KINETIC AND THERAPEUTIC ASPECTS OF ZINC METABOLISM

VOLUME I

by

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CONTENTS

VOLUME I

Chapter		Page
1	Review of Previous Work on Zinc Metabolism and Purpose of the Present Study	1
2	Study of the *in Vitro* Binding of Zinc-65 Added to Plasma, Using Separation by Electrophoresis on Cellulose Acetate	20
3	Investigation into the Suitability of Cross-Linked Dextran Gels for the Study of Plasma Zinc Binding	43
4	Distribution of the Endogenous Zinc in Plasma and Serum	63
5	Study of the Variation in the Zinc Binding Pattern in Plasma with Time after an Oral Dose of Zinc-65	85
6	Relationship between the Clearance of Zinc from the Plasma Proteins and the Uptake and Clearance by the Tissues	102
7	Effects of Ingestion of Therapeutic Amounts of Zinc on Plasma Zinc Levels and on the Distribution of Zinc among the Plasma Proteins	130
8	Review and Discussion of Results	147

CONTENTS

VOLUME II

	Page
Appendix I	
Properties of Zinc	1
Appendix II	
Techniques for Protein Separation Using Electrophoresis	3
Appendix III	
Method for Protein Separation Using Sephadex Gels	7
Appendix IV	
Estimation of Total Protein and Albumin Levels using Spectrometric Methods	11
Appendix V	
Method for Measurement of Protein Concentration by Radial Immunodiffusion	14
Appendix VI	
Estimation of Stable Zinc Levels Using Atomic Absorption Spectrometry	18
Appendix VII	
Calculation of the Transfer Function for the Passage of Zinc through the Gut Wall	20
References	23
Tables	35
Figures	43

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PREFACE

The work described in this thesis formed a self-contained study into some of the more fundamental aspects of zinc metabolism and was carried out as part of a project on the role of zinc in wound healing involving the Department of Oral Medicine and Pathology, Glasgow Dental Hospital and School, the Department of Clinical Physics and Bio-engineering, Western Regional Hospital Board and the Departments of Dermatology and Nuclear Medicine, Glasgow Royal Infirmary. The work was carried out mainly in the Department of Oral Medicine and Pathology, Glasgow Dental Hospital but also in part in the Department of Nuclear Medicine, Glasgow Royal Infirmary.

SUMMARY

Zinc is one of the essential trace elements for man and there is now considerable evidence to show that a deficiency of zinc is related to the poor wound healing found in some patients, particularly those with varicose ulcers. Oral zinc therapy has been used successfully in the treatment of these patients but as zinc is toxic in large doses many workers have regarded it with disfavour, particularly since little is known about the metabolism of either dietary or therapeutic doses of zinc in man. The aim of the present work has therefore been to study the manner in which both trace and therapeutic amounts of zinc are transported by plasma to the organs and tissues and to relate the uptake and clearance of zinc from plasma to that of the various tissues.

An initial study was carried out on *in vitro* binding of zinc to the plasma proteins to compare the results obtained using protein separation by electrophoresis on cellulose acetate and by chromatography using Sephadex gels. Chromatography on Sephadex gels was found to be the more suitable method, particularly for study of endogenous zinc binding and of the binding of trace amounts of radiozinc administered to subjects *in vivo*. While albumin was found to bind virtually all the zinc added to plasma *in vitro*, the endogenous pattern of zinc binding has two main components, albumin and a second high molecular weight protein fraction coincident with

α_2-macroglobulin, which has been shown to be a zinc metalloprotein by other workers. A study of the endogenous zinc distribution in normal subjects showed that approximately one third of the plasma zinc was bound to α_2-macroglobulin and the remaining two thirds to albumin.

The variation of binding of a tracer dose of radiozinc administered orally to two normal volunteers was studied as a function of time. It was found that at peak plasma zinc-65 level about 90% of the zinc-65 was bound to albumin but this level dropped during the first 24 hours to about 75%, a value approximately equal to the endogenous proportion. When intravenous clearance of zinc-65 was studied it was found that the major part of the zinc-65 disappeared very rapidly from plasma, with a half-life of about 20 minutes. As this could be related to clearance from the albumin fraction it was shown that albumin is a transport protein for zinc.

When the uptake of zinc-65 by liver and muscle was compared with the disappearance of zinc from plasma it was found that the zinc-65 was cleared very rapidly into the liver during the first 24 hours, at a rate which corresponded to the clearance of zinc-65 from the albumin fraction. Clearance of zinc-65 in plasma into the liver stopped when the fraction of the zinc-65 bound to α_2-macroglobulin reached about one third of the total. Uptake by muscle was much slower and evidence

has been produced to show that this is due to transfer of zinc from liver to muscle by plasma.

The clearance of a therapeutic dose of zinc from plasma was then studied in normal volunteers after an oral dose of zinc sulphate. It was found that the resultant increase in plasma zinc was bound to both α_2-macroglobulin and albumin fractions, in some cases more than doubling the original amounts bound to the proteins. After about 6 hours both albumin and α_2-macroglobulin zinc levels had dropped significantly, in some cases back to the original levels, showing that α_2-macroglobulin is also a transport protein for zinc.

There are thus two transport proteins for zinc, albumin and α_2-macroglobulin, which apparently have separate functions in the control and clearance of absorbed zinc to the tissues.

CHAPTER 1

CHAPTER 1

REVIEW OF PREVIOUS WORK ON ZINC METABOLISM AND PURPOSE OF THE PRESENT STUDY

		Page
1.1	Zinc Deficiency in Biological Systems	2
1.2	Zinc Content of Body Tissues	4
1.3	Biochemical Role of Zinc	6
1.4	Zinc and Wound Healing	7
1.5	Altered Zinc Metabolism in Pathological Conditions	9
1.6	Toxic Effects of Zinc	11
1.7	Dynamic Studies of Zinc Metabolism	12
1.8	Transport of Zinc by Plasma	14
1.9	Recognition of Zinc Deficiency States in Man	16
1.10	Aims of the Present Work	18

1.1 Zinc Deficiency in Biological Systems

Zinc is an essential constituent for all forms of plant and animal life. Although it only constitutes about 0.005% of the earth's crust it is present in living organisms in higher concentration than several elements which are more plentiful. Zinc has been found in measurable quantities in all of the human and animal tissues so far examined and this fact alone suggests an important biological role for the element.

