen, besitzen die biologische, nicht aber die gleiche immunologische Aktivität der hochgereinigten Präparate.

2. Die immunologische Aktivität ist besonders and den letzten Teil (20-39 Aminosäuren) des Moleküls gebunden.
3. Antikörper gegen Schaf-ACTH reagieren auch mit Schweine- und Menschen-ACTH; in Kreuzreaktionen zeigen Schaf-, Schweine- und Menschen -ACTH ähnliches immunologisches Verhalten, wobei Schweine- und Menschen-ACTH sich nicht unterscheiden.

Als erster benutzte Felber [1] für die Bestimmung von ACTH im Blut das von Yalow und Berson [13] für die Bestimmung von Insulin angegebene Prinzip. Bei Anwendung des Doppelantikörper-Systems von Hales und Randle [3] fand Felber bei Stoffwechselgesunden sehr hohe Werte von 30-50 mμg/ml Plasma.

Ebenso wie für Insulin, Wachstumshormon und Parathormon verwenden Yalow und Mitarb. [12] zur Trennung des gebundenen vom freien ^{131}J-ACTH die Chromatographie und die Papierelektrophorese. Die Methode dieser Autoren ist sehr empfindlich; mit ihr können bis 0,06-0,12 mE ACTH/100 ml Plasma gemessen werden. Bei Stoffwechselgesunden schwanken die Werte von Yalow und Mitarb.

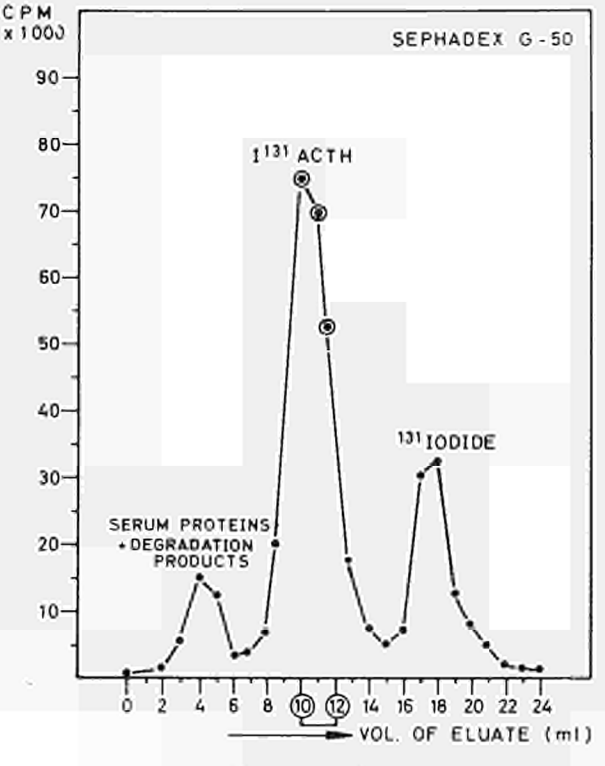

ABBILDUNG 1.

zwischen 0,15 und 0,38 m μg/ml Plasma mit einem Mittelwert von 0,26 m μg/ml (0,6 mE/100 ml). Diese Werte entsprechen den Werten der biologischen Methoden.

Um das freie und das antikörpergebundene ^{131}J-ACTH zu trennen, haben wir Anionenaustauscher verwendet (Amberlite CG 400 I). Die Anionenaustauscher wurden schon von Meade und Klitgard [7] und von uns selbst [8] für die Bestimmung von Insulin im Serum benutzt. Die Trennung beruht auf der Eigenschaft des Kunstharzes freies ^{131}J-Hormon zu binden (Free). Die nicht an den Anionenaustauscher gebundene Radioaktivität entspricht antikörpergebundenem ^{131}J-Hormone (Bound).

Das anti-ACTH-Serum wurde von Kaninchen gewonnen. Die Tiere wurden in Abständen von drei Wochen — über einen Zeitraum von sechs Monaten — mit 1 ml einer Emulsion immunisiert, die zu gleichen Teilen aus Schweine-ACTH-Lösung und Bacto Adjuvant Complete (H 37 Ra, Difco Laboratories, Detroit, USA) bestand.

ACTH wurde mit ^{131}J nach der von Greenwood und Mitarb. [2] angegebenen Methode markiert. Diese Methode verwendet Na-^{131}J (IBS 3 von Radiochemical Center Amersham, England) mit einer Gesamtaktivität von 2-4 mC und Chloramin T als Oxydationsmittel. Die spezifische Radioaktivität von ^{131}J-markiertem ACTH lag bei unseren Versuchen zwischen 300 und 500 mC/mg (0.4-2 Atome Jod/mol ACTH).

Bei der Markierung entstehen durch die Strahlung und andere chemische Alterationen des Hormonmoleküls radioaktive Degradationsprodukte, die sich an Serumproteine binden. Zur Vermeidung von Fehlern bei der radioimmunologischen

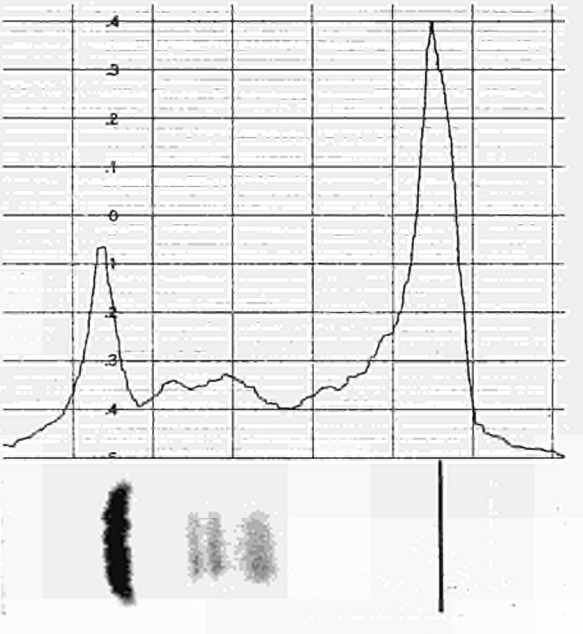

ABBILDUNG 2.

Bestimmung muß das verwendete ^{131}Jod-Hormonpräparat von solcher unspezifischen Radioaktivität befreit werden. Zur Reinigung wurde das ^{131}Jod-ACTH nach der Markierung mit Bromphenolblau-gefärbtem Serum kurz inkubiert und dann auf einer Sephadex-G-50-Säule filtriert [9]. In dem milliliterweise gesammelten Eluat findet man drei deutlich abgegrenzte Radioaktivitätsgipfel (Abbildung 1). Der erste entspricht der an die Serumproteine gebundenen unspezifischen Radioaktivität, der zweite dem ^{131}J-ACTH, der dritte dem Jodid-131, das nicht an das Hormon gekoppelt wurde. In der Papierelektrophorese wandert die unspezifische Radioaktivität mit allen Serumproteinen, während das intakte Horminmolekül, von der Zellulose des Papierstreifens adsorbiert, am Startpunkt bleibt (Abbildung 2).

Nach ein oder zwei Reinigungen des ^{131}J-ACTH in Sephadex G-50 findet man im Papierelektrophoretogramm nur einen scharfen Radioaktivitätsgipfel. Nach Inkubation mit normalem Serum liegt er am Startpunkt (Abbildung 3, oben), während er nach Inkubation mit anti-ACTH-Serum im Bereich der Gammaglobuline zu finden ist. (Abbildung 3, unten).

Entsprechend sind die Ergebnisse mit Amberlite CG 400 I. Das Harz wurde vor Gebrauch aus der Chloridform in die Hydroxylform umgewandelt. In dieser

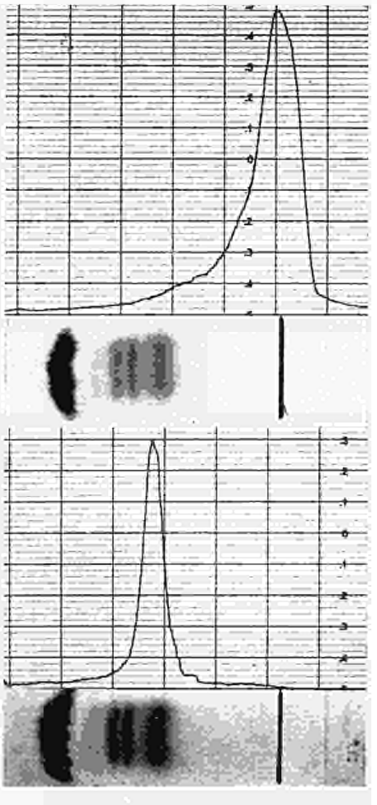

ABBILDUNG 3.

Form kann Amberlite CG 400 I das freie ^{131}J-ACTH binden, nicht aber die an Albumin oder an Serumproteine adsorbierte unspezifische Radioaktivität und nicht das antikörpergebundene Hormon.

Die Abbildung 4 zeigt die Adsorption des ^{131}J-ACTH und der unspezifischen Radioaktivität an Amberlite in Abhängigkeit von der Konzentration des Albumins oder des Serums. ^{131}J-ACTH wurde in 2 ml Veronal-Puffer (0,1 M, pH 8,6) verdünnt und 15 Minuten mit 200 mg Amberlite geschüttelt; danach wurde die Radioaktivität im Überstand gemessen.

Die Verteilung der unspezifischen Radioaktivität auf Amberlite und Überstand ändert sich mit der Konzentration des Albumins oder der Serumproteine im Lösungsmittel. Bei Gebrauch eines Puffers ohne Albumin oder mit niedriger Albuminkonzentration (0,5 %) werden die Degradationsprodukte zum großen Teil von dem Harz adsorbiert (Abbildung 4). Steigt die Albuminkonzentration im Puffer, so steigt auch die Radioaktivität im Überstand. Mit Albuminkonzentrationen von 2-5 % erreicht die Adsorption einen Grenzwert, der durch weitere Zugabe von Albumin nicht mehr deutlich überschritten wird. Wie mit 1:4 verdünntem Serum sind praktisch alle Spaltprodukte an Protein adsorbiert und können auf diese Art als konstanter Faktor im Überstand gehalten werden. Mit einem gut gereinigten ^{131}J-ACTH wird, wie die Abbildung 4 zeigt, das freie ^{131}J-ACTH auch bei einer Albuminkonzentration von 20 % von Amberlite völlig adsorbiert.

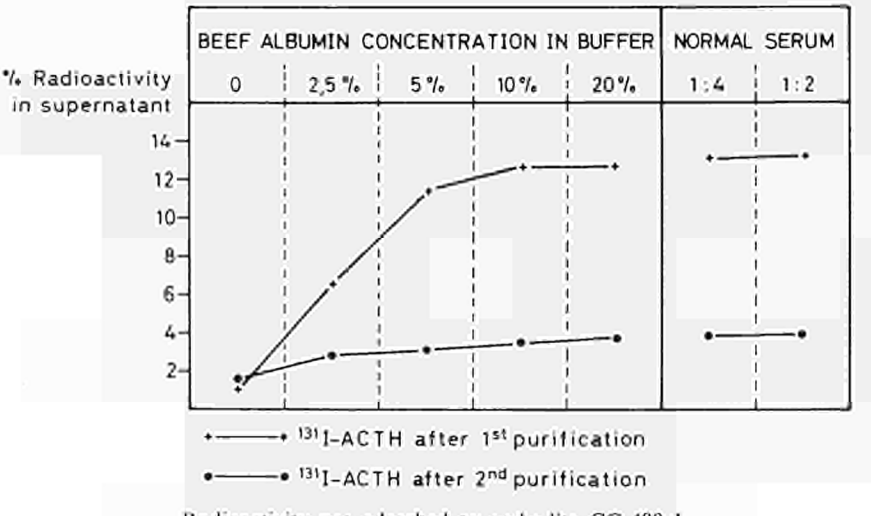

ABBILDUNG 4.

Die Voraussetzungen für die radioimmunologische Messung des ACTH sind in der Abbildung 5 dargestellt. Freies ^{131}J-ACTH muß im Serum ebenso wie im Puffer von Amberlite adsorbiert werden.

Die Zugabe von Serum (1:2, 1:4 verdünnt) darf die Verteilung der Radioaktivität auf Amberlite und Überstand nicht beeinflussen. Das Antiserum muß so verdünnt werden, daß 70-80 % des ^{131}J-ACTH an Antikörper gebunden sind.

Radioactivity in supernatant 0,05-0,1 mμg ^{131}I-ACTH	
Buffer-2 % alb.	3-5 %
Serum 1:2, 1:4, 1:8	3-5 %
Dilution of antiserum 1:40,000	70-80 %

ABBILDUNG 5.

Die Ansätze für die Standardkurve und für die Serumbestimmung enthalten :
1 ml verdünntes Antiserum (1:40.000 — 1:60.000) mit 0,01 — 0,03 m μg/Jod^{131}-ACTH.
1 ml unmarkiertes ACTH (2,5 bis 0,05 m μg) oder Serum (1:2; 1:4; 1:8 verdünnt).

Für die Ansätze haben wir entweder Veronal-Natrium-Puffer (0,1M, pH 8,6) oder physiologische Kochsalzlösung mit Zusatz von Rinderalbumin (1-2 %, normalem Kaninchenserum (1 %) und 0,01 % Merthiolat verwendet.

Nach drei Tagen Inkubation wurden jedem Wassermannröhrchen 200 mg Amberlite zugesetzt. Nach 15 Min. Schütteln und kurzem Zentrifugieren wurde die Radioaktivität von 1 ml Überstand gemessen. Sie entspricht der Hälfte des antikörpergebundenen ^{131}J-ACTH (1/2 B).

Die Abbildung 6 zeigt eine typische Standardkurve. Ohne Zusatz von unmarkiertem ACTH sind 80 % der Radioaktivität an Antikörper gebunden. Schon mit nur 0,05-0,1 mμg unmarkiertem ACTH hat man einen signifikanten Abfall der Radioaktivität im Überstand (gebundenes ^{131}J-ACTH). Mit 2,5 mμg ACTH sind nur 36 % der Radioaktivität an die Antikörper gebunden.

Mit verschiedenen Schweine-ACTH-Präparaten fanden wir bei der Aufstellung der Standardkurven keinen signifikanten Unterschied.

Die Abbildung 7 zeigt, da der Verlauf der mit drei Schweine-ACTH-Präparationen (75-84,5-100 E/mg) gebildeten Standardkurven völlig identisch ist. ACTH, einem Serum zugesetzt, wurde bei verschiedenen Serumverdünnungen zu 80-100 % wiedergewonnen.

Bei einer Serumverdünnung von 1:2, 1:4; 1:8 messen wir bei Stoffwechselgesunden Werte im Bereich von 0 bis 0,4 mμg/ml (3,4 mE/ml). In Gewichtseinheiten

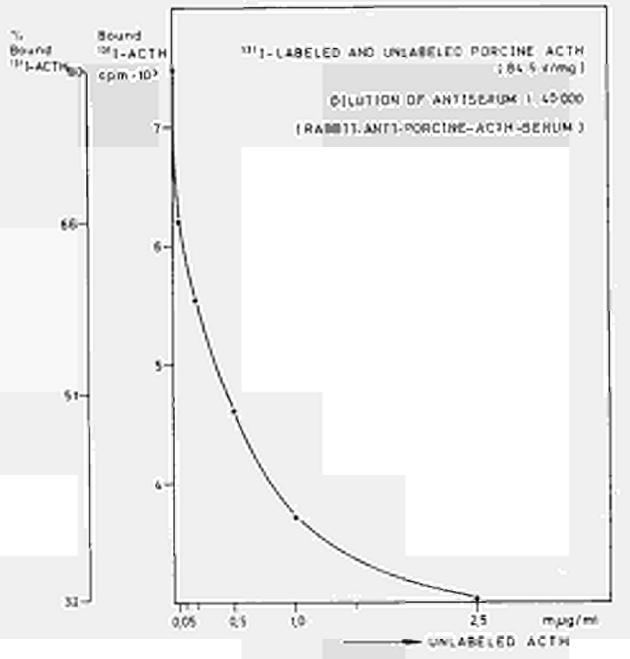

ABBILDUNG 6.

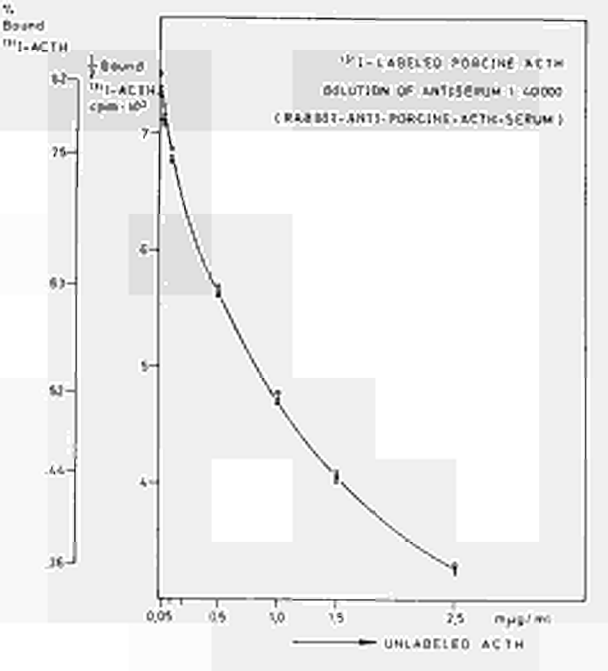

ABBILDUNG 7.

angegeben, entsprechen unsere Werte denen von Yalow und Mitarb. [12]. In biologischen Einheiten sind sie etwas höher. Bei Stoffwechselgesunden werden in unserem Laboratorium mit der biologischen Methode [5, 10, 11] Werte im Bereich von 0,25-1 mE/100 ml Plasma mit einem Mittelwert von 0,4 mE/100 ml gemessen.

Erhöhte Werte von ACTH im Serum fanden wir bei einem Addison-Patienten (2 mμg/ml; 16,8 mE/100 ml Serum) und bei zwei operativ behandelten Cushing-Patienten (2,2-2,6 mμg/ml; 18,5-21,8 mE/100 ml Serum).

ZUSAMMENFASSEND :

1. Die radioimmunologische Methode, die wir benutzt haben, ist sehr empfindlich und technisch einfach.
2. Die Werte bei Stoffwechselgesunden entsprechen in mμg berechnet denen von Yalow und Mitarb.
3. Die kleinen Unterschiede, die bei der Umrechnung in biologische Einheiten auftreten, liegen wahrscheinlich in der nicht einheitlichen Standardisierung der verschiedenen ACTH-Präparationen.

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DISCUSSION

J. P. FELBER (*Lausanne*) : Dr. Melani's method seems very interesting, being simple and precise. The plasma values obtained are similar to the ones published by Yalow *et al.* (*J. Clin. Endocrinol.*, **24** : 1219, 1964) and slightly lower than ours since we have started using precipitation in the double antibody system.

What seems striking in this paper is that Dr. Melani's method does not seem to suffer from the difficulties due to ACTH degradation in plasma. The ACTH estimations are done in serum instead of plasma. Would it be possible that the degradation of ACTH which certainly does occur during the period of coagulation would affect the biological activity without affecting the immunological activity ?

It is also of interest that this method does not seem to suffer from incubation damage, which obliged other autors (Yalow *et al.*) as well as ourselves, to use mercaptoethanol to prevent this degradation.

R. S. YALOW (*New York*) : I have been asked to comment on the potential interference of ACTH fragments in the immunoassay.

In our first report on the immunoassay of ACTH in plasma we noted that synthetic and highly purified α-MSH and hormonally active synthetic tricosapeptide inhibited the binding of labeled synthetic pork ACTH to antipork ACTH serum (Figure 1) (R. S. Yalow, S. N. Glick, J. Roth, S. A. Berson, *J. Clin. Endocrin. and Metab.*, **24** : 1219, 1964). But at least a thousand to ten thousand fold higher quantities are required to produce the same effect as intact ACTH. The higher reactivity of native β-MSH may be related to contamination with ACTH since the preparation used was not highly purified. Since that time Imura, Grodsky *et al.* have reported that, with their antiserums, hormonally inactive peptide fragments reacted more

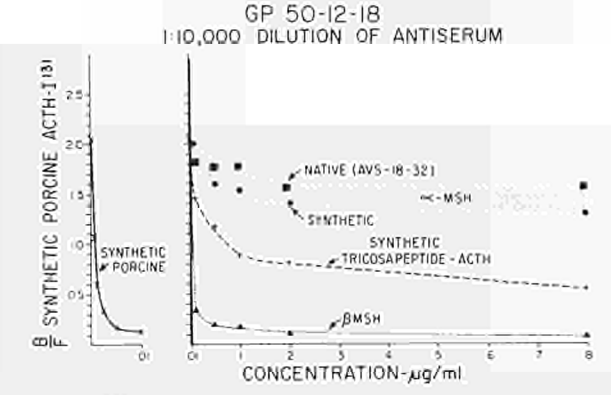

FIGURE 1. —B/F ratios for ^{131}I-labeled synthetic porcine ACTH (Schwyzer) as a function of concentration of ACTH (left), various MSH's and ACTH tricosapeptide (Hofmann) (right). The synthetic α-MSH was prepared by Dr. K. Hofmann. The heavy vertical line adjacent to the ordinate axis of the right-hand graph shows the entire concentration range of the left-hand graph (Reproduced from *J. Clin. Endocrin. and Metab.*, **24** : 1219 (1964)).

strongly that intact ACTH. As I emphasized earlier, this must be tested individually for each hormone and with each antiserum.

With the antiserum we now employ for the assay of ACTH, both hormonally active (1-23 and 1-24) and inactive (11-24 and 25-39) ACTH fragments reacted hundred to thousand fold less strongly than intact ACTH; so that these fragments would have had to be present in very high concentration to be of significance. Furthermore, no constant factor will tansform the cross-reactivity curves of the fragments to that of the intact preparation. For the latter reason, when there is present in plasma a substance which cross-reacts in the immune system, but which is not identical

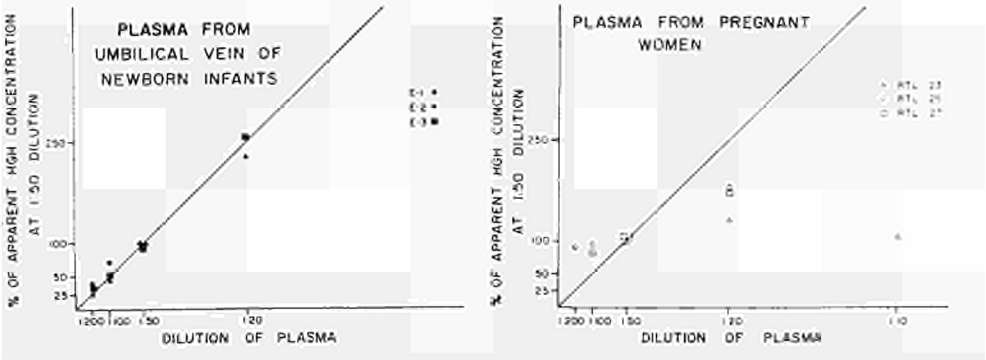

FIGURE 2. — Effect of dilution of plasma on apparent HGH plasma concentration. Values should fall along the diagonal line if reacting material is immunochemically identical with pituitary HGH used as standard. Plasma from normal adults and from acromegalic subjects behaves like plasma from umbilical veins of newborn infants. Plasma from pregnant women does not behave properly on dilution. (Reproduced from *Rec. Prog. in Hormone Res.*, XXI : 241 (1965)).

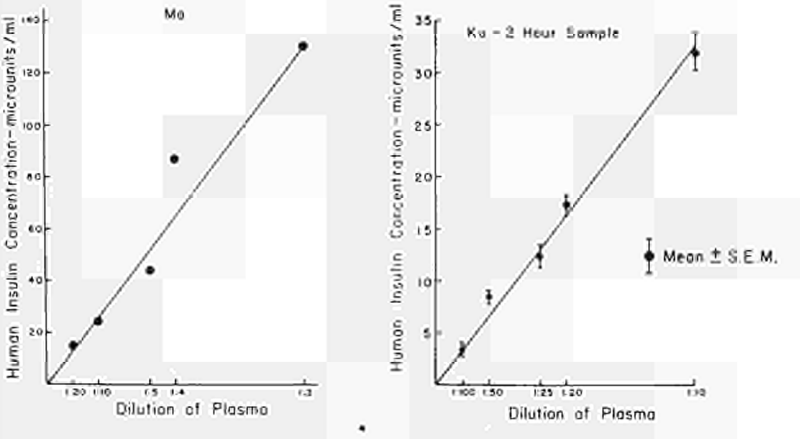

FIGURE 3. — Effect of dilution of plasma on measured concentration of endogenous plasma insulin. For replicate determinations were made for each point in the experiment on the right. (Reproduction from *J. of Clin. Invest.*, **39** : 1157, 1960)).

with the hormone used as the standard, dilution of the plasma does not produce a proportionate decrease in the apparent concentration of hormone. For instance, plasma from the umbilical cord blood of the new born, from acromegalic subjects or from normal subjects stimulated to secrete high levels of growth hormone all show proportionate decrease in hormone concentration on dilution (S. M. Glick, R. S. Yalow, J. Roth, S. A. Berson, *Nature*, **199** : 784, 1963). But plasma from pregnant women does not behave properly on dilution because of the presence of placental lactogen, a substance which cross-reacts with, but is not identical with, human growth hormone (Figure 2) (S. M. Glick, J. Roth, R. S. Yalow, S. A. Berson, Rec. Prog. In Hormone Res., XXI : 241, 1965).

In the case of insulin, we earlier demonstrated proportional decrease in endogenous hormone concentration on dilution (Figure 3) (R. S. Yalow and S. A. Berson, *J. Clin. Invest.*, **39** : 1157, 1960). With ACTH also, dilution of plasma from 1 : 2.5 to 1 : 100 results in proportionate decrease in hormone concentration.

It would appear as if a dilution experiment similar to those described above would provide the most useful direct demonstration of immunologic identity of reactive material in plasma with the hormone used in the preparation of the standard curve.

E. F. PFEIFFER (*Chairman*) : I should take the opportunity for commenting on the comparisons between the ACTH values measured by immuno- and by bioassay. We are familiar with the bioassay, based on corticosterone production of the dexamethasone (pituitary) blocked and hypophysectomized rat, measuring the corticosterone increase either in the peripheral or in the adrenal venous blood since several years (Pfeiffer *et al.*, 1960; Retiene *et al.*, 1962; Pfeiffer *et al.*, 1963). As a matter of fact, it has turned out to be our best assay, running with an index of precision of $\lambda = 0.18$ over 4 years. This was due to the fact that we cannulated the adrenal vein of the hypophysectomized rat from the femoral vein, drawing the blood before and after injection of the standard and the serum, each animal serving as its own control. One of my associates, Dr. Retiene was invited by Dr. Lipscomb to join him for one year time, running the Lipscomb and Nelson procedure and our own, without having any differences. Hence, I feel any differences observed cannot be attributed to the method but rather to the different ACTH content on a weight basis of the various ACTH preparations used as standards. Moreover it seems to us that the immunoassay *per se* always gives a little bit higher values, and this can be due to the fact that the 1-39 aminoacid residues containing molecule is measured immunologically, whereas the bioassay (steroidogenic capacity taken as parameter of ACTH activity) mostly evaluates the N-terminal portion.

RADIOIMMUNOASSAY OF GLUCAGON

K. F. WEINGES (*)
II. Med. Universität Klinik und Poliklinik
Homburg/Saar, Deutschland

ABSTRACT

A short review is given of the method for radioimmunoassay of glucagon, which was first described by Unger and co-workers. Glucagon antibodies have been demonstrated in rabbit, guinea-pig and goat. Various immunization techniques have been tested. The immunization with P. V. P. or C. D. I. conjugated glucagon mixtures given weekly seems to be most successful and reliable. Like insulin antibodies, glucagon antibodies are of the nonprecipitating type and can, therfore, be detected only by such method as the paper chromato-electrophoretic, precipitation or ionic exchanger techniques.

The preincubation of nonradioactive glucagon with glucagon antiserum reduces binding of ^{131}I-glucagon to a degree related to the quantity preincubated, thereeby providing the basis of a radioimmunoassay for glucagon.

Other peptide hormones do not influence ^{131}I-glucagon binding, implying a high degree of specificity for the assay. Cross reaction between human or canine glucagon and antibodies to beef-porc glucagon has been proved.

Therefore the radioimmunoassay for glucagon is highly specific and exquisitely sensitive and facilitates the measurement of circulating glucagon in concentrations as little as 150-200 μμg/ml. Glucagon levels have been determined in the plasma of dog and man in different metabolic situations to study the physiologic rôle of this hormone.

The labelling of proteo-hormones with ^{131}I has been discussed competently. The procedures employed for radio-iodination of glucagon are a modification of the Pressman-Eisen technique [1, 2], the method of Hunter and Greenwood [3], and the one described by Niemann (**).

These techniques provide a high specific activity without undue damage to the glucagon molecule. Biologic assays of ^{131}I-glucagon failed to demonstrate a greater loss of biologic activities despite weeks of storage in frozen state.

The specific activities used are in a range between 10 and 600 mCi/mg. The degree of specific activity has to be considered when different techniques of separation between free and antibody-bound hormone are applied.

The main problem of the glucagon-assay is the production of specific antiserum, probably due to the relatively low molecular weight of 3483.

Various procedures of immunization with different adjuvants, precipitations and the conjugation with larger molecules, as well as different injection techniques and various animals have been used. They are summarized in Figure 1 [4-7].

(*) The own investigations were supported by Deutsche Forschüngsgemeinschaft.

(**) These Proceedings, p. 43.

Antigens	Animals (No.)	Method of Inject.	No. of Inject.	Cases with Antibodies (Authors)
Beef-pork glucagon in complete Freund's adjuvant (1 mg/2 ml) (emulsified or alum-precipitated)	white female rabbits (29)	0.2 ml foot pad 1.8 ml subcutaneously (hindquarter)	monthly for 3 months	27 (Fig. 2) (Unger, R. H. *et al.* : J. Clin. Invest., (1961) 40 : 1280)
Recrystallized porcine glucagon suspended in Bayrol-Alarcel or Freund's adjuvant	male albino rabbits (4)	subcutaneous 3-10 mg	varying intervals over a 2-year	4 (after 1-3 months) (Grodsky et al. *Proc. Soc. Exp. Biol.* (1961) 107 : 491).
or conjugated to ovalbumin with bis-diazo benzidin suspended in adj.				
Beef-pork glucagon alum-precipitated in Freund's adjuvant.	female goat (1)	subcutaneous (neck) 10 mg	1 inj. every two weeks	1 (after 3 months) (Weinges et al. : *Verh. Dtsch. Ges. Inn. Med.*, Bd. 71 (1965) 713).
Beef-pork glucagon + Freund's adj. (emulsified or alum-precipitated).	rabbits (8) chickens (5)	subcutaneous 1 or 2 mg	5 5	0 (7) (Assa, R. et al. : 0 (3) Lancet 1965 II, 590)
Beef-pork glucagon + Freund's adjuvant.	rabbits (3) chickens (2) guineapigs (5)	» » subcutaneous + foot pad 1 or 2 mg	3 3 3	1 (3) » 0 (2) » 4 (4)* »
Beef-pork glucagon (10 mg/2 ml H_2O) + polivinyl pyrrolidone (3 ml; 25 %) + Freund's adjuvant (5 ml)	guineapigs (5)	subcutaneous + foot pad 1 or 2 mg	3	3 (4)* »
Conjugated beef-pork glucagon (10 mg) rabbit or guineapig serum albumin (100 mg in 0.5 ml H_2O) with carbodiimide (100 mg).	rabbits (4) guineapigs (1)	subcutaneous subcutaneous + foot pad 1 or 2 mg	3 3	3 (3) » 1 (1) »

( ) No. of animals tested
* one animal died — anaphylactic shock

The demonstration of antibodies after immunization with emulsified or precipitated glucagon with or without Freund's adjuvant remains inconstant. The immunization with conjugated glucagon, however, seems to be more successful and reliable, as described by Assan. The antigenicity of glucagon seems to increase by association (conjugation or adsorption) with larger molecules, proteinic or not. This may be related to the greater stability of the molecule and to the well-known fact that insoluble antigens are more immunogenic than soluble antigens. Preparations of long-acting glucagon are shown in Figure 2 (alium-precipitated) and Figure 3 (conjugated with CDI).

Solutions (sterile) :

1. 0.35 g borax in 25 ml water (stock solution).
2. 10.0 g KAl $(SO_4)_2$.12 H_2O in 100 ml water.
3. 5 n sodiumhydroxide (NaOH).

Preparation : (mixed in a 100 ml glass tube, work under sterile conditions).

1. 0.8 g of eggalbumin in 12.0 ml normal saline.
2. 70 mg glucagon dissolved in 30 ml solution of (16 g (ml) glycerol, 2.27 (ml) phenol, 981.73 g (ml) H_2O, titrated to pH 2.85 with HCl).
3. add. a mμg ^{131}I-glucagon in 0.1 ml (in order to determine the loss of glucagon).
4. add. 45 ml 10 % KAl $(SO_4)_2$ — solution 2.
5. titration to pH 6.5 with 5 n NaOH — solution 3.
6. spin at 2000 RPM 5 min, discard supernatant.
7. wash twice with 50 ml borax solution — 1.0 ml of stock solution 1. in 99.0 ml normal saline.
8. dissolve again with an adequate volume of normal saline.
9. count glucagon-disappearance rate — recovery of 80 per cent is sufficient.
10. prepare for injection the suspension (1-2 mg glucagon in 1 ml) can be injected to animals with or without Freund's adjuvant.

FIGURE 2. — Preparation of long-acting glucagon (Unger *et al.*)

1. rabbit or guineapig serum albumin 100 mg in 0.5 ml of water.
2. beef-pork recrystallized glucagon 10 mg (powder).
3. carbodiimide ("Morpho CDI", Aldrich Chemical) 100 mg (powder).

(work sterile, mix, conjugation mixture must be dialysed before injection to prevent the death of the animals).

Linkage was checked using labelled glucagon. Fractionation by "Sephadex G-75", by paper electrophoresis, and by immuno-precipitation with an anti-albumin serum proved that 40-70 % of radioactivity was bound to albumin.

Guinea-pigs received 1 or 2 mg glucagon weekly. The animals were bled 14 days after the third injection.

FIGURE 3. — Preparation of long-acting glucagon (Assan *et al.*)

Like insulin antibodies, glucagon antibodies are of the nonprecipitating type and can, therefore, be detected only by such methods, which permit separation of free ^{131}I-glucagon from antibody-bound ^{131}I-glucagon.

Detection and study of antibodies can be carried out by the Berson, Yalow and Volk technique adapted for ^{131}I-glucagon [8] (Figure 4). This method is based

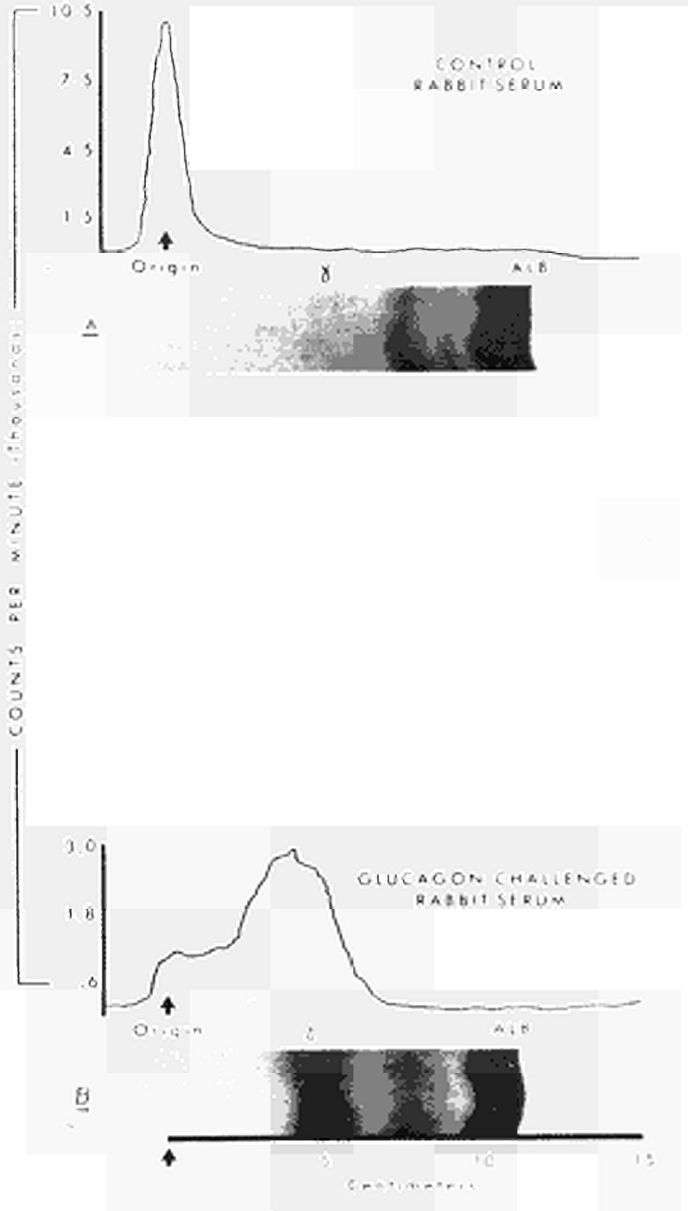

FIGURE 4 (Unger *et al.*)

upon the observation that ^{131}I-glucagon, when incubated in normal serum and then applied to a filter-paper strip, is adsorbed to the paper at the point of its application (origin) and will not, under these circumstances, migrate either chromatographically or electrophoretically, thereby permitting its separation from the serum proteins. In the presence of glucagon antibodies, however, ^{131}I-glucagon is expected to bind to and migrate with globulin in a manner similar to that described for ^{131}I-insulin and insulin antibodies [9]. Whereas the insulin-binding antibodies of humans and guinea pigs are in the inter-$\gamma\beta$-zone, the glucagon-binding antibodies of rabbits and guinea pigs appear to migrate electrophoretically as a γ-globulin.

Berson, Yalow and Volk have shown that a small fraction of ^{131}I-glucagon damaged by irradiation or other factors, during or after the iodination procedure, migrates non specifically with serum proteins. Therefore, parallel control runs with undiluted serum from unimmunized rabbits are necessary. The damaged moity ranges from 4.0 to 20 per cent, but is almost always less than 10 per cent.

The antiserum also can be tested by salt precipitation (Na_2SO_4) or by ionic exchanger (Amberlite).

Protein content	Curve	Protein content	Samples
.014	1.0 ml albumin free buffer 0.2 ml 7 % albumin (pH 7.6)	—	1.0 ml albumin free buffer
.001	0.1 ml antiserum (1:20) in 1 % albumin-glycine buffer dilution	.002	0.2 ml antiserum (1:40) in 1 % albumin-glycine buffer dilution
.001	0.1 ml standard in 1 % albumin-glycine buffer dilution	.014	0.2 ml sample (unknown serum)
	one hour preincubation		
.002	0.2 ml ^{131}I-glucagon in 1 % albumen-glycine buffer dilution	.002	0.2 ml ^{131}I-glucagon in 1 % albumen-glycine buffer dilution
.018	1.6 ml	.018	1.6 ml
	incubation 4 days at 4° C		

FIGURE 5. — Preparation of spot-curve and samples for radioimmunoassay of glucagon *(0.2 m glycine buffer, pH 8.8)*

A dilution of antiserum able to bind 60-80 per cent of the iodine-labelled glucagon permits the measurement of as little as 150-200 μμg/ml of glucagon with confidence and with a reproducibility approaching 2 per cent.

The preparation technique carried out for the radioimmunoassay of glucagon is demonstrated in Figure 5.

An identical protein content in both, samples of standards and samples of unkonwn serum, is very important.

The mixture is incubated at 4° C for at least 24 hours, better even for 2 to 4 days.

Free and antibody-bound glucagon can be separated with different techniques similar to the method to separate free and antibody-bound insulin :

I. paper chromato-electrophoretic method of Berson and co-workers [9, 10, 11],

II. Na_2SO_4-precipitation [12, 13],

III. ionic exchanger [6].

The preparation of specimens for salt precipitation is shown in Figure 6. The procedure is simple, but there is the disadvantage of co-precipitation of free glucagon in amounts of approximately 6 to 10 per cent.

We are using the ionic exchanger Amberlite CG 400 I. A sufficient amount of Amberlite is added to the antibody-glucagon mixture samples after the incubation, shaken at room temperature for 1 hour and then centrifuged. Free glucagon will be adsorbed to the ionic exchanger (F) and the antibody-bound glucagon remains in the supernatant (B). The activity of aliquots of the supernatant is counted.

Specimens	Curve	Sample
1	1.6 ml (0.2 ml 7 % albumin)	1.6 ml (0.2 ml plasma)
	add : mixture of "carrier protein".	
	0.2 ml EDTA plasma	0.2 ml 7 % albumin
	1.0 ml human plasma	1.0 ml human plasma
	1.0 ml glycine buffer	1.0 ml glycine buffer
	3.8 ml final volume	3.8 ml
2	mix and stir well; allow to set at room temperature for 1 hour	
3	spin at 2,000 RPM for 30 min.	
4	remove supernatant carefully with a capillary pipette.	
5	wash with 6 ml of 15 % Na_2SO_4, stir well	
6	spin again, remove supernatant	
7	count radioactivity of precipitation.	

FIGURE 6. — Preparation of samples for Na_2SO_4 precipitation (separation between free and antibody-bound glucagon)

After incubation the mixtures were allowed to reach room temperature. Place 8-12 samples in waterbath at 30° C

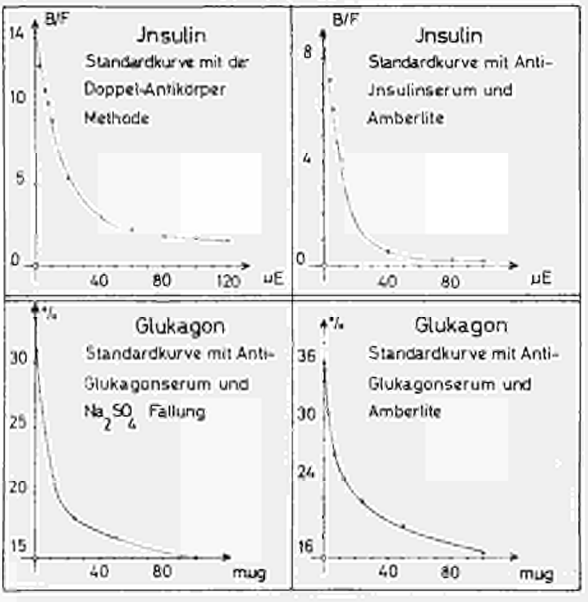

FIGURE 7

Good separation can be achieved as compared with the paper-chromato-electrophoretic method. The advantage is the possibility to use a ^{131}I-glucagon with the relatively low activity of 10 to 20 mCi/mg.

Standard curves of salt precipitation and separation by means of ionic exchanger are given in Figure 7.

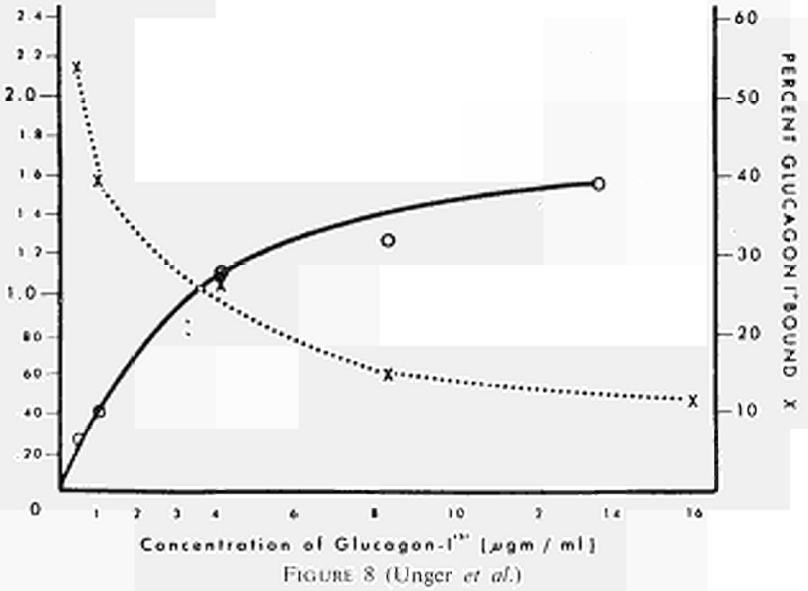

FIGURE 8 (Unger *et al.*)

Studies of kinetics were done [10]. Similarities between insulin-binding and glucagon-binding reactions were observed. First, the reaction of glucagon and its antibody was found to be reversible. Second, like insulin-binding, glucagon binding is governed by the law of mass action; at a constant antibody concentration the amount of ^{131}I-glucagon bound increases with the concentration of ^{131}I-glucagon, although the percentage of ^{131}I-glucagon bound decreases progressively (Figure 8).

There is no evidence of cross reactivity between insulin and glucagon, the binding of ^{131}I-insulin to globulins in anti-glucagon serum and of ^{131}I-glucagon in anti-insulin serum being no greater than in non-immune sera. The ability of other peptide hormones to lower the B/F ratio of ^{131}I-glucagon was tested and compared with that of glucagon. Only glucagon causes a significant decline in the B/F ratio of ^{131}I-glucagon, suggesting a high degree of specifity for the assay method [10].

Curves made with different lots of ^{131}I-glucagon and different lots of diluted antiserum differ from each other to a varying degree. Excellent reproducibility can be noted when the same standards and other materials are set up in duplicate on the same day. Under these circumstances Unger [10] found in 35 consecutive duplicate B/F ratios of glucagon standard solutions r mean standard deviation of $\pm$ 0.-0436.

In order to test the ability of the immunoassay to measure exogenous beef-pork glucagon *in vivo*, 3 mg unlabelled glucagon were injected intravenously and blood specimens were drawn at intervals for one hour thereafter. Measurements

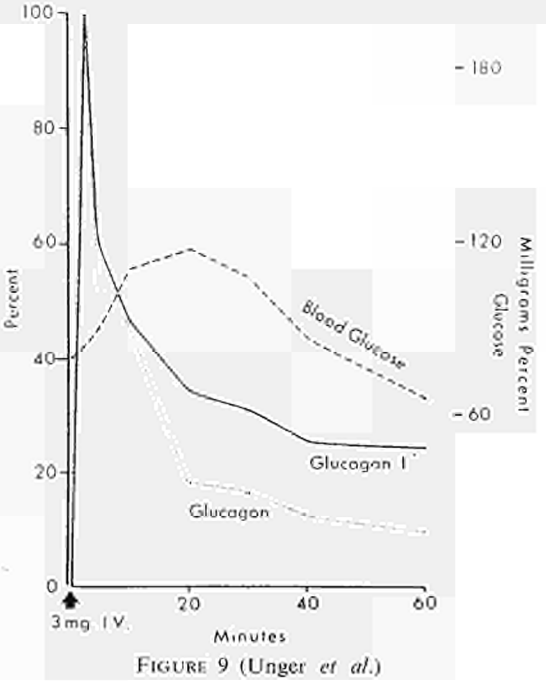

FIGURE 9 (Unger *et al.*)

of glucagon concentration by means of the immunoassay gave a disappearance curve for exogenous glucagon quite similar to that obtained by radioactivity measurements after radioglucagon administration (Figure 9).

A higher plateau of trichloroacetic acid-recipitable radioactivity is attributed to the altered ^{131}I-glucagon which binds to serum proteins and disappears less rapidly than undamaged ^{131}I-glucagon.

The high glucagon concentrations, measurable in extracts of human and canine pancreas, indicate a cross reaction between the glucagon of these species and antibodies to beef-pork glucagon.

These results of Unger and co-workers are the bases for the study of circulation endogenous human glucagon in health and disease.

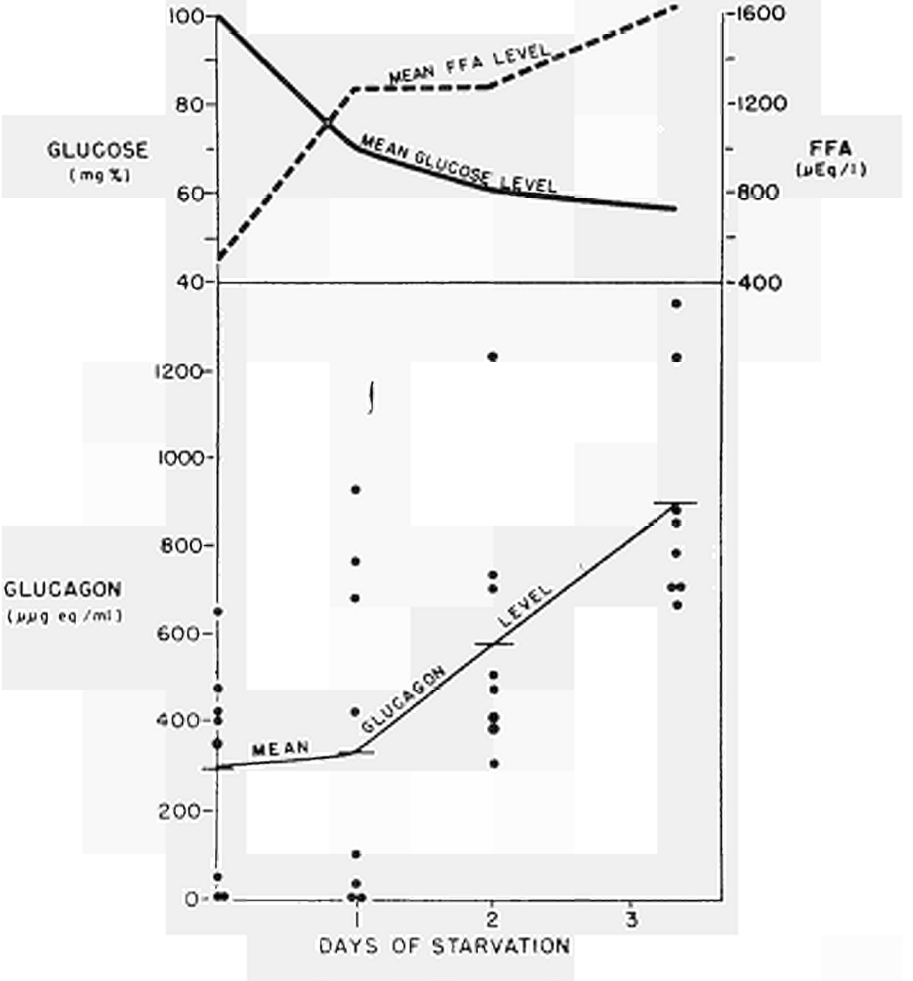

FIGURE 10 (Unger *et al.*)

By means of the exquisitely sensitive and highly specific radioimmunoassay, glucagon has been measured in the plasma of dogs and man for the first time. The glucagon concentration in dogs ranges from 0 to 800 μμg/ml mean 496 μμg/ml) and in man from 0 to 650 μμg/ml (mean 292 μμg/ml) (*).