Although Raulin showed that zinc was essential for the growth of the organism Aspergillus niger as early as 1869 (Raulin, 1869) it was not until the 1930s that Todd, Elvehjem and Hart showed that the element was also necessary for the growth and development of animals. They showed that when weanling rats were fed a diet in which the zinc content was considerably reduced their rate of growth was severely restricted compared with that of normal controls (Todd et al, 1934). Subsequent work showed that many species of animals, notably pigs and cattle, are subject to zinc deficiency and that this deficiency is fairly common. It was found that zinc deficiency produces a similar group of symptoms in virtually all species. Besides retarded growth, these include skeletal abnormalities, anorexia, fragile skin and poor wound healing, and parakeratosis, a skin condition producing scaly, flaking skin which has some resemblance to psoriasis.

While the addition of zinc to the soil to combat

zinc deficiency in crops had been tried and found to work in the 1920s the widespread incidence of zinc deficiency in plants has been established only in the last 20 years. Although the symptoms of zinc deficiency are different in different species, these are all indicative of stunted growth and altered metabolism.

While the commercial importance of zinc deficiency in crops and domestic animals encouraged a large volume of work in these fields no evidence of a similar deficiency of zinc in humans was found for some time. It was not until 1961 that the first evidence of a possible zinc deficiency state in man was described by Prasad and his co-workers. Groups of adult male villagers from Iran and Egypt were discovered who were suffering from retarded growth and arrested sexual development, accompanied by anaemia and rough hyperpigmented skin. These dwarfs were thought to be suffering from malnutrition caused by a diet consisting almost entirely of wholemeal bread. However, only slow improvement in growth occurred when they were given a diet containing adequate animal protein. It was found that when 27mg zinc (as zinc sulphate) was given daily as a dietary supplement a highly significant increase in growth rate occurred, together with a dramatic onset of sexual maturity (Prasad et al, 1961). While it was originally assumed that the zinc deficiency syndrome was confined to males, later studies by

Halsted and his colleagues showed that females could also be affected (Halsted et al, 1972).

The comparatively rare occurrence of the zinc deficiency syndrome described by Prasad and his colleagues is probably due to the ubiquity of zinc, which is normally present in adequate amounts in most foods. Balance studies based on analysis of the zinc content of normal diets over a considerable period have shown that the average dietary zinc intake of an adult is in the range 10 - 15 mg elemental zinc per day (Osis et al, 1972; Schlage and Wortberg, 1972), though considerable variation from day to day occurs. It has been shown that normal adults require a minimum daily intake of approximately 7 mg zinc per day (Engel et al, 1966; Spencer et al, 1972). Although the average Western diet easily provides this amount of zinc it is probable that zinc deficiency can occur in countries where the supply of animal protein in the diet is inadequate. It has been shown that although the zinc levels in animal and soyabean protein are approximately the same, twice as much zinc is absorbed from the animal protein (Forbes and Yohe, 1960). Hence zinc deficiency might be expected to be associated with malnutrition and is thought to be an accompaniment of conditions such as kwashiorkor (Sandstead et al, 1965).

1.2 Zinc Content of Body Tissues

With the development of sensitive methods for trace

element analysis, particularly activation analysis and atomic absorption spectrometry, the zinc content of many tissues and body fluids has been accurately measured. It is found that zinc is distributed fairly uniformly in human tissues, within a range of values of about 20 - 400 parts per million (ppm). The highest tissue zinc levels are found in the choroid and retina of the eye and particularly high values are found in teeth, with much higher levels in enamel than in dentine (Nixon, 1969). Liver, kidney and muscle all show similar high zinc levels of approximately 100 - 200 ppm (Liebscher and Smith, 1968). While the zinc content of of skin is fairly low the level in epidermis is high, similar to that in liver and muscle (Molokhia and Portnoy, 1969). The zinc content of hair has been found to vary according to the zinc status of the subject and it has been suggested that this might be useful as an indicator of possible zinc deficiency (Reinhold et al, 1966; Strain and Pories, 1966). An analysis of the trace element content of several cadavers gave values for the total zinc content of the adult body in the range 1.0 - 2.4 g (Widdowson et al, 1951).

Plasma zinc levels for normal adults have been found to be in the range 80 - 130 μg / 100 ml plasma (Greaves and Boyde, 1967; Halsted and Smith, 1970). Normal erythrocytes contain much higher concentrations of zinc, from 450 - 800 μg / 100 ml whole blood. However leucocytes contain an even higher concentration,

approximately 1.4 x 10^{-14}g zinc per cell, a level which is comparable with the level of iron in erythrocytes (Dennes et al, 1961). Zinc in erythrocytes is almost entirely associated with the enzyme, carbonic anhydrase and leucocyte zinc appears to be firmly bound to a protein whose function is as yet undiscovered but has been found to contain 0.3% of zinc (Hoch and Vallée, 1952) which is not readily exchangeable.

1.3 Biochemical Role of Zinc

The fairly uniform distribution of zinc in tissues has stimulated much work on the biochemistry of zinc compounds. Being high in the electrochemical series zinc is a highly electropositive metal and reacts readily to form compounds as the cation Zn^{++}. The first conclusive evidence of a definite biological function for zinc was produced in 1940, when Keilin and Mann isolated carbonic anhydrase, an enzyme found to contain approximately 0.3% zinc which is necessary for the biological activity of the enzyme (Keilin and Mann, 1940). In the 1950s a second zinc-containing enzyme, carboxypeptidase, was isolated (Vallée and Neurath, 1954). Subsequent work has shown that these and many other enzymes need zinc for their biological activity, either as a cofactor or as an integral part of the molecule. Among the enzymes found to be zinc metalloproteins are several aldolases, several dehydrogenases and alkaline phosphatase. Many other enzymes are activated by the presence of zinc,

for example, enolase, amino-peptidase and tripeptidase.

Convincing evidence has also been produced that zinc is necessary for the synthesis of both ribonucleic and deoxyribonucleic acids. Wacker and Vallée showed that RNA and DNA obtained from diverse sources always contained significant amounts of several metals, among them zinc (Wacker and Vallée, 1959) suggesting that some metals may be necessary for protein synthesis. Later experiments showed that when zinc was removed from rat liver DNA synthesis was reduced and that infusion with zinc could reverse this process (Fujioka and Liebermann, 1964). It has also been shown that zinc deficiency affects DNA and protein metabolism in the rat (Williams and Chesters, 1970).