Dogs made acutely hypoglycaemic by a rapid administration of glucagon-free insulin, generally show a gradual rise in glucagon secretion. Severe chronic hypoglycaemia in dogs induced by the administration of phloridzin is accompanied by a more striking and consistent rise in glucagon secretion. The mean pancreaticoduodenal venous glucagon level in the hypoglycaemic dog is approximately 4 times that of the control group [14].

In man the chronic glucose need induced by total starvation is associated with a 3-fold rise in mean peripheral venous glucagon concentration after 72 hours of fasting (Figure 10) [15].

These findings reveal the presence of endogenous glucagon in pancreatic venous blood, and indicate that glucagon secretion is enhanced during glucose need, irrespective of cause, and that sudden glucose replenishment abruptly abolishes this hyperglucagonaemia [16].

Glucagon mobilizes not only glucose from liver glycogen but also non-esterfied fatty acids in adipose tissue and provides the organism with two most important substrates with high energy potentials [17].

In its physiologic rôle glucagon seems to be a "starvation hormone". Epinephrin, in comparison, which has very similar metabolic effects could be called a "stress hormone".

More studies on glucagon secretion are necessary to gain further knowledge of its physiologic significance (**). Roger Unger wrote in his last letter that he has found a patient with a glucagon producing tumor with the extremely high peripheral glucagon level of 50 mμg/ml.

I think he will report on the metabolic stiuation of this patient later on this year.

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(*) Samols found glucagon concentrations in plasma of man range from 1.0 so 5.0 mμg/ml.
(**) Samols has some new points and I am very interested of his studies and results.

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DISCUSSION

E. SAMOLS (*London*) : Dr. Weinges is to be congratulated on his presentation, at short notice, of the admirable work of Dr. Unger. The availability of an anti-glucagon antiserum with a suitable binding energy for endogenous glucagon is of course a critical requirement for the radioimmunoassay of plasma endogenous glucagon levels in man. In the absence of a human glucagon preparation, it is important that serial dilution of plasma should give a proportional decrease in glucagon concentration, to show that human endogenous glucagon cross-reacts perfectly with the beef pork glucagon used as a reference standard. Neither Dr. Weinges nor Dr. Unger have, to the best of my knowledge, presented information on plasma dilution curves. We have recently reported (E. Samols, J. Tyler, G. Marri and V. Marks, *Lancet* ii, 1257, 1965) mean overnight fasting endogenous glucagon concentrations in man of 2 mμg/ml, almost ten times greater than the mean level of 0.29 mμg/ml mentioned by Dr. Weinges. We were therefore concerned about the validity of our assay values, but our results have been supported by plasma dilution studies. Over a range of plasma dilutions from 1 : 2 to 1 : 50 the decrease in endogenous glucagon is almost proportional. Dilution curves enable us to construct standard curves for endogenous glucagon which show, with our antiserum, a virtually identical cross-reaction between human endogenous glucagon and beef pork glucagon. It is possible that our fasting glucagon levels are higher that those of Dr. Unger in part because the cross-reaction between endogenous and exogenous glucagon is better in our antiserum. Dr. A. M. Lawrence from Chicago has also recently reported fasting endogenous glucagon concentrations in agreement with our results.

On the other hand the lower fasting glucagon levels reported by Dr. Unger are, in one respect, more plausible to me than our own fasting assay values. I shall be presenting data to show the potent insulinogenic effect of constant infusions of relatively small amounts of glucagon (1-5 μg/min). It is difficult to understand how glucagon infusions promote such hyperinsulinaemia with a rise in circulating glucagon levels (by immunoassay or by calculation) which is relatively small compared with our fasting values, so that the lower fasting glucagon values reported by Dr. Unger might be more appropriate.

The immunoassay is complicated by the damaging effect of human plasma on glucagon ^{131}I. We obtained our higher fasting glucagon values and dilution curves only after reducing incubation damage. At present correct absolute values of circulating glucagon remains an open question, but relative changes (i.e. increase or decrease) in endogenous glucagon after provocative tests are detectable with confidence by our immunoassay. We have recently reported (Samols *et al.*, 1965, *Lance*t, ii, 1257) the new observation that endogenous glucagon levels rise after 100-200 g glucose by mouth. We have suggested that the alimentary absorption of glucose stimulates glucagon secretion by the gut or the pancreas, thereby promoting insulin secretion.

K. F. WEINGES : For the present I have no real comment on Dr. Samols results. We have to compare our glucagon antiserum, the separation technique of free and antibody bound ^{131}I-glucagon and we have to discuss the possibility of labeled glucagon damage in human plasma during the incubation. Unger and we found a cross-reaction between extracted human's pancreas glucagon and beef pork glucagon antiserum. There was no possibility to test cristallized and purified human glucagon because we could not get one. I was listening with great interest Dr. Samol's communication. There are a lot of problems. It is very hard to believe that glucagon stimulates the secretion of endogenous insulin directly.

As you know Dr. Pfeiffer and coworkers found a stimulation of insulin secretion by STH and secretin. We found a considerable increase of cortisol in plasma after glucagon administration and there is correlation between cortisol and insulin levels in plasma.

The most remarkable effect of glucagon is its effect on the liver. May be the changing of the liver metabolism (increase of glucose concentration in the liver cells) has an inactivation influence on endogenous insulin.

E. SAMOLS : I doubt that Dr. Weinges would observe a rise in plasma cortisol, 1-2 minutes after the start of the injection of glucagon, nor have we observed dramatic changes in plasma control after constant infusions of smaller amounts of glucagon. We have previously shown (E. Samols, J. Ryder, *J. Clin. Invest.*, 1961) that, in subjects with porta-caval anastomoses, the hepatic uptake of insulin is relatively constant even when insulin levels are increased by infusing exogenous insulin, so that the insulin response to a rapid injection of glucagon represents a spur of insulin secretion.

APPLICATION OF INSULIN RADIOIMMUNOASSAY IN DIAGNOSIS AND CLINICAL INVESTIGATION

E. SAMOLS (•) and V. MARKS (○)

(•) *Department of Medicine, Royal Free Hospital, London, Great Britain*

(○) *Area Laboratory at West Park Hospital, Epsom, Great Britain*

ABSTRACT

Insulin radioimmunoassay permits precise reproducible estimations of endogenous plasma insulin in man, and is valuable clinically in the differential diagnosis of spontaneous hypoglycaemia.

Compared with control subjects, excessive inappropriate insulin secretion was demonstrated in all of 28 patients with fasting hypoglycaemia. Insulin levels were normal, or more often low, in most other diseases causing spontaneous hypoglycaemia, including 11 patients with non-islet cell tumours (unless the tumours were invading the pancreas). Overnight fasting hyperinsulinism, demonstrable in simple obesity, diabetes mellitus and hepatic cirrhosis, did not occur in conjunction with hypoglycaemia in obesity and diabetes.

The insulin responses to tolbutamide, L-leucine and glucagon were frequently exaggerated in patients with insulinomas but the response to intravenous glucose was usually normal or subnormal. This led to the discovery that glucagon normally stimulates insulin secretion, independently of its effect on arterial blood glucose. With constant infusions of relatively small amounts of glucagon the insulinogenic effect, usually apparent only when the blood glucose was raised, was greater than that induced by simple hyperglycaemia. The insulinogenic action of glucagon was enhanced by hyperglycaemia. Evidence is presented to suggest that the insulinogenic effect of glucagon may be physiological.

In a series of outstanding reports dating from 1956, Berson and Yalow [1-3] pioneered the principles, development and application of a radioimmunoassay technique for insulin. Its value in elucidating homeostatic mechanisms and in clinical diagnosis has been attested to in numerous publications [4-6], including a recent review [5]. It is our intention in this communication to demonstrate the usefulness of insulin immunoassay in (*a*) the differential diagnosis of spontaneous hypoglycaemia in man, and — arising from these studies — (*b*) establishing the insulinogenic property of glucagon.

In compiling this report we have relied largely upon our own experience.

METHODS

It was requested that this report should not discuss the technical features of immunoassay. According to the principles of radioimmunoassay, the amount of labelled insulin which can be bound by a constant quantity of antibody is decreased by additional unlabelled insulin in a standard or a plasma sample. Beef or pork ^{131}I-insulin with a specific activity of 150-600 μC/μg was prepared by the method

of Greenwood and Hunter [7], which is vastly superior to the method of Samols and Williams [8], used before 1963. Insulin was bound by guinea-pig anti-beef or anti-pork insulin antibodies with high equilibrium constants, and the soluble antibody-bound insulin complex precipitated with a rabbit anti-globulin serum by the immunoprecipitation method of Samols and Bilkus [9]. We still use the original chromatographic separation of Yalow and Berson [1] as the final arbiter in doubtful assays, and extensive tests have shown the precise agreement between the two techniques.

Because of the variable species specificity of the antigen antibody reaction, human insulins — prepared [9] by Dr. Fisher, Connaught Laboratories, Toronto, and by the Wellcome Laboratories, Beckenham — were used as reference standards until 1964, after which Novo twice crystallised human insulin (23.6 U/mg) was used.

95 % confidence limits of 3.0-7.8 % were obtained for concentrations of human insulin between 1.0-30.0 μU/ml and were ± 10 % for plasma insulin values measured in duplicate in the range 5-400 μU/ml. The reproducibility of the assay is shown when each plasma sample was assayed only once on two different occasions (Table I). Blood glucose was measured by glucose-oxidase and plasma glucagon by radioimmunoassay.

TABLE I. — Comparison of Single Plasma Insulin Estimations on Different Occasions

Fresh plasma before freezing μU/ml	Stored frozen plasma 4 weeks later μU/ml
33	31
40	40
90	91
121	120
130	119
130	111
140	97
121	120
200	190

FASTING PLASMA INSULIN LEVELS.

a) Control Subjects.

In 1963 we reported [4] the range for overnight fasting plasma insulin in 100 control subjects as 2-63 μU/ml, with a mean value of 19 ± SD 7.5 μU/ml. Subsequent recognition that obesity alone may lead to elevation of the overnight fasting plasma insulin concentration, led to a re-evaluation of the normal range. After an overnight

fast, the mean plasma insulin level in 350 venous blood samples from 150 non-diabetic, non-obese normoglycaemic men and women in the U. K. and U. S. A. was 18 ±SD 5.7 μU/ml in 1963-64 [5]. In 1965 mean overnight fasting plasma insulin

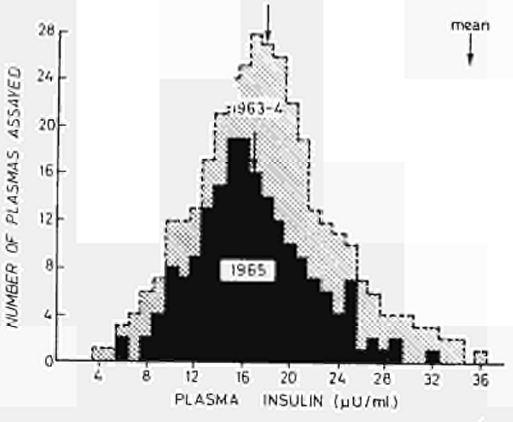

FIGURE 1. — Overnight fasting plasma insulin levels in control subjects, 1963-64 and 1965

was 17 ±SD 4.1 μU/ml in 189 venous samples from 75 control subjects in the U.K. (Figure 1). The histogram of these results approximates normal distribution, and in both series the vast majority of levels fall in the range 8-31 μU/ml. The small shift to the left in the second series — which may have been accounted for by the use of a fresh standard, or by minor changes in technique — is not clinically significant. In contrast to non-obese control subjects, the fasting plasma insulin concentration is — despite normoglycaemia — more than 30 μU/ml (usually 30-50 μU/ml) in over 30 % of subjects who are more than 20 % above their ideal weight. This is important clinically because patients with spontaneous hypoglycaemia may also be obese.

b) Modification of Plasma Insulin Concentration by Hypoglycaemia.

When the blood or plasma glucose concentration is depressed in normal individuals — either by severe carbohydrate restriction or prolonged fasting — there is a parallel fall (Figure 2) in plasma insulin ($r = 0.89$; $p = <0.001$). Similar parallelism between blood glucose and plasma insulin is observed in obese subjects under the same conditions. These observations suggest that insulin levels would be lower than normal in "spontaneous hypoglycaemia" unless endogenous hyperinsulinism was responsible for, or contributing to, the hypoglycaemia. Thus, it can be shown that the hypoglycaemia resulting in susceptible subjects from the ingestion of salicylates, fructose, galactose or alcohol is not insulin mediated [10-13], plasma insulin levels being invariably low under these circumstances.

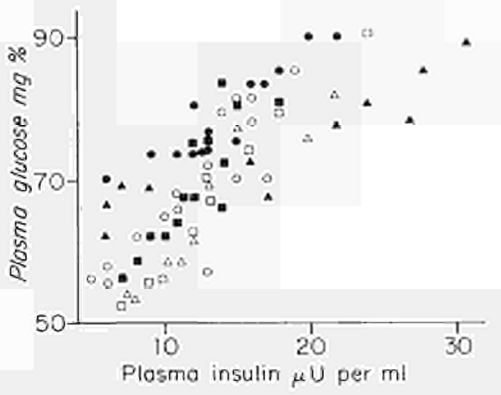

FIGURE 2. — Plasma glucose and insulin concentration before and during prolonged fasting. Each symbol represents one subject. (Reproduced from *On the Nature and Treatment of Diabetes*, Ed. B. S. Liebel and G. S. Wrenshall, by permission of the publishers).

c) Insulinoma.

Our results of fasting plasma insulin assays in patients with insulinoma and those of others [3, 6], show that inappropriate excessive insulin secretion is the cause of hypoglycaemia in this condition. With realisation that the upper limit of normal for fasting plasma insulin is lower than formerly believed, and that hypoglycaemia *per se* lowers the concentration still further, we have reviewed all of our data and

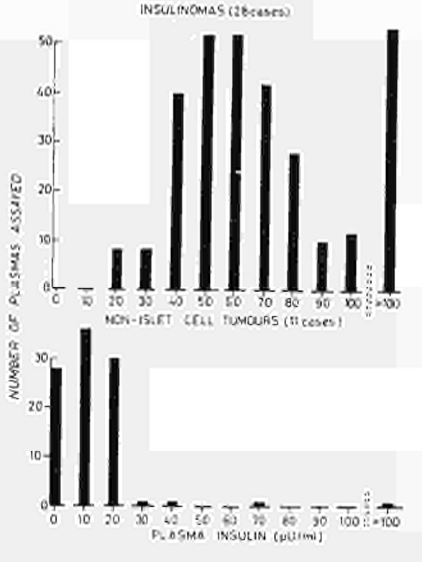

FIGURE 3. — Plasma insulin concentrations during spontaneous fasting hypoglycaemia in 28 patients with insulinomas (above) and 11 patients with non-islet cell tumours (below).

find clear-cut evidence of fasting hyperinsulinism, i.e. plasma insulin levels of 40 μU/ml or more, in every one of the 28 cases (Figure 3) of proven insulinoma we have examined. This high detection rate is attributed to our practice of taking multiple samples on several different days, as we previously reported [4] that occasionally, despite hypoglycaemia, the plasma insulin level may be completely normal. This apparent contradiction to the view expressed above that *inappropriate insulin secretion* is the cause of hypoglycaemia in insulinoma, is at least partially explained by the demonstration [4] that in some, though not all cases of insulinoma, large spontaneous fluctuations in plasma insulin levels occur within periods of 10-120 minutes. Another explanation is that insulin concentrations in portal blood may be elevated without a corresponding elevation of peripheral insulin concentration [4]. In many of the patients with insulinoma, the plasma insulin level remains constant (±15 %) for several hours, and it is not uncommon to obtain similar elevated values from day to day in the same subject. Thus, it seems that while all tumours secrete insulin inappropriately, some secrete it in spurts and others — particularly malignant ones — secrete it more or less constantly.

d) Other Conditions Associated with Spontaneous Fasting Hypoglycaemia.

Plasma insulin levels are normal or, more commonly, low in patients with non-islet cell tumours causing hypoglycaemia [14, 15] showing that hyperinsulinism is not the cause of hypoglycaemia (Figure 3). Nevertheless, in two of 11 personal cases, plasma insulin levels were high during hypoglycaemia. In each of these, the tumour — a retroperitoneal mesenchymal fibrosarcoma in one case and a pancreatic acinar carcinoma in the other — involved the pancreas itself, suggesting that under these circumstances there may be true "inappropriate" insulin secretion.

We have invariably found fasting plasma insulin levels to be normal or low during hypoglycaemia in patients with essential reactive hypoglycaemia, Addison's disease (4 cases), hypopituitarism (5 cases), and various types of glycogen storage disease (5 cases). High fasting plasma insulin levels occasionally occur with normal blood glucose levels in mild, and particularly, obese, diabetics who at *other times* may also have spontaneous hypoglycaemia. Hypoglycaemia and high plasma insulin levels do not coincide however, i. e.there is not inappropriate insulin secretion. In patients with cirrhosis of the liver, particularly those with porto-caval shunts, plasma insulin levels in excess of 40 μU/ml are occasionally encountered, suggesting that hyperinsulinism may contribute to the production of hypoglycaemia in this condition. Our experience with children with idiopathic hypoglycaemia of childhood (IHC) is limited, but like that of Yalow and Berson [3], suggests that hyperinsulinaemia is uncommon, but does occur.

INTRAVENOUS TOLBUTAMIDE TEST.

In normal individuals, the rapid intravenous injection of 1 g sodium tobultamide causes a moderate increase in plasma insulin and a fall in blood glucose [5].

In patients with insulinomas, the insulin response to tolbutamide is often exaggerated and was diagnostically useful in 14 out of 16 patients. In most the plasma insulin rose excessively within ten minutes of the tolbutamide injection and fell thereafter, but frequently failed to return to normal levels (Figure 4). In two cases, the rise in plasma insulin following intravenous tolbutamide was less pronounced and slower to appear but persisted for up to 180 minutes. In three of the cases the abnormal plasma insulin response was unaccompanied (Figure 4) by the characteristic fall in blood glucose concentration [16] and in two patients, neither the glucose nor insulin response was typical of insulinoma. An exaggerated plasma insulin

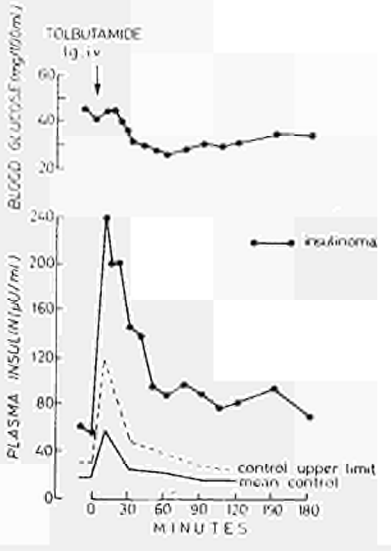

FIGURE 4. — Plasma insulin and glucose values during a tolbutamide test in a patient with insulinoma.

response to tolbutamide cannot be considered pathognomonic of insulinoma however, as a similar response occurs in a small percentage of patients with cirrhosis and paradoxically, some obese maturity-onset diabetics [5].

Despite encouraging earlier reports [16, 17] abnormal glucose responses to intravenous tolbutamide are common in other varieties of spontaneous hypoglycaemia, being encountered in patients with hypoglycaemia due to cirrhosis, adrenal and pituitary insufficiency, non-islet cell tumours, IHC, and in those with alcohol- induced hypoglycaemia. The insulin response, on the other hand, is generally normal except in cirrhotics, in whom it may be excessive.

L-LEUCINE.

L-leucine is normally a stimulus to insulin secretion, but with a standard oral dose (150 mg/kg body weight) the mean normal rise in plasma insulin is only 9 μU/ml. In half the patients with insulinoma tested, the same dose of L-leucine causes

a rapid rise in plasma insulin of 40 μU/ml or more, and an acute fall in blood glucose concentration. Both responses are restored to normal by removing the tumour. Increased sensitivity to L-leucine, judged by its effect on plasma insulin, does not occur in other types of "spontaneous" hypoglycaemia, apart from that due to the leucine sensitive variety of IHC and factitious hypoglycaemia due to sulphonylurea ingestion.

ORAL AND INTRAVENOUS GLUCOSE TOLERANCE TESTS.

The typical insulin response to provocative tests, summarised in Table II, shows that the insulinomas behave like an exaggerated caricature of normal cells when stimulated by tolbutamide, L-leucine and glucagon. It is therefore of interest that hyperglycaemia, the best known stimulus to insulin secretion, rarely provokes

TABLE II. — Characteristic Insulin Responses to Provocative Tests in Insulinomas and Non-islet Cell Tumours

	Insulinomas	Non-islet cell tumours
Tolbutamide 1 g I.V.	Exaggerated	Sub-normal or normal
L-leucine	Exaggerated (50 %)	Sub-normal or normal
Glucagon	Exaggerated	Sub-normal or normal
Oral Glucose	Exaggerated or normal	Sub-normal or normal
I.V. Glucose	Sub-normal or normal (rarely exaggerated)	Sub-normal or normal

an excessive insulin response, as is shown by the typically normal or sub-normal insulin response to intravenous glucose. The insulin response to oral glucose does not reflect the insulinogenic effect of hyperglycaemia alone, as insulin secretion is normally augmented by other factors after the ingestion of glucose.

These results explain why blood glucose studies during oral or intravenous glucose tolerance tests do not provide useful information in the diagnosis of insulinoma. While a normal or sub-normal insulin response helps to exclude generalised islet cell hyperactivity, an excessive insulin response to glucose is compatible with either generalised islet cell hyperactivity or localised islet cell tumour(s).

THE GLUCAGON TEST.

The insulin response to intravenous glucagon was excessive in 10 out of 12 patients with insulinoma, as shown by the example in Figure 5. This provides a rational explanation for the report by Marks [18] and our further observations that

in patients with insulinoma, the injection of glucagon typically provokes reactive hypoglycaemia after 90-180 minutes.

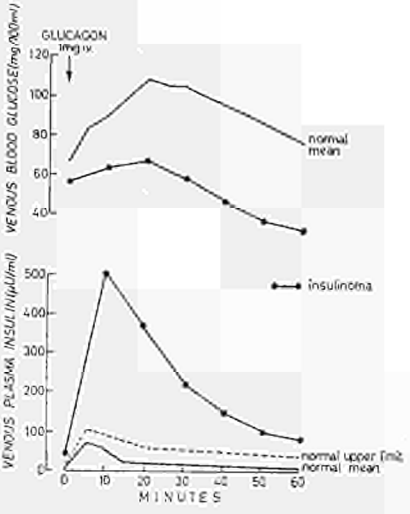

FIGURE 5. — Plasma insulin and glucose values during a glucagon test in a patient with insulinoma.

STIMULATION OF INSULIN SECRETION BY GLUCAGON IN NORMAL PEOPLE.

The possibility that the excessive rise in plasma insulin we observed in patients with insulinoma resulted from a direct effect of glucagon on the abnormal islet cells was considered, and led to the discovery [19] that in man glucagon is a potent stimulus to insulin secretion by normal as well as by tumorous β-cells, and has a previously unsuspected rôle in glucose homeostasis [20].

The second part of this report describes how the insulinogenic effect of glucagon was clearly established and separated from its hyperglycaemic effect. Although it was known with the advent of radioimmunoassay that glucagon caused a rise in insulin levels, this response was, until recently [19], considered to be secondary to arterial hyperglycaemia resulting from accelerated hepatic glucose release. Arterial blood (brachial) was sampled in these studies as there may be large arterio-venous blood glucose differences during hyperglycaemia, and it is conventionally thought that it is the *arterial* blood glucose perfusing the pancreas that determines the rate of insulin secretion.

It is apparent from Figure 5, that the maximum insulin response to 1 mg glucagon given by rapid intravenous injection normally *precedes* maximal hyperglycaemia, in contrast to the situation when hyperglycaemia was induced by administering glucose [5]. The time relations of glucagon administration and the rise in blood glucose and plasma insulin occasioned by it, were amplified in 7 healthy volunteers by frequent arterial sampling during and after the administration, at a constant rate over a 2 minute period, of 1 mg glucagon intravenously.

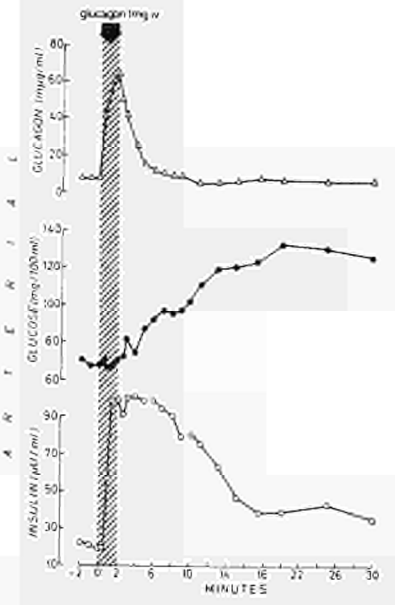

FIGURE 6. — Changes in arterial glucose, insulin and glucagon concentrations in a normal subject given glucagon 1 mg intravenously (Reproduced by permission of the Editors, Minerva Med. Marri, Tyler, Marks and Samols. To be published).

In a representative subject (Figure 6) there was a striking and highly significant rise in arterial insulin at 1, 1.5, 2 and 2.5 minutes after the start of the glucagon injection, even though arterial blood glucose levels did not change during this period. The maximum insulin response occurred during the first ten minutes, in contrast to the maximum rise in blood glucose which was observed after 15-30 minutes. Thus, glucagon was directly responsible for the earlier larger rise in plasma insulin which reflects a spurt in insulin secretion. This early insulinaemic response was so rapid and so large that it could have been due neither to a reduction in the rate of insulin degradation, nor to a decrease in its rate of disappearance from the blood. There is also conclusive evidence that insulin contamination of the glucagon preparation used [19] was not responsible for the rise in plasma insulin.

These results clearly show that glucagon in relatively large amounts — i.e. 1 mg — stimulates insulin secretion directly and independently of its effect upon the arterial blood glucose concentration. That the insulin so released is also biologically active was intimated by the observations in patients with islet cell tumours, and confirmed by the demonstration that in normal subjects the rate constant (K) for glucose disappearance during the intravenous glucose tolerance is significantly increased by concommitant administration of glucagon [19].

During the phase of maximal hyperglycaemia, 15-30 minutes after glucagon injection (Figure 6), insulin levels were still two to three times the fasting level. This later, smaller insulin rise was secondary to hyperglycaemia as it did not occur

in certain patients — including diabetics — who were known, from previous experience, to have a negligible insulin response to glucose.

The pattern of change of glucagon levels after injection of 1 mg of glucagon intravenously was similar in four subjects. Plasma glucagon rose immediately at 0.5 minute, and increased further to peak values of 45-70 μmg/ml at the end of the two minute injection. Thereafter, glucagon levels fell rapidly to reach basal values after 10 to 20 minutes. The half-life of exogenous glucagon was 5-10 minutes, but these figures, although derived from an exponential slope, may reflect disappearance of glucagon before it reached its volume of distribution.

A second intravenous injection of 1 mg of glucagon, 40 minutes after the first and while the arterial blood glucose concentration was still raised, caused a greater rise in plasma insulin despite a similar elevation of the plasma glucagon level. This finding is consistent with other evidence that the insulinogenic effect of glucagon is enhanced by hyperglycaemia and suggests that during the hyperglycaemic phase following glucagon injection, there is no obvious inhibition of insulin secretion, e.g. by catecholamine release [21].

During constant infusions of glucagon in doses of 1, 2, 10 or 20 μg/min plasma insulin levels rise and fall *parri passu* with the arterial glucose concentration. This correlation between glucose and insulin levels contrasts with the effect produced by a rapid injection of large (i.e. 0.25-1 mg) amounts of glucagon, when the highest plasma insulin levels precede maximal hyperglycaemia.

In order to compare the hyperinsulinaemic effect of smaller amounts of glucagon with glucose, it is necessary to obtain similar arterial blood glucose concentrations. This was achieved in several ways. Successive infusions of glucagon (10 μg/min), glucose (500 mg/min) and a mixture of glucose and glucagon in half the previous strengths, were given to six healthy volunteers. Representative results in one subject, shown in Figure 7, clearly indicate that the insulin response to the glucagon or glucose-glucagon infusion, was at least twice as large as the insulin response to the glucose infusion alone, even though the arterial blood glucose levels were similar. When, in the same subject, the order of infusion was reversed and glucose given before the glucagon, the hyperinsulinaemic effect of glucagon (Figure 8) was much more rapid and much greater (Figure 7). This agrees with other evidence [19, 20] that raising the blood glucose either with intravenous glucose or glucagon enhances the insulinogenic effect of glucagon. The greater insulin response during the glucagon infusion caused enlargement of the arterio-venous (A-V) blood glucose difference in the forearm (Figure 8).

The possibility that the insulinogenic effect of glucagon represents a pharmacological, rather than a physiological effect of glucagon was considered but discounted for the following reasons. The amount of glucagon infused was progressively reduced and the smallest amount of glucagon (0.2 μg/min) that we have infused, produced a 40 % mean rise in arterial insulin (Figure 9) and a 10 % mean rise in arterial blood glucose in four healthy subjects. As the increases in blood glucose and insulin were very small in individual subjects, the insulinogenic effect

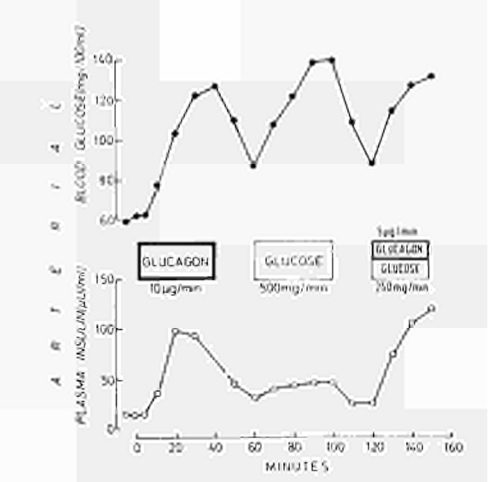

FIGURE 7. — Comparison of the effects of glucagon and glucose infusions on arterial blood glucose and insulin concentrations in a healthy subject.

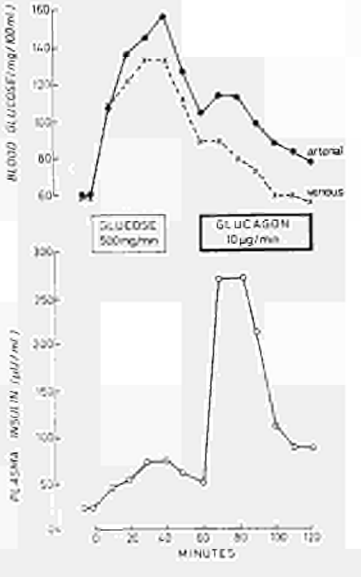

FIGURE 8. — Comparison of the effects of glucose and glucagon infusions on arterial blood glucose and insulin concentrations in the same subject as Figure 7.

of glucagon was not clearly demonstrable with a very small rise in circulating glucagon. Because of the relatively small or negligible effect of glucagon 0.2 μg/min, it is plausible that physiological increases in glucagon concentrations were similar to those produced by infusions of glucagon 0.5-5 μg/min [20]. With 1-5 μg/min glucagon infusion, the insulinaemic effect was two to three times greater than during similar hyperglycaemia produced by an intravenous glucose infusion (Figure 10).

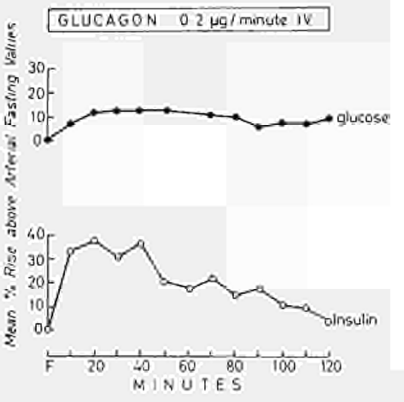

FIGURE 9. — Mean percentage rise above arterial fasting values of glucose and insulin in four subjects during a constant intravenous infusion of glucagon 0.2 µg/min.

These doses of glucagon are "physiological" since they raise the blood glucagon level to values no higher than those observed after oral glucose, which we have recently shown [20] increases endogenous glucagon levels.

In any one subject (Figure 10) glucose 100 g by mouth also produced a greater rise in plasma insulin than does intravenous glucose at similar arterial glucose concentrations. It should be noted (Figure 10) that there is a correlation between blood glucose and plasma insulin levels after oral or intravenous glucose and glucagon in the same subject, but that insulin secretion is augmented both by intravenous glucagon and when glucose is taken by mouth.

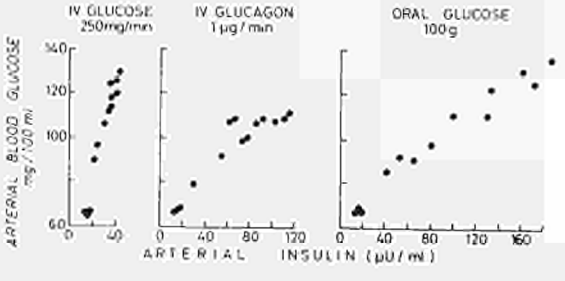

FIGURE 10. — Comparison and correlation of arterial blood glucose and insulin concentrations in the same individual given intravenous glucose, intravenous glucagon and oral glucose on different occasions.

Thus, the ingestion of glucose in man stimulates the secretion of endogenous glucagon in amounts sufficient to augment insulin secretion, and may explain why glucose by mouth, compared with intravenous glucose, causes a large rise in plasma insulin for similar blood glucose levels. The physiological and pathophysiological implications of the insulinogenic effect of glucagon are of some interest and may

be relevant not only to certain types of diabetes mellitus (glucagon deficiency ?) and reactive hypoglycaemia — particularly after gastrectomy — (glucagon excess?) but also to the fundamental control of insulin secretion. Elsewhere, evidence has been summarised to suggest [22] that the first step in the promotion of insulin secretion by glucagon is stimulation of glycogenolysis within the β-cell.

Finally, the present report has presented two aspects of the use of the radioimmunoassay of insulin in metabolic studies. The diagnostic value of the assay in hypoglycaemic states has been illustrated. Prompted by results in these diagnostic studies, insulin assay was used to show that glucagon directly stimulates or augments insulin secretion.

ACKNOWLEDGEMENTS.

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DISCUSSION

H. DITSCHUNEIT (*Frankfurt*) : I was really impressed by your data but they give rise to some questions. If you stop the glucagon infusion then the immunoinsulin will decrease very quickly and this means that the half time of the measurable insulin will be only of a few minutes. When you inject crystallized insulin you will find a much longer half time of about 30 minutes. How would you explain this discrepancy ?

E. SAMOLS : We have conclusively shown that insulin contamination of the glucagon preparations used could not have caused the insulinogenic effect of glucagon for a number of reasons. Firstly with an insulin contamination of 0.2 mU insulin per mg Lilly beef pork glucagon, the injection of 1 mg of glucagon would not increase the plasma insulin level by more than 0.6 μ/ml, by simple calculation, and we have proved this experimental by injection of 0.3 mU pork insulin in human subjects (E. Samols, G. Marri, V. Marks, *Lancet*, ii, 415 /1965/), by the same technique used for the rapid injection of glucagon. Secondly a rapid injection of insulin would cause a peak insulin value at the end of the injection followed by a rapid fall in insulin levels, unlike the insulin response to the injection of glucagon. Thirdly there is no insulin response to glucagon in some diabetics with very high fasty blood glucose values, but when these same diabetics are controlled with chlopropamide and diet, an insulin response to glucagon becomes apparent. The rapidity of the fall in insulin levels on cessation of the glucagon infusion is quite compatible with a half life of about 10 minutes that we obtain for endogenous insulin. After stopping constant I. V. infusions of glucose the rate of fall of endogenous insulin is frequently very similar to that observed after glucagon infusions are ended.

F. C. GREENWOOD (*London*) : As Dr. Yalow has pointed out the labeled hormone did not react in an identical manner of the unlabeled hormone or plasma hormone (Yalow, Berson, 1960, *J. Clin. Invest.*, 39, 1157). Dr. Samols figures illustrate the validity of this concept and show that a slight loss of precision and sensitivity has to be accepted but that specificity is not impaired.

E. F. PFEIFFER (*Chairman*) : The findings of Dr. Samols pointing at the capacity of glucagon to stimulate insulin secretion, are particularly fascinating. I wonder whether these results might have been obtained also in the *in vitro* preparation. When we, last week and without knowing that Dr. Samols is going to bring up that point, were adding glucagon to slices of pancreatic tissue from rabbits and dogs, no increase in insulin labeled activity and immunoinsulin secretion was obtained. On the other hand, in the same preparation, secretin for example was fully active in inducing insulin release in amounts comparable to those mobilized follow-

ing 200 mg glucose concentration in the medium (Pfeiffer *et al.*, Dtsch. Med. Wscht. 1965).

E. SAMOLS : I think that the observation of Prof. Pfeiffer of the insulinogenic effect of glucagon *in vitro* with rabbit pancreas may have been due to the absence of glucose in the medium. The human being, even when hypoglycaemic, always has some glucose perfusing the pancreas. Prof. Pfeiffer's observations are consistent with our hypothesis that glucagon promotes insulin secretion by stimulating intra-β-cell glycogenolysis, and that glucose entering the β-cell is not metabolised until it has passed through glycogen (i.e. 2 pools of glucose-6-phosphate). For this reason raising the blood glucose enhances the insulinogenic effect of glucagon. Also during prolonged starvation the intra-β-cell glycogen stores are depleted and the insulin response to glucagon is diminished or abolished. Thus during prolonged starvation glucagon levels may rise, but insulin levels fall parallel with blood glucose levels.

E. F. PFEIFFER : If glucagon is active on β-cell tissue only in presence of a certain glucose concentration whereas secretion is working also in the glucose free medium, then we should be justified in concluding that the two effects are different. This seems to be an important point.

A. LUYCKX (*Liège*) : J'aimerais signaler ici les résultats d'une étude réalisée à l'Institut de Biochimie Clinique de Genève (Prof. A. Renold) par Mlle D. Vecchio et moi-même. Nous avons utilisé le pancréas de rat foetal, prélevé le 17^{e} jour de gestation, cultivé 4 jours puis incubé dans un tampon bicarbonate pendant deux heures. Dans un tel système, nous avons observé une nette augmentation de la quantité d'insuline libérée dans le milieu, mesurée par méthode radioimmunologique, en présence de glucagon. Nous avons étudié des doses de 0,2 à 0,3 μm/gl et avons constaté l'existence d'une relation dose-effet entre ces valeurs.

LABELLED PROTEIC HORMONES FOR METABOLIC STUDIES

Chairman : C. A. ROSSI
Istituto Chimica Biologica, University of Pisa, Italy

DOSAGE RADIO-IMMUNOLOGIQUE DES GONADOTROPHINES FOLLICULO-STIMULANTE ET LUTEINISANTE

P. FRANCHIMONT (*)

Institut de Médecine, Département de Clinique et de Pathologie Médicales, Université de Liège, Hôpital de Bavière, Liège, Belgique

ABSTRACT

A radioimmunoassay of the follicle stimulating hormone (FSH) and of the chorionic and pituitary luteinizing hormone is now proposed.

1. *The follicle stimulating hormone extracted from human pituitaries is labelled by ^{131}I using Greenwood and Hunter method. The specific activity is around 100 and 350 μC/μg. This labelled preparation of FSH is purified and the contaminants discarded by chromatography and often by horizontal starch gel electrophoresis. So, a pure preparation of ^{131}I-labelled FSH was obtained which in radio-immunoelectrophoresis shows only one line.*

   Antiserum against FSH is treated by serum proteins from hypophysectomised subjects to discard aspecific antibodies. This treated antiserum shows only one precipiting line in immunoelectrophoresis and neutralizes completely the biological effect of FSH.

   To separate free ^{131}I-FSH from complex ^{131}I-FSH-antibodies the chromato electrophoresis on paper and the chemical reaction with ions exchangers are not available. We use a good separating method based on starch gel electrophoresis. With this technique, free FSH migrates in the postalbumine zone and the complex FSH-antibodies in the fraction of starch gel between the origin and the slow α_2-globulins.

2. *Purified chorionic luteinizing hormone is obtained. This hormone is labelled by the Greenwood and Hunter method. Controls of purity were made by chromatography and radio-immunoelectrophoresis. The antiserum against this pure preparation of HCG contains only one type of antibodies which neutralizes the biological activity of the hormone.*

   Starch gel electrophoresis can separate free ^{131}I-labelled HCG from ^{131}I-labelled HCG-Antibodies.

   In this condition, we may appreciate the human chorionic gonadotrophin in serum and urines from pregnant women. The radioimmunoassay of HCG can be used to test the human luteinizing pituitary hormone (LH). Indeed, there is a cross reaction between HCG and LH.

   Some results are reported in this communication.

RÉSUMÉ

Une méthode radio-immunologique de dosage de l'hormone folliculo-stimulante et des hormones lutéinisantes chorionique et hypophysaire est proposée.

1. L'hormone folliculo-stimulante extraite d'hypophyse est marquée par ^{131}I en utilisant la technique de Greenwood et Hunter. Cette préparation de ^{131}I-FSH

(*) Chercheur Qualifié du Fonds National de la Recherche Scientifique.

est purifiée et les contaminants écartés par chromatographie sur DEAE-cellulose puis par électrophorèse horizontale en amidon. Nous obtenons ainsi une préparation pure de ^{131}I-FSH.

Le sérum anti-FSH est incubé préalablement par des protéines sériques de sujets hypophysectomisés afin d'écarter les anticorps aspécifiques.

Pour séparer la FSH* libre de la FSH*-Ac nous utilisons l'électrophorèse en amidon. Avec cette technique la FSH libre migre dans la zone des postalbumines tandis que le complexe FSH-Anticorps se situe du côté cathodique des α_2- globulines lentes.

2. L'hormone chorionique gonadotrope lutéinisante peut être obtenue à l'état pur. Le marquage de cette hormone par ^{131}I s'effectue en utilisant la méthode de Greenwood et Hunter. Les contrôles de pureté ont été réalisés par chromatographie sur Séphadex G 200 et par radio-immuno-électrophorèse. L'antisérum anti-HCG contient un seul type d'anticorps qui neutralise l'activité biologique de l'hormone.

L'électrophorèse en amidon permet de séparer ^{131}I-HCG libre de ^{131}I-HCG fixé aux anticorps.

Dans ces conditions nous pouvons apprécier le taux de l'hormone chorionique gonadotrope dans le sérum et les urines de femmes enceintes.

Le dosage radio-immunologique de l'HCG peut être utilisé pour apprécier le taux de l'hormone lutéinisante hypophysaire (LH) dans le sérum ou les urines car il existe une parenté antigénique entre HCG et LH.

Les premiers résultats obtenus à l'aide de notre technique sont ici rapportés.

Depuis quelques années, l'étude de la sécrétion des hormones protidiques s'est considérablement développée grâce à l'introduction des techniques immunologiques dans les méthodes de dosage de ces hormones. La méthode radio-immunologique est la meilleure car dans les conditions idéales, elle associe à la spécificité rigoureuse de la réaction antigène-anticorps l'exquise sensibilité des techniques isotopiques. Ces méthodes radio-immunologiques sont actuellement utilisées pour explorer la sécrétion de l'insuline (Yalow et Berson, 1959), de l'hormone de croissance (Utiger *et al.*, 1962, Greenwood, 1962), de l'ACTH (Felber, 1963; Yalow *et al.*, 1964), du glucagon (Unger *et al.*, 1961) et de la parathormone (Berson *et al.*, 1963).

L'application de ces techniques radio-immunologiques aux gonadotrophines est rendue difficile par l'absence de préparations parfaitement purifiées de ces stimulines hypophysaires et du fait de l'ignorance de la structure chimique des gonadotrophines physiologiques.

Depuis plusieurs années, nous nous sommes attachés au problème du dosage des gonadotrophines. Nous avons proposé, en 1962, une technique immunologique reposant sur l'hémagglutination (Franchimont, 1962). En 1965, nous avons mis au point une méthode de dosage radio-immunologique de l'hormone folliculostimulante (Franchimont et Van Cauwenberge, 1965; Van Cauwenberge et Franchimont, 1965) et de l'hormone lutéinisante chorionique et hypophysaire (Franchimont, 1965).

Dans cette communication, nous désirons prouver la valeur des techniques radio-immunologiques que nous avons proposées pour le dosage des hormones

folliculo-stimulante (FSH) et lutéinisante (LH). Nous nous attacherons en particulier à démontrer le respect dans ces cas particuliers, des conditions indispensables à la réalisation de tout dosage radio-immunologique, à savoir :

— l'obtention d'un antisérum spécifiquement dirigé contre la seule hormone à doser;

— l'utilisation d'une préparation d'hormone marquée à l'état pur;

— l'emploi d'une méthode valable de séparation de l'hormone marquée libre et de l'hormone marquée fixée aux anticorps.

Nous avons en outre vérifié l'identité immunochimique de l'hormone étalon et de cette même hormone présente dans les milieux biologiques.

I. Dosage radio-immunologique de l'hormone folliculo-stimulante

A. Obtention d'un antiserum specifiquement dirige contre la seule hormone folliculo-stimulante.

La préparation d'hormone folliculo-stimulante utilisée, a été extraite d'hypophyses humaines par la méthode de Steelman *et al.*, (1959). Son activité FSH est de 2.250 unités HMG_{21} et LH de moins de 25 U.I. HCG par mg. Elle a servi à induire la formation d'anticorps chez le lapin.

L'étude immuno-électrophorétique de cette préparation et des antisérums correspondants a montré qu'il existe, dans ces derniers, trois types d'anticorps

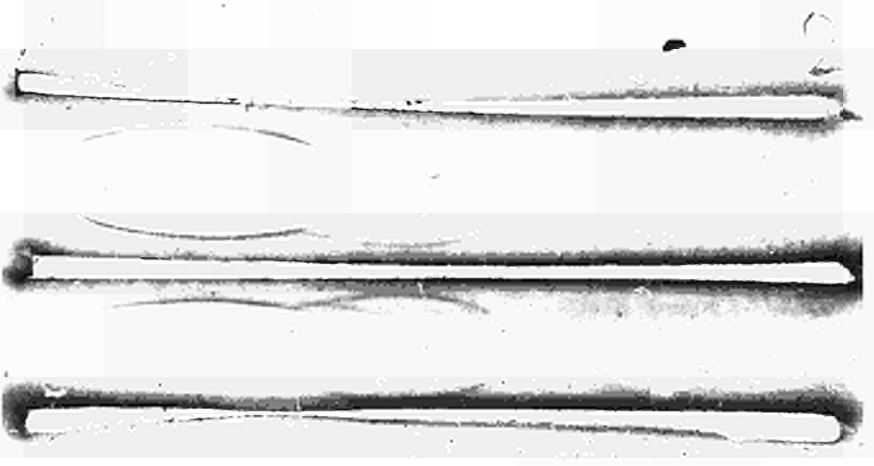

Figure 1. — Diagramme immuno-électrophorétique obtenu lors de la réaction entre la préparation d'hormone folliculo-stimulante déposée dans les logettes et soumise à l'électrophorèse et l'antisérum correspondant, soit non traité (se trouvant dans les rigoles supérieure et moyenne) soit incubé avec des protéines sériques de sujet hypophysectomisé (déposé dans la rigole inférieure).

dirigés contre des protéines migrant respectivement dans la zone des albumines, des α_1- globulines et des α_2- globulines (Figure 1).

Les albumines et les α_1- globulines sont des contaminants aspécifiques. En effet, lorsque ces antisérums sont épuisés par les protéines sériques de sujets hypophysectomisés suivant une technique précédemment décrite (Franchimont, 1964), puis mis en contact avec notre nouvelle préparation purifiée de gonadotrophines hypophysaires (*) nous n'obtenons plus qu'un seul trait de précipitation en α_2- globulines (Figure 1). L'antisérum ainsi épuisé ne donne plus aucune réaction soit en immuno-électrophorèse, soit par la technique d'Ouchterlony, soit par l'hémagglutination, ni avec les albumines humaines purifiées ni avec l'ensemble des protéines sériques. Par ailleurs, la préparation gonadotrope purifiée mise en contact avec un sérum antisérum humain fait apparaître deux traits de précipitation en albumines et en α_1- globulines. Les albumines et les α_1- globulines sont donc des contaminants aspécifiques tandis que les α_2- globulines ne sont pas des constituants majeurs du sérum.

Biologiquement, l'antisérum ainsi épuisé inhibe l'activité tant FSH (test de Steelman et Pohley, 1953) que LH (accroissement de poids de la prostate ventrale et déplétion de la vitamine C et du cholestérol ovarien) d'une préparation de gona-

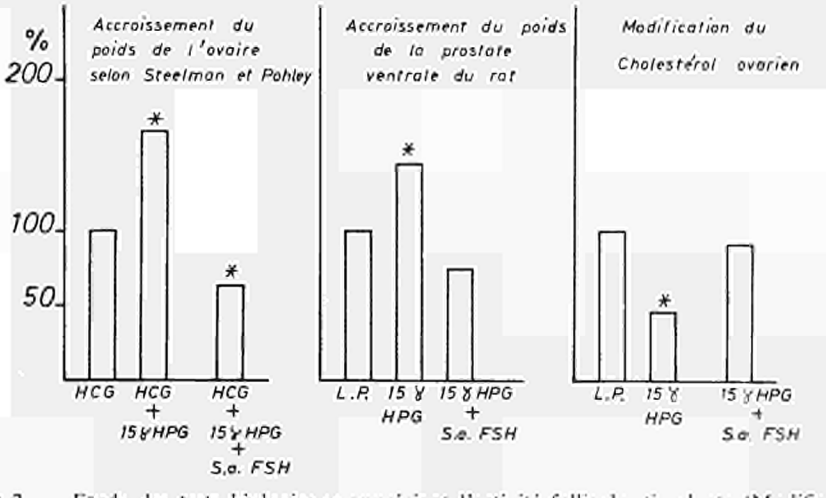

FIGURE 2. — Etude des tests biologiques appréciant l'activité folliculo-stimulante (Modifications pondérales des ovaires de rates saturées par de l'hormone chorionique gonadotrope : HCG) et lutéinisante (Augmentation du poids de la prostate ventrale du rat immature et variations de la concentration du cholestérol ovarien) sous l'action de 15 µg d'une préparation d'hormone gonadotrope hypophysaire (HPG) et de cette même quantité de HPG préalablement incubée avec 0,25 ml de sérum anti FSH épuisé par des protéines sériques d'un sujet hypophysectomisé (S.a.FSH). L'incubation avec l'antisérum inhibe toutes les activités biologiques de la préparation de HPG manifestes lorsqu'elle est injectée seule.
* marque les variations statistiquement différentes par rapport aux valeurs témoins.

(*) Préparation gracieusement mise à notre disposition par les laboratoires de Recherches Organon que nous remercions très vivement.

dotrophines hypophysaires humaines (HPG) moins pure dont l'activité FSH est égale à 400 unités HMG_{24} et LH de 66 U.I.HCG/mg et utilisée afin d'épargner notre préparation hautement purifiée (Figure 2).

L'antisérum épuisé par les protéines sériques d'un sujet hypophysectomisé ne donne donc plus qu'un seul trait de précipitation en α_2- globulines. Celui-ci correspond à la réaction gonadotrophines-anticorps antigonadotrophines, car il inhibe l'activité FSH et LH d'une préparation de gonadotrophines hypophysaires. Il importe de voir s'il existe des anticorps spécifiquement dirigés contre des groupements antigéniques déterminants propres à l'hormone folliculo-stimulante et s'il est possible d'écarter les anticorps anti-LH.

Wide et Gemzell (1961) puis nous-mêmes avons démontré qu'il existe une communauté antigénique entre la LH hypophysaire et l'hormone chorionique extraite d'urine de femmes enceintes (HCG). En effet, le sérum anti-HPG inhibe l'action biologique de HCG, ce qui permet d'envisager la neutralisation, par l'hormone chorionique, des anticorps anti LH de notre immun-sérum déjà traité par des protéines sériques de sujets hypophysectomisés. Dans ce but nous avons recherché la quantité de HCG capable de neutraliser les anticorps dirigés contre l'hormone lutéinisante en déterminant l'effet de quantités identiques de HCG non traitées et préalablement incubées avec le sérum anti-FSH sur la déplétion de cholestérol ovarien (Van Cauwenberge, Franchimont, 1965).

A partir de 200 unités, le HCG retrouve sa propriété spécifique de diminuer le taux de cholestérol ovarien en dépit de son incubation préalable avec 1 ml de

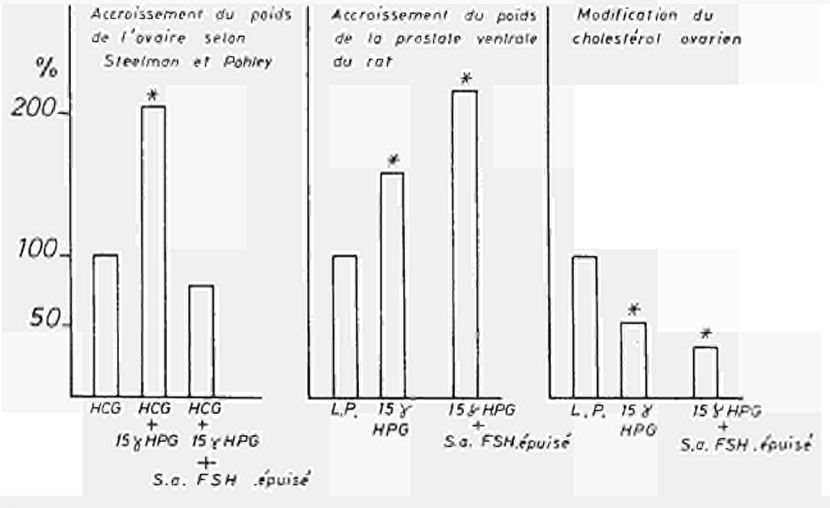

FIGURE 3. — Etude des mêmes tests biologiques que dans la figure 2 sous l'action de 15 µg de HPG seul puis de cette même quantité d'hormone préalablement incubée avec 0,25 ml de sérum anti-FSH traité à la fois par les protéines sériques de sujet hypophysectomisé et par 5000 U.I. de HCG; seule l'activité folliculo-stimulante est inhibée par l'incubation de la préparation avec l'antisérum ainsi traité.

sérum anti-FSH qui inhibe l'activité biologique spécifique du principe lutéinisant pour les doses inférieures utilisées.

Désirant assurer à cette réaction d'épuisement des anticorps anti-LH la plus grande sécurité, nous avons ajouté par ml 5.000 U.I. de HCG. L'antisérum ainsi traité fait encore apparaître en immuno-électrophorèse le trait de précipitation décelé avant la mise en contact avec l'HCG et conserve la propriété d'inhiber l'activité FSH des préparations de HPG. Par contre, il ne modifie pas l'activité LH de ces préparations (Figure 3).

Ainsi donc nous avons obtenu un antisérum spécifique, dirigé uniquement contre la seule hormone folliculo-stimulante. La première condition pour la réalisation d'un dosage radio-immunologique de l'hormone folliculo-stimulante est ainsi obtenue.

B. Obtention de l'hormone folliculo-stimulante marquée a l'état pur.

La préparation d'hormone folliculo-stimulante en notre possession contient au moins trois types d'antigènes générateurs d'anticorps après injections aux lapins. Les albumines et les α_1 globulines sont des contaminants tandis que les α_2-globulines correspondent bien à l'hormone folliculo-stimulante. Il importe donc de marquer la préparation par l'iode radioactif puis d'écarter les contaminants marqués en même temps que l'hormone folliculo-stimulante.

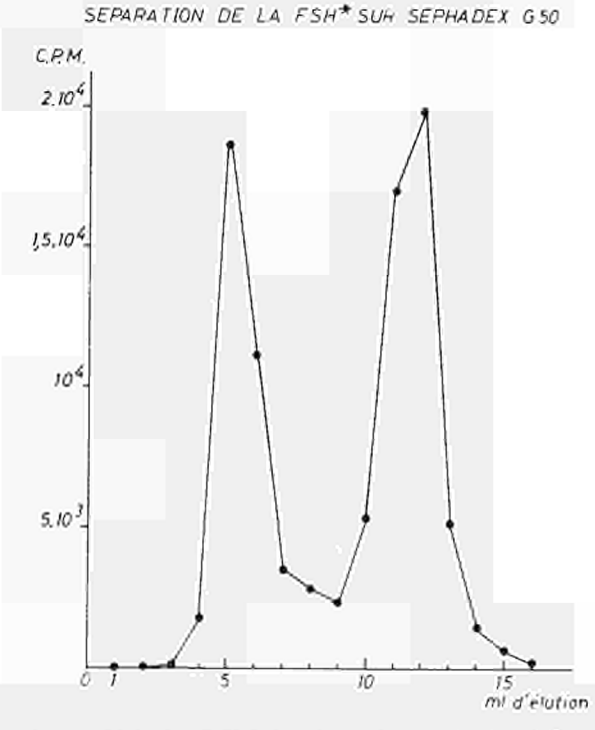

Figure 4. — Filtration sur Séphadex G 50 de la préparation de FSH immédiatement après marquage

1. *Marquage de la préparation d'hormone folliculo-stimulante par* ^{131}I.

Nous avons utilisé la méthode de Greenwood *et al.* (1963). L'hormone marquée est séparée des sels iodés radioactifs par filtration sur Séphadex G 50. L'hormone gonadotrope qui n'est pas retenue dans les mailles du gel passe dans le premie pic tandis que les sels radioactifs se retrouvent dans le second (Figure 4).

Nous avons déterminé la récupération de la préparation d'hormone folliculo-stimulante utilisée après les opérations de marquage. Des 5 µg de départ, la quantité moyenne récupérée varie entre 1,64 et 0,97 µg avec une valeur moyenne de 1,27 µg. L'activité spécifique des préparations d'hormone folliculo-stimulante marquée (FSH*) oscille entre 100 et 350 µC/µg.

2. *Purification de la préparation d'hormone folliculo-stimulante marquée.*

Pour écarter les contaminants albuminiques et α_1- globuliniques marqués en même temps que l'hormone, nous pratiquons en deux temps :

Une chromatographie sur DEAE cellulose (colonne de 10 cm de hauteur-diamètre 1 cm) équilibrée par K_2HPO_4 0,01 M est réalisée en premier lieu. Le volume recueilli après marquage est porté au sommet de cette colonne et l'élution s'effectue par 20 ml de K_2HPO_4 0,01 M puis 0,05 M. Comme l'illustre la figure 5, nous observons un pic de radioactivité au cours de l'élution par K_2HPO_4 0,01 M et une élimination plus importante de radioactivité lors de l'élution par K_2HPO_4 0,05 M. L'activité biologique se localise uniquement au niveau du second pic. En effet, après chromatographie, dans des conditions rigoureusement identiques, de 0,2 mg d'une préparation moins purifiée de FSH, nous avons injecté à l'animal saturé en HCG (Test de Steelman et Pohley, 1953) les volumes d'élution compris entre les flèches et correspondant à ces deux pics. Les volumes d'élution du premier pic ne provoquent pas d'accroissement du poids de l'ovaire tandis que ceux du second pic provoquent une augmentation pondérale de ces ovaires de l'ordre de 194 %.

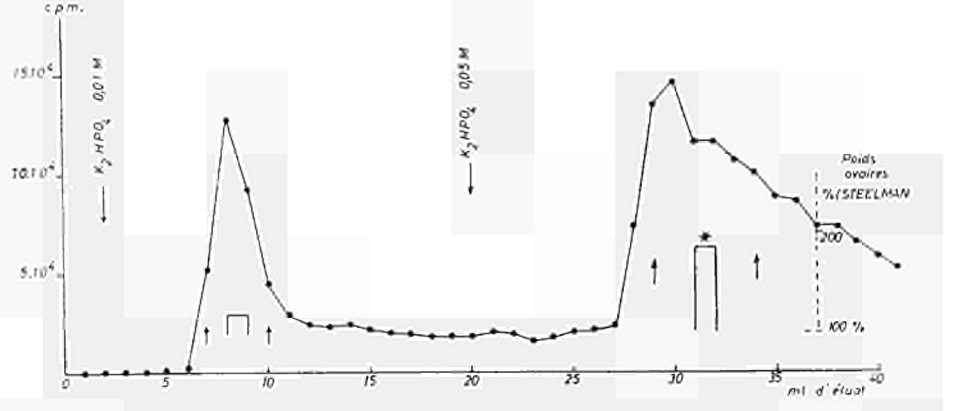

FIGURE 5. — Chromatographie de la préparation de FSH* sur DEAE cellulose. Elution par K_2HPO_4 0,01 M puis 0,05 M

Les deux ml correspondant au sommet du second pic d'élution sont alors soumis à l'électrophorèse en amidon selon la technique de Ferguson et Wallace (1961). La durée de l'électrophorèse est de 5 heures et la différence de potentiel de 220 volts. La région des postalbumines où se localise l'hormone folliculo-stimulante après électrophorèse (Franchimont, 1966) est prélevée et les protéines qui s'y trouvent sont récupérées soit par filtration-congélation soit par électro-dialyse (Moretti *et al.*, 1958).

La préparation ainsi obtenue peut être utilisée pour le dosage radio-immunologique de l'hormone folliculo-stimulante car elle nous donne des garanties de pureté suffisante pour aborder cette technique. En effet, lorsque la préparation de FSH* obtenue après marquage, mélangée avec de la FSH non marquée, réagit en immunoélectrophorèse avec le sérum anti-FSH non encore neutralisé par les protéines sériques de sujets hypophysectomisés, trois traits de précipitation apparaissent. Cette immunoélectrophorèse, mise en contact avec une plaque photographique impressionne celle-ci suivant ces trois mêmes traits de précipitation. Si l'hormone folliculo-stimulante marquée, puis purifiée, réagit en même temps que la FSH non marquée, avec le même sérum anti-FSH, la plaque photographique est

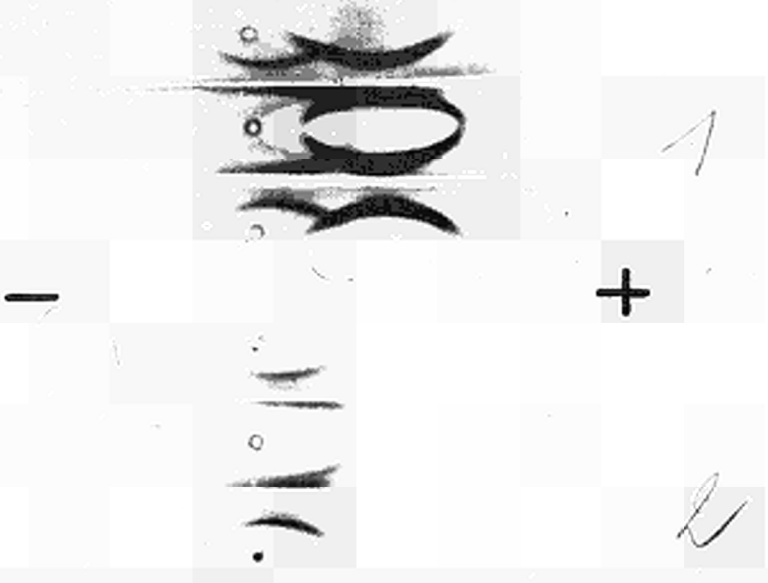

FIGURE 6. — Diagramme auto-radio-immunoélectrophorétique obtenu en présence de la FSH* marquée non purifiée et l'antisérum anti-FSH non traité (1). On note 3 traits d'impression de la plaque photographique correspondant aux traits de précipitation en albumines, en α_1- et en α_2- globulines obtenus en immunoélectrophorèse. Le diagramme inférieur (2) est celui de l'auto-radio-immunoélectrophorèse obtenu lors de la réaction de la FSH marquée et purifiée en présence du même antisérum. Il n'existe plus qu'un seul trait d'impression correspondant à la précipitation de l'hormone folliculo-stimulante dans la zone des α_2- globulines.

impressionnée selon un trait unique correspondant aux traits de précipitation situés dans la zone des α_2- globulines (Figure 6).

Ainsi nous possédons une préparation de FSH* à l'état suffisamment pur pour ne plus impressionner la plaque photographique que suivant le seul trait de précipitation de l'hormone folliculo-stimulante. La seconde condition est réalisée.

C. Methode de separation de l'hormone folliculo-stimulante marquee (FSH*) et du complexe hormone folliculo-stimulante marquee-anticorps (FSH*-Ac).

Dans le but de réaliser la séparation de la FSH* libre et de la FSH*-Ac, nous avons tenté d'appliquer l'électrophorèse sur papier comme Yalow et Berson l'ont réalisé pour le dosage de l'insuline (1959) et la fixation chimique de l'hormone libre sur un échangeur d'ions à l'instar de Melani *et al.*, pour l'hormone de croissance (1964).

La séparation par électrophorèse sur papier 3 MM nous paraît insuffisante pour assurer une séparation valable de la FSH* libre et de la FSH*-Ac. En effet, le principe de la séparation est en défaut. Celui-ci postule que l'hormone marquée libre doit subsister au point d'origine tandis que, seuls les complexes « hormones-anticorps » vont migrer avec les γ-globulines. En fait, l'hormone folliculo-stimulante marquée libre ne persiste que partiellement au point d'origine lors de l'utilisation du papier 3 MM Whatman. Avec le papier de D. E. 81 la migration existe mais est beaucoup moins importante.

Par ailleurs, la présence de protéines sériques humaines dans le milieu accroît encore la migration de l'hormone folliculo-stimulante marquée à partir de la région de départ. Ces éléments sont illustrés par la figure 7 où l'on constate une diminution de la radioactivité dans la zone de départ, en l'absence de tout anticorps, au

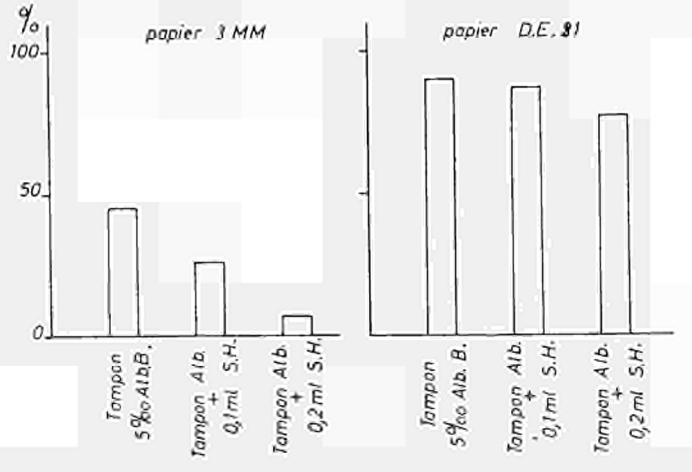

Figure 7. — Influence des protéines sériques humaines (S. H.) sur la persistance de la radioactivité au point d'origine au cours de l'électrophorèse sur papier

fur et à mesure que la concentration en protéines sériques humaines est accrue : 0,1 ml et 0,2 ml de sérum humain (S. H.) dans 0,5 ml d'incubation. Le reste du volume est constitué par du tampon phosphate de Sorensen pH 7,5 0,05 M contenant 5 g ‰ d'albumines bovines. Cette méthode de séparation n'a donc pas été retenue.

Par ailleurs, aucune réaction chimique entre l'hormone folliculo-stimulante libre et un échangeur soit d'anions soit de cations n'a pu être exploitée comme méthode de séparation de la FSH* libre et de la FSH*-Ac. Il n'est donc pas possible d'utiliser semblables techniques.

Seule l'électrophorèse en amidon permet une migration propre de la FSH* libre dans la zone des postalbumines et du complexe FSH*-Ac dans la région située entre les α_2- globulines lentes et le point de départ. Il est très important d'effectuer cette électrophorèse pendant 5 heures sous une différence potentielle de 220 volts. A la fin de cette migration, il suffit de réaliser une coupure dans la bande d'amidon au niveau des α_2- globulines lentes. La radioactivité du fragment anodique provient de l'hormone folliculo-stimulante marquée libre et correspond à 98,4 % de la radioactivité totale en l'absence d'anticorps anti-FSH. Celle de la fraction cathodique provient du complexe FSH*-Anticorps. En utilisant l'électrophorèse en amidon nous réalisons la troisième condition.

D. VARIATION DU RAPPORT $\frac{\text{FSH*-Ac}}{\text{FSH*LIBRE}}$ SOUS L'ACTION DE DOSE CROISSANTE DE FSH NON MARQUEE.

Pour établir la courbe standard de décroissance du rapport $\frac{\text{FSH*-Ac}}{\text{FSH* libre}}$ sous l'action de quantités connues de FSH non marquée nous mettons incuber pendant 90 heures à 4°, 0,25 mμg de FSH*, l'antisérum à la dilution finale de 1/25.000 et des doses croissantes de FSH non marquée : 0, 0,1; 0,5; 1; 2 et 5 mμg. Le liquide de dilution est constitué par le tampon phosphate pH 7,5-0,05 M contenant 5 g ‰ d'albumines bovines. Lorsque le délai d'incubation est écoulé, chaque tube reçoit immédiatement avant d'être soumis à l'électrophorèse en amidon 0,1 ml de sérum de cheval de façon à réduire à moins de 1 % la quantité de FSH libre adsorbée au point d'origine. L'électrophorèse est alors réalisée pendant 5 heures. Après coloration des bandes d'amidon, celles-ci sont coupées en un endroit bien précis et la radioactivité des deux fragments appréciée. Pour obtenir le rapport $\frac{\text{FSH*-Ac}}{\text{FSH* libre}}$ il suffit d'établir le rapport entre $\frac{\text{la radioactivité du fragment cathodique}}{\text{la radioactivité de fragment anodique}}$.

La sensibilité de la technique permet encore de doser 0,5 mμg de FSH soit 1,17 m. U HMG_{24}.

II. DOSAGE RADIO-IMMUNOLOGIQUE DE L'HORMONE LUTEINISANTE CHORIONIQUE ET HYPOPHYSAIRE.

L'hormone lutéinisante hypophysaire (LH) n'est pas disponible, à l'heure actuelle, à l'état suffisamment pur pour permettre un dosage radio-immunologique.

Par contre depuis les travaux de Got et Bourillon (1959-2 et 11) il est possible d'obtenir l'hormone chorionique gonadotrope lutéinisante (HCG) à l'état chimiquement pur. Par ailleurs, on connaît, à la suite des travaux de Wide et Gemzell (1961) la communauté antigénique qui existe entre l'hormone lutéinisante hypophysaire et l'hormone chorionique gonadotrope lutéinisante.

Au cours de cette seconde partie du travail nous voudrions vérifier les conditions indispensables à la réalisation du dosage radio-immunologique de l'hormone chorionique gonadotrope et démontrer que ce dosage radio-immunologique de l'HCG permet d'apprécier la concentration de l'hormone lutéinisante hypophysaire dans le sérum de sujets normaux ou atteints d'affections diverses.

A. Obtention d'un anti-serum spécifiquement dirige contre la seule hormone chorionique lutéinisante.

Les laboratoires Organon nous ont fourni des préparations chimiquement pures de HCG dont l'activité biologique est de 12.500 à 14.000 U.I./mg. Ce sont ces préparations qui ont servi à induire la formation d'antisérum chez le lapin.

L'analyse de la composition en anticorps des antisérums obtenus a été réalisée par les techniques immunologiques classiques : la double diffusion en gélose, l'hémagglutination et l'immunoélectrophorèse.

De cette étude il ressort que nos antisérums ne contiennent qu'un seul type d'anticorps. En effet, en immuno-électrophorèse, lorsque la préparation pure de HCG réagit avec l'antisérum correspondant, nous n'observons qu'un seul trait de précipitation dans la zone de migration des α_2- globulines (Figure 8). Le sérum de femmes enceintes et un extrait placentaire donnent un trait de précipitation identique en présence de ce même antisérum. Ce trait de précipitation correspond

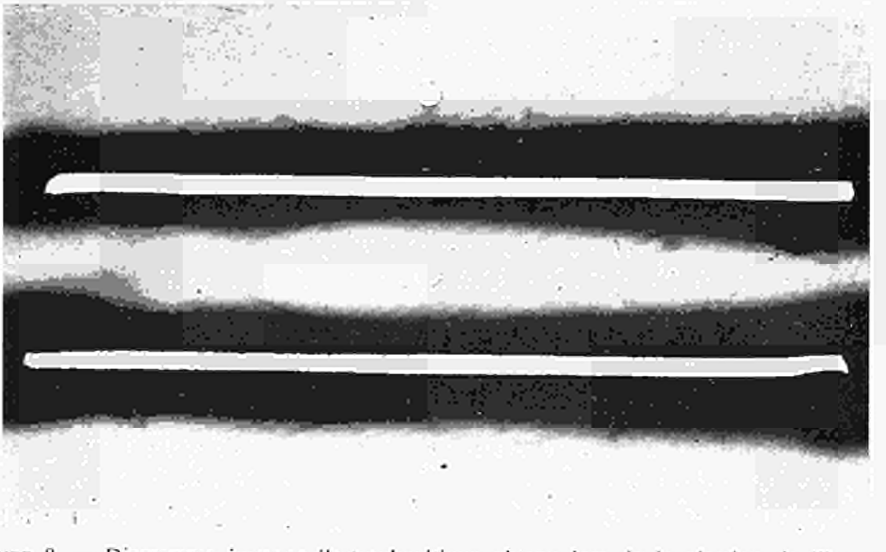

Figure 8. — Diagramme immuno-électrophorétique obtenu lors de la réaction de l'hormone chorionique gonadotrope et du sérum anti-hormone chorionique gonadotrope

bien à la réaction HCG-Anticorps anti-HCG, car les effets biologiques lutéinisants normalement obtenus avec l'HCG seule sont inhibés par l'incubation préalable de l'hormone pure avec le sérum anti-HCG (S. a. HCG) (Figure 9). Notre antisérum est spécifique et la première condition d'un dosage radio-immunologique est ainsi réalisée.

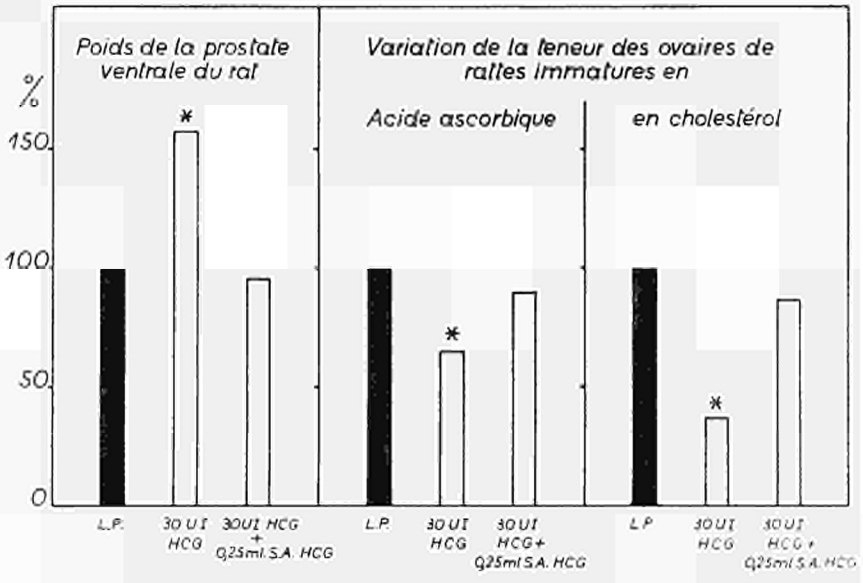

FIGURE 9. — Modification du poids de la prostate ventrale du rat immature et de la teneur des ovaires en acide ascorbique et en cholestérol, sous l'action de 30 U. I. d'hormone chorionique (HCG) et de cette même quantité de HCG préalablement incubée avec 0,25 ml de sérum anti HCG (S.a.HCG).

* marque les variations significativement différentes par rapport à la valeur témoin (rats recevant L. P. = NaCl 9 ‰).

B. UTILISATION DE L'HORMONE CHORIONIQUE MARQUEE A L'ETAT PUR.

La préparation purifiée de HCG a été marquée par ^{131}I en utilisant la technique de Greenwood *et al.* (1963). L'activité spécifique obtenue est de 150 à 300 $\mu C/\mu g$.

En chromatographiant cette préparation de HCG marquée (HCG*) sur Séphadex G 200 nous obtenons un pic unique de radioactivité. Nous conservons les tubes d'élution correspondant à l'élimination maximale de la radioactivité : du 19e au 22e ml (Figure 10). Il s'agit bien de l'hormone chorionique gonadotrope, car l'injection de ces éluats provoque, chez le rat, les manifestations lutéinisantes classiques.

Un argument en faveur de la pureté de la préparation marquée et de la conservation de ses propriétés immunologiques après marquage est fourni par l'autoradio-immunoélectrophorèse. Lorsque l'immunoélectrophorèse, obtenue en présence de l'hormone chorionique marquée et de l'antisérum correspondant, est mise en contact avec une plaque radiographique, nous n'obtenons, en effet, qu'un

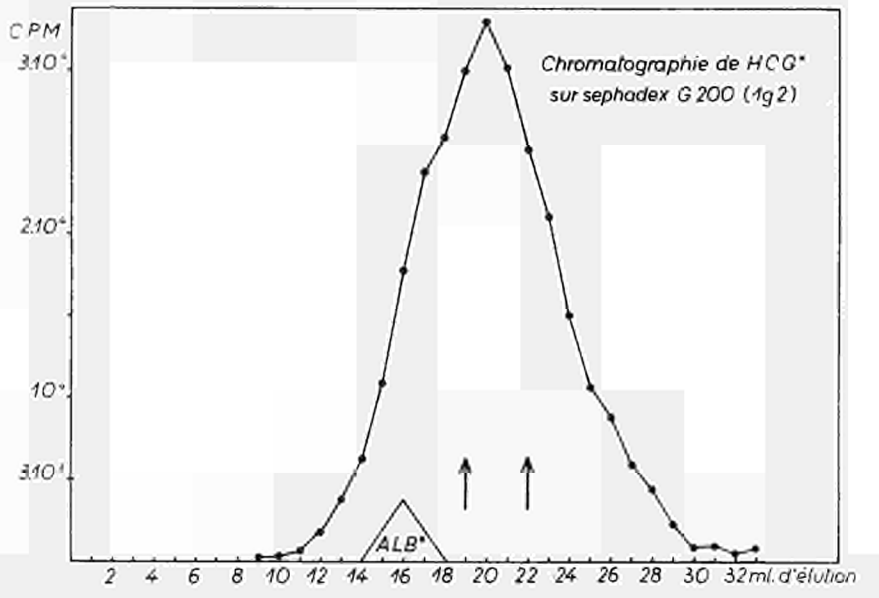

FIGURE 10. — Chromatographie de HCG* sur Séphadex G 200. Les volumes d'élutions compris entre les deux flèches sont conservés et serviront au dosage radio-immunologique

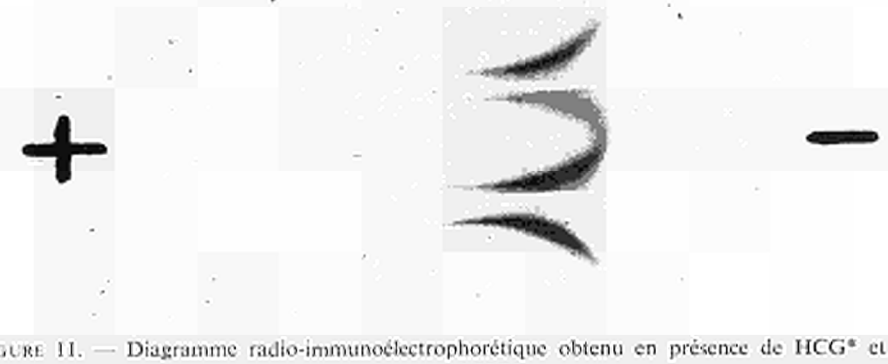

FIGURE 11. — Diagramme radio-immunoélectrophorétique obtenu en présence de HCG* et du sérum anti-HCG

seul trait d'impression correspondant à la ligne de précipitation relevée en immuno-électrophorèse (Figure 11).

La préparation de HCG marquée puis filtrée sur Séphadex G 200 est pure puisque l'impression de la plaque photographique s'effectue uniquement suivant le trait de précipitation de l'hormone en immuno-électrophorèse. Par ailleurs elle paraît bien conserver ses propriétés antigéniques lui permettant de réagir avec son anti-sérum. La seconde conditions est réalisée.

C. Methode de séparation de l'HCG* libre et de l'HCG* fixée aux anticorps.

Différentes techniques de séparation ont été tentées. Nous n'avons pas pu obtenir de bons résultats avec l'électrophorèse sur papier ni avec les échangeurs d'ions du type Amberlite 500, 50 ou DEAE cellulose (Franchimont, 1965).

Par contre, l'électrophorèse en amidon constitue une bonne technique de séparation. En effet, l'hormone chorionique marquée libre migre dans la zone des α_2-globulines rapides comme l'indiquent les expériences biologiques et radio-immunologiques, tandis que, en présence d'anticorps, la radioactivité de HCG* se situe au point d'origine et dans la zone comprise entre les α_2- globulines lentes et le point d'origine (Figure 12).

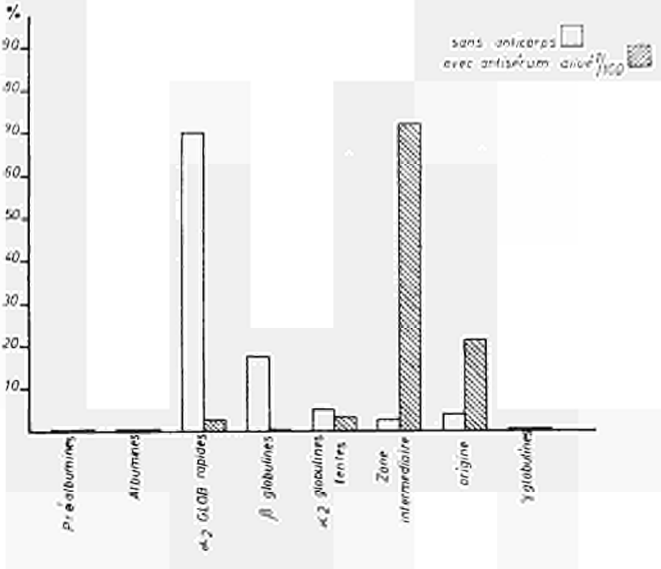

Figure 12. — Répartition de la radioactivité, après électrophorèse en amidon, de l'HCG* non traitée ou préalablement incubée avec l'antisérum correspondant.

Pour réaliser une séparation de l'hormone marquée libre et combinée aux anticorps il suffit de faire une électrophorèse en amidon à 220 V pendant 5 h 30, de colorer et fixer la plaque, puis de la couper au niveau du trait des α_2- globulines lentes. La radioactivité de la fraction anodique provient de l'HCG* libre et celle de la fraction cathodique dépend du complexe HCG*-Anticorps.

En l'absence d'anticorps, la radioactivité au point d'origine et entre celui-ci et les α_2- globulines lentes ne dépasse pas 3 %. C'est cette technique de séparation que nous utilisons.

D. IDENTITE ANTIGENIQUE ENTRE L'HORMONE CHORIONIQUE ETALON PURIFIEE ET L'HORMONE CHORIONIQUE PRESENTE DANS LE SERUM OU LES URINES DE FEMMES ENCEINTES.

Lorsque nous recherchons les courbes de décroissance du rapport $\frac{\text{HCG* fixée aux anticorps}}{\text{HCG* totale}} \times 100$ sous l'action soit de la préparation purifiée d'HCG non marquée soit d'urines (U♀E) soit encore de sérum (S♀E) de femmes enceintes, nous notons un parallélisme de ces courbes. Ce dernier signe un comportement immunologique identique de l'hormone chorionique gonadotrope, qu'elle soit purifiée ou présente dans les milieux biologiques, urines ou sérum (Figure 13).

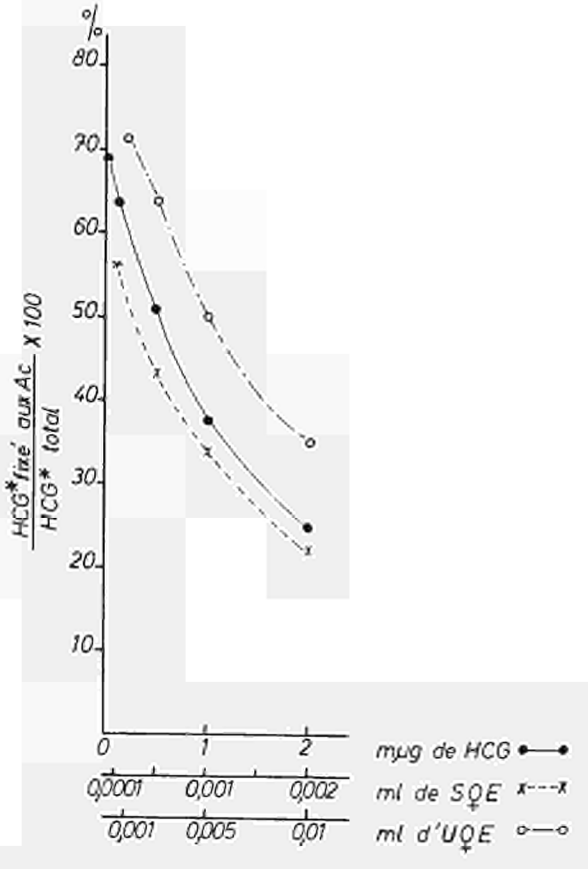

FIGURE 13. — Etude du rapport $\frac{\text{HCG* fixée aux anticorps}}{\text{HCG* totale}} \times 100$ sous l'action de quantités croissantes de HCG non marquée et de volumes différents de sérum (S♀E) ou d'urine de femme enceinte (U♀E).

E. Communauté antigénique entre l'hormone lutéinisante hypophysaire et l'hormone chorionique gonadotrope.

Peut-on utiliser la méthode de dosage radio-immunologique que nous proposons pour apprécier le taux de l'hormone lutéinisante hypophysaire chez des sujets non gravides ? La réponse sera affirmative pour autant qu'il existe une communauté antigénique entre l'hormone chorionique gonadotrope (HCG) et l'hormone hypophysaire lutéinisante (LH) et que tous les anticorps présents dans le sérum anti-HCG réagissent avec des groupements antigéniques déterminants communs à ces deux hormones.

La communauté antigénique entre HCG et LH a été découverte par Wide et Gemzell (1961).

Pour notre part, nous avons montré que le sérum anti-HCG agglutine les hématies de moutons recouvertes d'une préparation de gonadotrophines extraites d'hypophyses et contenant de l'hormone lutéinisante. Par ailleurs, ce sérum anti-HCG inhibe l'activité biologique lutéinisante de cette même préparation de gonadotrophines hypophysaires (Figure 14).

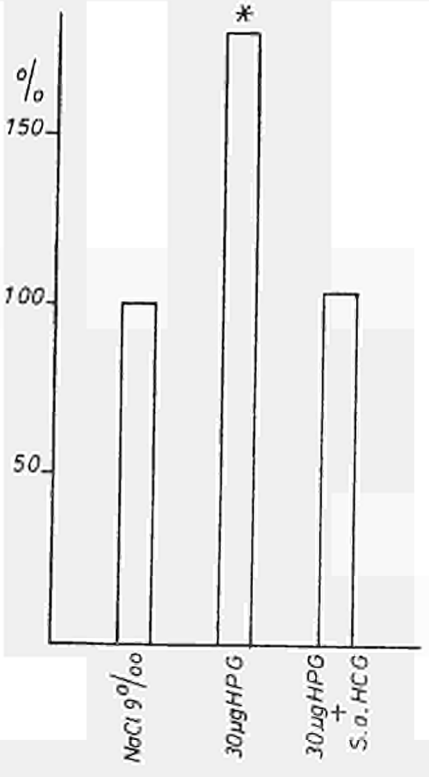

Figure 14. — Modification du poids de la prostate ventrale du rat sous l'action de 30 µg de gonadotrophines extraites d'hypophyse humaine (HPG) non traités puis préalablement incubés avec le sérum anti HCG (S. a. HCG).
* Marque les variations significatives par rapport à la valeur témoin.

Enfin, les courbes de décroissance du rapport $\frac{\text{HCG*-Ac}}{\text{HCG totale}} \times 100$ sont parallèles en présence soit de HCG non marquée soit de gonadotrophines extraites d'urine de femmes ménopausées (Human menopausal gonadotrophin : HMG) soit de gonadotrophines extraites d'hypophyses humaines (Human pituitary gonadotrophin : HPG), préparations contenant l'hormone lutéinisante d'origine pituitaire (Figure 15). La valeur la plus basse de ce rapport est identique lorsque l'on utilise des quantités importantes de HCG, de HMG et de HPG. Aussi, pouvons-nous confirmer la communauté antigénique entre l'hormone lutéinisante chorionique et l'hormone hypophysaire lutéinisante et démontrer que, dans nos conditions expérimentales, les groupements antigéniques déterminants de l'hormone chorionique qui ont donné naissance aux anticorps de l'antisérum, sont communs à l'hormone lutéinisante hypophysaire.

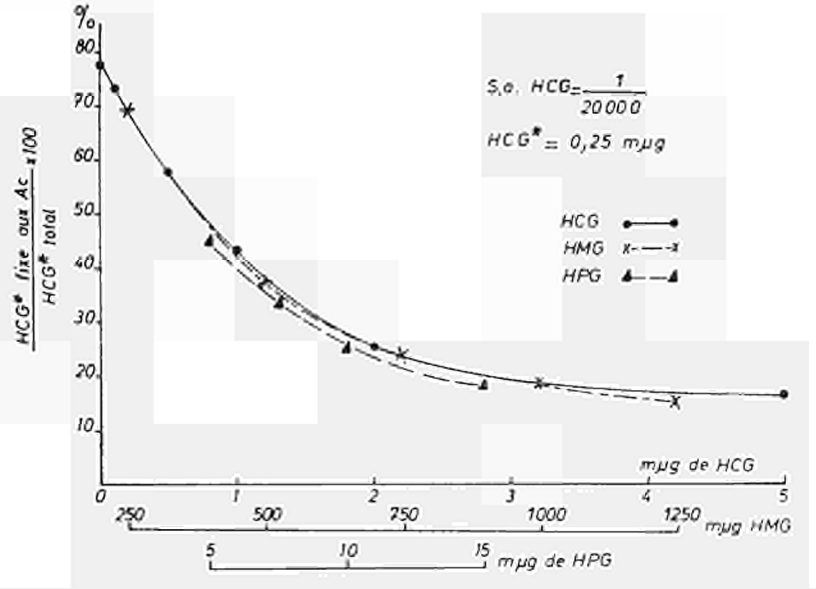

FIGURE 15. — Etude du rapport $\frac{\text{HCG* fixée aux anticorps}}{\text{HCG* totale}} \times 100$ sous l'action de quantité croissante de HCG non marquée, de gonadotrophines extraites d'hypophyses (HPG) et de gonadotrophines extraites d'urines de femmes ménopausées (HMG).

En utilisant l'hormone chorionique purifiée marquée et l'antisérum correspondant, nous pouvons donc réaliser un dosage radio-immunologique des hormones lutéinisantes chorionique et hypophysaire.

III. Resultats obtenus par les methodes radio-immunologiques

Les explorations réalisées à ce jour concernent principalement des femmes jeunes en bonne santé. Le taux sérique de l'hormone folliculo-stimulante est très élevé dans les premiers jours du cycle menstruel. C'est ainsi que nous relevons une concentration très importante les 2e, 3e et 4e jours du cycle. La valeur moyenne calculée lors de ce premier pic est de 42,2 mμg/ml. Par la suite, elle diminue, puis

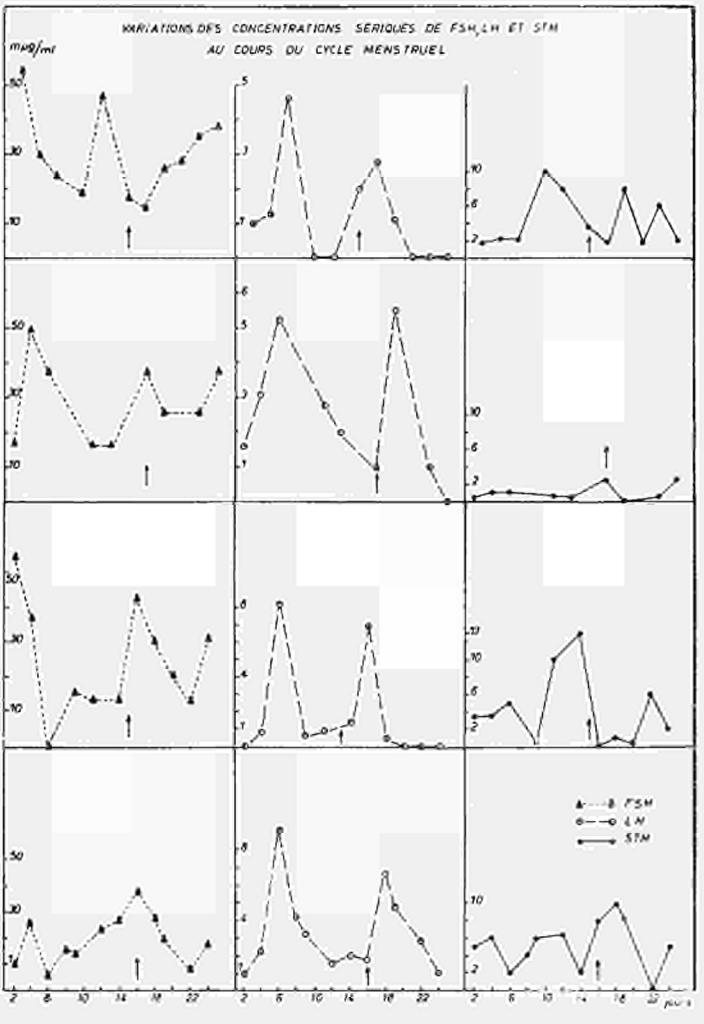

Figure 16. — Evolution du taux de l'hormone folliculo-stimulante (▲ - - - ▲), de l'hormone lutéinisante (⊙ - - - ⊙) et de la somatotrophine (●—●) au cours d'un cycle menstruel de quatre jeunes femmes

(La flèche marque le décrochement thermique).

augmente à nouveau immédiatement avant ou au moment de l'élévation thermique. La valeur sérique de cette hormone rediminue alors pour s'accroître une fois encore dans la seconde partie du cycle, aux environs du 24e jour (Figure 16).

Par ailleurs il existe deux pics de sécrétion de l'hormone lutéinisante, le premier suivant de peu celui de la FSH et l'autre se situant entre le 13e et le 18e jour du cycle.

La figure 16 donne les taux de FSH et de LH présents dans le sérum au cours d'un cycle menstruel chez quatre jeunes femmes.

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DISCUSSION

F. C. GREENWOOD (*London*) : Dr. Franchimont have you carried out parallel inhibition curves between your standards and a crude pituitary extract ? Similarly have you compared your standard curve in your FSH assay with the inhibition effect of plasma ?

P. FRANCHIMONT : La préparation d'hormone lutéinisante hypophysaire que nous possédons est très impure puisqu'elle possède une activité FSH de 970 U. HMG_{24} (appréciée par le test de Steelman et Pohley) et une activité LH de 172 U. I. HCG. La difficulté d'obtenir une préparation pure de LH hypophysaire nous a obligés d'entreprendre le dosage de cette hormone en utilisant le système hormone gonadotrope chorionique-anticorps antihormone gonadotrope chorionique. La préparation hypophysaire dont l'activité biologique vient d'être signalée provoque une courbe de réduction du rapport $\frac{^{131}\text{I HCG} - \text{Ac}}{^{131}\text{I HCG libre}}$ parallèle à celle produite par l'hormone chorionique non marquée. C'est un des arguments qui nous paraît établir la similitude immunologique de l'hormone lutéinisante hypophysaire et chorionique.

En ce qui concerne la FSH, nous n'avons pas encore tous les résultats de l'étude relative à la variation du rapport B/F. Les expériences qui sont en cours utilisent soit la préparation purifiée de FSH soit les milieux biologiques riches en FSH comme le sérum ou les urines de femmes ménopausées physiologiquement ou chirurgicalement.

RADIOIMMUNOASSAY OF HCG

P. G. CROSIGNANI and F. POLVANI

Clinica Ostetrica e Ginecologica,

Università di Milano, Centro di Endocrinologia Ginecologica, C. N. R., Milano, Italy

ABSTRACT

A simplified HCG radioimmunoassay method is described.

A preparation of HCG labelled with ^{125}I by means of electrolysis was used.

Rabbit anti-sera against HCG were obtained. They have been found capable of agglutining HCG coated red cells and of reacting with ^{125}I labelled HCG. The complex during the latter reaction is soluble, furthermore antibodies carefully precipitated (coagulated) with organic solvents are capable of binding ^{125}I-HCG, and bound radioactivity can be separated by centrifugation. When ^{125}I-HCG and unlabelled hormone are mixed, the specific activity of the former is decreased. By adding to the above mixture a given amount of coagulated anti-HCG γ-globulin, this will bind an aliquot of the ^{125}I-HCG and unlabelled HCG. The radioactivity bound to the coagulum is directly related to the specific activity of the mixture, and the unknown amount of hormone can be measured.

This method was applied to radioimmunoassay of human chorionic gonadotrophin in pure solution, and it would also appear suitable for assay in biological fluids.

The following immunological methods are currently in use for the determination of HCG in biological fluids : haemagglutination inhibition [1], inhibition of "latex particle agglutination" [2], and complement deviation [3].

The first two methods are more widely employed, but are only semiquantitative. The third method is quantitative, although the various parameters of the haemolitic system which acts as index of the reaction have to be constantly controlled.

Wilde and co-workers [4] have recently applied to the determination of HCG the radioimmunological method developed for insulin by Hales [5].

The method consists of addition of anti-HCG antibodies to a mixture of unlabelled and labelled HCG and measurement of the specific activity in the supernatant after precipitation of the antigen-antibody complex; the amount of unlabelled HCG in the system is calculated from this specific activity.

The complex HCG-rabbit antibodies is completely soluble at low concentration; it can be separated from the unbound hormone either by adding a second antibody, an anti-rabbit γ-globulin serum, as proposed by Hales, or by making the complex insoluble. With the latter method the time required is much shorter, and the possibility of incomplete precipitation of the antigen-antibody complex by the second antibody is avoided. In fact, γ-globulin can be coagulated by means of various agents without destruction of the combining sites. A coagulum obtained in this way has already been employed in microbiology as specific antigen adsorbent [6].

Owing to the advantages afforded by the use of coagulated antibodies, we decided to use this method.

Material and method

Antisera HCG.