1.4 Zinc and Wound Healing

The importance of zinc in such fundamental processes as enzyme activity and protein synthesis would suggest that even minor reductions in the amount of zinc available for these processes could have important consequences. If zinc is necessary for protein synthesis it is also essential for the processes of tissue repair. It has been shown, using radioisotopes of zinc, that zinc concentrates in healing wounds in rats (Savlov et al, 1962) and poor wound healing is a prominent feature in zinc-deficient animals.

The topical application of zinc compounds to assist healing in superficial wounds has been used for over two thousand years but interest in the use of systemic

zinc to accelerate wound healing has only been aroused in the last 20 years, as a result of the work of Pories and Strain. After initial studies showed that oral zinc supplements accelerated the healing rate of excised wounds and of thermal burns in otherwise normal rats (Strain et al, 1960), Pories and his colleagues carried out a clinical trial on the effects of such supplements on wound healing in patients after excision of infected pilonidal sinuses. They found that the healing time was 46 days for the zinc supplemented group and 80 days for the control group. As these patients were young, otherwise healthy airmen it was concluded that zinc is not only essential for wound healing but that the body zinc stores are inadequate to provide the amount required for optimal healing (Pories et al, 1967).

The suggestion that, in a majority of cases, oral zinc therapy can accelerate wound healing, has resulted in a number of studies on the effect of zinc therapy on the rate of healing, with conflicting results. Many workers have felt that adverse effects may be caused by prolonged zinc therapy and therefore that in cases where healing proceeds normally zinc therapy is not justified. However in cases where the patient has already been shown to have a wound healing problem, oral zinc supplements might be of benefit. Patients with chronic varicose ulcers fall into this category and

many studies have been carried out on the effects of zinc therapy on patients with chronic leg ulcers, on the whole with encouraging results. In a study of 104 patients with such ulcers Husain found that the ulcers of patients receiving 150 mg zinc daily healed in an average time of 32 days, compared with 77 days for the control group (Husain, 1969). Significant improvement has also been found by several other workers (Greaves and Skillen, 1970; Hallböök and Lanner, 1972), though other studies have shown no demonstrable improvement (Myers and Cherry, 1970; Greaves and Ive, 1972).

The success of zinc in promoting healing in some patients with wound healing problems would suggest that there is a minority suffering from latent zinc deficiency which only shows up when an excessive demand is made on the zinc stores and that in the majority the zinc stores are adequate. Henzel and his associates (Henzel et al, 1970) found a significantly increased output of zinc in urine (zincuria) in patients during weight reduction for obesity, after severe burns and following major operations. This was paralleled by reduced zinc levels in serum, erythrocytes and skin but patients with post-operative healing problems were found to have wound tissue zinc levels which were approximately half those of patients where healing was proceeding normally.

1.5 Altered Zinc Metabolism in Pathological Conditions

Low plasma zinc levels have been found in several disease states, among them myocardial infarction, peptic

ulcers, pulmonary infections, alcoholic cirrhosis and Down's syndrome (Halsted and Smith, 1970). The decrease in plasma level in alcoholic cirrhosis has been found to be accompanied by an increase in urinary zinc excretion (Vallée et al, 1956). A rise in plasma zinc level is found in the acute phase of acute intermittent porphyria (Roman, 1969). It is also generally accepted that patients suffering from psoriasis have altered zinc metabolism. A large number of patients with severe psoriasis are found to have low serum zinc concentrations and the zinc level in the psoriatic scales is higher than that in normal skin (Voorhees et al; 1969; Molokhia and Portnoy, 1970; Greaves, 1971). In appearance psoriasis is similar to parakeratosis in animals, which is cured by zinc therapy, but attempts to treat psoriasis using oral zinc therapy have been unsuccessful (Voorhees et al, 1969).

In pregnancy significantly lowered plasma zinc levels are found, possibly reflecting a demand made on the maternal zinc stores by the developing foetus. A similar effect is found in women taking oral contraceptives and the fall in zinc level in paralleled by a rise in plasma copper level (Johnson, 1961; Halsted and Smith, 1970).

The zinc content of tissue, plasma and urine in patients suffering from neoplastic disease has been studied by several investigators and while significant

differences from normal levels have been found no consistent overall pattern has so far emerged (Talbot and Ross, 1960; Vallberg et al, 1966; Györkey et al, 1967). However a definite and consistent change in leucocyte zinc is found in patients with various types of leukaemia, where the zinc content of the cells is found to be approximately half that in normal leucocytes (Dennes et al, 1961).

1.6 Toxic Effects of Zinc

Zinc, like many other heavy metals, is toxic when ingested in large amounts, produced by, for example, cooking or storing foods with a high acid content in galvanised containers. The symptoms of zinc poisoning are described as vomiting, stomach cramp and diarrhoea (Brown et al, 1964). However studies in rats have shown that the toxic effects are mainly due to suppression of other essential elements by the greatly increased zinc intake. Reductions in magnesium, copper and iron stores and alteration in the calcium to phosphorus ratio in bone have been observed (Sutton and Nelson, 1937; Sadasivan, 1951; Stewart and Magee, 1964; McCall et al, 1961).

High mineral concentrations in the atmosphere, in soil and in water supplies have been found in areas where cancer incidence is high (Stocks, 1960; Allen-Price, 1962) and it has been suggested that higher than average levels of zinc absorbed from these sources

may be related to the incidence of stomach cancer (Stocks and Davies, 1964). These results, together with the animal studies, would suggest that caution must be exercised in the use of oral zinc therapy, particularly for long periods.

1.7 Dynamic Studies of Zinc Metabolism

The recognition of zinc deficiency states in man and the development of efficient means for its correction can only be achieved from a basic knowledge of the various stages of the metabolic cycle of zinc in the body. The use of radioactive isotopes of zinc enables valuable information to be obtained on the biological fate of zinc in the body, as the variation of radiozinc levels in tissues and body fluids can be followed, provided suitable samples can be obtained for counting. While the bulk of the work published on the absorption and clearance of radioactive zinc *in vivo* has been carried out in experimental animals several studies on the absorption and excretion of trace amounts of the radioisotope, zinc-65, in human subjects have been published (Ross et al, 1958; Graig and Seigel, 1960; Prasad et al, 1963; Spencer et al, 1965; Spencer et al, 1966; Sullivan and Heaney, 1970). These results are mostly confined to measurement of zinc-65 activity in blood, urine and faeces.

It was found that intravenously administered zinc-65 disappeared very rapidly from plasma, 90% disappearing

in less than two hours and the remaining 10% more slowly, though it was found that this later clearance was significantly faster in zinc-deficient subjects (Prasad et al, 1963). Zinc-65 appeared in erythrocytes and leucocytes fairly rapidly, with activity remaining in the erythrocyte pool for several hundred days. Excretion studies showed that the percentage of the dose excreted in the faeces was much higher than that in urine, showing that the main pathway for excretion of absorbed zinc in man is via the intestine. This level is higher than that to be expected from zinc secreted in bile and pancreatic juice (Spencer et al, 1965).

Studies on the loss of zinc-65 with time in the total body mass have shown that the biological half-life of zinc in the body is of the order of several hundred days (Furchner and Richmond, 1962; Spencer et al, 1965; Newton and Holmes, 1966). Measurements of tissue levels of zinc-65, obtained post mortem at varying times after administration of the dose, showed that initially levels were highest in liver but after about 70 days the activities in muscle and liver were similar (Spencer et al, 1965). In studies of the uptake and clearance of orally administered zinc-65 it was found that the plasma zinc-65 level reached a peak value at between 1 - 4 hours after administration of the dose (Spencer et al, 1966; Husain and Bessent, 1971). Calculation of the percentage of the dose absorbed gave values in the range 20 - 90%.

1.8 Transport of Zinc By Plasma

While these studies have shown that the major portion of the absorbed zinc is rapidly incorporated into the tissues from plasma and that it remains in the body for a considerable time, little is known about the mechanism whereby zinc is shared among the tissues or about the subsequent fate of the tissue zinc, whether it forms an easily mobilised reservoir of zinc or whether it is tightly bound and largely unavailable. Information of this kind depends on a knowledge of the method by which the plasma zinc level is controlled and the different forms in which zinc exists in plasma, since the plasma is the medium by which all absorbed zinc is transported to and from the tissues. It has been shown that homeostatic control is exercised over the plasma zinc level, since individual plasma levels, studied over a considerable period, show little variation from the mean (Halsted and Smith, 1970). The existence of such a control suggests that normal plasma levels may occur in cases where the body zinc stores are considerably reduced.

Plasma is a heterogeneous solution containing a vast number of constituents of widely varying molecular weight and complexity, from simple ions to proteins whose molecular weight is in excess of one million. Many of these substances are in transit to various parts of the body or are essential for the transport of other

substances. While some essential elements are carried to the tissues in the ionic form many minerals are transported as a complex of the ion with one or more of the plasma proteins, and several of the proteins have no other known function apart from the transport of one particular substance, for example, transferrin, a globulin which transports iron absorbed from the gastro-intestinal tract and from the cells of the reticulo-endothelial system to the bone marrow. Albumin, a single protein of molecular weight 69,000 which forms approximately 70% of the total plasma protein concentration, is involved in the transport of a number of substances, both essential nutrients and compounds such as drugs, and it is important in the transport of several elements, in particular, calcium and copper.

While there are measurable amounts of several zinc metalloenzymes in plasma, such as lactic dehydrogenase and alkaline phosphatase, the concentrations are so low that they account for a negligible fraction of the total plasma zinc level. Vikbladh showed that almost all of the endogenous zinc in plasma is bound to protein and that about one third of the bound zinc was firmly bound to protein while the remaining two thirds was more loosely bound and probably associated with albumin (Vikbladh, 1951). Later work by Vallée and his colleagues demonstrated the existence of several zinc-containing fractions among the plasma proteins. Himmelhoch and his associates isolated three zinc-containing protein

fractions, one of which was found to have a molecular weight of approximately 120,000 (Himmelhoch et al, 1966). However, Parisi and Vallée identified only two major zinc-containing fractions in serum, one of which was identified as albumin and the other was found to be a zinc metalloprotein containing 320 - 770 μg zinc per gram of protein which had a molecular weight of about 800,000. They identified this protein as α_2-macroglobulin, a protein which is present in relatively large amounts in normal serum but of unknown function (Parisi and Vallée, 1970). They suggested that the transport proteins for zinc were albumin and α_2-macroglobulin and that α_2-macroglobulin was a specific transport protein for zinc, analogous to transferrin for iron.

Other workers have used the radioisotope, zinc-65, to study the binding of the zinc-65 to the plasma proteins *in vitro*, on the assumption that this procedure is analogous to the uptake by plasma of zinc absorbed from the gut. Although all studies report binding of the major part of the added zinc to albumin there is disagreement about which, if any, of the globulins bind zinc (Dennes et al, 1962; Suso and Edwards, 1969; Prasad and Oberleas, 1970; Boyett and Sullivan, 1970; Parisi and Vallée, 1970).

1.9 Recognition of Zinc Deficiency States in Man

Although zinc has been recognised as an essential element for man only within the last decade considerable

advances have been made in the elucidation of its biological role, notably the discovery of a definite zinc deficiency state in man and a significant relationship between zinc and healing. However these discoveries have themselves raised many fundamental questions concerning the utilisation and metabolism of the element, which become of particular importance with the increasing use of zinc as a therapeutic agent.

The most important clinical problem is the recognition of latent zinc deficiency, particularly in patients with healing wounds. If zinc deficiency is defined as a reduction in the body store of available zinc below the level necessary for both normal requirements and the needs of such processes as tissue repair then the recognition of zinc deficiency requires some means of estimating this zinc store. To date no single parameter has been found which acts as an indicator of zinc deficiency. While some workers have suggested that patients who respond to zinc therapy have low plasma zinc levels (Hallböök and Lanner, 1972) it has been shown that some patients who benefit from zinc therapy have plasma levels which are within the accepted normal limits (Husain, 1969). While other criteria, such as excessive zincuria or deficiency of any of the zinc metalloenzymes, might suggest disordered zinc metabolism, so far the only reliable criterion for zinc deficiency has been a positive response to zinc therapy. As a diagnostic test, this is far from

ideal, particularly since zinc has been shown to be toxic in large doses and the absorption of large amounts of the element is known to cause a reduction in the intake of other essential minerals. It would seem, therefore, that at present our knowledge of the basic processes involved in zinc metabolism are inadequate and that much further work is needed in this field before recognition of zinc deficiency in routine clinical work is possible.