Antisera to HCG were prepared by injecting into rabbits a crude preparation of the hormone (Lot No. 51 M. ISEC) containing 5,300 I.U./mg, together with Freund's complete adjuvant. The sera so obtained were pooled and adsorbed with an extract of urine of a child, in the proportion of 1 ml of antiserum to an extract corresponding to 100 ml of urine, as suggested by Goss [7]. The haemagglutination title of the antiserum was 1/5,120.

The antiserum γ-globulin was separated by salting with Na_2SO_4, and then redissolved in a volume of water twice the original volume of serum.

The γ-globulin was coagulated by addition of 90 % aq. ethanol at pH 7, leaving 30 min at 34° C, as described by Sutherland [6]. The coagulum so formed was washed three times with buffered saline (*), suspended in a volume of buffered saline two-four the volume of the γ-globulin solution and merthiolate added.

Human Chorionic Gonadotrophin (HCG).

A commercial preparation containing 2,000 I.U./mg (Pregnyl Organon), was labelled with iodine ^{125}I by Dr. Rosa (Sorin, Saluggia, Italy) using an electrolytic method [8]. The specific activity was 5 mC/mg. The labelled hormone was lyophilized and kept in the dry state.

Procedure

Preliminary experiments were performed to establish the amount of coagulated anti-HCG globulin sufficient to remove approximately half the total radioactivity of a fixed aliquot of ^{125}I-labelled HCG. Immediately prior to use, the solution containing the coagulated antibodies was thoroughly shaken to insure homogeneous suspension, and then let to stand for 2 min. 0,1 ml aliquots were distributed in two series of conical bottom test tubes. The tubes were centrifuged at 4.000 rpm for 5 min and the supernatant discarded. The coagulated antibodies were incubated for 60 min at room temperature with 0,1 ml of standard or unknown solution of unlabelled HCG in duplicate.

Subsequently, 0.05 ml of a solution containing 100 mμg ^{125}I-HCG per ml, equal to 500 μμg, was added, the test tubes shaken one by one and left at room temperature for 60 min. After this, they were centrifuged at 4,000 rpm for 5 min and the supernatant transferred to another test tube; the precipitate was washed

(*) Solution containing 0.15 M NaCl and 0.11 M Phosphate Buffer, pH 7.4.

with 0.5 ml buffered saline, again centrifuged and the supernatant added to that obtained after the previous centrifugation. The radioactivity was measured in both the precipitate and the supernatant by means of a crystal scintillation counter.

Results

With coagulated anti-HCG γ-globulin in excess only 65-70 % of the radioactivity was recovered in the precipitate in each experiment; the radioactivity in the supernatant is not attributable to free ^{125}I, since this accounts for less than 1 % of the total radioactivity. The linkage of labelled HCG to coagulated anti-HCG globulin took place very rapidly and was practically complete within 30 min (Figure 1). The effect of varying amounts of unlabelled hormone on the adsorption

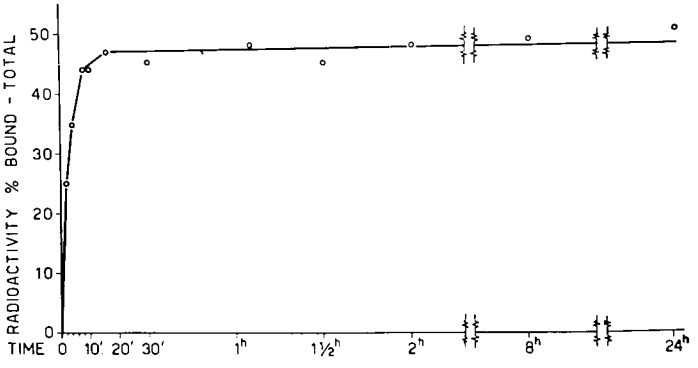

FIGURE 1. — Binding of ^{125}I-HCG with anti-HCG coagulated γ-globulin in relation to incubation time

of labelled HCG to the antibodies is illustrated in Figure 2. Within a certain range, there was a linear relationship between log amounts of unlabelled hormone preincubated with the antibody and the percentage of radioactivity present in the precipitate. By comparing with a standard scale the ratio bound/unbound radioactivity in the sample, i.e. the radioactivity in the precipitate and in the supernatant respectively, it is possible to calculate the amount of unlabelled hormone present in the sample.

We prefer to measure the radioactivity present in both supernatant and precipitate and not only in the supernatant as in other methods, because the bound/unbound radioactivity ratio is also dependent upon the specific activity of the hormone in the sample, and in addition has the advantage of decreasing technical errors which may occur in the procedure.

The method described above is simple and rapid. A series of determinations can be accomplished within 4-6 hours. The sensitivity and precision of the method depend upon the specific activity of the labelled HCG — reliable results cannot be obtained with a specific activity less than 2-3 mC/mg —, and upon the relative

proportions of antibody and antigen incubated together; an excess of antibodies reduces both the precision and the sensitivity of the method. The sensitivity of the method would appear to be greater than that of the haemagglutination inhibition reaction which is generally considered satisfactory for clinical purposes.

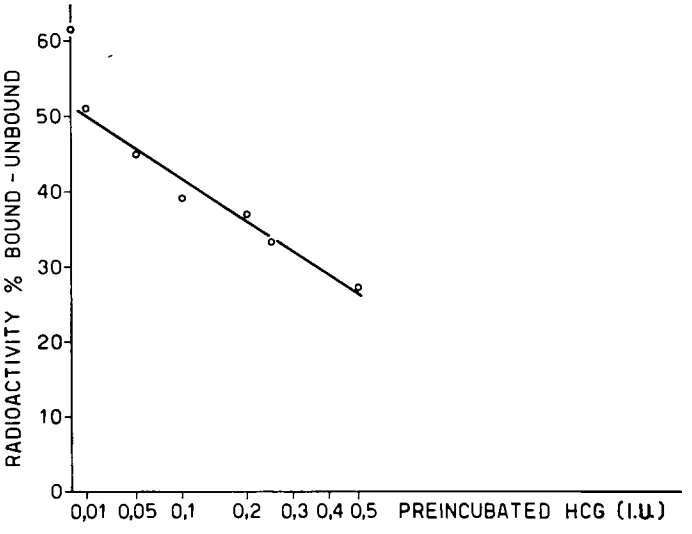

FIGURE 2. — Standard assay curve for HCG. Effect of preincubated unlabelled HCG on the binding of ^{125}I-HCG to anti-HCG coagulated γ-globulin

It must be noted, however, that when HCG is assayed in urine containing less than 300-400 I.U. HCG/liter, the presence of LH, which can cross-react with anti-serum HCG[1] could interfere with the assay.

Results of duplicate radioimmunoassays performed on pregnancy urine showed good agreement; the values were comparable with those obtained on the same samples by means of the heamagglutination method [9].

However, these results in urine must be considered only preliminary and further work is needed in order to confirm the validity of the method.

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DISCUSSION

P. FRANCHIMONT (*Liège*) : Je voudrais faire trois remarques :

1) L'antisérum obtenu par le Dr. Crosignani en utilisant une préparation de HCG d'une activité biologique de 5.000 U. I./mg n'est certainement pas spécifique. En effet, nous avons déterminé que semblable préparation contient au moins 4 types d'antigènes dont un correspond à l'hormone gonadotrope chorionique et les 3 autres à des contaminants aspécifiques. Il faut donc nécessairement effectuer un épuisement.

2) Le Dr. Crosignani marque par ^{131}I une préparation de HCG ayant une activité de 2.000 U. I./mg. Nous avons fait l'étude de la composition antigénétique de ces préparations et avons montré qu'elle contient 7 à 8 substances antigénétiques différentes dont une seule est l'hormone chorionique. Ces protéines aspécifiques seront donc marquées également par ^{131}I et perturberont le dosage. En effet, avec un antisérum spécifique, le rapport $\frac{^{131}I \text{ HCG liée}}{^{131}I \text{ HCG libre}}$ doit rester très bas par suite de la présence de ces contaminants marqués qui ne réagissent plus avec l'antisérum épuisé. L'auteur envisage-t-il une purification consécutive au marquage ?

3) Le pourcentage de l'hormone chorionique marquée, fixée aux anticorps serait, selon le Dr. Crosignani, modifié pour 0,05 U. I. HCG. Nous avons observé dans nos expériences une modification significative de ce pourcentage pour 0,1 m μg d'une HCG dont l'activité biologique est de 12.500 U. I./mg, soit pour 0,00125 U. I. de HCG. La sensibilité que nous obtenons est donc meilleure.

P. G. CROSIGNANI : Rispondo nell'ordine alle tre domande :

1) L'antisiero utilizzato presentava 6-7 contaminanti specifiche e si è infatti provveduto alla loro eliminazione mediante assorbimento con "Peduex" in accordo con Goss (*J. Clin. Endocrinol.*, **24** : 408, 1964).

2) Concordo pienamente con il Dr. Franchimont sull' utilità di una purificazione della gonadotropina marcata, tuttavia le elevate quote di ormoni presenti nelle urine della donna gravida permettono una valutazione quantitativa della HCG anche nelle condizioni descritte.

3) La sensibilità del sistema rivelatore utilizzato dal Dr. Franchimont appare indubbiamente molto più elevata della nostra. Il suo problema però è quello di misurare l'LH ipofisario (poche decine di unità internazionali per litro di urina); il nostro è quello di determinare l'HCG in gravidanza (alcune migliaia di unità internazionali per litro di urina).

^{125}I-INSULIN AS A TRACER OF INSULIN IN DIFFERENT CHEMICAL PROCESSES

K. JØRGENSEN and C. BINDER

Novo Research Institute, Copenhagen, Denmark

ABSTRACT

A method for the preparation of ^{125}I-insulin is briefly described.

The radioactivity of the ^{125}I-insulin almost completely followed carrier insulin in the crystallization process. Nearly 100 percent of the radioactivity could also be bound to antibodies against insulin.

However, the radioactivity did not follow carrier insulin in all experiments of electrophoresis. Furthermore, in a certain partition chromatography about half of the radioactivity followed the single peak of carrier insulin, while the other half of the radioactivity appeared as « trailing ».

The radioactivity of ^{125}I-insulin, prepared by an other method, also followed carrier insulin in the crystallization process and could also be bound to antibodies against insulin. However, in the partition chromatography this labelled insulin behaved quite differently from both insulin and ^{125}I-insulin prepared by the present method.

The results demonstrate the need of investigating the behaviour of radioiodinated insulin in comparison with that of insulin in the specific process, for which the labelled insulin is intended as a tracer of insulin.

A description of such an investigation is given, which shows the reliability of using ^{125}I-insulin prepared by the present method for studies of the absorption of some subcutaneously injected pharmaceutical insulins.

For use in the present study insulin was radioiodinated by mixing carrier free $^{125}I^-$ and insulin in acid solution, and subsequently adding an excess of iodate. Purification was performed by precipitation with zinc acetate, filtration, and washing. The specific radioactivity of the final product was between 10 and 15 mC/mg insulin corresponding to a degree of iodination of about 5 atoms of iodine per 100 insulin monomers. The yield of radioactivity was about 70 percent.

With fresh preparations it was found that nearly 100 percent of the radioactivity followed carrier insulin of the same species as the labelled insulin through at least four crystallizations. With preparations stored for two months at -25° C in a solution containing 0.1 percent albumin, having a pH of 3, and with an initial radioactivity concentration of about 10 μC/ml, 95 percent or more of the radioactivity still followed added insulin through the crystallizations.

Nearly 100 percent of the radioactivity could also be bound to antibodies against insulin.

However, the radioactivity did not follow carrier insulin in certain experiments of electrophoresis, while it did follow in other experiments of electrophoresis. Furthermore, discrepancies appeared when a mixture of the ^{125}I-insulin and carrier

insulin was submitted to a reversed-phase partition chromatography performed according to a modification of the method described by Andersen [1].

The chromatogram A in Figure 1 shows how one part of the radioactivity curve followed the insulin curve while the rest appeared as trailing.

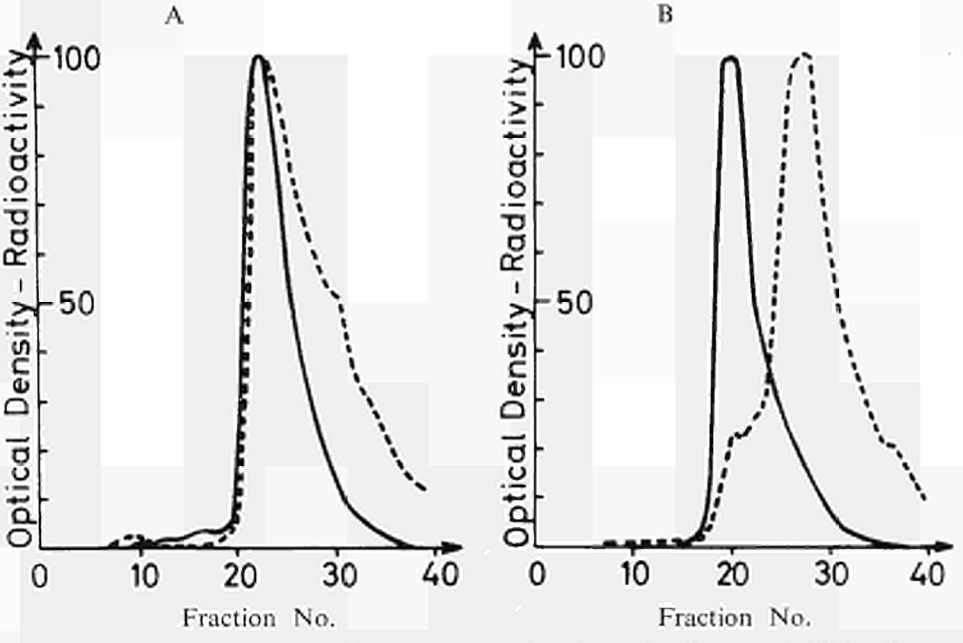

FIGURE 1. — Reversed-phase partition chromatography of pork insulin + pork ^{125}I-insulin prepared by the present method (A) and the method of Greenwood *et al.* (B). Degree of iodination for both ^{125}I-insulin preparations : 5 percent. Optical Density at 276 mμ in percent of max. value : ——— Radioactivity in percent of max. value : - - - - - - - System : 500 ml 0.04 M trichloroacetic acid and 200 ml 2-butanol. Stationary phase : 4 ml organic phase supported by 6 g silane-treated kieselguhr (2) in a column (13 × 1.2 cm). Fractions of 2 ml, diluted with 1 ml water before measurements. Flow rate : 0.5 ml/min. Temperature thermostatically maintained at 21° C. Application : 10 mg pork insulin + ^{125}I-insulin (trace amount) in 1 ml aqueous phase.

For the sake of comparison, ^{125}I-insulin was prepared according to the method of Greenwood, Hunter and Glover [3] using chloramine T as the oxidizing agent at pH 7-8 and gel filtration on Sephadex for purification. Nearly 100 percent of the radioactivity of this preparation also followed carrier insulin through four crystallizations and could also be bound to antibodies against insulin. However, as seen in the chromatogram B in Figure 1 the radioactivity curve was quite different both from the insulin curve and from the radioactivity curve in the chromatogram A.

These results demonstrate that it is possible to prepare radioiodinated insulin which follows insulin in the crystallization process and which is immunologically active. However, on this basis alone no predictions can be stated as to the pro-

perties of the labelled insulin as a tracer of insulin in other chemical processes. Therefore, the reliability of using a given kind of radioiodinated insulin as a tracer of insulin must be investigated for each intended kind of application.

In the following a description will be given of such an investigation which shows the reliability of using ^{125}I-insulin prepared by the present method for studies of the absorption of subcutaneously injected pharmaceutical insulins.

The point was to inject ^{125}I-insulin containing preparations and to follow the absorption by measuring the radioactivity above the site of injection by means of a scintillation detector.

In order to investigate whether the externally measured count rates truly reflected the absorption of insulin, experiments were carried out in animals.

Pork and beef ^{125}I-insulin were prepared separately by the present method. The preparations were diluted with carrier pork and beef insulin, respectively. The mixtures were then treated in the prescribed ways for the preparation of five different, pharmaceutical insulins as given in Table I.

TABLE I. — Results of sheep experiments

SI = Soluble Insulin, an acid solution of pork insulin.

A = Actrapid, a neutral solution of pork insulin with a low content of zinc.

SL = Semilente, a neutral suspension of amorphously precipitated pork insulin with a high content of zinc.

CII = Crystal II, a neutral suspension of beef insulin crystals with a low content of zinc.

UL = Ultralente, a neutral suspension of beef insulin crystals with a high content of zinc.

Kind of insulin injected (1 ml ~ 40 i.u.)		SI	A	SL	CII	UL
Percent rest-radioactivity at the time of excision, externally measured		*a*	34	46	37	49
Percent rest-radioactivity in excised tissue, measured in well-defined geometry		49	28	46	33	47
Percent insulin-bound radioactivity	In preparation . .	98	97	96	100	99
	In extract	98	105	98	98	99
Specific radioactivity of extracted insulin / Specific radioactivity of injected insulin		1.02	0.85	0.91	0.92	0.92

[a] The determination failed.

1 ml of each preparation was injected subcutaneously in five different sheep. The radioactivity was measured by a scintillation counter above the skin. When the apparent radioactivity had fallen to about 50 percent of the initial value the injected tissue was excised and extracted with acid aqueous ethanol. The radioactivity of the extract plus the small amount of un-extracted radioactivity was determined in percent of the injected radioactivity on the basis of measurements in well-defined geometry. From Table I it appears that the externally measured rest-radioactivities at the time of excision are in fairly good accordance with the measurements performed in well-defined geometry after excision.

The relative insulin-bound radioactivities were determined in the insulin preparations and in the extracts as the percentages of radioactivity following added insulin of the corresponding species through four crystallizations. It appears that all the percentages are close to 100.

Finally the specific radioactivities were determined in the preparations and in the purified extracts. The single specific radioactivity was computed as the ratio between the concentration of insulin-bound radioactivity and the concentration of insulin determined by immunoassay, performed as a modification of the method of Heding [4]. In Table I the ratios between the specific radioactivity of the extracted and of the correspondingly injected insulin are given. As all of the ratios are close to one, it is concluded that if there are differences in the rates of absorption for the labelled and the unlabelled insulin such differences must be very small.

These animal experiments thus show that the externally measured count rate is proportional to the amount of residual radioactivity. This is equal to the amount of insulin-bound radioactivity, which is again proportional to the amount of residual insulin. Thus the externally measured count rate is proportional to the amount of residual, unabsorbed insulin.

In summary, the results of the present work demonstrate how radioiodinated insulin can fail as a tracer of insulin in some processes and still be an excellent tracer of insulin in other processes.

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DISCUSSION

K. BRUNFELDT (*Gentofte*) : I should like to ask whether you can give an explanation of the different behaviour of the 2 iodinated preparations. As our knowledge of the fate of the insulin in the organism is restricted, experiments, as reported by you, demonstrating differences in the chemical behaviour between labeled and non labeled insulins are very useful. Thus the distribution of the insulin in the organism may be heavily influenced by changes due to the labeling of the insulin molecule.

K. JØRGENSEN : I must answer that I have not investigated that point, but since it seems that the radioiodine is really protein-bound in both preparations the chromatographical differences may be due to different distributions of iodine in the insulin molecule. I can only say that in the ^{125}I-insulin prepared by the present method nearly all of the radioactivity is present in the A-chain.

R. S. YALOW (*New York*) : Can labeled pork insulin be used as tracer for beef insulin or vice versa in crystallization experiments — i.e. can you crystallize to constant specific activity with the heterologous species ?

K. JØRGENSEN : I must answer that I have only performed some few experiments of the mentioned kind, but it seems like mixtures of labeled insulin of one species and carrier insulin of an other species do not crystallize with constant specific radioactivities. Therefore, when I check my ^{125}I-insulin preparations I always use carrier insulin of the same species as the labeled insulin in the crystallization process.

VERGLEICH ZWISCHEN BIOLOGISCHER UND RADIO-IMMUNOLOGISCHER INSULINBESTIMMUNG

H. DITSCHUNEIT, J. BEYER, F. MELANI, K. SCHÖFFLING, M. TELIB und E. F. PFEIFFER

Abteilung für Klinische Endokrinologie, I. Med. Univ. Klinik
Johann Wolfgang Goethe-Universität, Frankfurt/Main, Deutschland

Abstract

From the results of comparative insulin assays in normal and pancreatectomized dogs deprived of their hypophyses, as well as in small pancreas portions incubated "in vitro" *and stemming from rabbits, and in human adults and newly born, such comparisons being made with isolated fat tissue, rat diaphragm and by the radioimmunological method, the following conclusions were arrived at :*

1) *The different insulin values obtained by the biological and the radioimmunological methods cannot be explained only by a different specificity of those methods. Probably they must for a large part have originated in qualitatively different properties of endogeneous insulin.*
2) *It will probably be immunologically impossible to measure all of the insulin present in the serum, while in case of application of biological methods still other substances having an insulin-synergistic effect on metabolism must be taken into consideration.*
3) *It will not be possible to achieve clarification of problems of insulin metabolism by the highly sensitive, very accurate and rather simple radioimmunoassay alone. For the time being, it is still unavoidable to have recourse to biological methods too, among which the fat tissue method offers a maximum of advantages.*

Zusammenfassung

Aus den Resultaten vergleichender Insulinbestimmungen an normalen und pankreatektomierten hypophysenlosen Hunden sowie an in vitro inkubierten Pankreasstückchen von Kaninchen und an erwachsenen Menschen und Neugeborenen mit dem isolierten Fettgewebe, Rattendiaphragma und radioimmunologischem Verfahren ergeben sich folgende Schlußfolgerungen :

1) Die unterschiedlichen mit biologischen und radioimmunologischen Methoden gemessenen Insulinwerte sind nicht allein mit einer unterschiedlichen Spezifizität der Methoden zu erklären. Sie werden zu einem großen Anteil wahrscheinlich auf qualitativ differente Eigenschaften des endogenen Insulins zurückzuführen sein.
2) Immunologisch läßt sich wahrscheinlich nicht das gesamte im Serum vorhandene Insulin messen, während bei Anwendung biologischer Methoden noch andere, auf den Stoffwechsel insulinsynergistisch wirksame Substanzen in Erwägung gezogen werden müssen.
3) Aufklärungen von Problemen des Insulinstoffwechsels werden nicht mit den hochempfindlichen, äußerst genauen und technisch sehr einfachen radioimmunologischen Insulinbestimmungsverfahren allein zu erreichen sein. Auch biologische Verfahren müssen z.Zt. noch eingesetzt werden, von denen die Fettgewebsmethode die größten Vorteile bietet.

Insulinbestimmungen mit biologischen Methoden führen bei Verwendung des isolierten Fettgewebes und Diaphragmas von Ratten zu unterschiedlichen Resultaten. Diese wiederum unterscheiden sich sehr wesentlich von den mit radioimmunologischer Methodik gewonnenen Ergebnissen. Die Ursache für diese Diskrepanzen könnte in einer unterschiedlichen Spezifität der einzelnen Methoden, aber auch in differenten Eigenschaften des im Blut zirkulierenden Insulins im Vergleich zu kristallisierten Insulinpräparationen begründet sein. Um näheren Aufschluß über diese Probleme zu erhalten, wurden vergleichende Untersuchungen mit dem isolierten Rattendiaphragma und Fettgewebe sowie dem radioimmunologischen Verfahren durchgeführt.

Die Bestimmungen erfolgten an normalen und pankreatektomierten Hunden, ferner an in vitro inkubierten Pankreasstückchen von Kaninchen sowie an erwachsenen Menschen und Säuglingen.

Methodik

Die Sera und Inkubationslösungen der isolierten Pankreasstückchen wurden unmittelbar nach der Gewinnung eingefroren und bis zur Insulinbestimmung bei —20° C aufbewahrt.

Die Insulinbestimmung mit dem isolierten Rattendiaphragma erfolgte exakt nach der zur Zeit von Vallance-Owen [1] angewendeten Arbeitsvorschrift.

Die Bestimmung am isolierten Rattenfettgewebe führten wir nach dem von Martin, Renold und Dagenais [2] angegebenen Prinzip in einer technisch leicht modifizierten Weise durch [3].

Die radioimmunologische Insulinbestimmung erfolgte nach dem von Yalow und Berson [4] entwickelten Prinzip in einer kürzlich von Melani und M. [5] beschriebenen Modifikation.

Bei den Untersuchungen an Hunden konnte nur ein Teil der mit dem Fettgewebe bestimmten Sera auch gleichzeitig mit dem Diaphragma und radioimmunologisch gemessen werden.

Die Anordnung der Versuche und der Houssaypräparationen sowie die Serumextraktionen erfolgten entsprechend den bereits früher von uns mitgeteilten Verfahren [6, 12].

Die Meerschweinchen-Anti-Insulin-Sera wurden in der früher von uns angegebenen Weise gewonnen [5].

Bei den in vitro-Versuchen über die Insulinfreisetzung isolierter Pankreasstückchen erfolgten die Insulinbestimmungen immer gleichzeitig mit der Fettgewebsmethode und dem radioimmunologischen Verfahren. Die verwandte Technik entsprach unserer früheren Mitteilung [13].

Bei den Versuchen am erwachsenen Menschen über den Einfluß intravenöser Glukosebelastungen (0,33 g/kg) auf den Seruminsulingehalt wurde die ILA nur am Fettgewebe und nicht am Diaphragma gemessen. Gleichzeitig erfolgte die Bestimmung aber auch in allen Serumproben mit dem radioimmunologischen Verfahren. Die Messungen wurden unmittelbar vor sowie 15 und 30 Minuten nach der

Glucoseinjektion vorgenommen. Zum 45-Minutenzeitpunkt bestimmten wir nur das Immunoinsulin.

Bei 104 Säuglingen stoffwechselgesunder Mütter, die nach dem Geburtsgewicht von weniger und mehr als 4.000 Gramm in zwei Gruppen unterteilt wurden und ferner bei 6 Säuglingen von diabetischen und prädiabetischen Müttern führten wir ebenfalls Insulinbestimmungen mit dem Fettgewebe und gleichzeitig auch mit dem radioimmunologischen Verfahren durch. Die Bestimmungen erfolgten im Nabelschnurvenenblut, das unmittelbar nach der Entbindung gewonnen wurde.

Ergebnisse und Diskussion

Im Blut von 34 Hunden, das an mindestens zwei verschiedenen Tagen innerhalb einer Woche entnommen wurde, ergab sich für insgesamt 98 Einzelproben mit dem Fettgewebe ein mittlerer Wert für 1: 2 verdünnte Sera von 236 $\pm$ 18 µE/ml. Bei 21 dieser Hunde wurde in den gleichen Proben auch das Immunoinsulin bestimmt. Für 37 Proben errechnete sich für konz. Serum ein Mittelwert von 21,6 $\pm$ 2,4 µE/ml, der damit weniger als 1/10 des ILA-Meßwertes beträgt.

Am Diaphragma steigern 33 unverdünnte Sera der gleichen 21 Hunde die Glukoseaufnahme um 8,1 $\pm$ 0,7 mg %/10 mg Trockengewicht. Dieser Effekt läßt sich wegen des erheblichen methodischen Fehlers der Diaphragma-Methode nur grob mit dem Effekt von krist. Insulin vergleichen. Die in jedem Versuchsansatz zur Kontrolle gleichzeitig bestimmte Wirkung von 1.000 µE/ml Insulin auf die Glukoseaufnahme von 3 Zwerchfellhälften betrug 12 bis 15 mg %/10 mg Trockengewicht, so daß unter Zugrundelegung der von Vallance-Owen *et al.* [14] für die Dosis-Wirkungsbeziehung am Zwerchfell gefundenen Gesetzmäßigkeiten dieser Serumeffekt schätzungsweise im Bereich von 500 µE/ml krist. Insulin liegt und damit ungefähr der Wirkung von unverdünntem Serum am Fettgewebe gleichkommen könnte. In Relation zu dem gemessenen Immunoinsulin liegt dieser Meßwert am Muskelgewebe aber bei den Versuchen am Fettgewebe wie um ein Vielfaches höher.

Nach Entfernung der Hypophyse und des Pankreas fallen die mit allen drei Methoden meßbaren Insulinaktivitäten ab. Aber während das Immunoinsulin praktisch vollständig verschwindet, persistiert ein großer Teil der ILA am Fettgewebe und auch am Diaphragma während der gesamten bis auf über 4 Monate ausgedehnten Beobachtungszeit. Dieser Anteil der ILA, ebenso wie die Differenz zwischen Immunoinsulin und ILA am Fett- und Muskelgewebe von normalen Hundesera, kann aber nicht allein einem unspezifischen Serumeffekt zugeschrieben werden. Diese Annahme wird durch die folgenden z. T. von Professor Pfeiffer gestern bereits genannten Untersuchungsresultate unterstützt .

1. Mit Insulin-Antiserum läßt sich die ILA von normalen Hunden am Diaphragma und auch am Fettgewebe um einen weit größeren Betrag hemmen, als immunologisch zu messen ist.
2. Die ILA des Serums von pankreatektomierten Hunden, in denen kein Immunoinsulin zu finden ist, kann durch Antiserumzusatz weiter vermindert werden.

3. Mit Insulinextraktionsverfahren (Salzsäure-Alkohol) kann ebenso wie aus Normalserum auch aus dem Serum von Houssay-Hunden ILA extrahiert werden, die nahezu komplett mit AS hemmbar ist.
4. Die ILA des Houssayserums läßt sich durch Cystein komplett inaktivieren (vgl. auch Steinke *et al.*, 1963 [15]).

Bei vergleichenden Untersuchungen mit dem Fettgewebe und der radioimmunologischen Methode über die Beeinflussung der Insulinaktivitäten durch Glukose-, Tolbutamid- und Wachstumshormonbelastungen ergeben sich bezüglich der zu beobachtenden Maxima Unterschiede (Abbildung 1). Nach Injektion von Glukose erreicht sowohl die ILA als auch das Immunoinsulin nach 15 Minuten das Maximum.

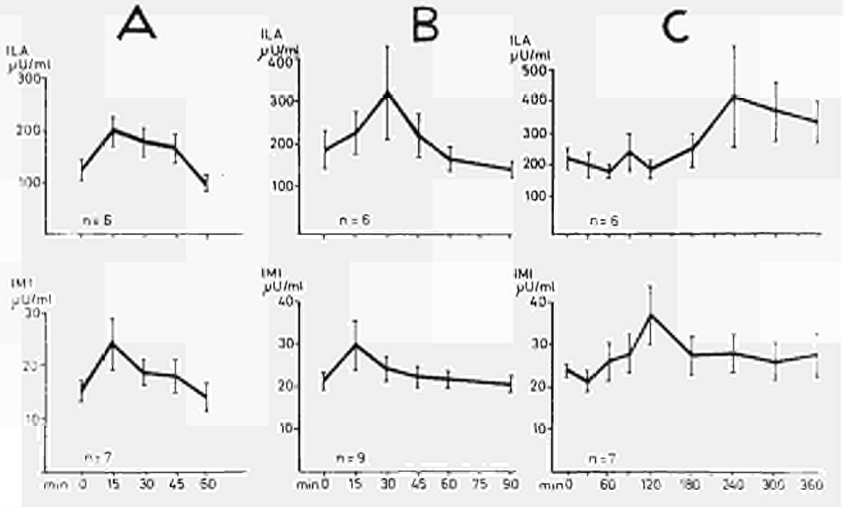

ABBILDUNG 1. — ILA and IMI values in peripheral venous blood of normal dogs following injection of glucose (1 g/kg i.v.) [A], tolbutamide (100 mg/kg i.v.) [B], and human growth hormone (5 mg i.v.) [C]. n = number of dogs.

Nach Tolbutamidgabe liegt das Maximum der ILA dagegen bei 30 Minuten, während das des Immunoinsulins bereits nach 15 Minuten erreicht wird. Weit größere Differenzen treten nach STH-Belastung auf. Der höchste ILA-Wert ist dabei erst nach ungefähr 4 Stunden der höchste Wert für das Immunoinsulin dagegen bereits nach 2 Stunden zu messen.

Diese Differenzen sind nach den Untersuchungsresultaten an Houssay-Hunden wahrscheinlich nicht einer unzureichenden Spezifität der biologischen oder radioimmunologischen Methoden, sondern irgendwie verschieden gearteter Insulinaktivitäten zuzuschreiben. Für diese Annahme sprechen auch unsere Resultate an in vitro in Pufferlösung inkubiertem Pankreasgewebe (Abbildung 2).

Das von dem Gewebe an das Inkubationsmedium abgegebene Insulin ergibt am Fettgewebe einen 6-fach höheren Wert als immunologisch zu erfassen ist. Nach Stimulation der Insulinabgabe durch Zusatz von Glukose steigt das Immunoinsu-

lin in 18 Versuchen im Mittel um 270 μE/ml an, während die ILA eine Steigerung um 1.000 μE/ml erfährt. Dennoch lässt sich durch Zusatz von Insulin-Antiserum die gesamte ILA hemmen, so dass die immunologischen Eigenschaften des aus dem isolierten Pankreasgewebe abgegebenen Faktors erwartungsgemäss kristallisiertem Insulin gleichen. Bezüglich der Reaktionsenergie mit dem Antikörper scheint aber gegenüber krist. Insulin ein wesentlicher Unterschied zu bestehen, da anderenfalls die biologischen und radioimmunologischen Messwerte identisch sein müssten.

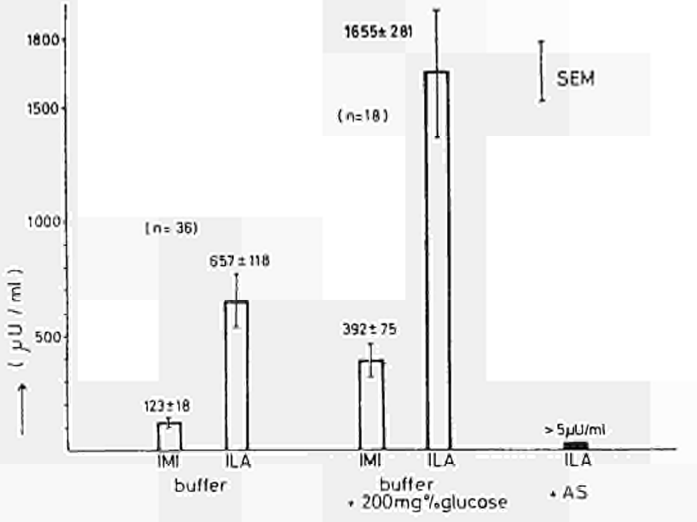

Abbildung 2. — IMI and ILA of the incubation medium of isolated pieces of rabbit pancreatic gland and the effect of guinea-pig anti-insulin (AS) on ILA. n = number of experiments.

Für die sehr unterschiedlichen absoluten biologischen und radioimmunologischen Messwerte beim Menschen haben verschiedene Insulinaktivitäten wahrscheinlich ebenfalls grösste Bedeutung. Im Nüchternserum von 98 Personen, bei denen es sich um Stoffwechselgesunde, Adipöse und Prädiabetiker handelt, ergab sich für die ILA am Fettgewebe ein Mittelwert von 237 μE/ml (Abbildung 3). Für das Immunoinsulin beträgt der Mittelwert dagegen nur 15 μE/ml. Nach intravenöser Glukosebelastung mit 0,33 g/kg steigt das Immunoinsulin innerhalb von 15 min. bedeutend stärker an als die ILA. Bei Aufteilung des Gesamtkollektivs nach dem aus der Glukosebelastung berechneten Assimilationskoeffizienten in zwei Gruppen finden wir bei 38 Probanden mit einem k-Wert unter 1,3 eine prolongierte, über den gesamten Beobachtungszeitraum von 45 Minuten anhaltende Steigerung des Immunoinulins, die aber gleichfalls auch bei der ILA zu erkennen ist. Bei der Gruppe mit k-Werten über 1,3 ist der Anstieg beider Messwerte nur kurzdauernd. Auf die Bedeutung dieser Befunde für den Zusammenhang zwischen

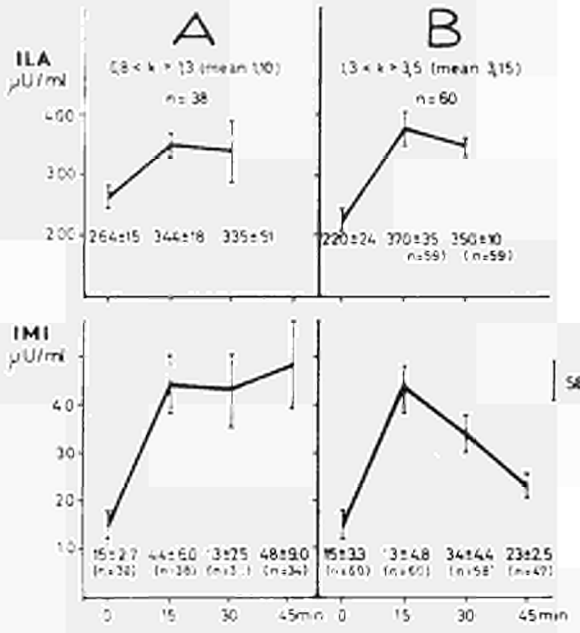

ABBILDUNG 3. — IMI and ILA following glucose injection (0.33 g/kg B. W., i.v.) in persons divided in two groups with k-values below (A) and above (B) 1.3. n = number of persons.

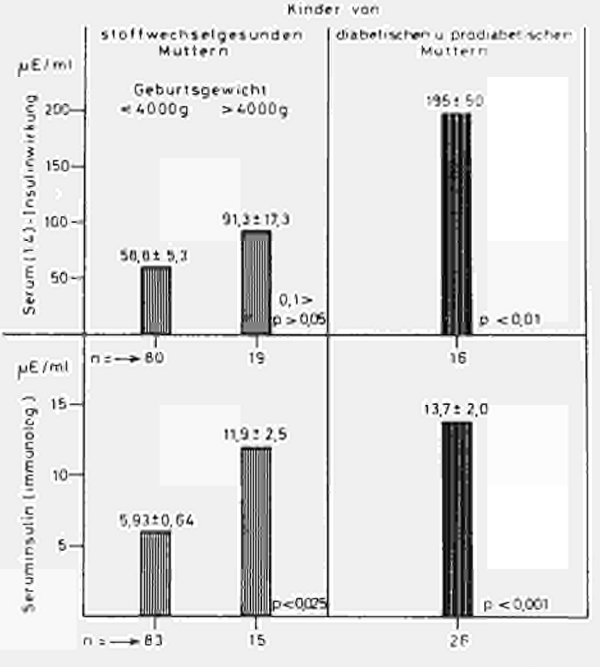

ABBILDUNG 4. — Insulin im Nabelschnurvenenblut bei Neugeborenen Bestimmung mit isoliertem Fettgewebe (oben) u. immunolog. (unten).

gestörter Blutzuckerregulation und erhöhtem Insulinspiegel bei Prädiabetikern soll hier nicht näher eingegangen werden.

Die Ergebnisse der vergleichenden Untersuchungen an Säuglingen sind in der Abbildung 4 wiedergegeben. Das Immunoinsulin beträgt auch in diesen Versuchen bei den verschiedenen Gruppen nur etwa 1/10 der gemessenen ILA. Bei Neugeborenen mit einem Geburtsgewicht über 4.000 g ergeben sich mit beiden Verfahren etwa zweifach höhere Werte als bei der Kontrollgruppe. Die Kinder von diabetischen und prädiabetischen Müttern weisen dagegen relativ höhere ILA-Werte als immunologisch messbare Werte auf.

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DISCUSSION

J. R. M. FRANCKSON (*Bruxelles*) : Je voudrais demander au Dr. Ditschuneit s'il a eu l'occasion d'étudier les modifications du taux sanguin de l'insuline immunologiquement dosable dans les heures qui suivent immédiatement la pancréatectomie. D'après les résultats fragmentaires que nous avons obtenus, il nous semble que la demi-vie que nous avons observée dans le sang portal est nettement supérieure aux valeurs usuellement signalées dans le sang périphérique.

H. DITSCHUNEIT : Die ersten Messungen des Insulinspiegels mit der immunologischen Methode ergfolgten erst 24 Stunden nach der Operation, und zu diesem Zeitpunkt konnten wir kein Insulin mehr nachweisen. Über die Halbwertszeit des Immunoinsulins lässt sich daher aus unseren Versuchen kein Anhaltspunkt gewinnen.

K. BRUNFELDT (*Gentofte*) : Haben Sie ein Kontrollversuch mit Extrakten von Pankreas ohne Inselgewebe ausgeführt ? Denn es muss kontrolliert werden, ob die Werte, die man mit der Fatpad-Methode erreicht, nicht von anderen Substanzen als Insulin bedingt sind.

H. DITSCHUNEIT : Kontrollversuche mit Pankreasgewebe, das keine Inseln enthält, haben wir nicht durchgeführt. Wir sind aber sicher, dass aus dem inkubierten Pankreasgewebe keine insulinpotenzierenden Substanzen abgegeben wurden.

A SIMPLIFIED INSULIN RADIOIMMUNOASSAY METHOD

L. G. HEDING

Novo Research Institute. Copenhagen, Denmark

ABSTRACT

Insulin-antibody-complex is separated from free dissolved insulin by precipitation with 80 percent ethanol. With this technique the sources of error found with the double antibody reaction are avoided and the procedure is rapid and easy. With the simplified method it was found that any of the ions Cl^-, NO_3^-, $HCOO^-$, CH_3COO^-, and I^- increases the insulin-antibody reaction rate and the amount of antibody-bound insulin. The following ions were found inactive : Na^+, K^+, NH_4^+, SO_4^{--}, HPO_4^{--}, BO_3^{---}, citrate, and oxalate.

The insulin content of serum and plasma from normal persons has been determined and the influence on the insulin content of different anticoagulants used for the preparation of plasma investigated.

The separation of free and antibody-bound insulin in the immunochemical determination of insulin has been performed in five different ways :

1) Electrophoretic and chromatographic separation, the original method introduced by Berson and Yalow [1, 2].
2) By means of the second antibody reaction between insulin-antibody-complex and anti-γ-globulin [3, 4, 5].
3) Sodium-sulfite precipitation of insulin-antibody-complex in the presence of 15 percent urea and carrier protein [6].
4) Separation of the free insulin from the antibody-bound insulin by means of an anion-exchange resin [7, 8] or dextran-coated charcoal [9].
5) Separation by a gel-filtration, using Sephadex G 75 [10].

Besides these five methods of separation, which have been used in the immunoassay, a few other separation methods have been described.

Moloney and Aprile [11] have reported a semi-quantitative precipitation of the insulin-antibody-complex by 25 percent ethanol at —5° C. The insulin concentration was approximately 1 unit/ml.

Gordis [12] has used 79 percent ethanol containing salt and potassium hydroxid and a temperature of —10° C for the precipitation.

The obvious simplicity of an ethanol precipitation in the immunoassay procedure motivated our present study.

As a reference we used an immunoassay method based upon the double-antibody principle.

All reaction-media contained albumin and 0.04 M sodium phosphate at a pH of 7.4 in the reference assay as well as in the experimental study.

First the solubility of free labelled insulin was examined using various concentrations of ethanol. The curve below in Figure 1 shows that the insulin (approximately 120 μU/ml) was completely soluble at concentrations of ethanol as high as 84 percent. The amount of precipitated antibody-bound insulin was then measured in another series with the results shown by the two other curves. The upper curve was obtained with anti-insulin serum diluted 1:400. The middle curve corresponds to the usual dilution 1:6,000. Precipitation was considered complete at concentrations above 79 percent ethanol since the upper limit reached at this concentration is identical with the amount of precipitated insulin found with the double-antibody method used as a check. The values obtained with the double-antibody method are plotted at the ordinate. Furthermore it was shown that the chromatographic method gave the same results as obtained with the ethanol method. A concentration of 81 percent ethanol was chosen for the separation of free and antibody-bound insulin. Other solvents, methanol, propanol, dioxan, and acetone have been tried, but none of them could be used for the separation.

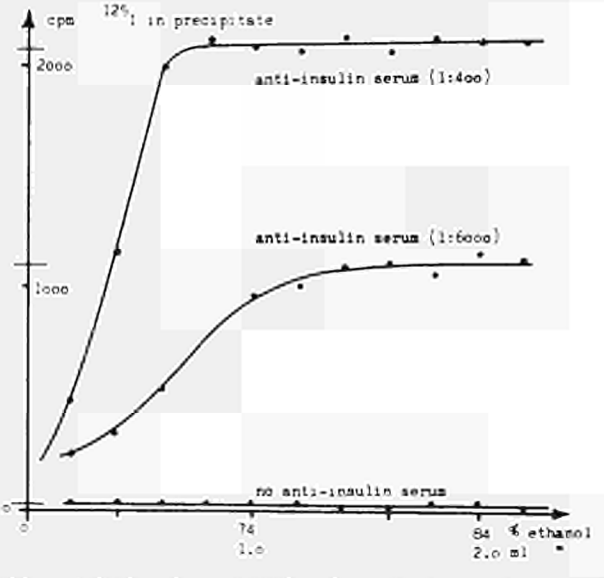

FIGURE 1. — Solubility of ^{125}I-insulin and ^{125}I-insulin-antibody-complex at various ethanol concentrations.

It is essential that the ethanol is added after the reaction of insulin with antibody has taken place since this reaction is immediately stopped by the addition of ethanol. Actually the course and rate of reaction can be studied by adding the ethanol at different times.

It could be suspected that the precipitated insulin-antibody-complex would break down releasing free insulin into solution, and this process was found to occur to some degree, but not within the first two hours.

In the experiments with standard solutions of insulin it was observed that the insulin antibody reaction was contigent upon the presence of specific ions.

As the anti-serum which was used in the reactions was diluted 1.6,000 with buffer it practically did not contain any of the ions from the serum, and as the procedure did not involve any addition of serum or plasma the reaction could be carried out in a pure buffer medium where all concentrations of ions could be controlled. In this way it was possible to investigate the influence of certain ions of the antibody reaction.

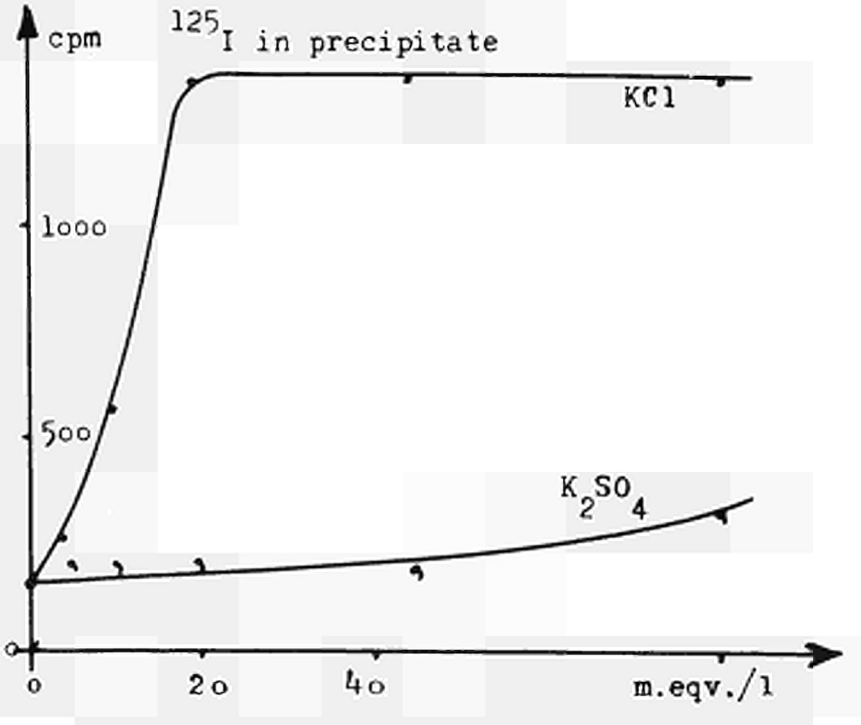

FIGURE 2. — Effect of KCl and K_2SO_4 on the insulin-antibody reaction

Figure 2 shows the effect of the addition of KCl and K_2SO_4 in equivalent amounts. It is seen that only the KCl increased the amount of antibody-bound insulin. There was a sharp increase in the interval 0-20 m. eq./l, and a Cl^--concentration above 20 m. eq./l gave no further rise in the amount of antibody-bound insulin.

Figure 3 shows the effect of NaCl and Na_2HPO_4 in equimolar concentrations. It is seen that Cl^-, but neither phosphate nor Na^+-ions were effective. It was found that other monovalent anions such as nitrate, formate, acetate and iodide had the same effect as chloride, whereas sulphate, phosphate, borate, citrate, oxalate, ammonium, sodium and potassium had none or very little effect. All the active ions men-

tioned gave the same maximum amount of antibody-bound insulin when the concentration of any of the ions was 20 mmol./l or higher.

It is therefore concluded that a certain minimum concentration of some specific ions is necessary for the completion of the reaction under the conditions of the assay.

Errors in the immunoassay are likely to occur if the concentration of active ions in the standard solutions is not sufficiently high.

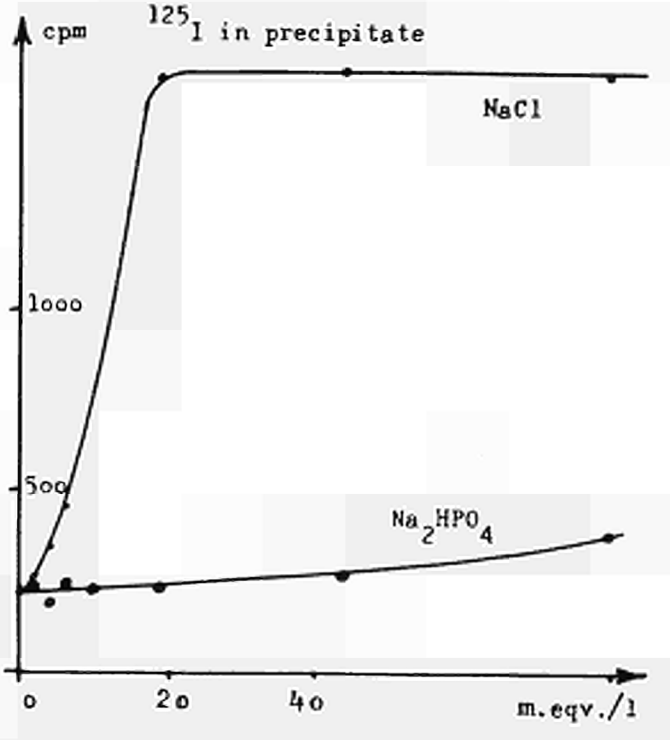

FIGURE 3. — Effect of NaCl and Na_2HPO_4 on the insulin-antibody reaction

If ethanol is used in the immunoassay procedure it is possible to determine the amount of free as well as antibody-bound insulin very easily. When plasma or serum is analysed a voluminous precipitate of plasma protein is formed after addition of ethanol, and some of the free insulin will naturally be present in the precipitate containing appreciable amounts of solution. Therefore, it is preferable to measure the activity in the supernatant liquid instead of in the precipitate.