1.10 Aims of the Present Work

The aim of the work described in the following chapters was to study certain aspects of the dynamics of zinc absorption and transport in man, with particular reference to the uptake and clearance of the element by the plasma components. Since plasma is the medium by which all absorbed zinc is carried to and from the tissues the distribution of zinc among the various zinc-containing plasma fractions is of special interest, as these fractions may reflect the various stages in its metabolism. For example, it is likely that newly absorbed zinc is associated with different plasma components from breakdown products which are to be excreted via the kidneys. In particular the existence of a specific transport protein for zinc would imply some measure of control by plasma of zinc uptake and clearance. It has therefore been the intention in the work described to develop a method whereby the main zinc-containing plasma fractions can be separated from each

other and their zinc content measured, as simply as is consistent with obtaining repeatable results. This method has then been used to study the absorption and transport of zinc in vivo, mainly in normal subjects, by the various fractions. The distribution in plasma of both trace amounts of radiozinc and therapeutic doses of stable zinc sulphate have been studied and these findings correlated with the uptake and clearance of zinc from various parts of the body. As this work is in essence a study of the transport of zinc no attempt has been made to study the biochemistry of the zinc-containing fractions. The degree of separation of the plasma fractions sought has been only sufficient for analysis of the main zinc-containing fractions and study of the variation of the zinc associated with each as a function of time. Where specific proteins were studied it was in the role of transport media for zinc.

In summary, therefore, the intention of the work to be described in the following chapters was:-

(a) To study the distribution of endogenous zinc among the components of plasma in man and the variation of the amounts of zinc bound to the various fractions as a function of time, using both trace quantities of radiozinc and therapeutic quantities of stable zinc.

(b) To relate these findings to the absorption and clearance of zinc by the various tissues of the body.

CHAPTER 2

CHAPTER 2

STUDY OF THE *IN VITRO* BINDING OF ZINC-65 TO PLASMA, USING SEPARATION BY ELECTROPHORESIS ON CELLULOSE ACETATE

		Page
2.1	Introduction	21
2.2	Materials and Methods	23
2.3	Measurement of Zinc Binding to the Low Molecular Weight Fraction in Plasma	26
2.4	Binding Pattern of Zinc to the Protein Fractions	28
2.5	Quantitation of Zinc Binding in Plasma	30
2.6	Relationship between Zinc Binding and Protein Concentration in Plasma	32
2.7	Effect of Dialysis on Protein-Bound Zinc in Plasma	36
2.8	Discussion	36
2.9	Conclusions	41

2.1 Introduction

A study of the binding of zinc to plasma *in vitro* is a useful preliminary to *in vivo* studies. Addition of the element to plasma *in vitro* allows the binding of accurately measured quantities of both zinc and plasma to be studied, unaffected by the many variations introduced by the *in vivo* situation and hence the binding capacity of the various plasma components can be investigated in a way which would be impossible *in vivo*. Also it seems reasonable to suppose that, provided zinc binding is a passive process unaffected by factors external to the plasma, the binding pattern of zinc added to plasma *in vitro* will be the same as that of zinc newly absorbed into plasma from the gastro-intestinal tract and this method therefore provides a simple method of studying the first stage of binding *in vivo*.

The most convenient way of studying the binding of added zinc is by the use of a radioactive isotope of zinc as a tracer and of the two radioisotopes of zinc (see Appendix I), zinc-65 has been used almost exclusively in metabolic studies to date. Several studies have been published on the binding of zinc-65 added to plasma *in vitro* and while there is general agreement that zinc is almost totally bound by the plasma proteins and that the major part is bound to albumin, there is some disagreement as to whether

the globulin fraction binds added zinc and, if so, which globulins are involved (Dennes et al, 1962; Suso and Edwards, 1969; Boyett and Sullivan, 1970; Prasad and Oberleas, 1970; Parisi and Vallée, 1970). In these studies the amounts of total zinc (stable zinc plus zinc-65) added to the samples of serum and plasma vary greatly. Some authors do not specify the actual amounts of zinc added in their radioactive solutions while others have added amounts which are at least equal to that already present in the plasma or serum sample.

The aim of the work described below was specifically to study the effect of adding increasing amounts of zinc (from 0.5 to 1000% of the endogenous plasma level) on the zinc binding pattern. Electrophoresis on cellulose acetate strips was chosen as the method of separation because it divides the proteins into the classic grouping of albumin, α_1-, α_2-, β- and γ-globulins and provides an inert support medium which is easily stained or cut into fractions for counting. High specific activity zinc-65 chloride solution was used to enable small amounts of total zinc to be added to plasma while still retaining adequate zinc-65 activity. Human serum albumin labelled with iodine-131 has been used as a marker for the distribution of albumin along the strips. Also, since it has been suggested that the iron-binding globulin, transferrin, might be

actively involved in the transport of zinc as well as iron (Dennes et al, 1962; Boyett and Sullivan, 1970) radioiron (iron-59) has been used to label the transferrin in plasma and this distribution compared with that obtained from zinc-65 added to the same sample.

Since in the <u>in vivo</u> situation competition for zinc by the plasma proteins occurs in a solution containing a considerable quantity of low molecular weight compounds dialysis of the plasma to remove this material prior to addition of the zinc limits binding to the proteins. However, as the low molecular weight components of plasma might not only bind zinc themselves but also affect protein binding, the zinc has been added to the physiological solution without prior dialysis. Dialysis has only been carried out to study the proportion of the added zinc which binds to the low molecular weight fraction and to determine whether the protein-bound zinc is removable by this method.

2.2 Materials and Methods

The zinc-65 was obtained as the chloride in 0.1N hydrochloric acid and was diluted to a concentration of 100 μg / ml. Since the specific activity of the solution was originally about 100 mCi / mg the diluted solution had an activity of 100 μCi / ml. Since zinc chloride tends to precipitate from a neutral solution no attempt was made to adjust the pH of the diluted

solution. Iron-59 as ferric chloride was obtained as a solution of specific activity approximately 10 mCi / ml and was diluted to a concentration of 1 mg iron per ml (100 μCi / ml). Human serum albumin labelled with iodine-131 was diluted to a concentration of 50 μCi / ml. All samples were diluted using double-distilled deionised water whose measured zinc content was ≯ 2 μg / 100 ml. All radioactive solutions were obtained from the Radiochemical Centre, Amersham.

Plasma samples were obtained from five normal volunteers, age 25 - 35 and from one patient suffering from suspected Crohn's disease with associated plasma protein abnormalities. Venous blood samples were withdrawn using a disposable syringe and needle and the samples transferred to vials containing lithium heparin. The lithium salt of EDTA, lithium sequestrene, is the anticoagulant usually used for stable zinc measurements but since it is a powerful chelating agent it was unsuitable for use in this study. To compare the plasma zinc levels obtained using containers treated with heparin and with sequestrene, equal volumes of the same blood sample were placed in one container of each type and the samples centrifuged. The plasma from each was then withdrawn into untreated sterile plastic vials. The zinc content of each was measured and the values obtained were the same, within the limits of experimental error. Blood samples were centrifuged

and aliquots of the plasma samples of the required volume were transferred to sterile disposable plastic vials. Solutions of stable zinc were made up using AnalaR grade zinc in hydrochloric acid, diluted to the required concentration using double-distilled deionised water. Any necessary adjustment of pH was made with hydrochloric acid. The zinc solutions were added to the plasma samples slowly using a disposable polypropylene syringe and a stainless steel needle. The samples were incubated for 10 minutes at 37°C before separation.

Pure dried human albumin and transferrin (Behringwerke, Ltd.) and dried α-globulins, designated Cohn Fraction IV (Miles Laboratories, Inc.) were used to increase the natural plasma levels of these proteins in some samples. The required amounts were either dissolved in isotonic saline and added to the plasma or usually, dissolved directly in the plasma samples.

Electrophoresis was carried out on 2.5 cm x 12 cm cellulose acetate strips using a barbitone / sodium barbitone buffer. After separation the strips were either cut into 2 mm sections along the length of the strip or cut along the divisions between the protein fractions. The techniques used for electrophoresis and for preparation of the cellulose acetate strips for counting are described in Appendix II. To reduce contamination the electrophoresis apparatus and all

glassware were soaked in a solution of 50 ml Decon-90 (Decon Laboratories Ltd) per litre of double-distilled deionised water for two hours and washed three times in double-distilled deionised water before use. All solutions used were made up using Analar grade chemicals where possible and in double-distilled deionised water.

Dialysis was carried out using Visking dialysis tubing at a temperature of 4°C. Metal contamination in the tubing was removed by boiling the cut lengths in a 10% solution of di-sodium EDTA for 10 minutes, washing twice in double-distilled deionised water and then boiling for 10 minutes in water. Plasma was used in most of the experiments, but, because of the fact that heparin's anticoagulant properties last for only a short time serum was used for all dialysis experiments.

2.3 Measurement of Zinc Binding to the Low Molecular Weight Fraction in Plasma

Zinc-65 chloride solution (2 μCi) was added to 2 ml normal serum and the sample diluted to 5 ml with isotonic saline. The resultant solution was dialysed against 500 ml isotonic saline at 4°C for 18 hours. Two μCi zinc-65 chloride in 5 ml isotonic saline was dialysed against 500 ml isotonic saline at the same time. The activity of 2 ml of the dialysate was measured in each case and the total activity lost from the samples calculated.

Percentage activity lost by zinc-65 chloride in saline = 58%

Percentage activity lost by serum = 3%

Therefore, assuming the same rate of dialysis from both samples,

Total dialysable activity in serum $= \frac{3}{58} \times 100$

$= 5.2\%$

Dialysis can only provide information about the low molecular weight compounds as a whole and gives no information on the amount of unbound zinc ions existing in the sample. Since zinc is a positively charged cation excess zinc should separate from the plasma proteins on electrophoresis, since they move towards the anode, and thus enable an estimate of the free zinc to be made. However, when zinc-65 chloride solution was added to an equal volume of the barbitone buffer and a sample of the solution separated using electrophoresis in the buffer solution it was found that the activity concentrated in a band towards the anode side of the origin, indicating formation of a compound of zinc which acquired a net negative charge on applying an electric field. The position of this compound was approximately midway between the albumin and α_1-globulin fractions of the plasma proteins. To check, therefore, whether any ionic zinc was present in the

active plasma, 5 μl samples were run using isotonic saline as the electrolyte. Although the solution was not buffered, stained strips showed that the proteins had migrated towards the anode, while the activity of a zinc-65 chloride-saline solution was found, as expected, at the cathode side of the origin. The mean activity of the (unstained) areas containing the proteins from a number of strips was compared with the mean activity of several 5 μl samples of the radioactive plasma. The values were found to be the same and no measurable activity was detected at the cathode side of the origin. Since all the added zinc appeared to have been bound by the plasma constituents it was decided that barbitone buffer could be used in further experiments to take advantage of the sharper separation obtainable.

2.4 <u>Binding Pattern of Zinc to the Protein Fractions</u>

An aliquot of 10 μCi zinc-65 solution in 0.1 ml (0.1 μg zinc) was added to 1 ml plasma. The endogenous plasma zinc level was 98 μg / 100 ml and the amount added therefore amounted to about 10% of the total plasma zinc level. 5 μl plasma samples were then separated using barbitone buffer and each strip cut into 2 mm sections as described in Appendix II. The zinc-65 activity of each 2 mm strip was plotted against distance from the origin, giving the distribution shown in Fig. 1, with a large peak coincident with the

albumin fraction and a second smaller peak apparently coincident with the α_2-globulins. To enable the position of this second peak to be assessed more accurately, human serum albumin labelled with iodine-131 was used to outline the distribution of the zinc-65 bound to albumin and hence the distribution of the remaining activity by subtraction. Fig. 2 shows the distributions of both iodine-131 and zinc-65 activities, obtained from plasma samples containing both 15 μCi zinc-65 and 10 μCi iodine-131 per ml of plasma. It can be seen that the mobilities of the iodine-131- and of the zinc-65-labelled albumin are in fact measurably different. However, though transposed slightly, the zinc-65 curve follows the iodine-131 curve well enough to use the iodine-131 distribution to calculate the tail of the curve of the albumin-bound zinc-65. When this distribution is subtracted it leaves a single peak coincident with the α_2-globulins, as shown in Fig. 2.

Solutions of zinc-65 were added to plasma, taken from the same subject each time, in amounts such that the total quantity of added zinc ranged from 0.5 μg to 1 mg per 100 ml plasma. This covered the range of from 0.5 to 1000% of the endogenous plasma level for this subject (98 μg / 100 ml plasma). 5 μl samples were separated and the distribution of radioactivity against distance migrated plotted in each case. In all samples studied the distribution gave two peaks

coincident with the albumin and α_2-globulin regions, though the relative height of the two peaks varied with the amount of added zinc. Serum samples containing added zinc-65 also gave distributions of zinc-65 activity having two peaks in the albumin and α_2-globulin regions.

5 μl samples of radioactive plasma containing 20 μg added zinc were separated and the strips dried and fixed to sheets of X-ray film. The films were left in the dark for five days and then developed. Fig.3 shows one of the autoradiographs obtained, together with a stained strip from 5 μl plasma without added zinc-65, separated at the same time. It can be seen that only two bands, coincident with the albumin and α_2-globulin fractions appear on the autoradiograph.