Some of the free insulin might be adsorbed to the ethanol protein precipitate. This possibility therefore was checked. Known amounts of ^{125}I-insulin were added to buffer in one series and to serum and plasma in another. Ethanol was then added

and ^{125}I-insulin measured in the supernatant liquids after centrifugation. The results were identical in the two series, and the amounts of adsorbed ^{125}I-insulin were small and insignificant. However, adsorption should be remembered as a possible source of error under other conditions than those already checked.

With the ethanol precipitation it is possible to avoid errors which are due to an incomplete second antibody reaction. Especially in serum samples it can be very difficult to obtain complete precipitation of the antibody complex even with a high concentration of precipitating anti-serum.

Figure 4 shows a standardcurve based upon counting of supernatant aliquots. The straight line relationship illustrates that the amount of antibody-bound ^{125}I-insulin is proportional to the amount of antibody not blocked by the standard insulin added in the first step.

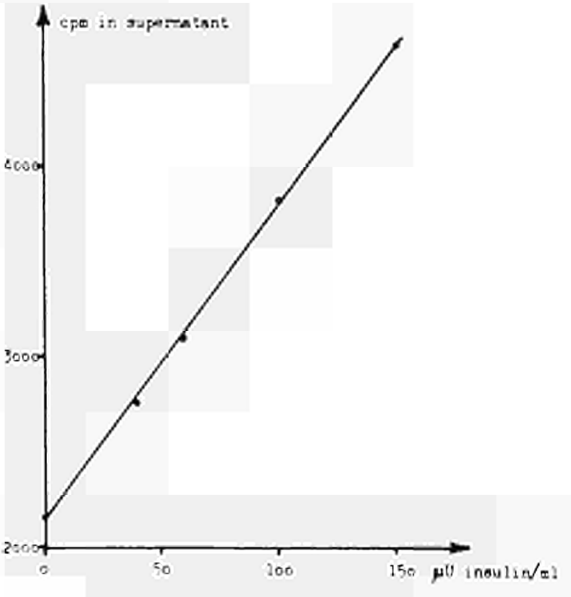

FIGURE 4. — Standardcurve

A number of serum and heparinized plasma samples from fasting normal persons have been analyzed after storage at —30°C. The values found were between 0 and 35 µU per ml.

Increasing amounts of different anticoagulants were then added to serum and standard-solutions of insulin in order to see if any of the added anticoagulants would interfere, e.g. by disturbing the antigen-antibody reaction. No influence on the immunological activity of the samples or on the antibody reaction could be shown as a result of the addition of heparin, citrate, oxalate and EDTA. The

maximum concentration of anticoagulant added was approximately 5 times the concentration normally used.

The conclusion is that in the immunoassay procedure insulin-antibody-complex can be separated from free insulin by precipitation with ethanol. The results found with this method were identical with the results obtained with the double antibody reaction and the chromatographic method.

It was found that monovalent anions in a concentration of 20 mmol./l or higher were necessary for the completion of the insulin-antibody reaction.

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DISCUSSION

C. N. HALES (*Cambridge*) : Did I understand you to say that if you are carrying out a methanol precipitation in the presence of plasma, some of the insulin was carried down with the plasma proteins and the error due to this was avoided by counting the supernatant ?

L. HEDING : The carried down free ^{125}I-insulin is a consequence of a certain amount of solvent present in the precipitate and not of a degradation or binding. But since the amount of labeled insulin in aliquotes of the supernatants, when ^{125}-I insulin and plasma in one series and buffer in another series are added, is identical, no unspecific binding of ^{125}I-insulin to the plasma proteins can be shown to occur.

K. BRUNFELDT (*Gentofte*) : I cannot understand your troubles with the two antibody-methods which have now been in use for two years in our institution. The reliability allows the calculation of the results to be carried out automatically, by conversion of the scaler decimal output to decimal binary code on punched tape for later computer treatment, also comprising statistical calculations based on the double determinations.

BINDING OF RADIOIODINATED INSULINS WITH THE RAT DIAPHRAGM *IN VITRO* (*)

W. MALAISSE and J. R. M. FRANCKSON

Laboratoire de Médecine Expérimentale, Université de Bruxelles, Belgium

ABSTRACT

In vitro studies of the rat diaphragm show that the fixation of different radioiodinated insulins can be divided into two stages : a rapid and reversible binding occurring in the early minutes of incubation and a slow process easily isolated from the 10th minute onwards. The results of numerous experiments are compatible with the hypothesis that the first phenomenon is an adsorption process, poorly specific.

Incubation or pretreatment of insulin with guinea-pig anti-insulin antibodies inhibits the tissue fixation of the labeled compounds, revealing an identical immunological behaviour of cristalline insulin and of the radioactive fraction which binds to the muscle.

On the contrary, cristalline insulin, whatever the concentration or the incubation conditions, does not reduce the fixation of labeled material on the diaphragm. This lack of competition between the hormone and the radioisotopes is discussed.

Previous studies *in vitro* carried out with labeled insulins, were intended either to detect their subcellular distribution [1, 3, 4] or to compare tissue binding with metabolic activities [12, 13, 14]. On the contrary, our aim was to characterize the rate of binding of iodo-insulins on muscle in a simplified model.

The experiments were performed on hemidiaphragms of fasted rats weighing 150-250 g, incubated at 37.6° C in Krebs-Ringer bicarbonate buffer in a Dubnoff metabolic shaker [5]. At the end of incubation, the diaphragms were rapidly dried between filter papers, washed twice for 10 seconds in buffer and dried again in order to avoid contamination due to the diffusion of the radioactive materials in the tissues interstial water [7]. The diaphragms were then put into glass tubes and the radioactivity directly counted in a crystal scintillator, well type [6].

The ^{131}I-insulins added to the incubation medium had a specific activity ranging from 2.5 to 10 mC/mg, a mean iodination degree of 1 atom per mole (M.W. 6,000), an immunological purity of 90-92 %, a biological activity always superior to 80 % of the original crystalline lot (diaphragm and fat pad bioassays). They were generally prepared by elementary iodine oxydation [10] (**) and sometimes by catalytic oxydation [11] (***). The ^{125}I-insulins were obtained by the catalytic procedure (***);

(*) Work performed under Association Contract EURATOM-ULB-University of Pisa, No. 026-63-4 BIAC.

(**) Farbwerke Hoechst, Frankfurt, Germany.

(***) Sorin, Torino, Italy.

their mean iodination degree was superior to 3 atoms per mole, their biological activity practically abolished and their immunological properties markedly impaired.

The tedious problem of measuring the surface of the diaphragms was ruled out according to data obtained in a preliminary work [5] where in a relationship of direct proportionality was found between the surface area of the diaphragms (S, expressed in mm^2) and a function of their fresh weight (P, expressed in mg) :

$$S = 1.24 \times P^{2/3}$$

The characteristics of the binding of ^{131}I-insulin to the diaphragm may be summarized as follows :

1. Binding is proportional to the duration of dipping of the diaphragm in the insulin medium (Figure 1), but the rate of binding decreases progressively and, for instance, between the 15th and the 90th minute represents only 1 % of the rate recorded during the first minute of dipping.

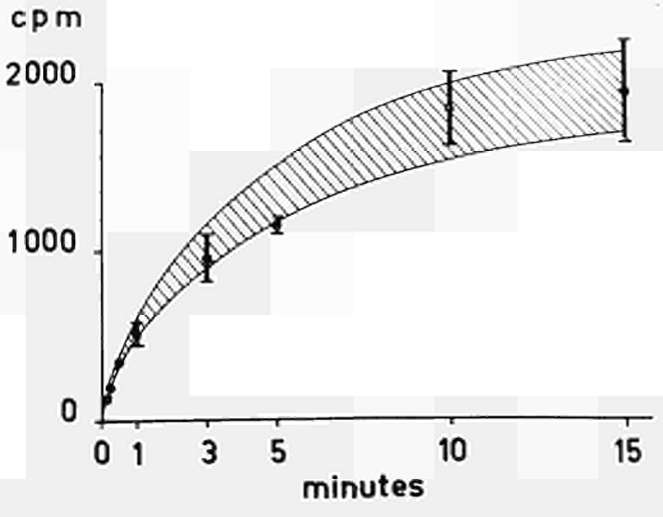

FIGURE 1. — Relationship between tissue binding of ^{131}I-insulin (expressed in cpm per hemidiaphragm and duration of dipping (min). Figures given are the mean and the extreme values.

2. Whatever the duration of the dipping, the binding is directly proportional to the surface area of the diaphragms, as estimated by the function $P^{2/3}$. Figure 2 illustrates the results observed for 2 different periods of dipping.

3. A straight line relationship between the amount of ^{131}I-insulin bound to the muscle and its concentration in the incubation medium can be demonstrated for a range of concentrations from 10 μU/ml up to 20 U/ml and for periods of dipping lasting from 30 seconds to 90 minutes (Figure 3).

4. The amount of bound radioactivity is very small : less than 5 % of the medium concentration being bound by surface unit. However this bound fraction comes mainly from ^{131}I-insulin and not from the accompanying impurities since an excess

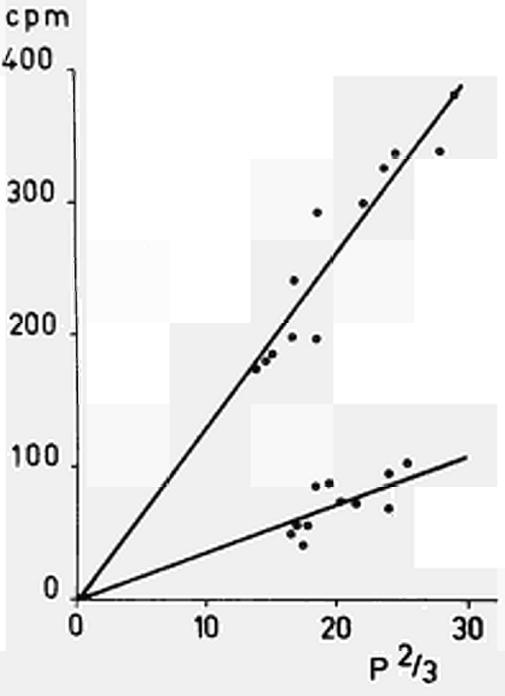

FIGURE 2. — Relationship between ^{131}I-insulin binding (cpm) and the surface area of the diaphragms ($P^{2/3}$). Lower regression : after 30 sec dipping; upper regression, 90 min.

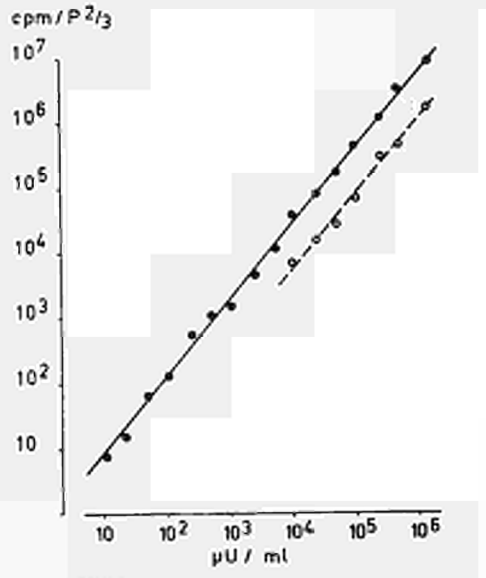

FIGURE 3. — Relationship between ^{131}I-insulin binding (expressed in cpm/surface unit) and ^{131}I-insulin concentration in the incubation medium (µU/ml). Clear circles correspond to 30 sec dipping; black circles, to 30 min).

of guinea-pig anti-insulin serum added to the medium prior to the dipping of the diaphragms, prevents the binding of the radioactivity up to 85 % of the control value [6].

5. The analysis of the curve expressing the binding of ^{131}I-insulin in respect to dipping time allows to define 2 exponential components (Figure 4) : the first one corresponding to a rapid association of the radioisotope with the tissue, only influences the curve during the 10 first minutes; the second one beginning with a delay of about 10 minutes represents a slow association process which tends to saturation around the 3rd hour of dipping [7]. None of these processes is the expression of diffusion as recently claimed [2], since the rapid washing and drying systematically performed before counting can clear the tissues interstitial fluids of the radioactive materials [7].

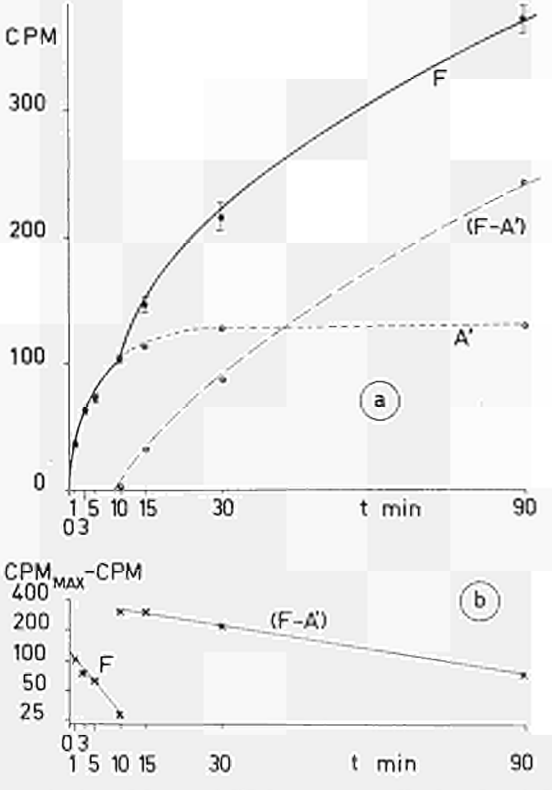

FIGURE 4. — ^{131}I-insulin binding (F in cpm/$P^{2/3}$) in respect to dipping time (min). In the upper part of the graph are indicated the evolution of the rapid (A') and the slow (F-A') association processes. In the lower part of the graph are plotted the semilogarithmic lines representative of these inverse exponential processes. Given figures are the mean and S.E.M.

The removal of the bound radioactivity can be achieved by washing diaphragms in buffer for prolonged periods. Figure 5 illustrates the decrease of the bound radioactivity in relation to the duration of the washing for a large number of diaphragms

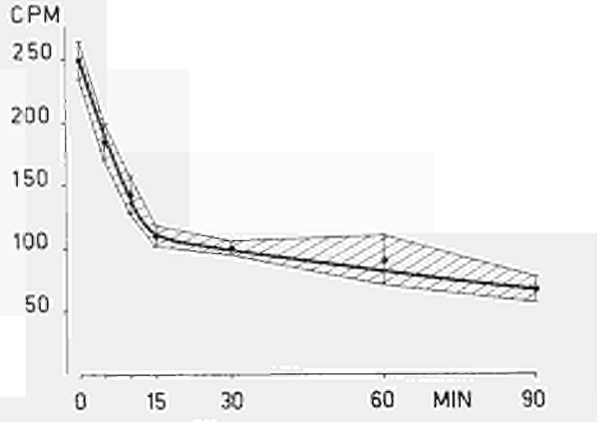

FIGURE 5. — Study of the removal of the ^{131}I-insulin bound to the diaphragm : relationship between the bound radioactivity (cpm) and the duration of the washing in buffer. Figures given are the mean and S.E.M.

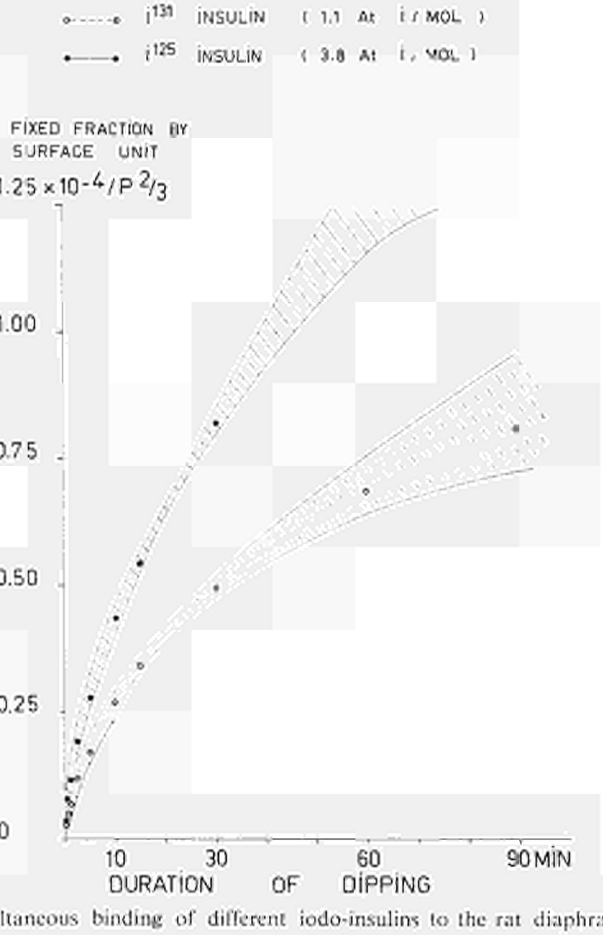

FIGURE 6. — Simultaneous binding of different iodo-insulins to the rat diaphragm (mean and extreme values).

pretreated with ^{131}I-insulin in identical conditions. As easily noticed, this curve is the reversed image of the binding curve shown in Figure 1; it can also be split up in 2 exponential components whose slopes come very near to those extracted from the binding curves.

6. The association rate of the iodoinsulins with the muscle does not run parallelly to their biological properties : incubation of diaphragms in buffer containing equal concentrations (in μgN) of ^{131}I-insulin (1 at./mol.) and ^{125}I-insulin (3 at./mol.) reveals that the binding of the heavy-iodinated compound is at any time the more marked one (Figure 6).

The poor specificity of these processes is also emphasized by its possible inhibition by non specific agents such as albumin added to the incubation medium [9] or by inert substrates [8, 16].

These data support the working hypothesis that the binding of iodoinsulins to muscle could be accounted by adsorption. All the characteristics required for this unspecific phenomenon are present : rapidity, reversibility (equal rates of adsorption and elution); direct proportionality to the surface (even for long periods of contact), stability of the ratio between the fraction bound to tissue and the medium concentration (equivalent to an actual partition constant), low value of this ratio (quantity bound to the interphase being negligible), possible inhibition by non specific competition. According to this hypothesis, *in vitro* association of I-insulin with muscle might be represented by a simple model : the membrane surface, locus of the rapid adsorption and desorption phenomena, would separate the incubation medium from a deeper membrane pool, where the exchanges of I-insulin or its catabolites and their entry inside the cell would proceed at lower rates. This concept is in concordance with autoradiographic studies [15] and with analysis of the intracellular distribution of iodoinsulins [1].

ACKNOWLEDGMENTS

We want to thank Drs. Niemann and Frühauf from the radiochemical laboratories of Farbwerke Hoechst and Dr. Rosa from the laboratories of the Sorin for the generous supply of the labeled insulins.

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ANALYSIS OF PLASMA DISAPPEARANCE CURVES OF CRYSTALLINE AND RADIOIODINATED INSULINS (*)

Y. ARNOULD, C. DELCROIX, E. RASIO, H. A. OOMS and J. R. M. FRANCKSON

Laboratoire de Médecine Expérimentale et Laboratoire de Médecine Nucléaire, Université de Bruxelles, Belgium

ABSTRACT

Different amounts of cristalline insulin (0.1 to 100 U/kg) or of radioiodinated insulins (0.4 to 8 mC) have been rapidly injected by the venous route to normal anesthetized dogs, maintained in normoglycaemia by glucose compensation. Blood samples were collected from the 2nd minute to the 6th hour following injection. Plasma insulin was estimated by radioimmunoassay and by bioassays; plasma insulinic radioactivity, by paper chromatography and immunochromatography. The blood disappearance curves have been submitted to mathematical analysis (digital computer).

The disappearance curves of cristalline insulin (radioimmunoassay) can be expressed by a sum of exponentials, variable according to the injected amounts. The shape of the radioinsulin curves are different, they tend to an assymptote differing from zero and can only be expressed by a sum of exponentials and of one constant.

Simultaneous injections of a same tracer of radioinsulin with graded loads of cristalline insulin, only produce a small change in the disappearance rate of the labelled compound. Several hypotheses can be forwarded to explain the poor competition between both types of insulins.

The kinetics of the blood disappearance of radioiodinated insulins following rapid intravenous injection has been but little studied. Most of the workers isolated one segment of the disappearance curve generally corresponding to the 2 first hours : they assumed it was a simple exponential and compared its characteristics in normal and diabetic subjects [1, 2, 3, 4, 5, 10, 11].

This procedure is rather arbitrary. Indeed, in semi-logarithmic coordinates, isolation of one segment in a fall-off curve may easily simulate a straight line whatever the equation of the whole curve. Moreover, no one is authorized to draw directly by graphical procedure an exponential out of a complex curve if the phenomenon has not been studied long enough to allow isolation of the final slope.

The aim of the work we present was to study the plasma disappearance of both crystalline and radioiodinated insulins for a long period of time in order to obtain a valid mathematical analysis of the curve. This was achieved by injecting in 10 sec into the femoral vein of healthy anaesthetized dogs (20-30 kg) 0.4 to 0.8 mC of iodinated insulin and by sampling arterial blood specimen from the 2nd up to the 240th

(*) Work performed under the Association Contract EURATOM-ULB-University of Pisa No. 026-63-4 BIAC.

or to the 420th minutes following injection. In most of the experiments, the ^{131}I-insulin, prepared by elementary iodine oxydation, had a mean specific activity of 10 mC/mg, a mean iodination degree of 1 atom per mole (assumed M.W. : 6,000), an immunological purity of about 90-92 % and a biological activity generally ranging from 80 to 90 % of the crystallin lot, as tested on the rat fat pad. Graded loads of crystalline insulin were simultaneously injected with the radioisotope in several groups of dogs so that the total amount of biologically active insulin ranged from 0.1 to 100 U/kg body weight. In every case normoglycaemia was maintained throughout the experiment by appropiate glucose infusions.

Plasma ^{131}I-insulin was isolated by classical paper chromatography [2] (Whatman 3 MC) in veronal buffer (0.2 M); the chromatography was allowed to run horizontally for about 4 hours at 12-16° C; the strips were cut in 0.5 cm parts and counted by crystal scintillation. The purity of the insulin spots isolated at the different times were cheked by chromatography with an excess of guinea-pig anti-insulin serum.

The shape of the disappearance curves of ^{131}I-insulin is illustrated by Figure 1. In cartesian coordinates these curves seemed to tend asymptotically to both axes, suggesting an hyperbola. This possibility was ruled out by a digital computer using the least squares method and successive iterations. Despite a satisfactory mean square

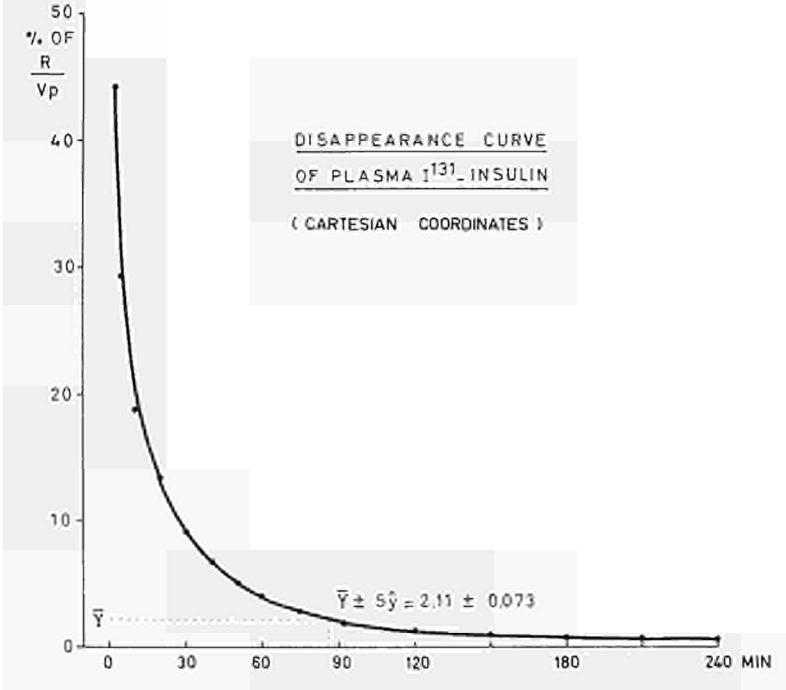

FIGURE 1. — Disappearance curve of plasma ^{131}I-insulin in cartesian coordinates. ^{131}I-insulin levels are expressed as percentage of ratio between injected amount of ^{131}I-insulin (R) and plasmatic volume (Vp).

deviation from the hyperbolic curve, this adjustment provoked a consistent distortion of the experimental curve as shown on Figure 2. This forced us to reject the hyperbolic function.

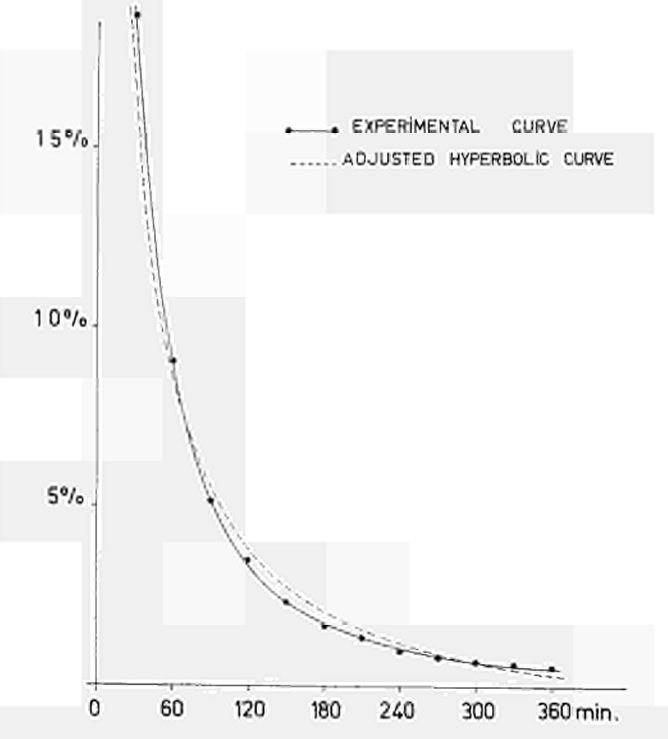

FIGURE 2. — Tail part of a plasma ^{131}I-insulin disappearance curve showing the distortion caused by the hyperbolic adjustment.

Therefore, another approach of the formula ruling these curves was attempted by the multiexponential analysis. After gross estimation of the different components by manual peeling off, a curve fitting was realized with the assistance of a digital computer IBM 7040 : a first estimate of the characteristics of each term was obtained by a subtraction-type method and the values were finally adjusted by the least squares method with a mean precision superior to 0.2 % of initial value (*). From these cal-

(*) Sample standard error of estimate $s = \sqrt{\frac{S(y-y)^2}{n-2}} \Big/ \sqrt{n}$, where y are the experimental values (expressed as percentage of initial value), y the corresponding values on the curvilinear regression, n the number of values.

culations, the plasma disappearance of ^{131}I-insulin (y) in respect to time (t) might be represented by a sum of 1 constant and 3 exponentials (Figure 3):

$$y = C_0 + C_1e^{-K_1t} + C_2e^{-K_2t} + C_3e^{-K_3t}$$

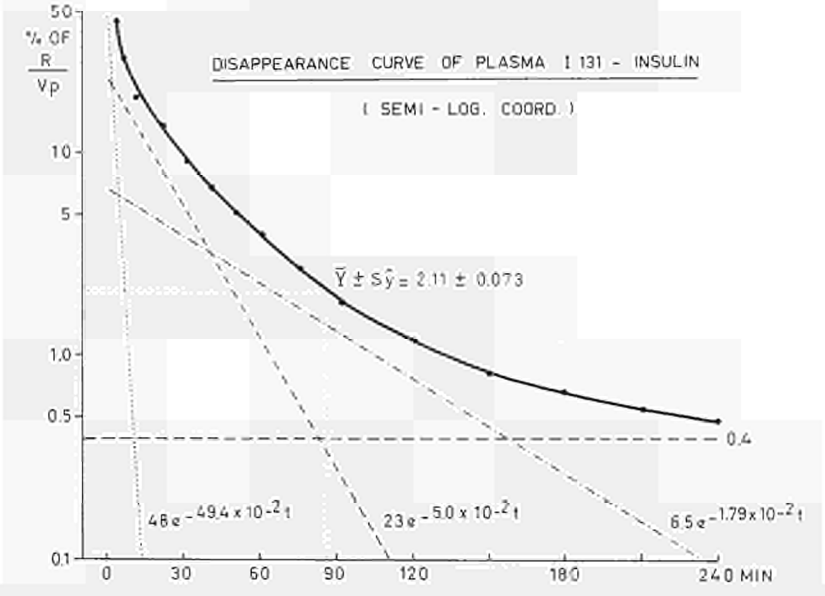

FIGURE 3. — Semi-logarithmic representation of a disappearance curve of plasma ^{131}I-insulin (Figure 1). Results of the multiexponential analysis are indicated in the lower part of the graph.

The final constant (C_0) has a small value; it approximated zero in 1/4 of the cases (from —0.1 to 0.1 % of initial value) and never exceeded 1.3 %. The first exponential removed by peeling off or computer subtraction ($C_1e^{-K_1t}$), is dominant from about the 100th minute up to the end of measurements (240-420 min); it can be adjusted with a precision of 0.016 % of initial value. The 3rd exponential ($C_3e^{-K_3t}$), corresponding to a rapid plasma disappearance rate mainly influences the curve during the first 15 min following injection. In the middle part of the curve (from 15th to about 100th min), the 2 exponentials $C_1e^{-K_1t}$ and $C_2e^{-K_2t}$ are summing their effects.

This multiexponential equation is not compatible with the simple generally accepted model in which, after mixing within the extracellular space, radioiodinated insulin would undergo irreversible disappearance by renal excretion, hepatic destruction and tissue uptake.

Indeed, in such a compartmental model, beyond the mixing period, the plasma disappearance should be ruled by a simple exponential whose intercept with the y axis should allow an estimate of the extracellular fluids. On the contrary, the multiexponential equation implicates feed back to the plasmatic compartment; conse-

quently each exponential is not a direct expression of an unique physiological process but represents the result of the different moves of the radioisotope from and to the plasmatic compartment. These implications are supported by the fact that the intercepts with the y axis of the 1st exponential and of the sum of the 1st and 2nd ones provide volumes of distribution which do not correspond to a physiological fluid compartment. Only from the sum of all intercepts one can approach the plasmatic volume, indicating early mixing within the plasma (Table I).

TABLE I. — Ratio between the injected amount of ^{131}I-insulin (R) and the various intercepts C_0, C_1, C_2, C_3. In the last column is given the plasma volume (Evans blue).

	$\frac{R}{C_0 + C_1}$	$\frac{R}{C_0 + C_1 + C_2}$	$\frac{R}{C_0 + C_1 + C_2 + C_3}$	Vp
Volume (% bodyweight)	73 ± 11	13.6 ± 1.3	5.6 ± 0.4	5.0 ± 0.2

The constants (K_1, K_2, K_3) of the exponentials representing the different exchange rates of the radioisotope between its pools are rather stable from one animal to another and are poorly influenced by the amount of crystalline insulin injected together with the labelled compound : the slopes of the regressions of the constants in relation to the dose of crystalline insulin are not statistically different from zero (Figure 4). Their mean values calculated for the whole group average :

$$K_1 = 1.36 \times 10^{-2} \pm 0.12 \times 10^{-2}$$
$$K_2 = 6.0 \times 10^{-2} \pm 0.8 \times 10^{-2}$$
$$K_3 = 29 \times 10^{-2} \pm 3 \times 10^{-2}.$$

This lack of influence of large amounts of crystalline insulin upon the exchange rates of labelled insulin can be interpreted in different ways : there might either be no competition between the 2 compounds, labelled insulin being not a valid tracer for *in vivo* metabolic studies, or no relationship between the size of the pool and the exchange rate constants, both crystalline and labelled insulins being assumed identical. These hypothesis were checked by studying the plasma disappearance rates of unlabelled insulin in conditions of administration, doses and time comparable to the first experiments.

Four groups of 3 dogs were injected with graded amounts of crystalline insulin ranging from 0.1 to 100 U/kg; plasma insulin was estimated by radioimmunoassay using a double antibody system [7]. The mean disappearance curves are illustrated in Figure 5.

Curve fitting was performed on all individual curves, except on those corresponding to the smallest dose (0.1 U/kg) owing to the early fall of plasma insulin level to

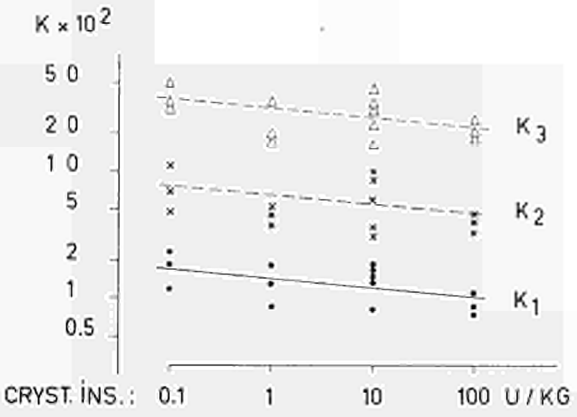

FIGURE 4. — Relationship between the amounts of crystalline insulin simultaneously injected with ^{131}I-insulin and the slopes of the different exponentials (K_1, K_2, K_3) obtained from the analysis of plasma ^{131}I-insulin curves.

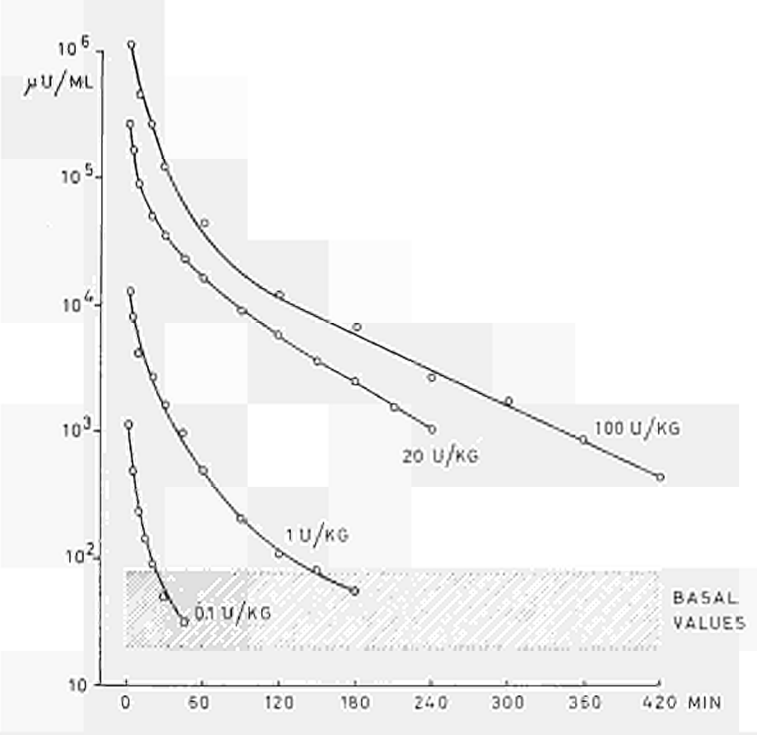

FIGURE 5. — Mean disappearance curves of plasma unlabelled insulin (radioimmunoassay) after rapid intravenous injection of different loads of crystalline insulin.

basal values and hence, to an insufficient number of available experimental points. In 7 out of 9 cases, the disappearance curves could be represented by a sum of 3 exponentials but without the constant term C_o (Figure 6). The characteristics of these exponentials (intercepts and constants) come very near to those found for labelled insulin :

$$K_1 = 1.65 \times 10^{-2} \pm 0.17 \times 10^{-2}$$

$$K_2 = 5.6 \times 10^{-2} \pm 0.43 \times 10^{-2}$$

$$K_3 = 26.5 \times 10^{-2} \pm 3.1 \times 10^{-2}.$$

Here too the volumes determined by the intercepts do not correspond to physiological fluid compartments and, from one animal to another, there is no relation between the amount of injected insulin and the constants of the exponentials. The major influence of the dose upon the disappearance rate of plasma insulin lays in the period during which the curve is the only expression of the slow exponential decay ($C_1e^{-K_1t}$) : the larger the dose, the longer the simple exponential return to basal level (Figure 5).

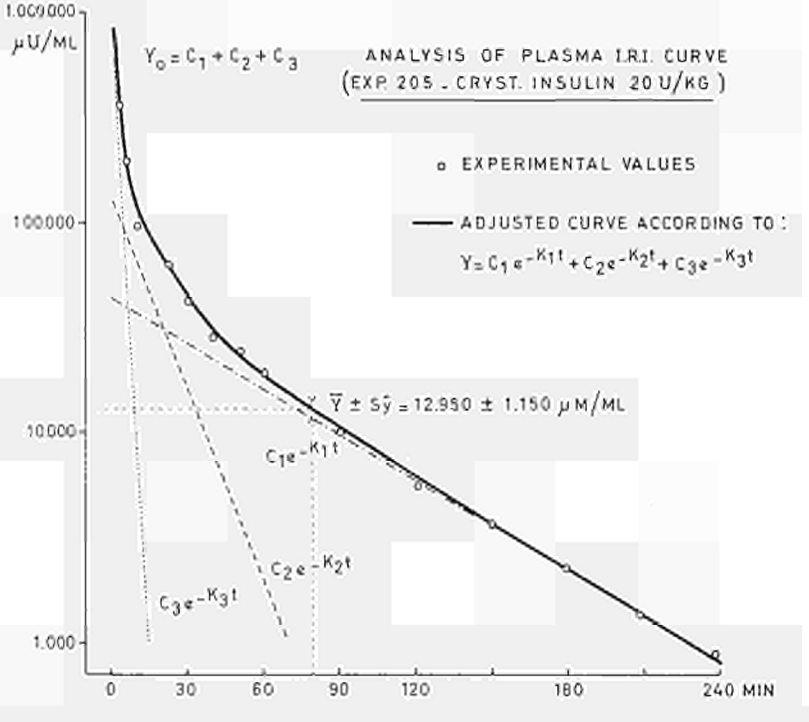

FIGURE 6. — Multiexponential analysis of a disppearance curve of unlabelled insulin after intravenous injection of crystalline insulin.

Thus, as far as the disappearance curve of crystalline insulin has a duration of at least 4 hours (injected amount superior to 1 U/kg), its mathematical analysis provides a multiexponential function similar to the one obtained from the decay of ^{131}I-insulin, with the exception of the constant term C_0. However, this type of disappearance curve is not specific to insulins still possessing the biological properties of the crystalline hormone. Indeed, works presently going on in our laboratory show that a similar sum of 3 exponentials and 1 constant can be drawn from the plasma decay of labelled insulins with a mean iodination degree superior to 3 atoms per mole, although those compounds exhibit practically no *in vitro* biological activity on the diaphragm [9] or epididymal fat pad and a marked impairment of their immunological properties [8].

To conclude : studied for periods lasting from 4 to 7 hours, the plasma disappearance of ^{131}I-insulin in relation to time can be represented by a multiexponential equation. Save for a constant term of small magnitude, this type of equation is similar to the one obtained with crystalline insulin. It is not specific of undegraded insulins. The characteristics of these exponentials are poorly influenced by the size of the insulin pool.

The results of our analysis are not compatible with a simple model comprising an extracellular mixing pool with irreversible losses; they implicate at least a 3 compartments mamillary model with feed back to the central compartment. These compartments more probably represent differing metabolic pools than physiological spaces.

These results are in concordance with *in vitro* data showing that the binding and the removal of the labelled insulins to and from the isolated muscle possess the characteristics of a non specific adsorption phenomenon [6].

The physiological significance of the mathematical terms is yet unknown.

ACKNOWLEDGMENTS

We want to thank Drs. Niemann and Frühauf from the laboratories of Farbwerke Hoechst and Dr. Rosa from the Sorin for the generous supply of the labelled insulins.

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DISCUSSION

K. BRUNFELDT (*Gentofte*) : Quels sont les calculs que vous avez employés pour justifier les deux lignes droites tirées sur la figure montrant l'adsorption sur le diaphragme de l'insuline marquée ?

J. R. M. FRANCKSON : L'impression que vous avez provient de ce que les résultats de l'expérience individuelle qui a été projetée sont représentés en graphique semi-logarithmique, ce qui donne une importance exagérée aux écarts entre valeurs extrêmes enregistrées aux faibles concentrations. Il ne nous a pas été possible par la méthode des différences finies de faire passer une seule courbe par tous nos points expérimentaux. Par contre, les 2 droites semi-logaritmiques représentées sont dans tous les cas comprises dans la zone fiduciaire 95 %. Il est bien entendu possible qu'une analyse poussée du phénomène puisse donner une équation différente.

PRELIMINARY DATA ON THE *IN VIVO* MEASUREMENT OF THE RATE OF DEGRADATION OF ^{131}I-INSULIN IN HUMANS (*)

R. BIANCHI, G. FEDERIGHI, F. VITEK and L. DONATO
Clinica Medica Generale dell'Università di Pisa, Italy

ABSTRACT

Using an original method which permits to measure in man the rate at which labeling iodine is liberated from rapidly degraded iodinated molecules, the rate of deiodination of ^{131}I labeled insulin has been measured in humans.

The method demands the simultaneous injection of ^{131}I labeled insulin and Na ^{125}I, and the continuous recording of thyroid uptake or urinary excretion of the two radioisotopes for two hours.

The results obtained with preparations at different degrees of iodination and the possibility of using the technique for physiological investigation are discussed.

Present methods for studying the metabolic degradation of iodoinsulin preparations are based on the estimate of the final slope of the plasma disappearance curve of the iodinated hormone after its intravenous injection [1, 2].

It is well known that the final slope method yields correct results only when the residual activity is uniformly distributed throughout the hormone pool in the body. To approach such a situation the metabolic degradation of the protein should take place at a rate much slower than the rate at which exchange between plasma and extravascular compartments occurs. Due to its degradation, in the case of insulin the final plasma slope is probably not representative of the catabolic rate of the hormone, and the actual relationship between slope value and rate of catabolism cannot be easily formulated.

In this paper we intend to illustrate the application of a new approach to the direct study of the *in vivo* degradation of iodinated insulin.

PRINCIPLE OF THE METHOD

The foundations of the method will be published in detail elsewhere [3]. In essence the method assumes that the kinetics of elimination of iodide derived from insulin breakdown is the same as the kinetics of iodide injected intravenously, except for the difference that the input to the iodide pool is represented in one case by the rate at which insulin is degraded, and in the other case by a single injection.

(*) Work supported by Euratom Association Contracts No. 026-4-63 BIAC and No. 48.65.1 BIOI.

From these assumptions, the method proceeds as follows :

^{131}I-insulin and ^{125}I-iodide are injected into the same patient;

the cumulative excretion of both isotopes is followed;

the cumulative excretion ^{131}I is a *convolution integral* of the function expressing the *rate at which iodide comes out of* ^{131}*I-insulin* and is fed to the iodide pool, and of the function expressing the *excretion of iodine from the iodide pool*, the latter is directly given from the cumulative excretion of ^{125}I;

after expressing both excretion curves as fractions of the respective doses, deconvolution of ^{131}I excretion into ^{125}I excretion curves directly gives the function representing the cumulative amount of iodine derived from iodoinsulin at any time, hence permitting the direct measurement of its rate of degradation.

The method can be used both measuring thyroid uptake or collecting urines in presence of a blocked thyroid uptake, and calculations can be made using an analog computer, or by direct analysis of the curve [3].

Preliminary results

The applicability of the method has been tested on iodo-insulin preparations, specially prepared by Rosa *et al.* [4], as a part of a study on the adequacy of iodoinsulin as a metabolic tracer. Studies so performed have indicated the feasibility of the method for direct measurement of the degradation rate of iodinsulin preparations in man. The results so far obtained can be summarized as follows :

Five iodoinsulin preparations having an average iodine content of less than 1 at I/mol were tested in normal subjects, and gave an average degradation rate of 2.61 ± 0.26(SD) % min^{-1} over the first two hours. At that time from 90 to 100 % of the preparation had already been degraded.

Six iodoinsulin preparations, having an average iodine content above 4 at I/mol were tested in normal subjects. Their average degradation rate was 0.98 ± 0.40 (SD) % min^{-1} over the first two hours, after which from 60 to 84 % of the preparation had been degraded.

When a second injection of a low-iodinated insulin preparation was made in a young normal subject, during i.v. glucose infusion, the mean rate of degradation increased from 2.91 to 7.28 % min^{-1}.

When the same experiment was made with a highly iodinated preparation, a modest increase took place from 1.48 to 1.73 % min^{-1}.

Conclusions

Too little experience has been collected to draw any conclusions concerning the practical value of the present method. The first results, however, seem to us very encouraging : the lower rates of degradation measured with high iodinated preparations, which are known to have suffered a loss of biological activity [4],

and the response to physiological stimuli, such as increase of blood glucose level, may all be suggestive of the practical value of the method as a simple and direct approach to the study of insulin metabolism *in vivo*.

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DISCUSSION

R. S. YALOW (*New York*) : The method described should prove a valuable tool for solving a problem that would otherwise be quite difficult. i.e. providing direct means for demonstrating that the terminal phase of the plasma disappearance curve of the labeled insulin is the insulin degradation rate as well as making it possible to calculate changes in degradation rates. Would it be possible now to combine your data with the plasma disappearance curve and see whether the increased rate of degradation after glucose infusion is related to change in space of distribution ?

L. DONATO : I will take it as an extremely interesting suggestion. It would certainly be worth to do such a sort of calculation. Something interesting could come out of it. Thank you.

ROUND TABLE ON APPLICATIONS OF TRACER THEORY TO PROTEIN TURNOVER STUDIES

Chairman : L. DONATO, Medical Clinic, University of Pisa, Pisa, Italy

Participants :

C. M. E. MATTHEWS, M. R. C. Cyclotron Unit, Hammersmith Hospital, London

B. NOSSLIN, Malmoe Allmanna Sjukhus, Malmoe

G. SEGRE, Institute of Pharmacology, Camerino

F. VITEK, Institute of Biophysics, Praha

S. B. ANDERSEN, Dept., Clin. Physiol., Glostrup Hospital, Copenhagen

A. S. MCFARLANE, National Institute for Medical Research, Mill Hill, London

P. PAVONI, Institute of Medical Pathology, University of Roma, Roma

R. S. YALOW, V. A. Hospital, Bronx, New York

L. DONATO : It is quite clear to everyone that no matter how good the experimental data are, there is always a good possibility that they will be spoiled by erroneous analysis. It is, therefore, critical to establish the information content of an experiment in a correct way.

This Round Table is an attempt to clarify this matter somewhat for the benefit of the investigators using labeled proteins for metabolic studies. We will first attempt to define what we would like to get out of the analysis of tracer studies with proteins. Let us first look at the experimental situation.

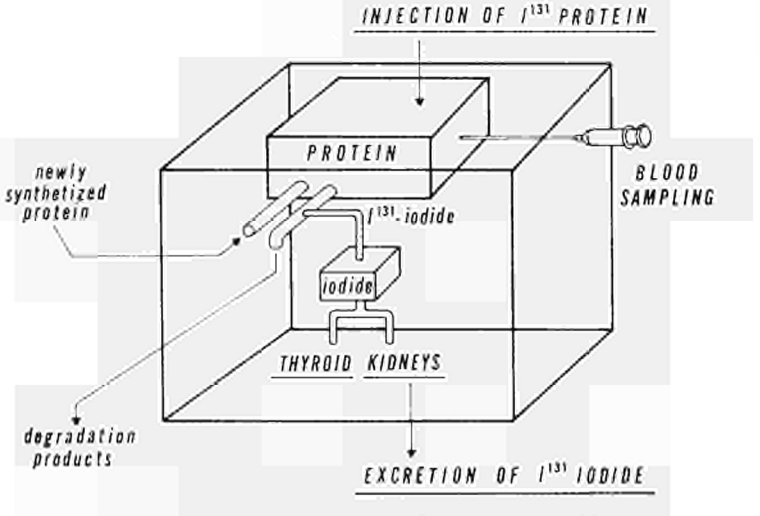

FIGURE 1. — Plasma proteins turnover studies.

In Figure 1, I have made an attempt to picture the system in purely descriptive terms. What we know is that after injection of the ^{131}I labeled protein, iodine is released and body radioactivity decreases because of its excretion.

We can measure the radioactivity remaining in the body either directly or by substracting the cumulative excretion of the tracer from the amount of tracer injected. As shown in Table I (Definition of terms) there are several synonimous terms for whole body activity.

We may add information by sampling blood and measuring the activity concentration in it.

We usually suppose that the mass of the protein that we want to study is in steady state conditions which are maintained by the balance between newly-synthetised protein and degradation rate. Our aim is that of measuring the protein mass and its rate of turnover, but to achieve this goal several requirements have to be met. First, our labelled protein has to mix with all the protein which is in the box. The second requirement is that iodine be released from the labelled protein

TABLE I. — Definition of terms

1. Pool = Space = compartment =
   = a part of the protein mass in which mixing occurs so rapidly that uniform specific activity can be assumed at any time.
   = a part of the protein mass in which all molecules have equal probability of transfer to other compartments (If no feed back to the pool exists, activity directly introduced in it would decay as a single exponential function).
2. "Turnover" generally indicates the process by which the substance is renewed through continuing synthesis and catabolism. Used with various meanings (rate, time).
3. Mass of unlabeled protein in pool M_P = mass in plasma
   M_E = mass in extravascular pool.
4. Rates of transfer : *a*) fractional : *fraction of pool* transferred per unit time out of the pool.
   b) absolute : *mass of substance* transferred per unit time (in or out of the pool) = fractional rate x pool mass
5. Kinds of rates : 1. Synthesis.
   2. Exchange (Distribution).
   3. Catabolism (= Synthesis in steady state).
   (Fractional catabolic rate = FCR).
6. Whole body activity = Retained activity (retention curve) =
   = injected activity = cumulative excretion =
   $= 1 - \int U dt = P + E.$

   where U = fraction of dose excreted per day.
   P = fraction of dose in plasma.
   E = fraction of dose in extravascular pool =
   = whole body activity — plasma activity.

only in connection with the degradation of the labeled protein. This requirement is pictured in Figure 1 showing the iodine released coming out from the pipe of the degradation products.

If we were dealing with a single big box in which the label could easily mix with all the protein content, it is quite obvious that just measuring the fractional amount of iodine coming out of the box per unit time we could estimate the fraction of the protein degraded. In these conditions it is easy to show that both blood and body radioactivity would decay as a nice single exponential function. However, even in such simple case, complications may arise because of the excretion of iodine. It is obvious from Figure 1 that the size of the iodide pool and the conditions of renal function will influence the way in which iodine is excreted.

All these points will be considered in this morning's discussion. But there is another point which I would like to consider briefly.

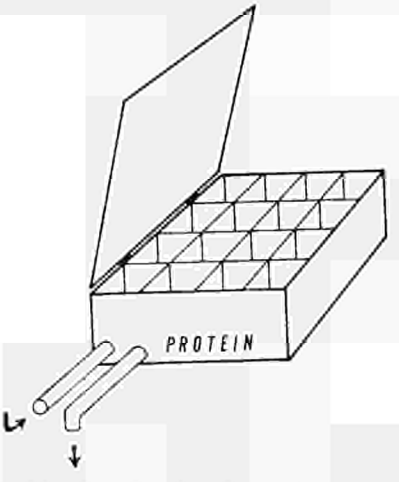

FIGURE 2. — Partition in sub-units of the plasma protein pool.

Sofar we have assumed that the activity could easily mix within the whole box : in fact this is not true, and instead of being a nice single big box if we could "open" the protein pool, we would find it more similar to the one which I have pictured in Figure 2. It is quite obvious that, if the pool is made up of several sub-units, just changing the sub-unit in which we actually inject the tracer will produce different effects on the rate at which the tracer will be eliminated from the box. Even if we assume that eventually the tracer will spread to the whole of the box, it is obvious, for instance, that if we inject it close to the outflow pipe, a greater amount of tracer will leave the system before all the boxes have been reached by it than in the case that it had been injected further away. It is also obvious that uniform concentration throughout the system will never be attained.

Even it we could think of starting out with uniform concentration in every sub-unit of the box, it is quite evident that the situation would be rapidly unbalanced

because the concentration in the sub-units closer to the input would be continuously lower than elsewhere, since the amount of tracer in them would be continuously diluted by new unlabeled protein coming in.

Fortunately we have some reasons to make some assumptions about the relationship between the location of the inflow and outflow pipe. We can assume that catabolism or degradation takes place in very close connection with the plasmatic space. The current assumptions in plasma protein studies are summarized in Table II. In Figure 3, I have pictured both catabolism and synthesis taking place close to the plasma pool; this simplifies our task of calculating the mass of the protein and its rate of turnover.

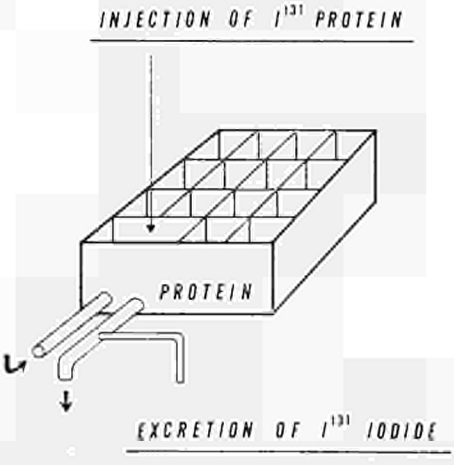

FIGURE 3. — Favourable conditions for tracer studies.

Unfortunately the process of protein degradation and label excretion is superimposed on the exchange processes that determine the transfer of the tracer between compartments or sub-units of the box. Our task would be much easier if we could split the exchange process, tending towards the attainment of an uniform concentration of the tracer throughout the box, from the turnover process, tending to eliminate the tracer from the box. All our efforts are directed to overcome the dif-

TABLE II. — Assumptions in plasma proteins tracer studies.

General assumptions	: tracer behaves as mother substance. newly synthetised protein enters the intravascular compartment before mixing with the main extravascular compartment.
Most models assume also	: Catabolism takes place in a compartment which exchanges rapidly with the intravascular pool [3]. rapid excretion of iodide.

ficulties arising from the fact that the two processes are in fact simultaneous and have to be dealt with together.

I think we should now proceed and I will ask Dr. Nosslin to start presenting to you the discussion on the first methods that have been divised to analyse protein tracer studies and that we have defined « approximation methods ».

B. NOSSLIN : The use of the word "approximation" for one of the groups may seem difficult to understand and I will admit that the word "approximation" in a way could be used for every method which we use since we all know, and we have just seen, that the true model in the body is always much more complicated compared to the technique we use in analyzing our results. However, those calculation methods which we have assembled in the first group — under the name "approximation methods" — have some special assumptions which are not necessary in the methods of the other two groups, as I think it will be made clear later on in this discussion.

Concerning calculation of the fractional catabolic rate, I will present three different methods often used. The first one is the well known use of the half life time, that is the half life of the final straight part of the curve when it is plotted on a semilog paper. I think you are all familiar with how to take it out graphically from the curve, and of course it has the dimension of time : in order to get a fractional catabolic rate from it you have to use the well-known expression that the fractional catabolic rate equals the natural logarithm of 2 over $T_{1/2}$ - that is $0.693/T_{1/2}$ The obtained fractional rate refers to the total pool. This estimation of the fractional rate is always an under-estimate of the true rate : that means that these half life times are always too long compared with the true half life of the protein. Unfortunately these half lives are often called the "biological half time" but since it is not identical with the true half life time of the protein I think the use of the word "biological" is not very good.

If you also have excretion values you can calculate two further expressions for fractional catabolic rate as the ratio of urine excretion over total radioactivity retained — U/(P + E) — or the content of activity in the plasma — the U/P ratio. This latter fractional rate refers to plasma pool and it is a correct estimate if the catabolism is intra-vascular.

The expression U/(P + E) or excreted amount per day over retained activity will, under steady state conditions, reach, sooner or later, a final constant value and this has been used as an expression for fractional catabolic rate. Of course it refers now to the total pool (that is a point that came up in the discussion two days ago) : there are then two different types of fractional rates calculated from urine excretion, one is referring to plasma pool, the other to the total pool. It can be shown that U/(P + E) always leads to an under estimation of the fractional rate of the mother substance, the protein.

I will now go over to the use of these methods for estimating the size of the total pool. The first, and the oldest one, I think, is the well-known "Sterling technique" [1] where the final straight line on the semilog paper is extrapolated back to zero

TABLE III. — Methods of analysis

Methods	Assumptions	Results and errors			References
		Pool masses	Fractional catabolic rate	Absolute catabolic rate	
A. — *Approximation methods*					
1. Final slope	Uniform specific activity throughout the protein pool	Total pool mass from intercept of final slope overestimation	As fraction of total pool from T ½ of final slope underestimation	From intercept and T ½ of final slope overestimation	Sterling, 1951 [1]
2. Distribution function (or retained activity)	Uniform specific activity throughout the protein pool when U/(P + E) is constant	Total exchangeable protein (albumin) TEA = $\frac{\text{Retained activity}}{\text{Plasma sp. act.}} = \frac{P + E}{P/M_P}$ (at equilibrium overestimation	As fraction of totalpool = $\frac{\text{Daily urine excr.}}{\text{Retained act.}}$ = U/(P + E) (at equilibrium) = FCR final slope underestimation	From TEA and FCR $\frac{P + E}{P/M_P} \times \frac{U}{P + E} = \frac{U}{P/M_P}$ Correct (if i.v. catabolism) since errors cancel out	Berson and Yalow, 1953 [2]
3. U/P clearance	Intravascular catabolism Rapid excretion of iodide	— —	As fraction of i.v. pool $\frac{U\ \%\ \text{dose/day}}{P\ \%\ \text{dose}}$ Usually correct; overestimation (except first days) if iodide excretion slow	$\frac{U}{P/M_P} = \frac{\text{Urine daily excr.}}{\text{plasma sp. act.}}$ usually correct overestimation as for FCR	Campbell et *al.*, 1956 [3] Berson and Yalow, 1957 [4]
4. Equilibrium time	Uniform extra-vascular pool	Extravascular pool mass from extravascular activity at peak time indeterminate	—	—	Campbell *et al.*, 1956 [3]

B. — *Specific models*

5. Multiexponential analysis of plasma curves	I.v. catabolism Albumin pool divided in definite number of mixing compartments	All pool masses from slopes and intercepts correct (specific compartment masses not necessarily meaningful)	From slopes and intercepts correct	Fom fractional catabolic rate and pool masses correct	Berman and Schœnfeld, 1956 [5] Matthews, 1957 [6]
6. Multiexponential analysis of plasma and whole body curves	Albumin pool divided in definite number of mixing compartments	All pool masses from slopes and intercepts correct (specific compartment masses not necessarily meaningful)	From slopes and intercepts correct	From fractional catabolic rate and pool masses correct	Berman and Schœnfeld, 1956 [5] Lewallen *et al.*, 1959 [7] Reeve and Roberts, 1959 [8]
7. Pipe model	—	Plasma and extravascular pool masses from slopes and intercepts correct	From slopes and intercepts correct	From fractional catabolic rate and pool masses correct	Reeve and Bailey, 1962 [9]

C. — *General methods*

8. Multiexponential analysis of plasma and whole body curves	Berman and Schœnfeld, 1956 [5]
9. Plasma curve integral	Nosslin, 1964 [10]
10. Plasma and retention curves integrals	Nosslin, 1964 [10] Matthews, 1965 [11]
11. Deconvolution techniques	Vitek *et al.*, 1966 [12]

time and the intercept is read : this gives a kind of dilution value from which the total pool can be calculated, knowing the size of the plasma pool. It can be shown that this approach always leads to an over-estimation of the true pool, sometimes a large over-estimation.

The next method in this group is the estimation of total exchanging albumin by dividing the retained activity by the plasma specific activity — a method used first by Berson and Yalow in 1953 [2]. It can be shown that this method also leads to an over-estimation, although in general, not as large as the Sterling technique.

A third method for estimating total pool is the so-called equilibrium time method [3] where the retained activity at peak time of extra-vascular activity is divided by the plasma specific activity at the corresponding time. This may lead to a correct estimation but that depends upon conditions in the extra-vascular space — whether the specific activity in the extra-vascular space is uniform or not.

So now we have three different methods of estimating fractional rate and three different methods of estimating total pool : in order to get the absolute rate, we now have to multiply the fractional rate with the corresponding estimate of the total pool.

L. DONATO : Thank you Dr. Nosslin. Dr. Matthews, would you like to comment on the approximation methods ?

C. M. E. MATTHEWS : These approximate methods may certainly give the right result under some conditions, but in other circumstances there may be a considerable error and so they must be used with caution. Taking the first method for the fractional catabolic rate, that is the half life method, this will always give an under-estimation and the reason can be shown by considering Figure 4.

Initially the intra-vascular specific activity falls and the extra-vascular specific activity rises and crosses it at the maximum point of extra-vascular specific activity, i.e. at the equilibrium time. After this time the specific activity in the extra-vascular pool is always greater than in the intra-vascular pool. Hence there is a net transfer of radioactivity from the extra-vascular to the intra-vascular pool, the specific activity in the extra-vascular pool being always higher than in the intra-vascular pool after this time. Thus there is a net transfer of radioactivity from the extra-vascular to the intra-vascular pool which reduces the slope of the intra-vascular specific activity curve. Therefore this slope does not correspond to the slope which would be obtained simply due to catabolism and if the slope is taken as a measure of catabolism it is an under-estimate.

When the fractional catabolic rate is low compared with the exchange rate between extra- and intra-vascular pools, the method may give approximately the right result, as for example with normal human albumin in normal humans, but when the catabolic rate is increased or total loss of protein from the intra-vascular pool is increased, as for example in nephrotic patients, the method does not give the right result. The method assumes in fact, a single pool, or that in all pools the fractional catabolic rate and the synthesis rate are the same : as we know this is not

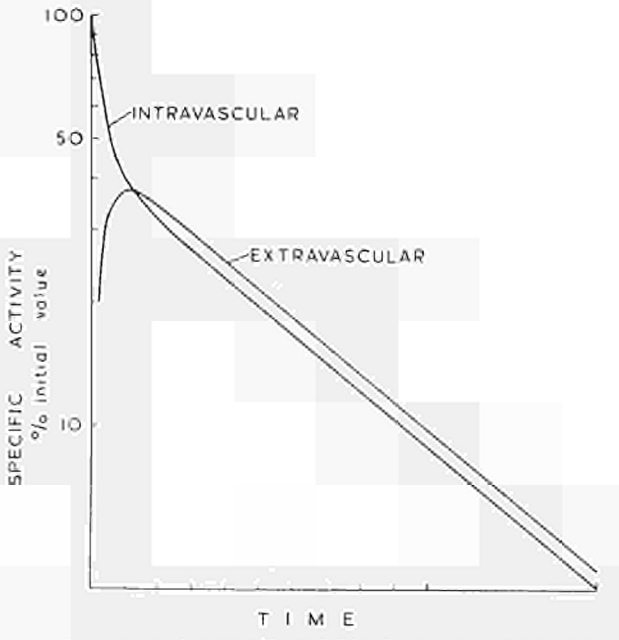

FIGURE 4.— Intra- and extravascular specific activities in a two compartment system with intravascular catabolism.

the case, the method, in general, is incorrect. For the same reasons method 1 will always give an over estimate of pool mass and of absolute catabolic rate. Method 2 also depends on the specific activity being uniform throughout the protein pool when the final slope is reached and so involves similar errors. However for the absolute catabolic rate these errors cancel out and the same result is obtained as in method 3.

Passing on now to the U/P ratio for fractional catabolic rate, this method is correct if the catabolism is in the intra-vascular pool or in a pool in rapid exchange with the intra-vascular pool, but there may be some error due to the effect of the iodide pool. If the iodide excretion is not sufficiently rapid compared with the catabolic rate, activity will build up in the iodide pool and the radioactivity excreted will not give a true measure of the radioactivity produced by breakdown. I have plotted some curves with an analogue computer to illustrate this and Figure 5 shows the kind of errors you may expect. A rather low iodide excretion rate of 80 %/day in assumed and the catabolic rate is varied from 7 to 100 %/day.

The production of radioactivity due to catabolism is shown and the excretion of radioactivity which would be obtained if the iodide was excreted very rapidly indeed. The actual excretion of the radioactivity is also shown. The ratio of the actual excretion to the iodide produced due to catabolism, is also included and the ratio of the measured catabolic rate to the true catabolic rate. As you see in the case where excretion rate is much higher than catabolic rate the ratio becomes almost one and so the error is small here except in the first few days (left of Figure 5).

With an increased catabolic rate (middle of Figure 5) the ratio of the two curves again rises to an approximately constant value but in this case the constant value is greater than one — about 1.5.

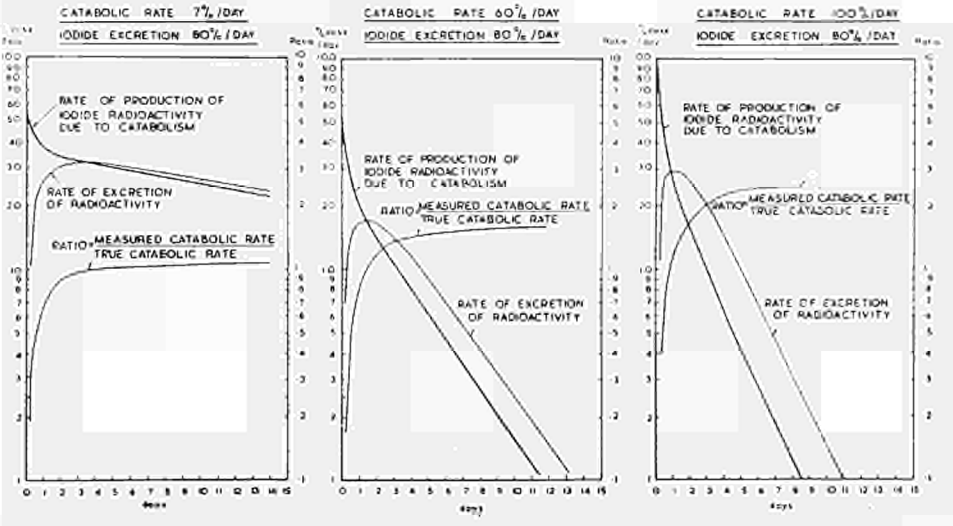

FIGURE 5.— Error in measured catabolic rate (U/P method) due to a constant slow iodide excretion for increasing albumin catabolic rates.

To the right of Figure 5 curves are shown for an even greater catabolic rate, this time 100 % per day and here the ratio reaches a constant value which is greater than 2. Therefore the error, as one might expect, depends on the values of the iodide excretion rate and the catabolic rate and with high catabolic rates and low excretion rates this error may be considerable. Even though the catabolic rate is constant it may still not be correct.

Figure 6 shows the effect on the whole body curve, with the curve which would be obtained without the iodide pool and also the total activity including the iodide pool. Again results are shown for three different catabolic rates with the same iodide excretion rate.

Figure 7 shows the effect of varying the excretion rate for a constant catabolic rate. To the left curves are shown for a catabolic rate of 7 % per day and increasing values of the excretion rate in per cent per day. The lower the excretion rate the longer the excretion curve takes to reach its maximum value. Hence the ratio of radioactivity excretion to radioactivity production takes longer to reach 1 when the iodide excretion rate is lower.

To the right of Figure 7 are shown curves for a very rapid catabolic rate of 200 % per day and in this case the error is very considerable for low excretion rates, for instance, for an excretion rate of 80 % per day, or 40 % per day the curves never become parallel and the activity tends to build up in the iodide pool. For an

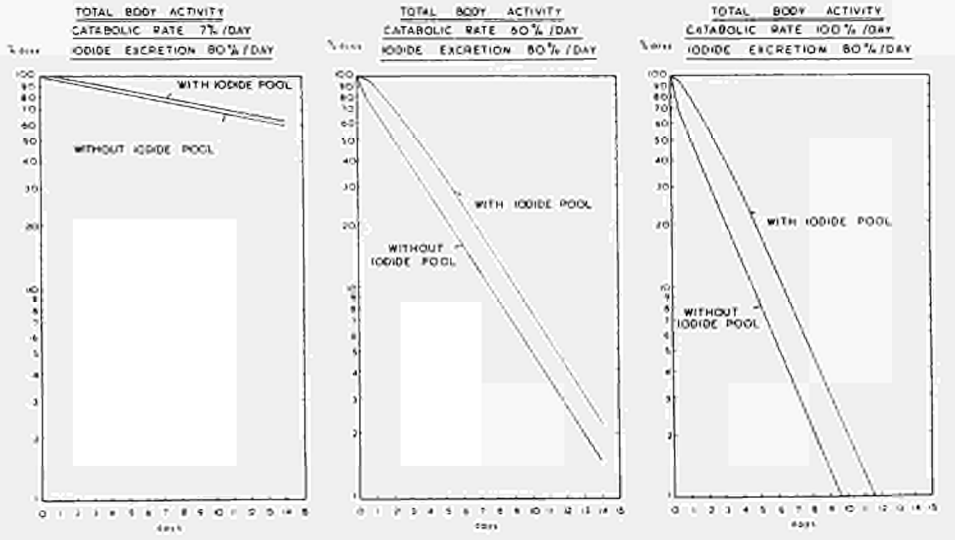

FIGURE 6.— Effect of slow iodide excretion on whole body curve for increasing albumin catabolic rates and constant iodide excretion rate.

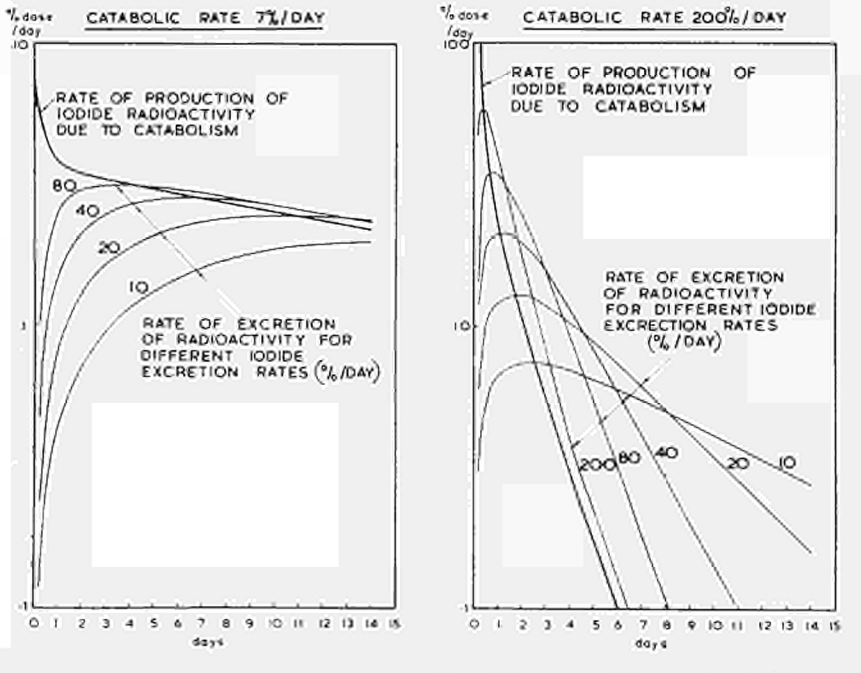

FIGURE 7.— Effect of slow iodide excretion on radioactivity excreted for varying iodide excretion rates with a constant catabolic rate.

excretion rate of 200 % per day the curves do, in fact, become parallel after a time and the error is only about 25 %.

Figure 8 shows the effect on total body activity, again for two different catabolic rates and for different values of the excretion rate. Here the effect is very large and could tend to make the whole body curve diverge from the intra-vascular curve.

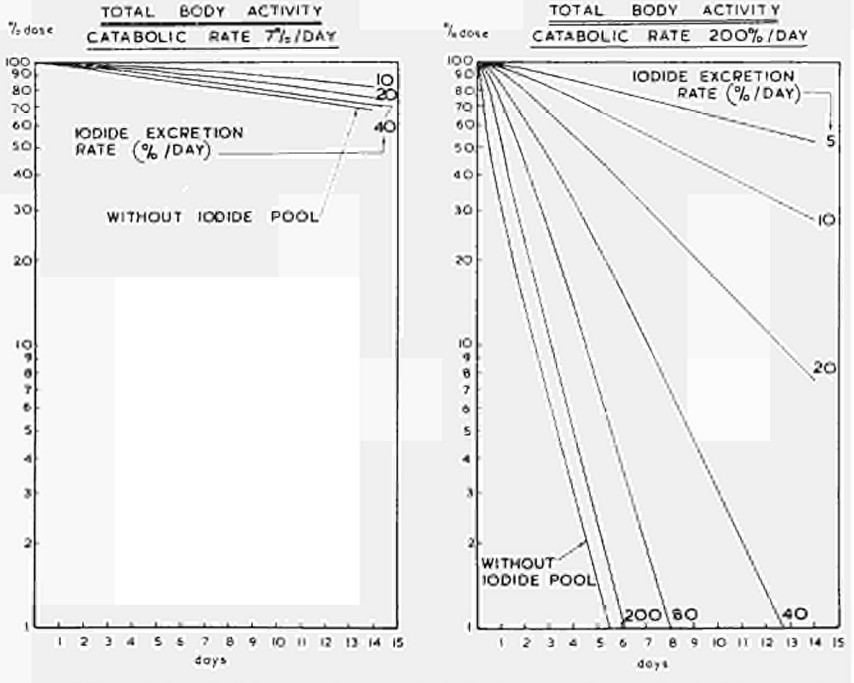

FIGURE 8.— Effect of slow iodide excretion on whole body curve for varying iodide excretion rates and constant catabolic rate.

The equilibrium time method depends on having only one extra-vascular pool, and if, in fact, there are two extra-vascular pools exchanging at different rates with the intra-vascular pool there will be some error, the extent of which is difficult to estimate as it will depend on the relative exchange rates between the two extra-vascular pools and the intra-vascular pool. Another disadvantage of this method is that the extra-vascular radioactivity curve may have rather a flat top and so it is difficult to determine the exact point of the maximum.

L. DONATO : Thank you Dr. Matthews. I would like to move now to the consideration of methods in which a specific number of compartments is assumed and analysis of the curves is performed on the basis of these assumptions. So I will then again ask Dr. Matthews to discuss the subject of specific compartment models.

C. M. E. MATTHEWS : I think you are probably all familiar with these models, I will only mention them rather briefly. These are all based on the analysis of the

curves into exponentials — I am sure you are all familiar with the method by which this is done by successive subtractions of the final slopes to obtain the slopes and intercepts of the component exponentials. From these slopes and intercepts the exchange rates between the pools and the catabolic rate can be calculated if a specific model is assumed.

The model assumed is usually the one in which the extra-vascular pools all exchange with a central intra-vascular pool with catabolism and synthesis in connection with the intra-vascular pool. Sometimes two extra-vascular pools and sometimes one extra-vascular pool are used. If the curve can be analysed into two exponentials, usually the simplest possible model is taken which would contain only one extra-vascular pool; for three exponentials one takes two extra-vascular pools.

It is important to emphasize that each of the slopes and intercepts depend on all the rate constants in the system and one cannot say that the fast exponential corresponds to the rapidly exchanging pool or the slow exponential corresponds to a slow pool. Each one of these exponential components depends on all the rate constants in the system, all the exchange rates and the catabolic rate.

Reeve and Roberts [8] have extended the two or three pool model to include the iodide pool and extra-vascular catabolism, and because the model is more complex they also have to use the total body radioactivity in order to have enough information to solve the equations and to obtain all the rate constants. With the simpler model it is only necessary to use the intra-vascular radioactivity curve, and the whole body curve, if it is available, can be used to check the results. With Reeve and Robert's method it is essential to have both of these curves, and from these curves one can calculate the exchange rates and both intra-vascular and extra-vascular catabolism if the iodide excretion rate is knwon.

Then another model is that of Reeve and Bailey [9] in which the extra-vascular pool consists of a series of pipes, that is the protein leaves the intra-vascular pool through a series of pipes which then return it to the intra-vascular pool so that the effect is one of a delay. The specific activity is not altered in travelling through the pipes but the protein of that specific activity returns to the intra-vascular pool after a delay and the series of pipes have a distribution of lengths. This is an extravascular pool in which there is no mixing. However, in fact rather fortunately the results by this method turn out to be much the same as by the compartment analysis that is if the intra-vascular radioactivity curve can be represented by a sum of exponential functions then Reeve and Bailey have to assume an exponential distribution of passage times through the pipes and this leads to exactly the same equations as one finds in a pool with complete mixing.

There is also the more general method of Berman and Schoenfeld [5] but we will be discussing that in the next section.

I will just make a small comment on these methods : sometimes the question is raised of what these pools mean and whether there is any point in finding these two extra-vascular pools. Perhaps they have no meaning and there is not much point in this calculation? Well I would like to show you that one can sometimes

find a meaning for these pools. In animals I have found a slow and a fast extra-vascular pool[13]. Figure 9 shows results for albumin in rabbits and the analysis into pools. There is a large slowly exchanging extravascular pool and a small rapidly exchanging one two extra-vascular pools and this gives curves like this for the radio-activity in the plasma in the slowly exchanging large extra-vascular pool and in the rapidly exchanging small extra-vascular pool are shown. The curve for the small extravascular pool reaches its maximum very rapidly and then comes down parallel to the intra-vascular pool.

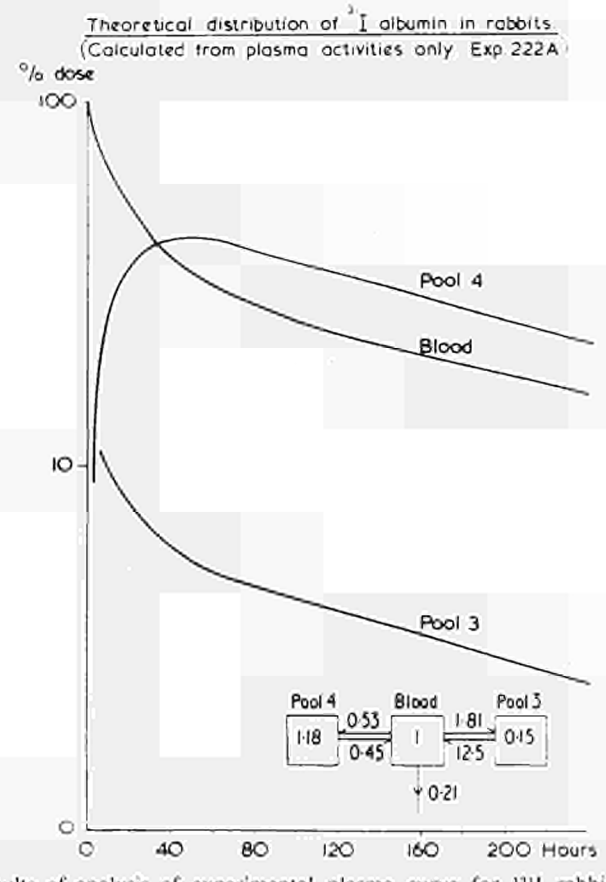

FIGURE 9. — Results of analysis of experimental plasma curve for ^{131}I rabbit albumin [13] — Theoretical curves also shown for extravascular pools 3 and 4.

Figure 10 shows experimental measurements of extra-vascular radioactivity in the pools in rabbits. The blood was labeled with ^{132}I and the intra-vascular radioactivity subtracted to obtain extravascular radioactivity. The muscle skin and bone pool (mainly muscle and skin) gives a curve rather similar to the theoretical curve which you saw on the previous slide for the large slowly exchanging pool and the gastro-intestinal tract gives a curve rather similar to that for the small

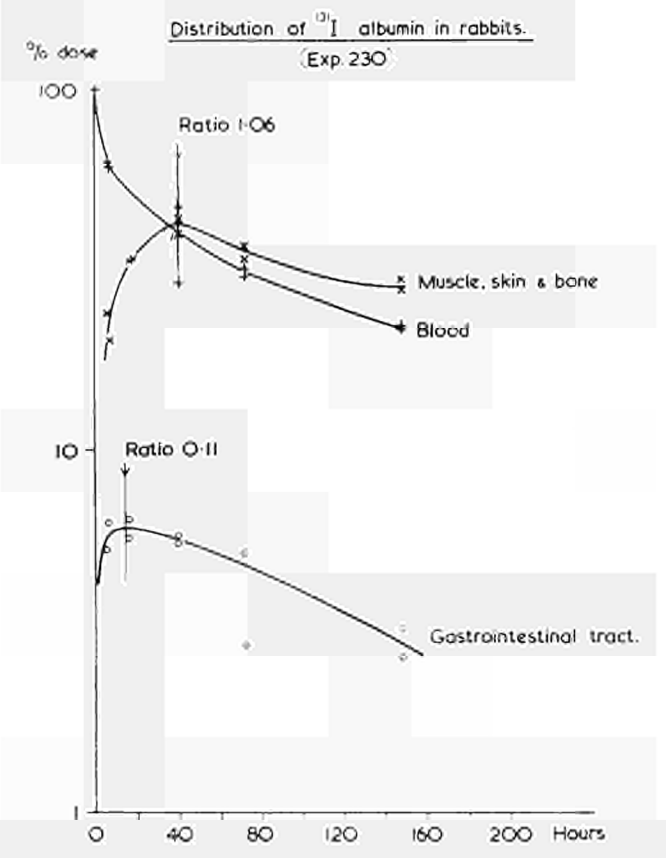

FIGURE 10. — Experimental measurements of extravascular radioactivity in rabbits [13]

rapidly exchanging pool. The other organs contained very small amounts of radioactivity and had a negligible effect on the intra-vascular curve.

L. DONATO : Thank you Dr. Matthews. Certainly the most elaborate step in dealing with compartment models is the Berman and Schoenfeld approach [5]. I would like Dr. Segre to make a comment about the Berman and Schoenfeld technique.

G. SEGRE : First of all I would like to set forth some probably obvious considerations about compartmental analysis because the method of Schoenfeld and Berman is connected to them. The multi-exponential analysis is usually a phenomenological description of complex phenomena; by carrying out compartmental analysis a homomorphism is performed between some part of the complex experimental structure and a mathematical model. Moreover every experimental design is endowed by a given "resolving power" : within a given experimental design it is possible to "see" only a given structure. For instance if 2n equally spaced points have been sampled from a given compartment then no more than n compartments

can be seen; in this case the resolving power of the experimental design corresponds to n compartments. In compartmental analysis however the most interesting problem is the determination of the transfer constants of the model; now for a system of n compartments n^2 transfer constants are possible and this number stops very soon the possibility of the analysis. The method of Schoenfeld and Berman represents a way to circumvent in a certain degree this impasse.

The experimental outcome of a given experiment is given by one or more multiexponential functions.

$$X_i(t) = \sum_{j=1}^{n} A_{ij} e^{-\alpha_j t}$$

The transfer constants (k_{rs}) of the model can be calculated through the matricial equation

$$|k| = |A|\ |a|\ |A|^{-1}$$

where

$$|k| = \begin{vmatrix} K_1 & -k_{21} & \dots & -k_{n1} \\ -k_{12} & K_2 & \dots & -k_{n2} \\ \dots & \dots & \dots & \dots \\ -k_{1n} & -k_{2n} & \dots & K_n \end{vmatrix} \qquad (K_i = \sum_{r=0}^{n} k_{ir};\ i \geqslant r)$$

$$|a| = \begin{vmatrix} a_1 & 0 & \dots & 0 \\ 0 & a_2 & \dots & 0 \\ \dots & \dots & \dots & \dots \\ 0 & 0 & \dots & a_n \end{vmatrix}$$

$$|A| = \begin{vmatrix} A_{11} & A_{12} & \dots & A_{1n} \\ A_{21} & A_{22} & \dots & A_{2n} \\ \dots & \dots & \dots & \dots \\ A_{n1} & A_{n2} & \dots & A_{nn} \end{vmatrix}$$

When the A_{ij} are not known, it is possible to substitute them by unknowns (x, y, z, etc.) and to solve the matricial equation; the matrix $|k|$ in this case will contain these unknowns, so that, by remembering that $k_{rs} \geqslant 0$ and that $K_i - \sum k_{ir} = k_{i0}$ (k_{i0} indicates the transfer constant from compartment i to the exterior), one can write a system of n inequalities.

For instance let us suppose that for compartment 1 one has

$$X_1/x_0 = (\tfrac{3}{8})e^{-3t} + (\tfrac{1}{4})e^{-2t} + (\tfrac{3}{8})e^{-t}$$

(where x_0 is the dose introduced into the system), and for the sum of compartment 2 and compartment 3 one has

$$(X_2 + X_3)/x_0 = -(\tfrac{3}{8})e^{-3t} + (\tfrac{3}{8})e^{-t}.$$

If one introduces two arbitrary parameters, x and y, then the system becomes

$$\left.\begin{aligned} X_1/x_o &= \tfrac{3}{8}e^{-3t} + \tfrac{1}{4}e^{-2t} + \tfrac{3}{8}e^{-t} \\ X_2/x_o &= -xe^{-3t} + ye^{-2t} + (x-y)e^{-t} \\ X_3/x_o &= (x-\tfrac{3}{8})e^{-3t} - ye^{-2t} + (\tfrac{3}{8}+y-x)e^{-t} \end{aligned}\right|$$

Then one has

$$|A| = \begin{vmatrix} \frac{3}{8} & \frac{1}{4} & \frac{3}{8} \\ -x & y & x-y \\ x-\frac{3}{8} & -y & \frac{3}{8}-x+y \end{vmatrix}, \quad |a| = \begin{vmatrix} 3 & 0 & 0 \\ 0 & 2 & 0 \\ 0 & 0 & 1 \end{vmatrix}$$

$$|A|^{-1} = \begin{vmatrix} 1 & \frac{1}{y}\left(\frac{2}{3}x - \frac{5}{3}y - \frac{1}{4}\right) & \frac{1}{y}\left(\frac{2}{3}x - \frac{5}{3}y\right) \\ 1 & \frac{1}{y}\left(-2x+y+\frac{3}{4}\right) & \frac{1}{y}(-2x+y) \\ 1 & \frac{1}{y}\left(\frac{2}{3}x+y-\frac{1}{4}\right) & \frac{1}{y}\left(\frac{2}{3}x+y\right) \end{vmatrix}$$

$$|K| = \begin{vmatrix} 2 & -1 & -1 \\ (-2x+y) & \frac{5}{2} - \frac{1}{y}\left(\frac{2}{3}x - y\right)\left(2x+y-\frac{3}{4}\right) & \frac{1}{y}(2x+y)\left(-\frac{2}{3}x+y\right) \\ \left(2x-y-\frac{3}{4}\right) & \frac{1}{y}\left(\frac{2}{3}x-y-\frac{1}{4}\right)\left(2x+y-\frac{3}{4}\right) & \frac{3}{2} - \frac{1}{y}\left(2x+y-\frac{3}{4}\right)\left(-\frac{2}{3}x+y\right) \end{vmatrix}$$

The two parameters x and y must satisfy to the following simultaneous inequalities :

$$\left.\begin{aligned} &\text{(I)} && 2x - y \geqslant 0 \\ &\text{(II)} && -2x + y + \frac{3}{4} \geqslant 0 \\ &\text{(III)} && \frac{1}{y}\left(-\frac{2}{3}x + y + \frac{1}{4}\right)\left(2x + y - \frac{3}{4}\right) \geqslant 0 \\ &\text{(IV)} && \frac{1}{y}\left(-x + \frac{5}{2}y + \frac{3}{8}\right) \geqslant 0 \\ &\text{(V)} && \frac{1}{y}(2x+y)\left(\frac{2}{3}x - y\right) \geqslant 0 \\ &\text{VI)} && \frac{1}{y}\left(-x + \frac{5}{2}y\right) \geqslant 0 \end{aligned}\right|$$

The above inequalities can be represented in a plane by the Figure 11, in which a straight line or a couple of straight lines delimitate the focus where the coordinates x and y satisfy the above inequalities. The hatched areas correspond to the points where all the six inequalities are satisfied; these areas correspond therefore to the physically possible models.

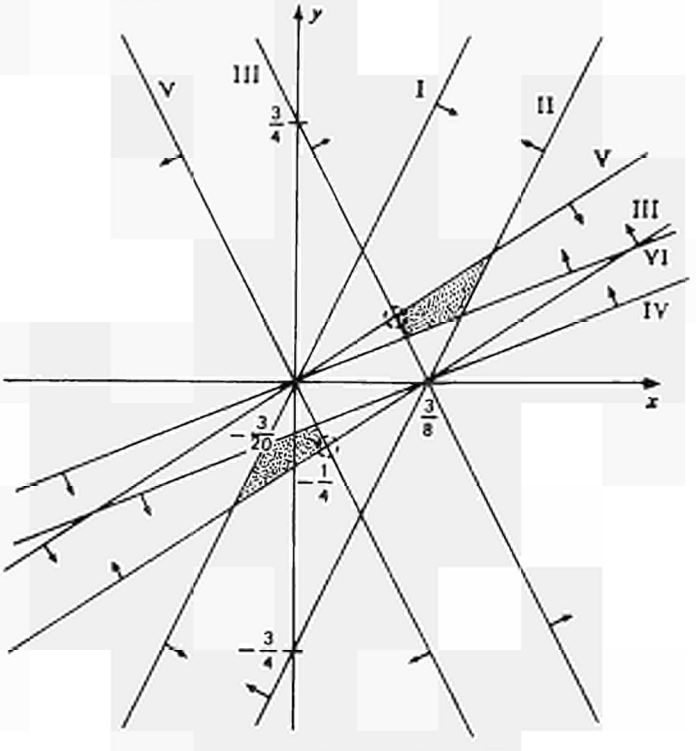

FIGURE 11. — See text (Segre) [22]

It is clear that this approach has great limitations : too many calculations are required and the method appears of practical use when the unknowns are only two, because in that case the representation requires a plane, as in the example given above. When the unknowns are three, a tridimensional representation is required.

L. DONATO : Thank you Dr. Segre. I know that Dr. Matthews has very precise opinions about the Berman and Schoenfeld approach and I would like to have her comments on this.

C. M. E. MATTHEWS : I think this method is very useful when the pool model is not known and the total body activity and the intra-vascular activity have been measured. For example the method can be used to show that from results of plasma protein analysis the catenary model is inconsistent with the data. The experimental results cannot be fitted by a catenary model, that is one in which the extra-vascular

pools are connected in series. Using Berman and Schoenfeld's method, one can work out all possible values of the exchange rates and the catabolic rates in all pools for this general three-pool model, with interconnections between each of the pools. This gives a series of models which would fit the experimental results. This may sound as though one was obtaining too much information which it would be difficult to deal with but, in practice, one may find for example that certain rates may only have very small values, so that there is a limited range of values for this rate for the model to be consistent with the experimental results. A particular rate may also turn out to be very small, for example the catabolism may be nearly all in the rapidly exchanging extra-vascular pool with very little actually in the intra-vascular pool. Hence one may find quite a narrow range for the different exchange rates and I think this method is useful because it is a more general analysis than merely assuming that certain rates must be zero, and it shows that there is only a limited range of models which will fit the experimental results.

L. DONATO : Thank you. We will certainly come back to multicompartment analysis during the discussion, but now I would like to move to the consideration of methods that seem to be less dependent on initial assumptions concerning the distribution of the mass in various compartments. We have called them "general methods" and I will ask Dr. Nosslin to start discussing this subject.

B. NOSSLIN : Figure 12 gives a description of a general technique of calculating the more important parameters of a system when you have access to the plasma curve only. It works with a minimum of assumptions and it can be shown, mathematically, with a rigorous proof, that these solutions are exact. The assumptions are, as usual, that synthesis is in the intra-vascular pool and in the case when we have only the plasma curve we also have to assume the site of the catabolism. The solutions given here refer to intra-vascular catabolism but it is possible to give comparable general solutions for other sites of catabolism. The information you need is the common one, the plasma curve described in a series of exponential functions; it does not matter how many, two or three or four or five or any number, since they are just used to describe the plasma curve completely, and after having done that graphically, with a common peeling-off technique, you have the information collected in a number of slopes and a number of intercepts, from which you can calculate three general magnitudes which are A, B and C under the picture. They are very easy to calculate, you divide each intercept by its slope, or by the square of its slope, or you just multiply the intercept and slope and then sum them for each term.

From these three expressions you can calculate the correct values for fractional and total catabolism and for the total pool and for the total exchange rate between plasma pool and all other pools in a way which is given in the legend of the figure. It is also possible to calculate a true value for the half time of the protein, the biological half time : if this value is compared with the $T_{1/2}$ of the final slope you will see that they will not come out equal.

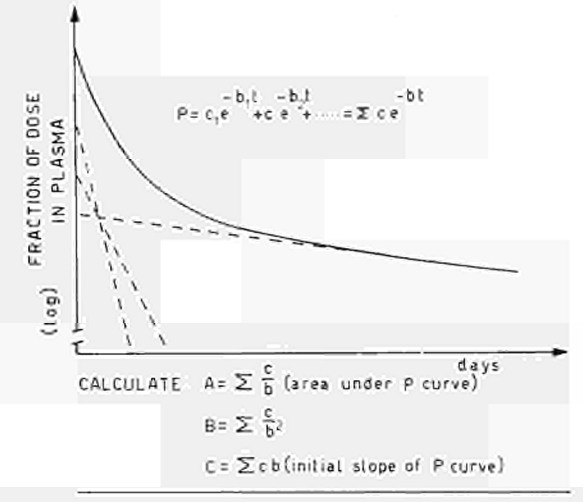

FIGURE 12. — *A general method for analysis of a plasma disappearance curve* ([10]). Assumptions : synthesis and catabolism in (or in close connection with) the plasma pool (P pool). No assumptions about number and arrangement of the extravascular pools. The plasma curve (P) must be followed until it has reached a final constant slope.

Calculations :

Fractional catabolic rate	1/A	Fraction of P pool per day
Rate of synthesis	P pool/A	grams per day
Total body pool	P pool × B/A^2	grams
Total exchange rate	C — 1/A	fraction of P pool per day
Half-life time	0.693 × B/A	days

This method should be used when only the plasma curve has been measured. The expressions for fractional catabolic rate and synthesis are independent of site of catabolism, while the total body pool, half-life and turnover time are correct only under the assumption of no extravascular catabolism.

I think that this general method should be used when you are interested only in the general properties of the system — total pool size, total catabolism, total rate of exchange. You do not need any more information in order to do this than you have in the other techniques discussed earlier.

If you have access to two curves, that means plasma curve and whole body curve — or retention curve — then it is possible to do the corresponding calculations, but now we do not have to assume the site of catabolism : this is then a real minimum of assumptions you need now and it is now possible to make correct estimates of the same parameters as earlier, just knowing the areas under the plasma curve and the retention curve (A_P and A_R in the figure). Also it is now possible to correct for the influence of the dead space between the system and the collection space, that is the iodide space. The area under these two curves can be measured or calculated in several ways : for example, by exponential analysis (and then it is easy to calculate the whole area under the curve) or by planimetry, adding an estimate

of the final part in the usual way with the use of the final slope. The two areas exactly define rate of catabolism and total pool size in the simple way shown in the legend of Figure 13.

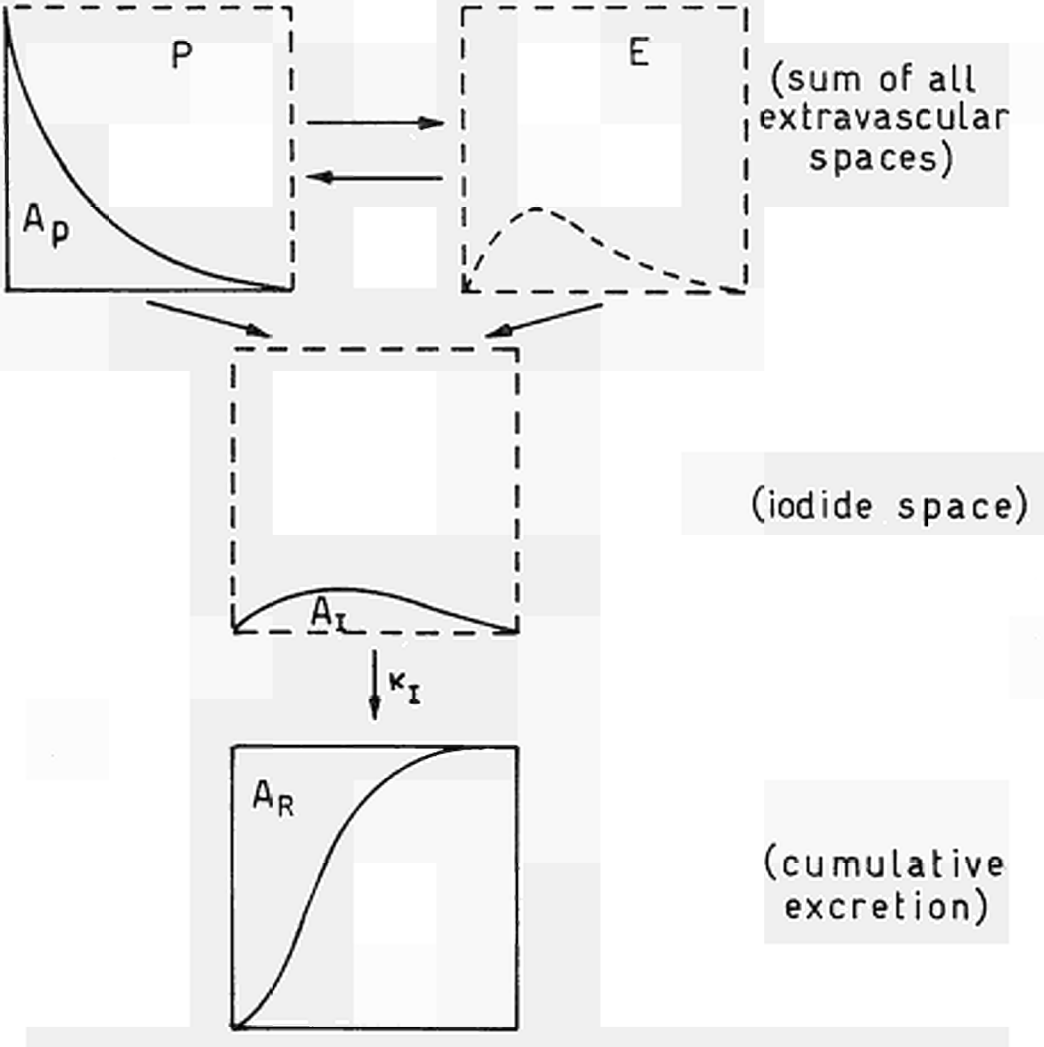

FIGURE 13. — *A general method for analysis of simultaneous plasma and retention (or whole body) curves* ([30]). Assumption : synthesis to plasma pool. Measure (or calculate) area under P curve (A_P) and area under retention or whole body curve (A_R). Area under the iodide space curve (A_I) is $= 1/k_1$ ($= 0.50$ days in man).