2.5 Quantitation of Zinc Binding in Plasma

Since the method used in this study detects binding only in the albumin and α_2-globulin regions the ratio of the area under the albumin peak to that under the α_2-globulin peak was used to quantitate the partition of zinc between the protein fractions, for various levels of added zinc. The distribution of zinc-65 activity was plotted as above and the distribution of iodine-131-labelled albumin used to estimate the tail of the distribution of albumin-bound zinc-65. The total area under the albumin peak was then subtracted from the total zinc-65 activity to give the area under the second peak. Table 1 shows the ratios obtained

from at least 10 different separations made on plasma samples, for 10% and 25% added zinc (10 μg and 25 μg per 100 ml plasma), taken from the same donor but at different times. It can be seen that though there is considerable variation among the individual values, the means do not differ significantly.

Since the separation obtained using electrophoresis is highly dependent on such factors as buffer composition, ambient temperature and plasma composition, the following method was used to eliminate variation introduced by differing experimental conditions, when comparing the ratios obtained on addition of 0.5%, 10%, 100% and 1000% of the endogenous plasma zinc level. Solutions of zinc-65 were prepared containing the required amount of total zinc in 0.1 ml solution, each solution having the same pH. 0.1 ml of each solution was added to 1 ml aliquots of the same plasma sample and 5 μl samples from the four aliquots were run together in the same tank, six runs being made in all. Since it had already been shown that measurable binding occurred only to albumin and the α_2-globulins, the ratios of albumin- to globulin- bound zinc-65 activities should be the same as those for albumin and the α_2-globulins throughout the range. The 5 μl samples were separated so that the maximum separation of albumin from globulins was achieved. The activity in each fraction was measured and the albumin/globulin zinc-65 ratios calculated. The results are shown in Table 2.

They show an increase in albumin/globulin ratio with increasing amount of added zinc. The probability that the differences in the mean albumin/globulin ratio for each of the distributions occurred by chance was calculated using the Student-t test. While differences between adjacent individual distribution have varying significance, the increase is found consistently throughout the whole range.

The binding patterns for zinc-65 for plasma samples from different normal donors were compared as follows. 20 μCi zinc-65 solution (equivalent to 20 μg / 100 ml plasma) was added to 1 ml plasma from each of five donors and the binding patterns studied. These were all found to have the general form of Fig. 1. Since it was not possible to run all five samples in the tank at once they were compared in groups of two, using four 5 μl samples from each aliquot per run, and making every attempt to keep the conditions of electrophoresis the same throughout. The results are shown in Table 3. Plasma zinc levels were in the range 85 - 160 μg / 100 ml plasma and none of the subjects had evidence of pathological conditions which might cause altered zinc metabolism. No significant difference could be detected between any of the distributions of added zinc-65, measured using the albumin/α_2-globulin ratios.

2.6 <u>Relationship between Zinc Binding and Protein Concentration in Plasma</u>

Since it has been shown that there are two

distinct fractions which bind zinc and which can be separated by electrophoresis it would seem reasonable to expect that there will be a relationship between the amounts of protein present in each fraction and the amount of zinc bound by each of them. To test this assumption the protein concentration of the plasma was altered by addition of known amounts of specific protein fractions and the binding pattern of zinc-65 added to the modified plasma compared with that of the original. This approaches the in vivo situation more closely than adding zinc-65 to the individual proteins in solution.

The albumin content of one sample was measured and an amount of dried human albumin equal to that already present in the plasma was dissolved in the sample (thus doubling the normal albumin level). 10 μCi zinc-65 solution was added to 1 ml of this solution and to 1 ml of the untreated plasma. Four 5 μl samples of each solution were run together as described above. The strips were stained using ninhydrin (Appendix II) and cut into albumin and globulin fractions. The ratio of albumin / globulin zinc-65 activities was then found for each strip and the mean values calculated. The ratios were found to be (1.73 ± 0.20) for the untreated plasma and (3.20 ± 0.08) for the sample containing twice the normal level of albumin, an increase of approximately a factor of two, suggesting that for albumin the amount of zinc bound is proportional to the quantity of the

protein present.

Since the α_2-globulin fraction is heterogeneous, this group could contain one or more proteins capable of binding zinc (for the purpose of the present study albumin has been considered as a homogeneous fraction). Hence it is necessary to increase the α_2-globulin fraction as a whole, rather than using a single protein. This was done in an approximate way by assuming a normal total α-globulin level of 800 mg / 100 ml in the plasma sample and adding this concentration to 1 ml plasma and thus approximately doubling the quantity of α-globulins in the sample. Since the added protein contains appreciable amounts of the other fractions, particularly albumin, the ratios obtained do not accurately reflect the change in protein concentration. However, when 10 μCi of zinc-65 was added to each 1 ml sample (modified and original plasma) and the mean albumin/globulin zinc-65 ratio calculated for eight 5 μl samples, values of (2.8 $\pm$0.7) for the normal plasma and (2.0 $\pm$ 0.5) for the plasma containing the additional α-globulins were obtained, showing a significant increase in binding to the α_2-globulins when the α-globulin level was increased.

To test the hypothesis that transferrin is a transport protein for zinc as well as for iron (Dennes et al, 1962; Boyett and Sullivan, 1970), zinc-65 and iron-59 were added to the same plasma sample. The amount

of iron added was chosen so that the total iron-binding capacity was not exceeded and hence all the iron added would bind to transferrin, producing a single peak in the β_1-globulin region. 10 μCi zinc-65 and 5 μCi iron-59 were added to 1 ml plasma and 5 μl samples separated. The cellulose acetate strips were cut into 2 mm sections and the activities of each isotope plotted, giving the graph of Fig. 4. This shows that at normal levels the binding of zinc to transferrin is too small to be measured using this technique and is negligible compared with the binding to the other fractions. The experiment was repeated using plasma from a second normal donor and similar results were obtained. However when the transferrin level of one plasma sample was raised to approximately four times the endogenous level (by dissolving the required amount of the dried protein in the plasma) and 10 μCi zinc-65 added to 1 ml plasma, measurable binding in the β-globulin region was obtained.

One plasma sample was obtained from a patient with severe malabsorption thought to be due to Crohn's disease and whose plasma protein levels were grossly abnormal. The binding pattern of zinc-65 to the plasma was studied and it was found that the pattern was noticeably different from the normal one, with a measurable peak in the β-globulin region, besides the albumin and α_2-globulin peaks found in healthy subjects.