Calculations :

Fractional catabolic rate	$1/A_P$	Fraction of P pool per day
Rate of synthesis	P pool$/A_P$	grams
Total body pool	P pool $\times$ $(A_R\text{-}A_I)/A_P$	grams
Half-life time	$0.693 \times (A_R\text{-}A_I)$	days

Corrections can also be made for sampling losses and for free iodide in plasma (not shown here).

These techniques, both of them, work with any number of extra-vascular compartments, and do not require any assumption about equal specific activity. The solutions must be equal to those in other techniques and they can be used as a check : for example, in the Berman and Schoenfeld technique, every other solution must come out with the same result as far as total size and total catabolism are concerned. As I said, there are proofs for this. These methods have been developed by several authors : Dr. Bergner in Sweden made it some years ago, I have done some work on it, Dr. Matthews has done it, and I think we can recommend them for more general use. They are not so well known today, because they haven 't been published in an easy form but I think we would like to recommend them for more common use. Thank you.

C. M. E. MATTHEWS : I would just like to express the same thing as Dr. Nosslin has said, but in terms of compartment analysis. The compartment analysis as Dr. Nosslin has said, will in fact, give you the same results as his method but these particular results are independent of the arrangement of pools.

Table IV shows some of the equations which are independent of the pool arrangement, these two equations are also independent of the site of synthesis or of catabolism. The last two equations are to indicate the notation; the intra-vascular radioactivity is expressed as three exponential components with coefficients A_{11}, A_{12} and A_{13} and slopes b_1, b_2 and b_3. The total body radioactivity curve has the same slopes with intercepts A_1, A_2 and A_3. The last two equations are just included to show the meaning of the symbols used.

TABLE IV. — Equations for protein studies, based on compartment analysis, but independent from pool arrangements, and site of synthesis and catabolism.

I.v. catabolism as fraction of i.v. protein mass $= A_1b_1 + A_2b_2 + A_3b_3$

Total rate of loss i.v. pool to e.v. pools $= (A_{11} - A_1)b_1 + (A_{12} - A_2)b_2 + (A_{13} - A_3)b_3$

I.v. radioactivity as fraction of dose $= A_{11}e^{-b_1t} + A_{12}e^{-b_2t} + A_{13}e^{-b_3t}$

Total body radioactivity as fraction of dose $= A_1e^{-b_1t} + A_2e^{-b_2t} + A_3e^{-b_3t}$

The first equation states that the intra-vascular catabolism — that is the part of the catabolism which takes place from the intra-vascular pool only (even if there is extravascular catabolism) can be found from the initial slope of the whole body curve. This equation is independent of the pool arrangement. The second equation shows that the total rate of loss from the intra-vascular pool to all the extra-vascular pools is the difference between the slope of the intra-vascular specific activity curve at time 0 and the slope of the total body activity curve at time 0.

Table V shows some equations which are also independent of pool arrangement but which do assume that the newly synthetised protein enters the intra-vascular pool. The first equation gives the total catabolism expressed as a fraction of the intra-vascular mass and this is just the usual equation for the catabolic rate by

the compartment analysis. If there are more than three pools more terms are added corresponding to the number of exponentials.

TABLE V. — Equations for protein studies based on compartment analysis but independent of pool arrangement. Assumption required : newly synthetised protein enters intravascular pool.

$$\text{Total catabolism as fraction of i.v. mass} = \frac{1}{\frac{A_{11}}{b_1} + \frac{A_{12}}{b_2} + \frac{A_{13}}{b_3}}$$

$$\text{Total protein mass as fraction of i.v. mass} = \frac{\frac{A_1}{b_1} + \frac{A_2}{b_2} + \frac{A_3}{b_3}}{\frac{A_{11}}{b_1} + \frac{A_{12}}{b_2} + \frac{A_{13}}{b_3}}$$

$$\text{I.v. radioactivity as fraction of dose} = A_{11}e^{-b_1 t} + A_{12}e^{-b_2 t} + A_{13}e^{-b_3 t}$$

$$\text{Total body radioactivity as fraction of dose} = A_1 e^{-b_1 t} + A_2 e^{-b_2 t} + A_3 e^{-b_3 t}$$

The second equation is for the total protein mass as a fraction of the intravascular protein mass, and this is obtained from the whole body radioactivity curve and the intra-vascular radioactivity curve and again is independent of the arrangement of pools.

L. DONATO : Thank you Dr. Matthews. There is a point that I would like to stress. Dr. Nosslin has discussed methods for measuring total mass of protein and catabolic rate which are independent of compartmental assumptions. But Dr. Matthews has just shown that total protein mass and catabolic rate can be derived just as accurately using multiexponential analysis. This is something that is very important to stress : some of the constants that may be calculated from labeled protein studies, such as size of individual extra-vascular compartments and individual exchange rates, strictly depend on the model assumed and do not necessarily have physical meanings. On the other hand, constants like total protein mass or catabolic rate, are not model-dependent, and can be obtained by various approaches. Dr. Nosslin has indicated how the significant constants can be obtained from the integrals of the curves. The same integrals enter in Dr. Matthews's equations, in term of intercepts and slopes of the exponentials in which the curves have been resolved. The problem is then whether a good fit of the experimental curve can be obtained using a sum of exponentials. If it so, then it is immaterial how the integrals are obtained. Do you agree on this Dr. Nosslin ?

B. NOSSLIN : Yes.

G. SEGRE : I would like to add a comment on compartmental versus non-compartmental analysis. If one fits a curve for instance by three exponentials, then one could obtain some information in addition to that deduced when the assumption

of compartmentalization is dropped. This information refers to the transfer constants of the model; by means of these transfer constants one might, for example, compare the clinical abnormal cases to the normal ones. The compartmental analysis in this case affords numbers which may be of value diagnostically. If one avoids the assumption of compartmentalization, then some information that might be of significance also from a practical point of view is lost.

L. DONATO : Thank you Dr. Segre. I am sure we will come back to this point.

The methods that Dr. Nosslin has discussed, and on which Dr. Matthews has commented are independent of assumptions on the system characteristics. They still require that the curves be entirely described since one has to calculate integrals from zero to infinity. This demands, in turns, experiments of long duration so that the final slopes of these curves are sufficiently well-defined to permit carrying out the extrapolation with minimal error. I would like Dr. Vitek to present us briefly another possible approach that might have the advantage of shortening the duration of the experiment.

F. VITEK : There is a possibility of arriving at the complete definition of the system (total extra-vascular pool, total intra-vascular pool and total transfer rates) under steady state conditions, in a time shorter than that necessary when analysis is based on the direct observation of the time course of activity. This possibility arises when one considers that the extra-vascular activity which can be calculated with the usual method can be treated as the convolution integral of the plasma activity and the transfer function which can be defined as net activity transfer function from plasma to extra-vascular space [12].

In Table VI, M_p and M_e denote total plasma and extravascular albumin, S the rate of albumin transfer from one to the other and viceversa, $P(t)$ and $E(t)$ are activity in plasma and extravascular space. The transfer function $G(t)$ is obtained by an analogue computer and it contains all the information required for the definition of the system except those concerning the degradation rate.

The method is valid whatever the arrangement of pools. The obtained transfer function obviously depends of the exchange constants and also of the initial conditions and initial distribution of the tracer. If at zero time no activity is present in the extra-vascular space the transfer function has some very important properties.

Its initial value equals the overall fractional efflux from plasma to the extravascular space, as it is seen in equation 2. The value of its integral from zero to

TABLE VI. — Deconvolution analysis of plasma protein turnover studies.

$$E(t) = \int_0^t P(\cdot)\, G\,(t - \tau)d\tau \qquad (1)$$

$$G(o) = S/M_p \qquad (2)$$

$$M_e/M_p = \int_0^\infty G(t)dt \qquad (3)$$

infinity equals the ratio of the extra-vascular to plasma albumina pool. Considering that the rate of degradation is obtained by a different approach the system is then, in our meaning, completely defined.

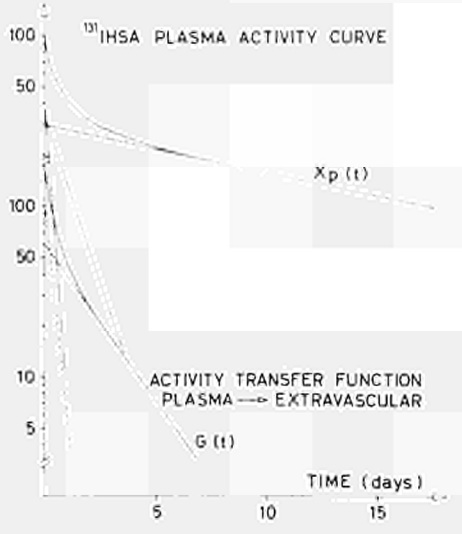

FIGURE 14. — Transfer function of radioactivity from plasma to extravascular space as determined by deconvolution

Figure 14 compares the time course of activity in plasma $Xp(t)$ and the calculated transfer function supposing two extra-vascular compartments. It is quite evident that the transfer function attains a constant rate of change much earlier than plasma activity. This is obvious because the effect of degradation does not come into play here. I would like to state that the case shown in this slide is a patient with nephrosis in which, due to the loss of plasma albumin and increased degradation rate, a constant rate of change of plasma activity is attained relatively soon. The difference between the time course of the transfer function and that of the plasma curve will be greater in a normal person. Supposing intra-vascular breakdown of serum albumin, the catabolic rate can be determined from the transfer function from the blood plasma into the urine. It is also possible to determine this value after repeated injections of the same tracer.

L. DONATO : I think it is time we stop for a moment the discussion between ourselves, and then I will ask Dr. McFarlane to come here and speak.

A. S. MCFARLANE : First of all Dr. Donato, I want to congratulate you on the organisation of this symposium, it has been quite delightful to listen, but I did get a little alarmed that time was getting on and we would not be allowed to ask questions.

What I wanted to ask about was in relation to the relative merits of compartmental analysis and the methods of Nosslin. In the first of Nosslin's methods, it is

apparently critically important that the curve must be followed until a final constant slope is reached. Now I think in practice this imposes too stringent a condition on the preparation of the labelled protein and on other matters. Once the retained dose in the plasma falls below 20 % of the injected dose — this is a matter of practical experience — the slope often becomes unreliable. It may be due to traces of other labelled proteins, it may be due to the thyroid beginning to put out labelled substances. But if that is a limiting condition, in your method, I think it is too stringent.

It is my impression, but this is a point I would like to be corrected on if I am wrong, that all methods of compartmental analysis also depend on a reliable terminal slope and therefore just at the moment I am immensely attracted to your last method in which the determination of the total body pool, which I think is one of the most critical measurements, is made from the ratio of the areas of the retention and plasma curve. Now if we measure those areas say up to a time of 20 % retention in the body, would that be, from your point of view, a satisfactory procedure and would we be free of the kind of errors that I have mentioned, which arise in the later stages ?

B. NOSSLIN : Concerning the first question about the importance of the final straight line, I think you have given the answer yourself. It is quite correct that this method depends upon a complete description of the curve to time infinity which means that one has to do an extrapolation using the final straight line. So far this technique depends on it just as much as all other techniques using final slopes, or slopes at all. That means the Sterling technique, all other techniques based on final constant rates, and so on, and also the compartmental analysis. All techniques requiring extrapolation are on an equal base and the error will be exactly the same. In other words there is no further critical dependence on extrapolation in my technique; I agree with you that in cases where for some reason the final straight line is not attained, then one has to choose other methods such as the equilibrium time method, the U/P or the U/(P + E) ratios which also give the visual impression from day to day on what is actually going on.

Concerning the second question about the other technique using areas under whole body curve and plasma curve, my answer to your question is, unfortunately, no — from a strict mathematical point of view — but in practice of course, the more you know the better is your estimate. So the longer the time you can go on measuring the more you come near to the correct value, but from a strict mathematical point of view it is the same situation as with a plasma curve, since you need to do extrapolation from the final slope.

L. DONATO : I know that Dr. Matthews would like to add a comment to Dr. McFarlane's question.

C. M. E. MATTHEWS : I only just wanted to say I agree with Dr. Nosslin, I think this is a basic difficulty in all methods of analysis. If you have only the first

part of the curve, you just have not got enough information to distinguish between different results and the longer you go on, as Dr. Nosslin says, the more information you have and the more accurate will be the results.

I think Dr. Vitek's method is interesting. I have not studied this method yet but it apparently does not seem to require quite such a long experiment as the other methods.

L. DONATO : I just want to add one further comment that might be of interest. These methods are very similar to those that have been used for a very long time in circulation studies. The mass of the protein is obtained actually multiplying the mean flow of the substance — in this case the rate of synthesis or breakdown of the protein times the mean circulation time — through the entire protein mass; and what Dr. Nosslin is deriving with his equation is exactly the mean circulation time through the whole system.

R. YALOW : Whether or not estimates from the intercept of the final slope of labeled protein disappearance curve represents an over- or an under-estimation depends on whether or not the degrading compartment is in rapid or slow equilibrium with the plasma pool [4]. In the former case the proper extrapolation should have been one with a curve concave upwards as it approached its ordinate, in the latter the extrapolation should have flattened as it approached the ordinate.

Actually although the general assumptions did not state this, the mathematical analysis presented today has assumed that degradation takes place in a compartment in rapid equilibrium with the plasma compartment, an assumption validated by previous experimental information [14]. It should be emphasized that the degrading compartment need not be and, almost certainly, is not the plasma pool itself.

A second comment concerns Dr. Matthews presentation concerning the significance of equilibration in the iodide pool. Her work shows very nicely the value of computer techniques in performing the often difficult calculations introduced by sophisticated mathematical analysis. However, we should not let this ease of mathematical manipulation blind us to some pertinent physiologic facts. In the normal subject, where catabolic rates average about 3 ½ %/day and iodide excretion rates average almost 200 %/day, the renal excretion curve becomes virtually identical with the iodioalbumin degradation curve after the first day or so and the error introduced by equating the two is generally insignificant [2].

Next I do want to remind those concerned with compartmental analysis of the debt we all owe to Sheppard and Householder [15] whose elegant analysis is basic to many of the problems still under consideration. Furthermore I am sure that Dr. Matthews did not mean to imply that her work in 1957 was the first general analysis of 2-3 compartment models in the context of protein turnover. I should like to refer her to some earlier publications in which a number of these problems were analyzed and experimental studies validating the analyses were described in detail [14, 16, 17].

L. DONATO : Thank you Dr. Yalow for your very interesting remarks. I would like to take just one for myself, that is the historical one.

I entirely agree with what you said about Sheppard and Householder but I would like to make another historical reference — even further away. Most of the methods that were covered today could be easily derived from the Stewart-Hamilton principles currently used in circulation studies for the determination of blood flow and circulating blood volumes from the integrals of indicator curves.

Dr. Yalow has posed a series of questions, and I think that Dr. Matthews should comment on them.

C. M. E. MATTHEWS : I think I agree with most of Dr. Yalow's remarks. However, we did not intend to give a historical review, and the references are intended only to identify specific methods of analysis.

As regards the iodide curves which I gave to show the effect of the iodide excretion, I quite agree that under normal circumstances with normal catabolism of human albumin the error is very small. However, these curves were intended to show that the error could be large in certain circumstances, either with proteins where the catabolic rate is very much higher than that of albumin or where the total loss from the plasma pool is greater or where the iodide excretion rate is reduced for example due to impaired kidney function.

I think I agree with most of the other remarks for cases where the catabolism is in the slowly equilibrating pool, but in fact all the evidence seems to indicate that this in fact is not so therefore we have not considered models in which the catabolism might be in the slowly exchanging pool for plasma proteins. On the other hand catabolism in a rapidly exchanging extra-vascular pool would give almost the same results as catabolism actually in the plasma pool.

L. DONATO : I would like to add a brief comment of clarification concerning the values that have been reported for the catabolic rates. I think that Dr. Yalow refers to the catabolic rate as a fraction of the total protein mass, while the values reported by Dr. Matthews refer to the intra-vascular mass only. This explains the numerical differences. I will call now on Dr. Pavoni.

P. PAVONI : All the methods that have been discussed here assume the attainement of a final straight line. However, the problem is not only that of continuing the experiment until the straight line is attained, but also that of obtaining values actually describing a straight line. Since during the last part of the experiment the radioactivity is very low, random errors play an important rôle : as a consequence, one is confronted with the problem of *interpolating*, and not simply of connecting the last points. Doubts may arise as to which points and how many of them should be considered in order to draw the straight line.

I would like to pose the following question : if the number of compartments and the biological behaviour of the system is unknown, which are the criteria and the methods followed by Dr. Donato and the other members of this panel in the

choice of the points for interpolation of the straight line, as well as in the choice of the type of model and of the number of compartments which are obviously influenced by the selected straight line. I would like to known if other mathematical procedures are available besides those that we published in 1962 [18]. Thank you.

L. DONATO : The problem posed by Dr. Pavoni is a technical one, strictly connected with the error of the experimental points. The interpolation is always somewhat arbitrary, whence the importance, for the methods based on the calculation of the integrals, of carrying on the experiment as long as possible, in order to minimize the contribution of the extrapolated part of the curve. As to methods for interpolation, I can only recall the classical techniques for best fitting, like the least square method. As it has been already pointed out, most of the methods depend on the description of the final course of the curves; it is obvious that no result will be better than the data from which it has been calculated.

The method discussed by Dr. Vitek may perhaps overcome some of these difficulties, since the calculation is based on the first part of the curve : the practical value of the method, however, is still to be demonstrated.

G. SEGRE : May I add some comment on the question of Dr. Pavoni ? Particularly when highly scattered experimental points are obtained there is a need for a criteria to evaluate the precision of the calculated parameters of the model. First of all every point ought to be weighted in the fitting process; this cannot be practically done by hand computation, or even by the analog computers, but only by digital computers. The scattering of the point from the theoretical curve gives high standard deviations of the calculated transfer constants of the system. These considerations are also of some relevance with respect to the problem raised by Prof. McFarlane : every truncated curve introduces an error into every value computed by means of that curve, either by using the method of Nosslin or by using the compartmental method. It is therefore necessary in any case to use a computing method that affords the standard deviations of each calculated parameter.

L. DONATO : Thank you Dr. Segre. Dr. Nosslin.

B. NOSSLIN : This is a very important question and it is often asked and I would like to summarise my view on it in the following way.

If you have two curves, both the plasma curve and the total body curve then there are methods which give correct, or almost correct, estimates of total size and total catabolism which are not very dependent on the final straight line : the deconvolution or other methods as we have discussed. But if you have only the plasma curve then there is no, and will never be, any method giving these correct estimates unless you know also the final slope; since we require the total area under the curve, the total form of the curve, then it is obvious that we will always need to know the final slope, and it will never be possible to calculate these parameters from just part of the plasma curve only. If the curve does not show a straight line then we have to use the final tangent and then we also have to be aware that we may be

under-estimating the area with a consequent influence on the values which we obtain. Another way of saying this is that it is very important, whenever you can, to measure not only plasma disappearance curve but also whole body or excretion. Thank you.

L. DONATO : Thank you Dr. Nosslin. Unfortunately we are running short of time, but before we have some more discussion I would like Dr. Segre amplify his statements concerning the use of computers in this sort of studies.

G. SEGRE : It is impossible even to summarize the topic in few minutes. The applications of the digital computers to the problems discussed in this round table will undoubtedly increase in the near future; the method of hand computation will be superseded because it is time consuming, does not give indications of the statistics of the parameters and it is expensive.

Programmes for digital computers are now available; the most versatile programme is that of Berman[19,20]; the fitting of a multi-exponential curve to a compartmental model, by using this programme and an IBM 7094 costs abouts 2 $. I think that every university in the next few years will have a computing center to which the biologists and the clinicians will have access; a good example of this trend is given by the Medical Clinics of the University of Pisa which works in connection with the computing center recently opened in Pisa.

The most important task for a research worker, before to procede to fitting the data to the model, is that of model building and of its validation.

In this respect there are three general approaches : the first is the multiexponential analysis, which would afford the number of compartments. The second is the determination of the transfer (or the weighting) function between two subsystems of the system, by calculating the deconvolution between them; the third method is given by the analysis of the precursor's order between two points of the system. All these preliminary steps are carried out by hand computation, but for the deconvolution there are programmes for digital computers (Phillips, of the Argonne National Laboratories).[21] The third method gives a quick indication on the number of arms which connect two compartments of the system[22]. If there is one arm only, then the weighting function between the two compartments is equal to

$$G(t) = A\, e^{-at}$$

and

$$\lim_{t \to 0} G(t) = A \neq 0;$$

in this case the two compartments are adjacent.

When

$$\lim_{t \to 0} G(t) = 0,$$

then the two compartments are not adjacent and at least one other compartment is placed between them.

If the above limit is equal to zero and

$$\lim_{t \to 0} dG/dt \neq 0$$

then the number of arms which connects the two compartments is equal to two, and so forth.

Also the analog computers can be of great help in this stage of model building.

After this stage has been performed, the approximate values of the parameters one is looking for are calculated; these values together with the experimental data are given to the computer. The Berman's programme includes many options and a great number of subroutines, and among the other problems, the following which are of interest for compartmental analysis may be processed :

curve fitting to a multiexponential function; the programme provides the standard deviations of the computed parameters (the exponents and the coefficients of the exponential terms), as well as the differences and the ratio of the observed and the calculated values, their plot and the sum of the squares of the differences between the observed and the calculated values;

the mapping, that is the solution of the previously discussed matricial equation

$$|k| = |A|\ |a|\ |A|^{-1};$$

the direct solution of the model, that is the computation of the transfer constants (with their standard deviation) directly from the experimental data.

All these subroutines employ a least squares method and an iterative procedure; a correlation matrix of the computed parameters is also provided by the programme.

L. DONATO : Thank you Dr. Segre. Before concluding I would like to ask the audience if there are more questions on the subjects that have been discussed. Dr. Andersen.

S. B. ANDERSEN : It would be interesting to hear a few words about which factors influence the error in the Berson and Yalow method of determining the total pool in comparison with the factors that determine the magnitude of the error in the Sterling method.

C. M. E. MATTHEWS : The factors are similar to the factors in the Sterling method: both methods basically assume that the specific activity is the same in the extra-vascular and in the intra-vascular pool and therefore they will only give the correct result when the exchange rate between the pools is very rapid compared with the catabolic rate, but not where this ratio is smaller.

L. DONATO : Would you like to comment on this Dr. Yalow ?

R. YALOW: The metabolic clearance method([1]) [metabolic clearance rate = *urinary excretion rate/mean plasma sp. activity* [16]] is generally applicable when degradation

([1]) Method 3 of Table III (No. E).

takes place in a compartment in rapid equilibrium with the plasma — the real physiologic situation in all experimental cases so far studied — and when the system is in what one can call quasi-static equilibrium i.e. when changes in the steady state conditions are slow. It would not be applicable if the specific activity in the degrading compartment were significantly different from that of plasma. The power of the metabolic clearance method is that it does not require long term sampling; for example, in the case of iodoalbumin it is sufficient to take, at 8-10 days, two plasma samples, 24 hours apart, and a single complete urine collection during this interval to get a reasonably good estimate of absolute catabolic rate. Absolute catabolic rates for iodoalbumin obtained using the metabolic clearance method do not differ significantly from that obtained by method 2, the one we used in our earlier work.

B. NOSSLIN : I would like to say that as far as absolute catabolic rate is concerned, the estimate with the Berson and Yalow technique is correct if there is intravascular catabolism or the catabolism is in a compartment in close connection with the intra-vascular compartment. It is not the same with the Sterling absolute rate because that is always giving an overe-estimate. Therefore the error is greater in the Sterling method. As far as fractional values are concerned, I can only repeat that the fractional catabolic rate is an under-estimate and the total exchangeable pool is an over-estimate but when you multiply the two, the errors exactly cancel in the Berson and Yalow method.

The total pool is always over-estimated as soon as there is a higher specific activity in the extra-vascular compartment. That is a question depending on the site of synthesis but if we assume that synthesis is intra-vascular there will always be higher specific activity in the extra-vascular space. This exchangeable mass is correct as far as the tracer is concerned, but unfortunately the distribution of the tracer and that of the mother substance are not the same because the mother substance is given as a continuous infusion while a tracer is given as a single shot and they do not end up with the same distribution ratio.

L. DONATO : Thank you.

I think we have to conclude this discussion, it could profitably go on for hours.

I would like to make a few remarks and I will start from Dr. Yalow's consideration that she rather likes methods that do not involve complex mathematical elaboration. I am sure that we all fully agree with her. I think it has been shown by this discussion that in fact there are reliable methods that do not involve too much mathematical elaboration of the data to provide useful data.

I think that we should make a strict separation between the constants that are inherent in the system (absolute catabolic rate of the system and total system mass) and those constants that strictly depend on the type of model assumed. Exchange rates between compartments and number of compartments will obviously depend of the type of model assumed, although Dr. Matthews has shown us an interesting correspondence between a given compartment and some parts of the body.

I would like to point out that as far as the fractional catabolic rate measurement is concerned the U/P ratio seems to stand quite strong. Of course there are limitations and possible sources of error, and I would like to emphasize once more the importance of the iodide kinetics, which should be looked at from two points of view : one is the size of the iodide pool and the other are the conditions of the kidneys, because an impairment of renal function will delay the excretion of iodine from the body thus resulting in possible important errors. As far as the evaluation of the protein mass is concerned it is quite evident that adequate methods are available, be they based on the direct computation of the areas of the curves as such, or on a preliminary multi-exponential fitting. They can provide us with the information we want, provided we can accurately describe the entire area : their limitation in fact is the accuracy with which we can obtain the full area and particularly the final slope. This, of course, bears directly on the duration of the experiment and on the accuracy of multi-exponential fitting. From the former point of view, I would like to point out that, if practical verification will be successfull, the method that has been described here by Dr. Vitek could be an interesting one on clinical grounds. From the point of view of multi-exponentail fitting of curves we have heard from Dr. Segre that computers can provide a much more rapid and efficient tool than simple peeling- off exponentials.

I think we can conclude feeling fairly relieved about the availability of relatively simple techniques to handle our problems. The possibility of measuring the catabolic rate and the total mass is probably the maximum that we should expect from present analytical techniques, since any additional information that we would like to derive from our experiments probably demands too many assumptions to be warranted on the basis of the available knowledge.

Although I regret it, I think that we have to conclude at this time, and I would like to thank all the members of this panel for the effort they have made to simplify their way of reasoning, and with them I would also like to thank the audience for its patience and contribution to the discussion.

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ROUND TABLE ON PROSPECTS IN RADIOIOMMUNOASSAY OF HUMAN GROWTH HORMONE AND PARATHORMONE

Chairman : G. MILHAUD, Institut Pasteur, Paris

Participants :

H. BRAUMAN, Institut Bordet — Bruxelles

P. M. COTES, National Institute for Medical Research — London

J. P. FELBER, Clinique Médicale Universitaire — Lausanne

P. FRANCHIMONT, Hôpital de Bavière — Liège

A. E. FREEDLENDER, Boston City Hospital — Boston

F. C. GREENWOOD, Imperial Cancer Research Fund — London

A. LUYCKX, Hôpital de Bavière — Liège

A. S. MACFARLANE, National Institut for Medical Research — London

R. MICHEL, Collège de France — Paris

J. T. POTTS, National Heart Institute — Bethesda

H. J. QUABBE, Universitätskliniken — Berlin

U. STEWART, Universitätskliniken — Frankfurt/Main

R. S. YALOW, Veterans Administration Hospital — New York

G. MILHAUD : To open the discussions at this round table on radioimmunoassay of growth hormone and parathormone, I should like to try to place the problem on a wider perspective.

In the early days of endocrinology the existence of the chemical messengers — what hormones are — was revealed by a particular physiological effect. A suitably chosen physiological effect was used to follow the purification of the hormone, which is the necessary step in the establishment of the chemical composition. As soon as the hormone was obtained in a pure or relatively purified state, the biochemical mechanisms responsible for hormone action were investigated. In the era of classical enzymology, detailed descriptions were given of the variations in the activity of numerous enzymatic systems under the influence of insulin, of growth hormone, of adrenalin and more recently, of parathormone. In the days of messenger RNA it was logical to attempt to relate hormone activity with the synthesis of enzymatic proteins. Consequently, in the case of growth hormone, parathormone and insulin, actinomycin D was used to see wether or not such particular biochemical effects were or not dependent on a synthesis or activation of enzymatic systems. In the case of parathormone the increase in calcemia is suppressed by the administration of actinomycin D. Furthermore, we can attempt to investigate the mechanism by which serum calcium drops after parathormone-induced hypercalcemia. In thyroparathyroidectomized animals it could be assumed that at a given threshold, hypercalcemia initiates the induction or activation of a calcemia-decreasing system, this being antagonistic to the calcemia-increasing system. In this case the administration of actinomycin D at the moment of the drop in hypercalcemia ought to inhibit the activity of the hypocalcemia-producing system, and the hyper-

calcemia should either be higher or longer. But this is not so, and actinomycin D depresses always the hypercalcemic response to parathormone. The hypercalcemia is neither intensified nor prolonged, and this leads to the assumption that the progressive lowering of the hypercalcemia is due to inactivation or elimination of parathormone itself [1].

However it may be advisable to consider the problem from another point of view, namely the relation between the hormone and the tissue receptor. Now if it is not at present possible to study the behaviour of the hormonal tissue receptor or receptors, we can at least study the variations in circulating hormone levels. This initial step can be accomplished either by means of biological assays or radioimmunoassays. The former are frequently less sensitive than the latter, which from the technical point of view, however, are often rather difficult to perform properly. Therefore I thought it desirable today to compare the experience of those who are mastering radioimmunoassays of two bone-acting hormones, namely parathormone and growth hormone, with the difficulties encountered by certain scientists, with the intention of promoting the development of radioimmunological techniques in Europe. I believe that this development has been delayed because very few biochemists can carry out radioimmunoassays by using only the published techniques : certain reagents are difficult to find and it is necessary to know certain tricks which are essential to the success of one step or another of the assay. These difficulties must consequently be overcome, and this round-table conference will have achieved its purpose if it makes possible a transfusion of technical know-how from highly qualified donors to recipients who are greatly desirous of becoming donors in what we hope will be the near future. The following four main subjects will be discussed :

1) Availability of highly purified growth hormone and parathormone,
2) Preparation of ^{131}I-labelled hormone of high specific radioactivity,
3) Preparation of antisera,
4) Assays techniques.

First I would like to ask Dr. Stewart to tell us something about the preparation of human growth hormone.

U. STEWART : For metabolic purposes and for the treatment of pituitary dwarfs we need large amounts of human pituitary growth hormone. We were thus forced to fractionate our own postmortem collected glands. Of all the procedures offered we most favoured and most successfully applied the one described by Gemzell *et al* [2]. The fresh frozen glands are extracted under physiological conditons with phosphate buffer. The active principle is then precipitated by the addition of ammonium sulphate and purified on Sephadex G 100 columns. From 100 g of fresh glands we obtain between 4 to 5 g of highly purified human growth hormone which contains neither serum protein impurities nor heme degradation products. The hormone was assayed in the laboratory of Dr. Li in Berkeley and had high biological activity in the

tibia test. With rabbit-antiserum against HGH we obtained a single antigen/antibody precipiting band. In starchgel electrophoresis, both in the conventional system of Smithies and in the modified system of Ferguson and Wallace, we have some heterogeneity. One major band is preceeded by a very faint more anodal band, both fractions migrate in the postalbumin region. On cellulose acetate as well as in disc-electrophoresis we observe a similar pattern. For the purpose of labelling with ^{131}I, we removed, about 8 to 10 % of aggregated protein by the procedure recently suggested by Hunter [3], namely filtration on Sephadex G 200 in alkaline buffer. The eluted active fraction is immediately employed for radioiodination without dialysis or freeze-drying. We obtained growth hormone with a specific activity of 150-300 mC/mg. A small sample of this iodinated hormone reapplied on Sephadex G 200 under the conditions of Hunter, elutes as a very symmetrical peak. Nervertheless, this material still shows unspecific binding to normal human serum. This problem will be dealt with in this panel.

G. MILHAUD : Thank you. There is also the good news which consist in the fact that you may not need to go through all the procedures described and I think that Dr. Cotes can tell us something about availability of highly purified growth hormone.

P. M. COTES : It has already been emphasised that the reference standard is an important reagent in any radioimmunoassay system. Ideally, this standard is derived from the same species as the hormone being estimated and after extraction and purification it should behave in the same way as the native hormone in the assay system. The ideal standard is not always easy to make and extensive testing is frequently necessary to establish the suitability of any one preparation to serve as a reference standard. The World Health Organization already provides a service by preparation and distribution of international standards of many hormones for bioassay purposes. It is proposed that this service should be extended by provision of international reference preparations, suitable to serve as radioimmunoassay standards, for some hormones which until recently could only be estimated by bioassay procedures. By international use of these services :

— Bioassay results can be expressed in international units which are identical from one laboratory to another. Radioimmunoassay results can be expressed in terms of (mass of), a single internationally accepted preparation. The suitability of each of these preparations for use as a standard or reference preparation is determined by international collaborative studies.

— When scarcity of certain biological reagents means that they must be used with the utmost economy, characterisation of these materials can be carried out in a few expert laboratories. Material needs not be wasted by replication of processing and testing in many centers.

— Continuity of units is established since, on replacement of an international standard or reference preparation, the new standard is calibrated in terms of the old by international collaborative assay.

The primary purpose of WHO international standards is for calibration of national and laboratory working standards. Certain international reference preparations may be available in limited amounts for use as laboratory working standards.

Batches of human growth hormone and human insulin have already been set aside by WHO for provision of international reference preparations for radioimmunoassay purposes.

The human growth hormone consists of highly purified hormone extracted from pituitaries collected by the British Medical Research Council and purified as part of a larger batch by Prof. A. E. Wilhelmi in the United States. Its suitability to serve as a standard has been studied in several laboratories including that of Dr. Yalow and Dr. Berson. Dr. Yalow is going to report some characteristics of a sample from the same batch of growth hormone from Prof. Wilhelmi. Tests on the final preparation will include studies of its long-term stability.

It is expected that by the end of the year the International Reference Preparations of Human Growth Hormone and Human Insulin will be available as ampoules containing approximately 200 micrograms of hormone, with 5 milligrams of sucrose, freeze-dried and filled with nitrogen. Ampoules may be obtained by writing to : The Director — Division of Biological Standards — National Institute for Medical Research — The Ridgeway — Mill Hill — London, N. W. 7.

It is hoped that these particular International Reference Preparations will be suitable to provide material for use as radioimmunoassay standards and for iodination. They will not be available for immunisation purposes.

F. C. GREENWOOD : In 1961 we developed a method for the routine preparation of high specific activity labelled hormones for use in *in vitro* radioimmunoassays. A preliminary note was published in 1962 [4] and a full publication in 1963 [5]. In the method some 2 mC of carrier-free ^{131}I are reacted with 5 μg of protein in the presence of excess chloramine T (50-150 μg). The reason for the excess chloramine T is not known. Then chloramine T is added to the mixture of isotope + protein. Metabisulphite is added to stop the reaction and should be added rapidly since Drs. Yalow and Berson have obtained evidence that delayed addition causes increased damage. Carrier potassium iodide was then added to insure complete recovery of unreacted iodine from a subsequent dextran column. This addition is not necessary if the reaction mixture is to be purified on starch gel.

We have carried out routine iodinations since 1961 and the method has been applied to a large number of hormones. The method was reliable until 1964 but we have subsequently obtained lower yields (30-50 % with an occasional 70 %)

using isotope samples from Amersham. Drs. Yalow and Berson, however,obtain yields from 80-90 % using isotope samples from Isoserve. In our experience damage to the hormone is more often present than not.

In our experience we feel that the cause of low yield and damage is due to unknown variations in the isotope samples. Dr. Yalow has indicated a correlation between percentage yield and the specific activity of the isotope samples. The development of a method to routinely measure the percent atoms excess of ^{131}I in the isotope samples by Drs. Yalow and Berson is extremely important. However, I would also agree with Dr. Yalow that there is still an additional factor responsible for damage which was not present in isotope samples from Amersham in the period from 1961-1964.

At the present time, therefore, purification of the labelled hormone must be considered essential although we feel that labelled hormone purified from a damaged sample is less satisfactory than a labelled hormone obtained undamaged.

Purifications of labelled hormones have been described using starch gel, Sephadex G 200 and 100 with or without added plasma and cellulose columns. We have found the cellulose columns of Yalow and Berson extremely effective for the purification of ACTH and we will certainly try their method for growth hormone using cellulose columns and elution with 20 % acetone in buffer.

A corollary of purification is that screening for damage is essential. In our opinion only the chromatoelectrophoretic method is available for this and this has predisposed us to use chromatoelectrophoresis for the subsequent assay. Cellulose acetate electrophoresis is less satisfactory for the detection of damage but we have retained it for routine growth hormone assays. Subsequent radioimmunoassays for ACTH, human prolactin, human placental lactogen and rat prolactin in our laboratories have used the Berson and Yalow chromatoelectrophoretic system. It must be pointed out that different hormones differ in the ease with which they may be radioiodinated and in their susceptibility to damage. Dr. Hughes has pointed out that the chemistry of iodine is not fully understood and it seems unreasonable, therefore, to expect the radiochemists to be fully aware of what is happening with radioactive iodine.

I would personally be delighted if a better method were available but in the meantime we willingly accept the difficulties involved for the output of 50-100 plasma determinations each week. In conclusion I would say that I would be very delighted if a more adjusted method were available, if we except the present difficulties, for the productivity of 50 to 100 plasma determinations each week with several hormones ?

G. MILHAUD : Thank you. I think that Prof. Michel, who is now in the happy state of having succeeded in getting some growth hormone of high specific radioactivity will tell us something about the time and the experience necessary to come to this point.

R. MICHEL : To iodinate growth hormone we use, with Job and Sizonenko, the method of Hunter-Greenwood after some minor modifications : in particular, we use a more dilute medium by adding two times more phosphate buffer and we

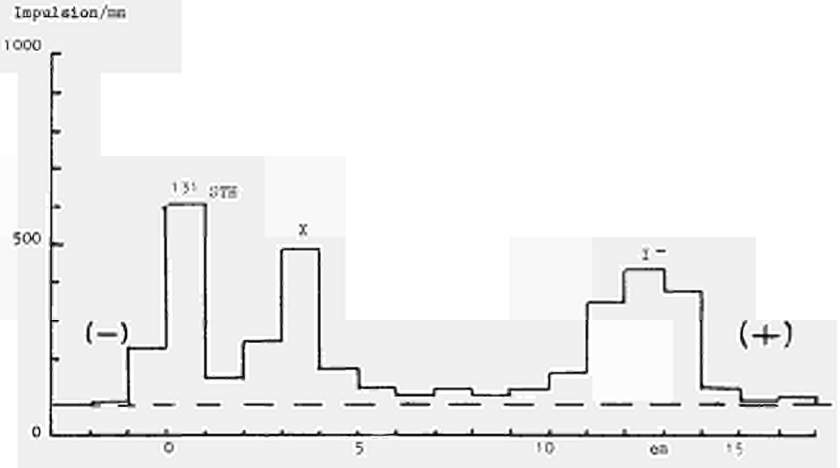


FIGURE 1. — Radioelectrophoretogram of iodination products of a human growth horomone preparation

Abscissa : length in cm; Ordinate : number of counts per minute of ^{131}I; ^{131}STH (labelled hormone); X : (damaged protein); I^- : (iodide).

Whatman 3 MM, 400 V, 1 ½ hour, 0.1 *M* sodium veronal buffer, pH 8.4, yield 43 % specific activity of the hormone, 182 $\mu C/\mu g$.

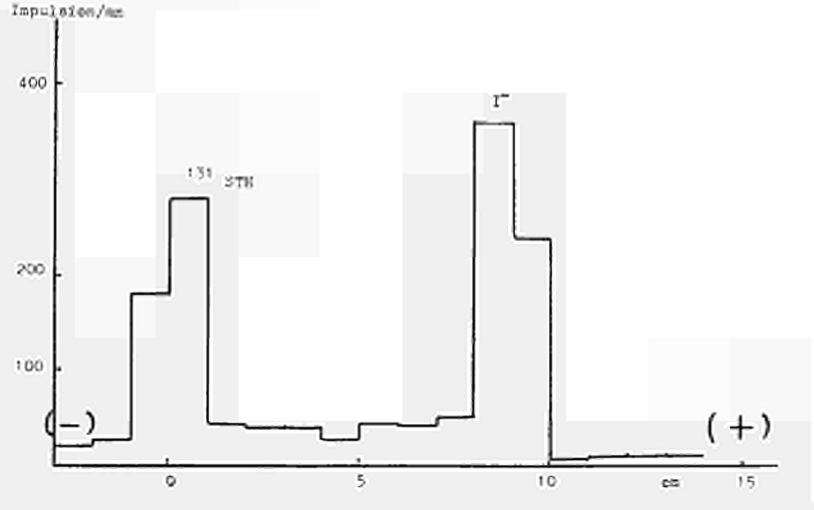


FIGURE 2. — Radioelectrophoretogram of iodination products of a human growth hormone preparation (Same conditions as in Figure 1).

operate at 2° C. As soon as the labelling is performed, a paper electrophoresis is carried out on an aliquot to establish the yield. The results obtained are of two types.

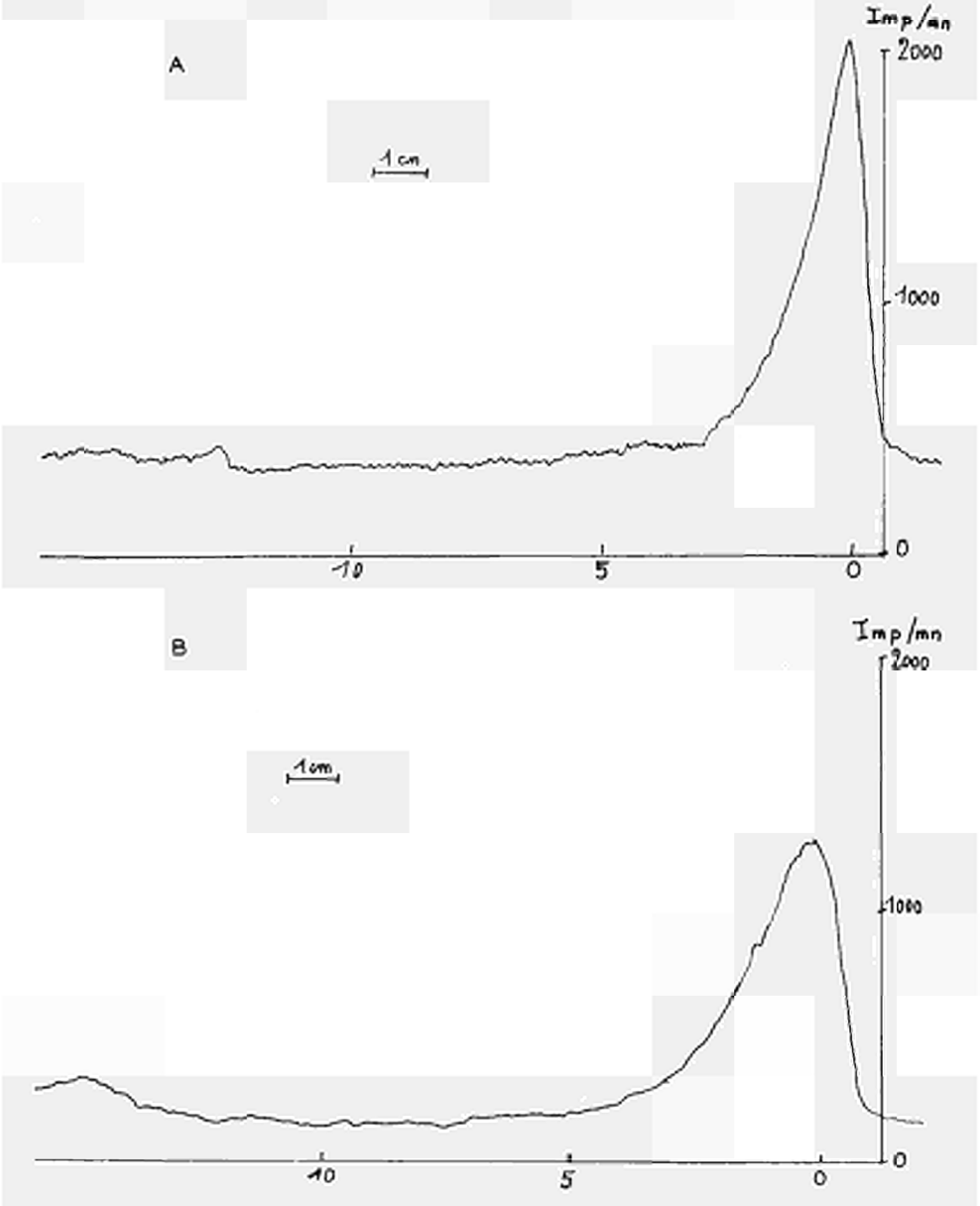


FIGURE 3. — Recordings of two radioelectrophoretograms indicating a preparation of undamaged growth hormone.

A. Immediately after preparation; B. After three weeks of storage at —20 °C (Same conditions as in Figure 1).

In Figure 1 three radioactive peaks are present; one at the origin, which is the labelled hormone, another corresponding to the damaged hormone and a third consisting of iodide.

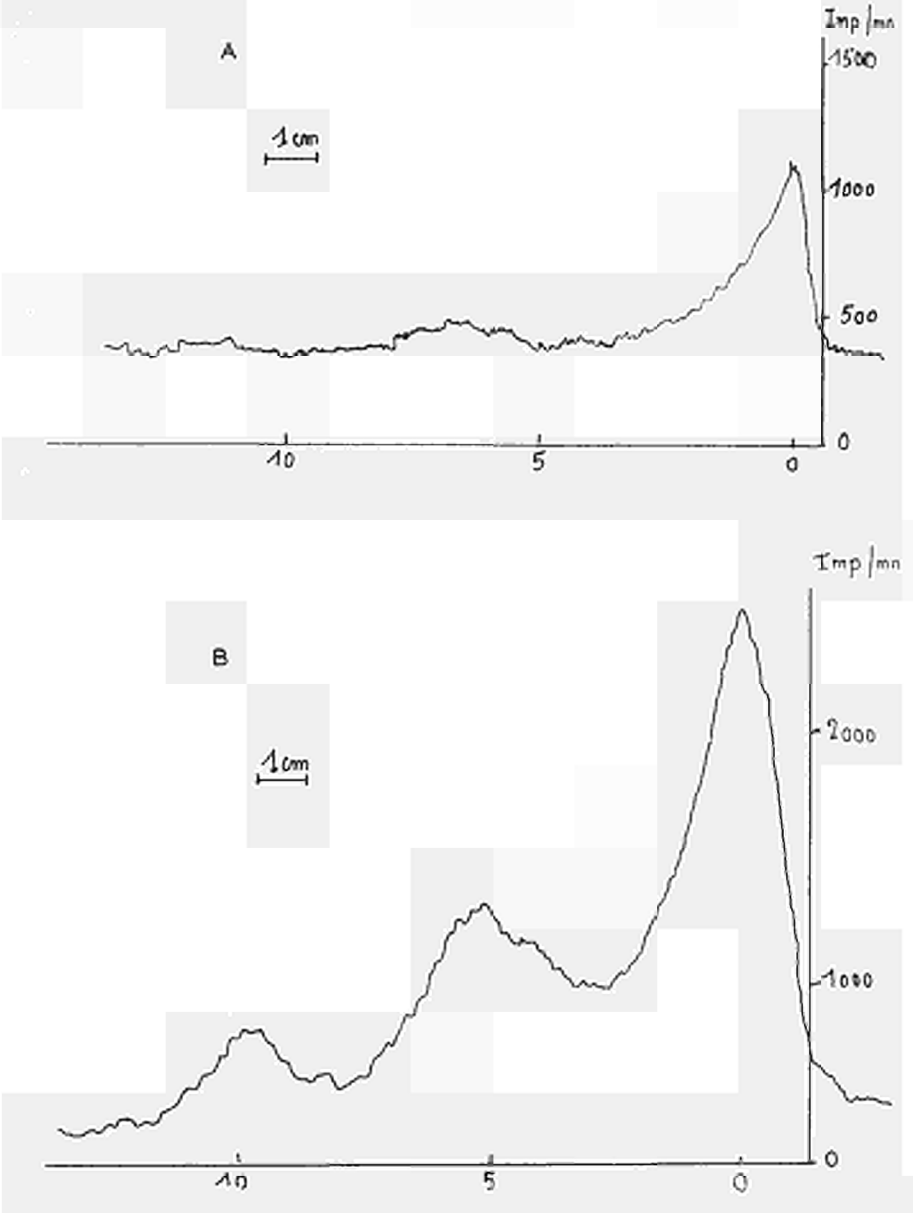


FIGURE 4. — Recordings of two radioelectrophoretograms of a growth hormone preparation indicating partial damage.

A. Immediately after preparation; B. After three weeks of storage at —20 °C (Same conditions as in Figure 1).

In Figure 2, two peaks are left : one remaining at the origin, which is the radioactive hormone and the other corresponding to the iodide.

As the two preparations have been made in exactly the same conditions, the damage may be attributed to two different radioiodine preparations.

After a G 50 Sephadex column the most radioactive eluate is subjected to electrophoresis. The homogeneous preparation stays at the origin. When stored in the frozen state at —20° C this pure preparation is unchanged three weeks later (Figure 3 and other samples of this preparation could be stored without damage for over 40 days).

If, on the other hand, a tube containing a minor amount of damaged hormone (Figure 4 A) was kept under the same conditions for 3 weeks, the damage became very important (Figure 4 B). A kind of autocatalytic decomposition must have occurred. These facts must be kept in mind by the preparation and storage of ^{125}I labelled hormone.

A. S. McFARLANE : In spite of the fact that it is not necessary that the labelled hormone should retain its biological activity for radioimmunoassay, I am sure that everyone here should like to obtain preparations that were less damaged. I think some of the damage which is attributable to chloramine T may in fact come from peroxides in the iodine shipment. I am surprised that the excellent procedure of Bale and his collaborators in Rochester [6] for getting rid of these peroxides, is not used more often. This is relatively simple and is based on the use of sulphite. If one wants to avoid chloramine T and at the same time to make use of iodate activity which is normally non-productive in labelling procedures then it is quite easy to convert the radioiodine into iodine monochloride, simply by adding hydrochloric acid. To do this quantitatively both for iodide and iodate it will be necessary to add a controlled amount of one or the other in inactive form which of course will produce a slight reduction in specific activity. It seems to me that a radioactive shipment treated in this way should be usable quantitatively for labelling purposes, but it will still be necessary to preoxidise any sulphydryl or other easily oxidisable groups in the hormone. Our procedure for doing this is one that Springell used [7] very nicely with insulin, which is to preoxidise with inactive iodine at an acid pH — at which the rate of substitution in tyrosine is very low. If the various hormone experts here are willing to permit this liberty being taken with the hormone then I think it should be possible to get less damaged preparations and more reproducible iodinations than at present.

P. FRANCHIMONT : The purity of the labelled growth-hormone preparation and the preservation of its antigenic properties are suggested by the following observation. On radioautography of immunoelectrophoresis of labelled growth-hormone purified on Sephadex G 200 and the corresponding antiserum a single line is observed. This is an argument in our opinion that labelled growth-hormone

contains only a single type of antigen reacting always with the corresponding antiserum (Figure 5). Furthermore this labelled growth-hormone does not react with a horse serum anti-human serum : no serum contaminants seem to exist.

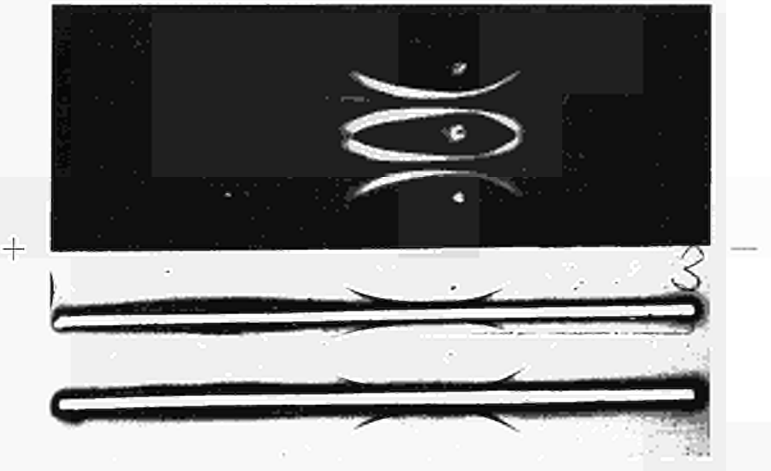


FIGURE 5. — Radioautography of immunoelectrophoresis of labelled growth-hormone

A. E. FREEDLENDER : Fortunately we, along with Dr. Yalow, have been using iodine from Isoserve. We have been iodinating for the past 8 months, with yields between 70 to 80 %. Stability studies on the final labelled preparation done by the method of Hunter and Greenwood have indicated that the preparation seems to be directly proportional to the amount of contamination in the protein used for stabilising. We have had our best results with the crystalline albumin. The material has maintened integrity of 95 % for periods of 3 weeks.

F. C. GREENWOOD : I thank Dr. McFarlane for his comments. Certainly the Springell technique has been shown to be suitable for low specific activity ^{131}I-labelled insulin required for the double antibody method [8]. We considered the addition of sulphite to decompose any hydrogen peroxide formed in iodine-131 samples. We couldn't follow the Bale method of sulphite addition followed by aeration since our isotope samples are only 0.075 ml. We did add metabisulphite and then a calculated amount of extra chloramine T during radioiodination. We got no detectable effect on yield or on percentage damage. If I remember correctly it is quite difficult to remove traces of hydrogen peroxide completely by any chemical method.

G. MILHAUD : Now we can come to the availability of antisera. Antisera can be obtained from two sources. The first one is William Latimer at Johns Hopkins at the National Pituitary Agency and he has rather large supply; antisera are also

commercially available at Mann. Furthermore we have some hope to get some Medical Research Council standards and I think Dr. Cotes could say a word on this.

P. M. COTES : The British Medical Research Council holds a small stock of rabbit antihuman growth hormone serum. This is a high titre serum with good avidity but, by mischance, the rabbit which provided it died after collection of only a small amount. This serum will be diluted and made available to users in the United Kingdom. Unfortunately, there is not enough of this reagent for it to be distributed internationally.

R. S. YALOW : Our laboratory has supplied Dr. William E. Latimer, National Pituitary Agency, 1900 McElderry Street, Baltimore Maryland 21205, with a high energy anti-HGH antiserum in quantity sufficient to assay 1-2 millions of unknown plasma samples. Supplies of this antiserum and directions for its use can now be obtained from Dr. Latimer. When the antiserum supplied to Dr. Latimer is exhausted, we will replenish the supply. However, these antisera are specially selected for high sensitivity for immunoassay and are not in unlimited supply. A suitable rabbit anti-HGH serum can be obtained from Mann Laboratories (136 Liberty Street, New York, New York 10006, Cat *1435* Price $ 15 per ml) for general laboratory purposes, such as testing ^{131}I-HGH for complete binding to antibody or practice in setting up the immunoassay. The sensitivity of the Mann anti-HGH serum is probably five- to tenfold less than the best available rabbit or guinea-pig antiserum but it has been used to study dynamic changes in HGH concentration in human subjects. Let me emphasize that the antiserum being distributed by Dr. Latimer is available in amounts sufficient only for immunoassay.

G. MILHAUD : This policy seems very reasonable. Now if we go to the procedure of immunoassay I think that Dr. Franchimont has a few words to say.

P. FRANCHIMONT : To separate free labelled growth hormone (STH*) from antibody bound STH* the following methods were used : chromatoelectrophoresis, fixation on an anion-exchanger (Amberlite 400) starch-electrophoresis.

1) Chromatoelectrophoresis was done with two types of Whatman paper (3MC and DE 21) containing DEAE-cellulose. Studies were performed to determine the influence of serum protein (SP) concentration and of migration time, on the persistance at the origin of free labelled growth hormone (in the absence of any antibody).

Figure 6 shows a reduction in the radioactivity at the origin when the concentration of SP increases and also for the same SP concentration, when the migration time rises. The question is raised whether this is due to a degradation of STH* during the incubation or to a combined migration with human SP.

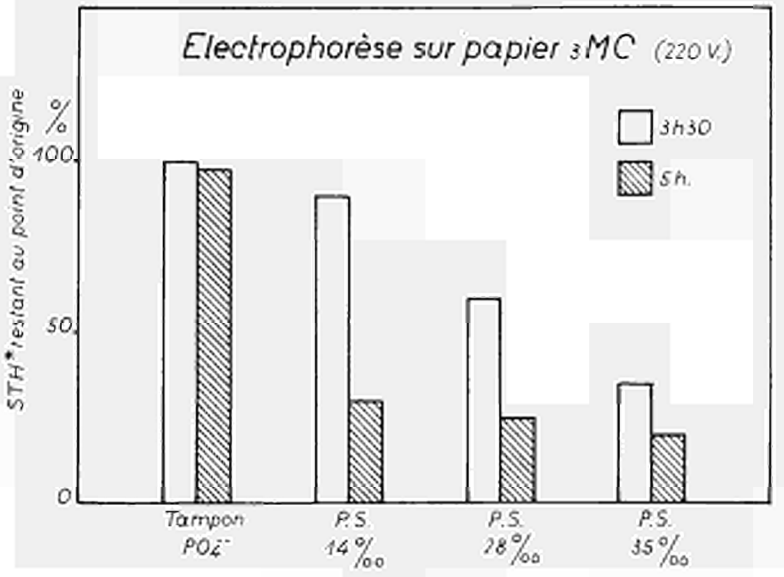


Figure 6

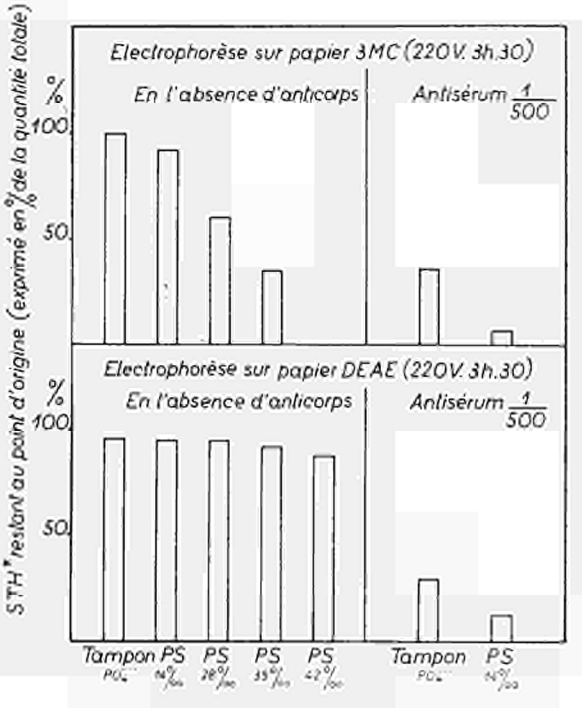


Figure 7

The two types of paper were used simultaneously (Figure 7). The reduction of the amount of free STH at the origin is observed only with 3MC paper. This difference may be attributed to a better quality of the batch 3 MC paper, which does not retain the labelled growth-hormone at its point of deposition. Further more when an excess of antibody is added the amount of antibody-bound STH is influenced by the concentration of human SP. It therefore appears that the concentration of SP in the medium changes the electrophoretic behaviour of both free STH and antibody-bound STH.

2) Amberlite 400 retains free STH* whereas antibody-bound STH goes through [9]. This is a good method of separation. It must be stated, however, that in the absence of antibody the resin fixation of growth hormone is influenced by the presence of human SP added, long before (three days) or immediately before the separation (Figure 8).

When 0.1 ml or less of human serum is added to the incubation medium, difference in the amount of hormone retained is less than 5 %. A check is always carried out by studying the fixation of STH* on Amberlite 400 in the absence of antibodies and in the presence of the same volume of each serum used for the assay; a correction cœfficient is thus calculated.

3) Finally, the method which we recommend consists of starch electrophoresis. In well-defined conditions free STH* migrates in the pre-albumin area, the STH*-antibody complex in the area situated between the slow α_2-globulin and the origin (Figure 9). Less than 6 % of free STH* migrates in STH-antibody complex area.

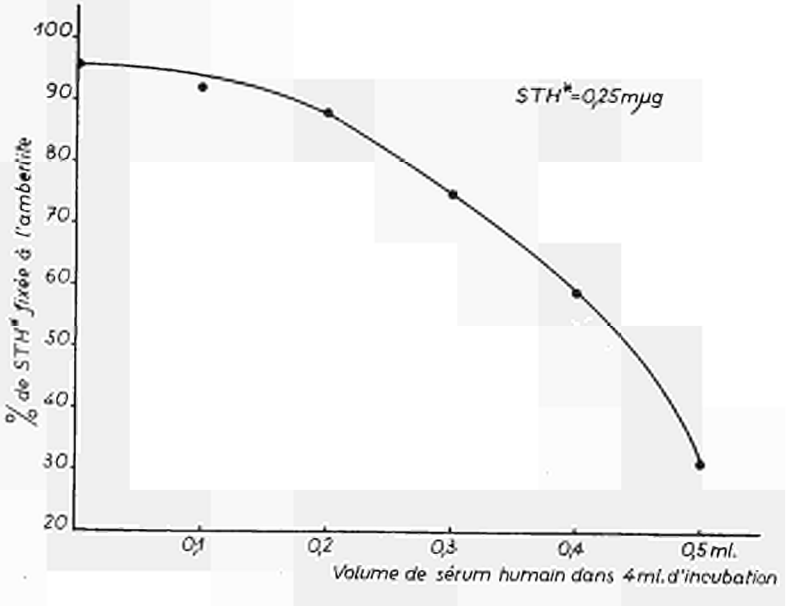


Figure 8

DISTRIBUTION DE LA RADIOACTIVITÉ DE LA STH APRÈS ÉLECTROPHORÈSE EN AMIDON (220 V — 5 H)*

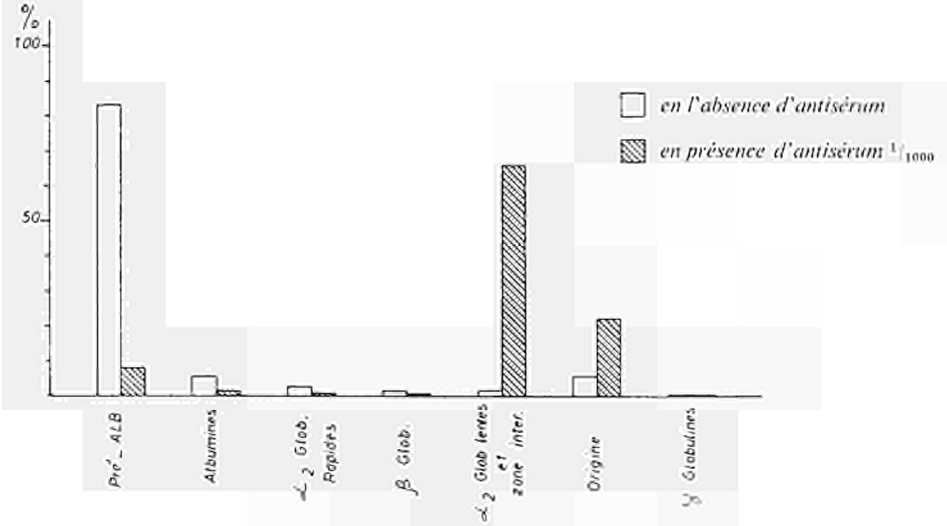
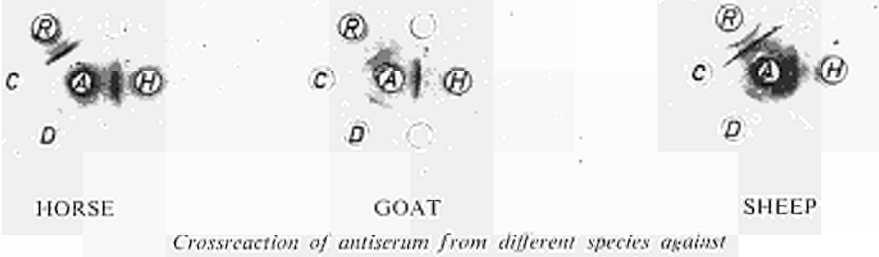


Figure 9

H. J. QUABBE : The difference between various methods for the radioimmunoassay of proteic hormones in plasma lies mainly in the technique by which antibody-bound and free hormone are separated after the completion of the antigen/antibody reaction. Immunoprecipitation with an anti-gammaglobulin serum offers a possibility of separation which combines easiness with the possibility to use a hormone of relatively low specific activity. For the assay of human growth hormone (HGH) it was first used by Utiger *et al.*[10]. Use of immunoprecipitation will sometimes yield falsely high serum values of the hormone, due mainly to incomplete precipitation of the first antibody. This may occur when some of the antigammaglobulin serum is lost from the reaction with its specific antigen by crossreaction with human gammaglobulin. Such a crossreaction has been described by several authors [8, 10, 11], while others failed to find it [12]. Figure 10 shows that the antigammaglobulin serum used in our laboratory (directed against rabbit gammaglobulin) crossreacts with human gammaglobulin. Titration of the antigammaglobulin serum should therefore be done in the presence of human serum.

For some sera the existence of an "inhibitor" of the immunoprecipitation has been postulated and Morgan *et al.* provided evidence that complement may be this inhibitor. When trying to apply their methods for elimination of the inhibitor to our sera it was found, that heat treatment indeed was effective, but treatment with EDTA in a final concentration up to 0.02 M never had an influence on the inhibition. The cause of this difference in our results and those of Morgan *et al.* is not known. The antigammaglobulin serum itself, when used in relatively high concentration (1/10 final concentration) seemed to supply enough complement



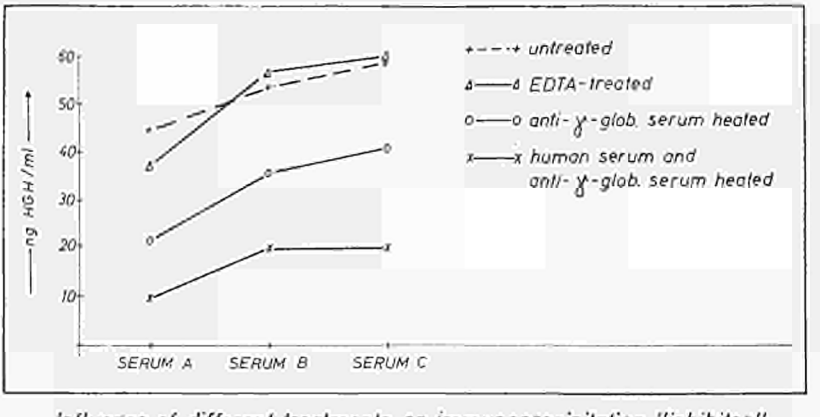
Crossreaction of antiserum from different species against rabbit γ-globulin with human serum protein. Ouchterlony gel diffusion 4 days.

A — anti-γ-glob. serum
H — human serum
D — diluted rabbit serum
C — cryst. bovine serum albumin
R — rabbit serum, undiluted

FIGURE 10

for some inhibition, since precipitation was further improved, when this serum was treated as well (Figure 11). Addition of guinea-pig serum rich in complement to treated serum restored the inhibition to pretreatment levels.

If proteic hormones are diluted to very low concentrations (in the order of micrograms or nanograms per ml) their nonspecific adsorption to the glass surface becomes relatively important. Since no data are available on this binding for HGH, its possible influence on the radioimmunoassay of this hormone was studied using ^{131}I-labelled HGH in the concentration which was used in the assay of the hormone (1 ng/ml). The labelled hormone was left in test tubes for 2 hours at + 4° C in the presence of different concentrations of protein. The tubes were then washed three



Influence of different treatments on immunoprecipitation "inhibitor". Heating: 45 minutes at 60° C. EDTA: 0.01 M final concentration.

Figure 11

times with different solutions which were designed to study the reversibility of the binding by different protein concentrations in the washing fluid. Table I shows that at higher protein concentrations in the tubes the binding to the glass surface is less important and also, that the binding is at least partially reversible by increasing the concentration of protein in the washing fluid.

TABLE I

Influence of protein concentration in incubation medium and washing fluid on binding of ^{131}I-HGH to the glass surface and its reversibility.

Incubation medium	Washing fluid	% Radioactivity remaining after three washings
1. CBSA 50 mg % in buffer	NaCl 0.9 %	5.9
2. CBSA 50 mg % in buffer + undiluted serum	NaCl 0.9 %	2.2
3. CBSA 1 g % in buffer	CBSA 50 mg % in buffer	3.9
4. CBSA 1 g % in buffer + undiluted serum	CBSA 50 mg % in buffer	2.4
5. CBSA 1 g % in buffer	CBSA 1 g % in buffer	3.3
6. CBSA 1 g % in buffer + undiluted serum	CBSA 1 g % in buffer	1.8

C. B. S. A. : crystalline bovine serum albumin.
Significance : 1 : 2 $p < 0.001$ 3 : 4 $p < 0.1$ 5 : 6 $p < 0.1$ 3 : 6 $p < 0.01$

In a small test series, not shown in the table, there was no decrease in the binding when siliconized tubes were used instead of normal glass tubes. The importance of these findings for the radioimmunoassay of HGH lies in the fact that usually the protein concentrations in the tubes of the standard curve and in the unknowns are different. The former are usually incubated in the absence of serum and a certain concentration of albumin is used instead. This may cause a higher degree of non-specifically bound radioactive hormone after the washing of an immunoprecipitate in the tubes of the standards. If this is not accounted for, falsely high hormone concentrations will be calculated for the unknowns. The error may be as high as 50 % or even more.

G. MILHAUD : Thank you. Dr. Felber, would you like to make a few comments on general problems and pitfalls which you have experienced.

J. P. FELBER : If one has a well-labelled hormone, potent antisera and good standard curve, some difficulties still remain in the estimation of the hormone in plasma : incubation damage, mentioned in Dr. Yalow's communication and failure to reach equilibrium in the incubation mixture. The incubation mixture contains free labelled hormone, free unlabelled hormone, free antibody, antibody-bound labelled hormone and antibody-bound unlabelled hormone. Any modification of the concentration of one of the components will have repercussions on the equilibrium. For example, dilution of the antiserum will delay the time needed to reach equilibrium.

The plasma to be tested will replace the unlabelled hormone in the incubation mixture. As it differs from the buffered solution of the hormone used for the standard curve, by the presence of the plasma proteins, a change may occur in the equilibrium, increasing the time needed to reach equilibrium. It seems, therefore, very important to make sure that complete equilibrium has been reached before starting the separation of bound and free labelled hormone.

Adding of plasma also has a different effect according to the system of separation used. It slows down the precipitation in the double antibody system, and this may produce falsely high values if the precipitation is not complete. If one uses cellulose (in powder form or in the form used in chromatoelectrophoresis) or resin for the separation of free and bound labelled hormone, the presence of plasma may decrease the binding of free labelled hormone to cellulose or resin, and therefore produce falsely low values. All these difficulties can be avoided, as long as one is aware of them.

G. MILHAUD : Thank you. Now the last speaker on the procedure of immunoassay is Dr. Freedlender, who is using a slightly different technique.

A. E. FREEDLENDER : We are using the double antibody radioimmunoassay and *à propos* of the comments just made by Dr. Felber, if one assays sera at a dilution of 1 to 20 the damage due to incubation is not greater than that in the buffer system alone. We are adding to our system of standard or unknown serum again diluted 1 to 20, 05 mμg labelled growth hormone, 1/10 mg of guinea-pig anti-growth hormone serum and maintaining the first reaction at 4° C for 7 days. Following this we precipitate with rabbit anti-guinea-pig globulin serum in the presence of carrier to form an adequate precipitate. In view of the report of Sharp and Parker who carry their first incubation for only 2 days in the double antibody method, we have investigated the time period necessary for achieving equilibrium and it should be noted that there is a sharp increase in the initial point of the curve resulting in a marked increase in both sensitivity and precision of the assay, as the incubation period is prolonged. I might point out that the curve does not look as deep as Dr. Yalow's because it is on a different plot but I think they are quite equivalent.

A propos of Dr. Quabbe's comments regarding the interference in precipitation we normally titrate our second antibody to achieve maximum precipitation

and then arbitrarily double it and have seen no interference by crossreaction with human gamma globulin.

The equilibrium is obtained in 18 hours and we have had again no trouble with interference in the immunoprecipitation. The reason for the dilution of 1 to 20, as suggested by Dr. Yalow, is that one human serum is representative of 20 recovery experiments, with the theoretical recovery line of added human growth hormone. When assayed at 1 to 20, these sera fall within experimental error, on the recovery line, however at 1 to 10 and, in particular, at 1 to 5, excess growth hormone was recovered presumably due to incubation damage with sera.

G. MILHAUD : Thank you. Now we shall go to another hormone, whose assay is still in experimental procedure, and first we will ask Dr. Potts to tell us something about the preparation of bovine parathormone.

J. T. POTTS : Dr. Milhaud indicated it might be of value to review our studies concerned with the isolation and chemical characterization of bovine parathyroid hormone. These studies represent the collaborative efforts of Drs. Gerald Aurbach, Louis Sherwood, J. L. H. O'Riordan and Henry Keutmann, working in Dr. Aurbach's group in the National Institute of Arthritis and Metabolic Diseases and our group in the National Heart Institute. The hormone is extracted from beef glands with phenol by the procedure of Aurbach [13], fractionated with salt and obtained as a partially purified product by trichloroacetic acid precipitation (this intermediate product, TCA-PTH, is active at 200-300 USP units/mg[14]). Complete purification requires gel filtration on Sephadex followed by chromatography on carboxymethylcellulose; the bovine hormone obtained in this manner (CMC-PTH) is free of non-hormonal contaminants [15]. Our studies of the amino acid sequence of the hormone and the relationship of structure to function have progressed sufficiently to provide a working model of the structure including regions that are important for both biological and immunological activity [16] (Figure 12). A region of 29 amino acids located at the carboxyl-terminus of the molecule contains a minimum structure requisite for both immunological and biological activity. Selective chemical modification of one of the two methionines and the single tryptophan and tyrosine residue of the polypeptide largely or totally destroys biological activity. These modified amino acid residues, shown with darkened borders in Figure 12 are contained within the 20 amino acid region at the carboxyl-terminus of the hormone.

Biological activity is retained when the last four amino acids at the COOH-terminus, 81 through 84, illustrated by cross-hatching in Figure 12, are removed from the native polypeptide by carboxypeptidase. The details of this work have been summarised elsewhere [15, 16]. Immunological activity has been estimated, so far, only by the radioimmunoassay techniques, employing two of the high-energy antisera used in the radioimmunoassays. Some dissociations between biological and immunological activity have been detected [15, 16] but it was gratifying to find that the same region of the molecule is important for both activities.

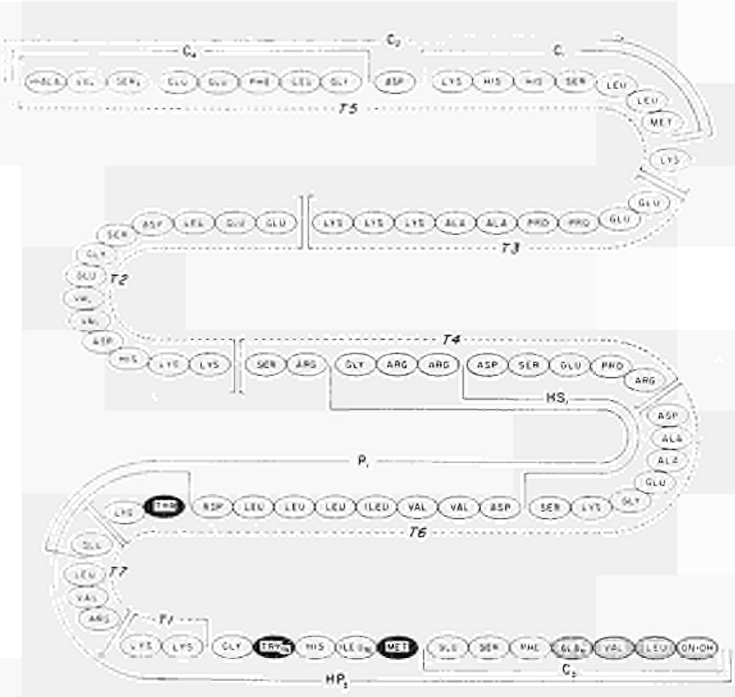


FIGURE 12.— Tentative model of the parathyroid hormone molecule illustrating regions important for biological and immunological activity. The regions corresponding to each of the tryptic peptides (T_1 through T_7) are outlined by the parentheses and dotted lines; absolute sequence within these regions is not known but the individual residues are arranged to reflect the partial sequence information deduced from exopeptidase studies, the characteristic specificity of tryptic attack, and the composition of fragments produced by other means of cleavage (indicated by the designations HP_3, P_1, C_1 etc.). The cross-hatched residues at the COOH-terminus were not necessary for biological activity but alteration of methionine, tryptophan and tyrosine residues (shown by symbols with darkened borders) caused marked loss of biological activity. The 20-amino region (HP-3) at the carboxyl terminus was a biologically and immunologically active fragment.

The single tyrosine of the polypeptide is not buried in the interior of the molecule but rather is solvent accessible[15]. Estimates made after iodination (performed by the technique of Hunter and Greenwood) indicate that this residue may be labelled to the extent of 50 % or more thereby providing a suitable tracer of high specific activity and purity. In preliminary tests it has been established that hormone isolated from bovine and human parathyroid tissue and the hormones circulating in the blood of the cow, goat and sheep are immunologically similar. Figure 13 compares the slope of the curves showing displacement of ^{131}I beef PTH from antibody by each of these unlabelled hormone preparations. Since only the beef hormone is sufficiently purified to use as a reference standard, the curves from the plasma samples and human hormone preparation, where hormone concentration is unknown, must be superimposed on the beef PTH standard curve to provide the best possible fit. It has been assumed that if such curves completely superimpose, the preparations

have identical immunological crossreactivity. By this criterion the hormone circulating in the blood of the cow and our purified beef hormone, CMC-PTH, are identical in their immunological reactivity; this would indicate that much of the essential chemical features of the native hormone have been preseved during isolation. (Figure 13). This comparison further suggests that immunologically human and beef parathyroid hormones are identical and that goat and sheep hormones are quite similar to beef PTH. This finding should eliminate the need to work with human parathyroid hormone for measurements in human plasma. However, it is not certain that the method of curve fitting is a sufficient test of the degree of immunological crossreactivity of two different hormone preparations. Accordingly, Dr. O'Riordan is continuing his efforts with us and with Drs. Berson and Yalow to purify and characterise the hormone from human adenoma tissue. It is hoped that a sufficient quantity of pure human hormone can be obtained to unequivocally establish the comparative immunological reactivity of known, identical quantities of human and beef hormone.

These studies are of great importance in determining the eventual success of efforts to measure hormone concentration in human blood. We have measured hormone concentration in the blood of the cow, goat and sheep, working with dilutions of plasma as great as 1 to 5. However, as Dr. Yalow will discuss, one can only infrequently measure hormone concentration in human plasma.

If the immunological crossreactivity of human and beef hormone is identical these difficulties must mean that hormone concentration in blood is lower in man than in ruminant animals; extraction from plasma would then be required for satisfactory immunoassay. On the other hand, if the immunological reactivity of human

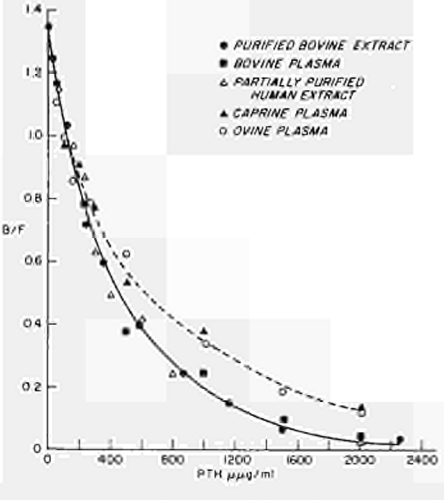


FIGURE 13.— Comparison of the immunological reactivity of parathyroid hormone obtained from several species.

and beef hormone is different, the problems might be circumvented by using human parathyroid hormone in the assay. This seems formidable at best in that the weight yield of hormone from parathyroid glands is probably only about 0.004 %; it will be difficult to obtain enough pure human hormone to use with the radioimmunoassay technique for tests in human subjects.

G. MILHAUD : Thank you Dr. Potts. As to the availability of the pure hormone no bovine hormone is available at present, to say nothing of the availability of any human hormone. So it seems to me that it would be very important to start collecting human adenoma and to have a central information storing system indicating the location and the weight of the adenoma. The fresh tissue should be put, immediately after removal, on dry ice and then kept in the deepfrozen state. When we obtain sufficient adenomas, then we should consider a joint project for extracting fractionating and getting some purified parathormone. At the National Institute for Health, they have now already 100 g of this material but you have to know that the yield may be 1 or 2 milligrams of pure human parathormone from 100 g fresh tissue. Therefore it would be really time for us to start to collect this in Europe.

As to the labelling I think there is no special problem and we could go over to the next point which is the availability of antiserum. Dr. Yalow would you say a few words on your experience in getting the antisera with impure preparations ?

R. S. YALOW : In addition to supplying Dr. Latimer with HGH antiserum, we have also been supplying insulin antiserum to a large number of investigators. However, the situation with respect to parathyroid hormone antiserum is quite different. The supply of antiserum suitable for detecting parathyroid hormone even in hyperparathyroid patients is at present in very short supply and it does not appear likely that such an antiserum will be available for general distribution for at least another year or two. If you plan to attempt to produce your own antiserum, perhaps you may profit from some of our experience. The antisera most suitable for assay purposes were produced in animals immunized with beef parathyroid hormone which assayed at about 200 U/mg. When the same number of animals were immunized with beef parathyroid hormone which assayed about 2000 U/mg, we did not get as sensitive an antiserum on similar immunization schedules. It would seem therefore that immunization with a very highly purified preparation probably offers no advantage. We have not investigated whether very low purity commercially available parathyroid hormone is suitable but it certainly seems worthwhile investigating this.

The following is the technique we have used for immunization of guinea-pigs : Beef parathyroid hormone is dissolved in 0.005 N HCl at a concentration equivalent to 1 mg pure beef parathyroid hormone per ml and homogenized with an equal volume Freund's complete adjuvant. Then each animal is injected subcutaneously (inside of thigh) with 0.5 ml of this mixture. Animals are re-immunized similarly at 2-5 week intervals for a total of 3-6 doses. About 8-12 days after the third or subsequent immunizations blood is taken by cardiac puncture with a heparinized syringe while the animal is under chloroform anesthesia. Animals producing sui-

table antisera are reimmunized at intervals and blood taken during the 8-12 day period following the booster. Antisera are not pooled. Guinea-pigs have survived for us as long as 4 years with as many as 20 cardiac punctures. It should be emphasized that a good animal should not be exsanguinated. We take about 10 ml blood for each puncture and the animal can be milked regularly every few months.

G. MILHAUD : I think that now we have to discuss some results. The results concerning animals will be presented by Dr. Potts, and the results concerning human sera will be presented by Dr. Yalow.

J. T. POTTS : Gerald Aurbach, Louis Sherwood and I, working with Dr. Anthony Care of the Rowett Research Institute in Great Britain and Drs. David Kronfeld, Pat Mayer and Charles Ramberg of the Veterinary School of the University of Pennsylvania have measured the concentration of parathyroid hormone in the blood of the cow, goat and sheep. The concentration in an unstimulated cow varies between 0.5 and 1.5 mμg/ml. The ease with which measurements can be made in the blood of the cow has permitted a variety of studies to define the factors which control the secretion of parathyroid hormone. The concentration of hormone in blood increases within minutes or less following induced hypocalcemia, increasing tenfold or more between suppression with high calcium and maximal stimulation with hypocalcemia [17]. A typical study with a cow is summarized in Figure 14.

In the studies with Dr. Care, the concentration of parathyroid hormone in the venous effluent from parathyroid in the sheep and goat was measured[18]. Results were similar to those seen in the blood of the cow, indicating actual changes in the secretion rate of hormone rather than only alteration of the distribution, excretion, or fate of hormone. Furthermore, it suggested that the parathyroid gland itself is the receptor tissue which responds to changing serum calcium. High concentrations of magnesium also suppress hormone secretion; phosphate, on the other hand, has no direct effect on the secretion of parathyroid hormone but only secondarily affects its secretion through changes induced in serum calcium concentration (Figure 15).

While we are continuing our collaborative efforts with Drs. Berson and Yalow on the application of the assay to measurements in human plasmas we are extending the experiments with animals.

We will study how rapidly hormone concentration changes in response to hypocalcemia as well as the relative importance of various ions in influencing hormone production by affecting release and/or synthesis. Also of interest is whether parathyroid hormone is continuously secreted and whether constant increments in secretion follow given degrees of hypocalcemia. Since blood calcium is a specific and direct regulator of parathyroid hormone production, these studies with the radioimmunoassay technique provide an unusual opportunity to carefully evaluate the physiological control of the secretion of this peptide hormone.

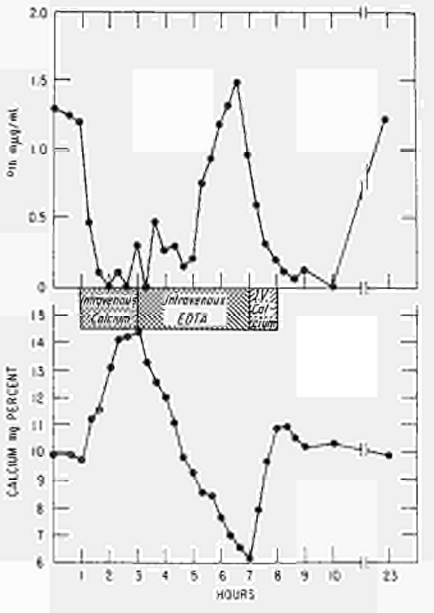


FIGURE 14.— Infusions of calcium to raise or EDTA to lower the concentration of calcium in the blood of the cow. The secretion of parathyroid hormone rises in response to hypocalcemia and is suppressed by hypercalcemia.

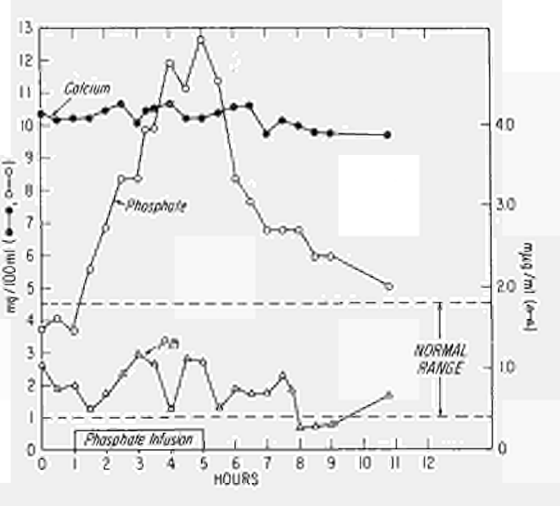


FIGURE 15.— Infusions of phosphate, sufficient to markedly elevate its concentration in blood (○—○), if accompanied by concomitant infusion of calcium to prevent hypocalcemia (●—●), does not significantly alter the concentration of parathyroid hormone in the blood of the cow (△—△).

R. S. YALOW : I will report briefly some of the results Dr. Berson and I have obtained in the immunoassay of human parathyroid hormone. We have been detecting elevated levels of plasma parathyroid hormone in cases of proven parathyroid adenoma, but frequently even higher levels in many cases of uremia. In the fasting state, normal subjects generally have values less than 0.5 mμg/ml. We have also studied the disappearance of endogenous parathyroid hormone following extirpation of parathyroid adenomas and have found turnover half-times of the order of 10-20 minutes, similar to those of endogenous ACTH, growth hormone and insulin. One interesting finding we have observed not infrequently is the marked stimulation of parathyroid hormone secretion associated with handling the tumour during surgery. Determination of parathyroid hormone levels following surgery has been of value in predicting whether or not complete removal of adenomas has been effected and whether clinical remission can be anticipated.

G. MILHAUD : Thank you Dr. Yalow. We have two more points to discuss concerning eventual prospects in radioimmunoassay. The first one concerns the use of ^{125}I. Would you like, Dr. Luyckx to comment on this ?

A. LUYCKX : For labelling human growth-hormone with ^{125}I we use the Hunter and Greenwood method, 10 μg of the Wilhelmi 612 A preparation being put in the presence of 4 mC of ^{125}I. The labelled product is immediately applied on a G 50 Sephadex column and the protein peak obtained is then filtered on a G 200 Sephadex column. If a comparison is made of the radioactivity in the eluted fractions of the G 200 column, it appears that in the case of ^{125}I-labelled hormone the radioactivity corresponding to the largest fraction is at exactly the same place as with ^{131}I-labelled hormone. However the radioactivity eluted before this main peak seemed to us in our first experiment to be appreciably lower than in the case of ^{131}I-labelled hormone.

By means of an immunoprecipitation technique usually used in our assays we have studied the binding of this preparation to the antibody. With ^{125}I-labelled hormone we obtained a maximum binding of 97 % whereas in similar conditions with ^{131}I-labelled hormone the binding was only 80-85 %. Finally we have used the same ^{125}I-labelled preparation for a series of standard successive curves over a period of three weeks. ^{125}I-labelling has obviously two advantages : a 60-day half-life and less radiation damage to the hormone.

At the bottom of the figure 16 is illustrated what I was saying just now, namely, the substantial reduction in the eluted material before the main radioactivity peak in the case of ^{125}I-labelled hormone. The top of the figure shows the usual radioactivity distribution for ^{131}I-labelled hormone.

G. MILHAUD : Dr. Greenwood would you like to comment?

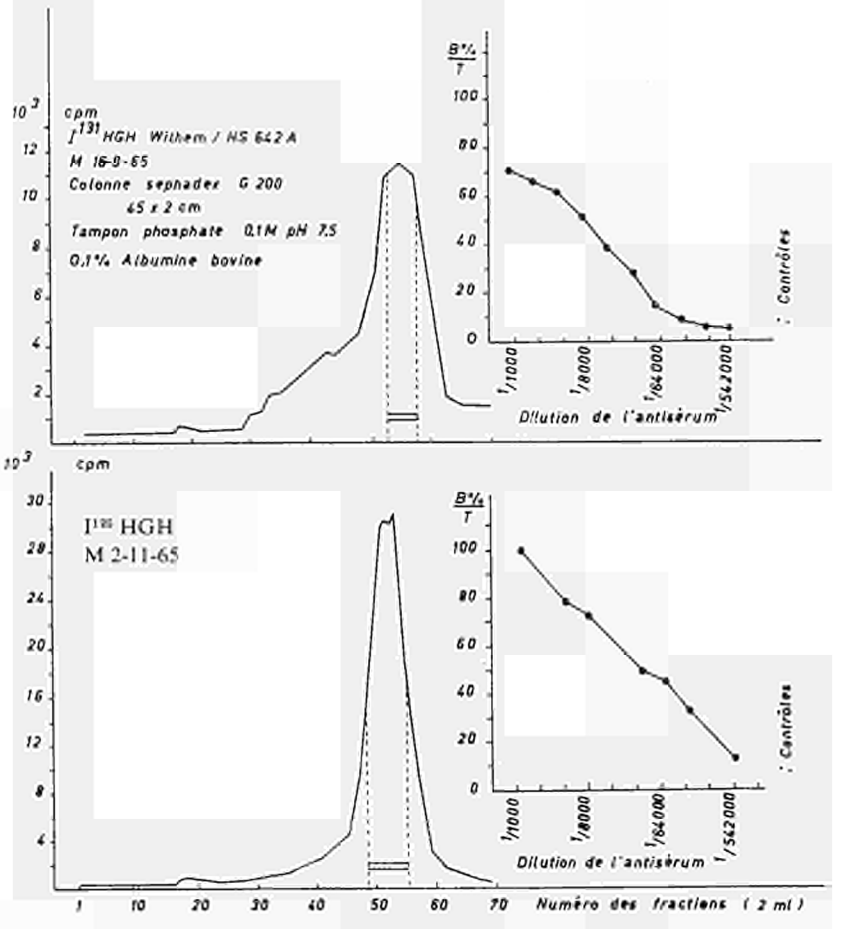


Figure 16

F. C. GREENWOOD : I would like to summarise briefly and dogmatically Dr. Hunter's experiences with ^{125}I. These were reported at the 9th Symposium on the use of radioactive isotopes in medicine (Oak Ridge Institute of Nuclear Science, October 1965). Essentially ^{125}I has given lower yields than ^{131}I in our method but, at least, a yield has always been obtained. The stability of different preparations of ^{125}I-human growth hormone is variable. A preparation may last a week or a month. The amount of isotope reacted has been to 0.75 mC otherwise damage is excessive. In our experience there is little to choose between the two isotopes. Certainly ^{125}I does not answer the problems outlined by Dr. Yalow and myself. A discussion of the radioimmunoassay technique, the labelling of protein hormones and the specificity of radioimmunoassays is to be published [19].

G. MILHAUD : Another possibility is to use ^{51}Cr and I think that Dr. Brauman has some experience to report with this isotope.

H. BRAUMAN : A batch of Raben's HGH was labelled according to the Hunter and Greenwood technique; the undamaged ^{131}I-HGH was successfully utilised in the Berson and Yalow radioimmunoassay.

Freshly prepared ^{51}Cr-chloride (Amersham or Saclay) had specific activities from 50 to 100 mC/mg. The labelling was obtained by incubation of small amounts of dissolved HGH at room temperature. All the radioactivity showed to be immediately fixed on the HGH with specific activites up to 100 mC/mg. No attempt has been made to obtain higher specific activities. When submitted to chromato-electrophoresis on Whatman 3 MM paper strips, following Berson and Yalow technique, the labelled product showed only one single symmetric radioactive peak at the origin. This single peak is not displaced by the addition of serum bovine albumin or horse serum. These results are comparable with those observed with undamaged ^{131}I-HGH.

Before drawing any conclusion for the future from these preliminary results, the reproducibility of the labelling procedure, the ability of the ^{51}Cr-HGH to react with specific HGH antibody and to be displaced by non-labelled HGH has to be tested.

G. MILHAUD : I should like to thank all the participants : those who came from far to give us the benefit of their experience and those who came to tell us of their successes and their difficulties. Finally, I would like to thank the institutions which made these discussions possible, Euratom and the University of Pisa and enabled such eminent experts to gather round this table. In this city, where every stone evokes a historical memory, we know that the past record of the University of Pisa and the efforts which have resulted in today's conference are the most reliable guarantees of the future for which we hope. It is time to conclude our discussions. The performance of the radioimmunoassay of human growth hormone should not involve major difficulties anymore and most european laboratories should be able to obtain very good results. The situation is unfortunately very different in respect to parathormone, as human parathormone has never been obtained in pure form. All the work is done with beef parathormone, which is scarcely available as well as the corresponding antisera. This means that one has to assume the identity of behaviour of the animal and human hormone for the immunoassay, even though this may well not be the case. These considerations emphasize the importance to collect as much parathyroid tissue as possible from patients operated upon in the european institutions. This material should be frozen and stored to be made available for fractionation and purification. I would be glad to centralise these informations from all interested investigators and we could meet again as soon as sufficient glands are available and discuss together the preparation of pure human parathyroid hormone.

There are two final comments I should like to make.

There is a source of error common to all immunological assays : we assay a protein by using its immunological reactivity, but it is not certain that the biolo-

gical activity behaves in the same way as the immunological activity, since it has not been established that the same chemical groups in the molecule take part in the two types of biological and immunological reaction. Whenever possible, therefore, the immunological assay should be supplemented by a biological assay, whereby the proportion of the hormone present in an inactived form can be estimated.

Finally, radioimmunoassays often represent an important stage in the elucidation of the relations between the chemical messenger and the receptor. Overproduction or underproduction of the messenger must give raise to a variation in the intensity of response of a particular receptor. Now the receptor will respond or fail to respond to variations in the stimulus provided by the hormone, according to whether or not it is intact. Consequently, with underproduction the response will always be smaller, with overproduction the increase in the response requires an intact receptor, which may not be the case [20].

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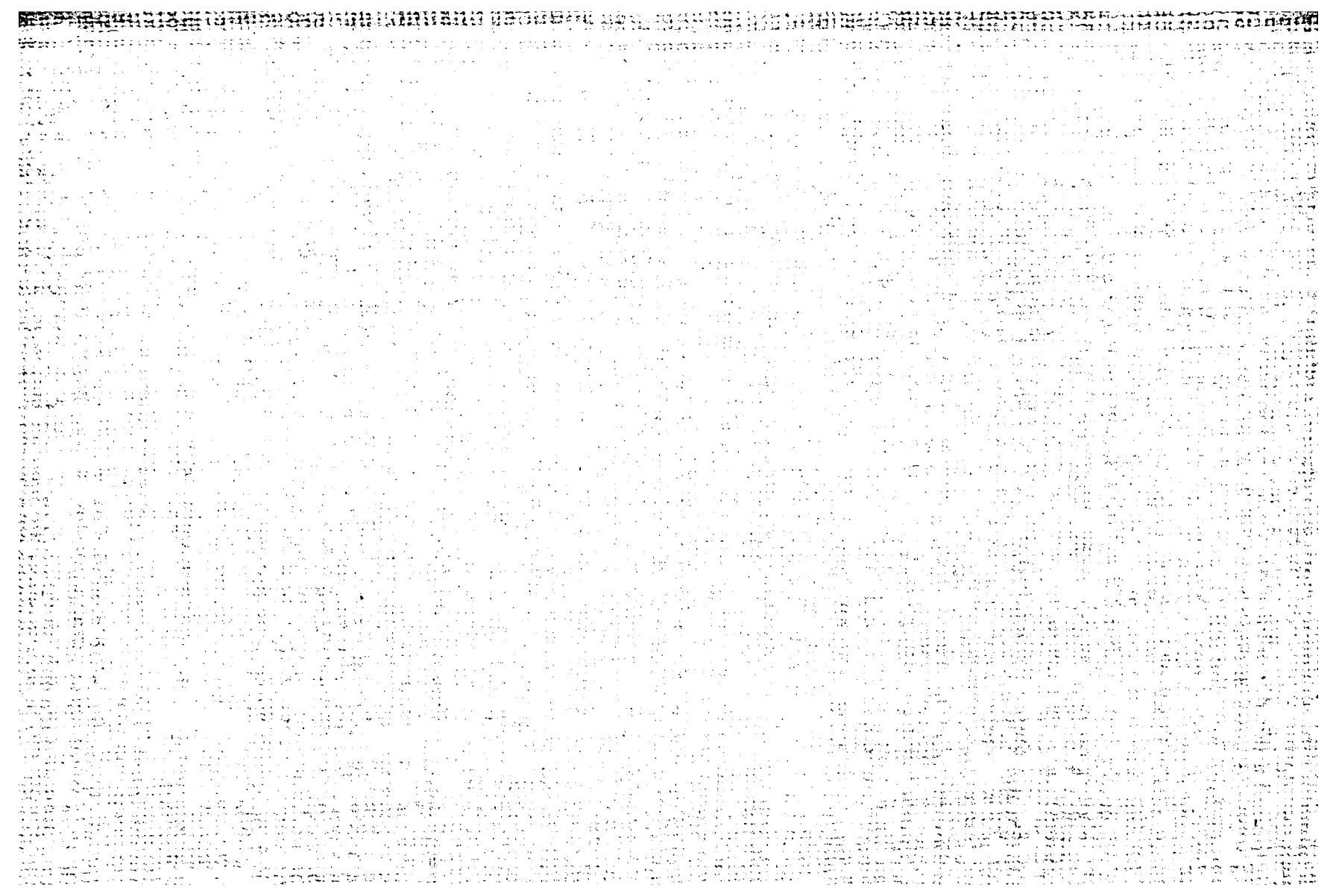

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