2.7 Effect of Dialysis on Protein Bound Zinc in Serum

Dialysis of 1 ml serum containing 20 µCi zinc-65 (0.02 µg added zinc) against 2 ml of the same serum containing no active material over a period of two weeks showed that zinc-65 was lost continuously through the membrane. Electrophoresis of samples of the active serum before and after dialysis and of the originally inactive serum after dialysis showed that the zinc was distributed as in Fig. 1 in each case. 0.25 µCi zinc-65 was added to 1.5 ml serum and the active serum dialysed against 2.5 ml of the same serum for five days. 0.25 µCi iron-59 was added to a second 1.5 ml sample and dialysed against 2.5 ml serum under identical conditions. The activity of the originally inactive serum samples was measured at intervals during this period and the gain of activity plotted against time for each sample, shown in Fig. 5. While no activity was gained from the sample containing iron-59 the zinc-65 was gradually lost from the other sample, suggesting a weaker binding of zinc to both protein fractions compared with the strong binding of iron to transferrin.

2.8 Discussion

The present study has shown binding of added zinc to two protein fractions, albumin and the α_2-globulins, when the proteins are separated using electrophoresis on cellulose acetate strips. Only those two fractions

bound measurable quantities of zinc, even when the amount of added zinc was ten times the normal level in plasma, suggesting that the capacity of the plasma proteins for zinc ions is very high, and unlikely to be exceeded even by therapeutic doses of zinc. Although only albumin and the α_2-globulins bind measurable amounts of the added zinc in a range of from 0.5 - 1000% of the normal level, the relative amounts bound to each vary with the amount of zinc added, from almost equal partition between the two fractions at the lower end of the range to almost two thirds binding to albumin at the upper end. This ratio is also dependent on the amounts of each protein present in the sample, though the variations in the plasma protein levels found in normal subjects were presumably too small to affect the ratio, since no difference in the ratios obtained from a group of normal subjects could be detected. However in one case where the plasma protein distribution was grossly abnormal a difference in zinc binding was detected.

The binding of zinc to two fractions, demonstrated using protein separation by electrophoresis on cellulose acetate, is consistent with the results of other studies which have used various forms of electrophoresis, all of which have shown binding to both albumin and to the globulins, the greatest affinity for zinc being shown by the α-globulins (Dennes et al, 1962; Prasad and Oberleas, 1970, Boyett and Sullivan, 1970).

The degree of separation achievable by electrophoresis is dependent on the support medium used and greater separation of the proteins can be obtained using support media such as starch and acrylamide gels which combine electrophoretic separation with molecular sieve effects. Dennes, Tupper and Wormall used filter paper as the support medium in their study of binding of zinc-65 to the plasma proteins in both rabbits and in human plasma _in vitro_. They showed that, when zinc-65 was added to human plasma the isotope bound to the albumin and to all of the globulin fractions, the α-globulins showing the greatest affinity among the globulins and the γ-globulins least. Prasad and Oberleas used starch gel and they also found that zinc-65 added to human serum _in vitro_ bound to both albumin and globulin fractions and that the α-globulins bound the major part of the globulin zinc-65. Boyett and Sullivan used an acrylic polymer as the support medium and found that zinc-65 added to human serum bound to both albumin and to a fraction containing mainly α_2-macroglobulin and transferrin, and, in some cases, to immunoglobulin-G. They found that when zinc-65 was added to serum samples from patients suffering from hepatic cirrhosis who had low serum zinc levels, the percentage of added zinc-65 bound by the albumin fraction was significantly lower than in normal controls. They postulated that the binding of zinc-65 to plasma samples _in vitro_ could

be used as an indicator of the manner in which the endogenous zinc in plasma is bound to the proteins, and they calculated the amount of stable zinc associated with each protein fraction from the percentage of the added radiozinc found in each fraction and the total serum zinc level. While it is of interest that they obtained maximum globulin binding in a fraction containing α_2-macroglobulin, their assumption that trace amounts of zinc-65 can be used to measure endogenous binding is open to question. It assumes, firstly, that added zinc will bind to all the zinc-containing proteins in serum and hence that all zinc-containing proteins obtain the zinc from ionic zinc absorbed into plasma, and, secondly, that binding of added zinc will reflect the partition of zinc among the proteins which already exists in the sample. It would seem much more likely that this method would indicate the capacity of the proteins concerned for zinc, rather than the amounts already bound to them.

In the present study the only globulin fraction found to contain measurable zinc-65 activity was the α_2-globulins. However this fraction was located by plotting the distribution of zinc-65 activity along the cellulose acetate strip, which gave two Gaussian distributions with peaks coincident with the albumin and α_2-globulin regions. A method which measures only the radioactivity associated with the protein

fractions as a whole would detect measurable activity from the major binding proteins in adjacent fractions also, since the protein distributions are continuous, which would explain the finding of smaller amounts of zinc-65 in both the α_1- and β-globulin fractions.

Boyett and Sullivan, who found that added zinc was bound by albumin and a fraction which contained both α_2-macroglobulin and transferrin, suggested that transferrin might be actively involved in zinc transport by plasma for this reason (Boyett and Sullivan, 1970). However the present work has shown that, when radioactive iron-59 is used to label the transferrin in plasma, no measurable activity associated with added zinc-65 could be found in the area of the iron-binding peak. This suggests that the zinc-65 in Boyett and Sullivan's second peak was bound only to α_2-macroglobulin.

The pattern of zinc binding obtained in this study agrees with that obtained by Parisi and Vallée for the partition of the endogenous zinc among the plasma proteins, with about two thirds of the zinc associated with albumin and one third with one of the α_2-globulins, the zinc metalloprotein, α_2-macroglobulin (Parisi and Vallée, 1970). However it is significant that those authors who have used methods of protein separation other than electrophoresis have not detected binding of zinc-65 to any other protein than albumin, when the isotope was added to the sample *in vitro*. In

particular, Parisi and Vallée found that zinc-65 added *in vitro* did not bind to the α_2-macroglobulin fraction, but only to albumin, using chromatography on Sephadex gel as the method of separation. Similar results were obtained by Suso and Edwards using the same method of protein separation (Suso and Edwards, 1969). It is possible that the electric field applied during electrophoretic separation affects the binding of added zinc in some way, and this is the more likely since the binding of zinc to albumin is known to be weak (Vikbladh, 1951; Parisi and Vallée, 1970). Comparison of the results obtained in the present work with separation using a chromatography technique is therefore essential, especially since electrophoresis on cellulose acetate strips is not suitable for the separation of large volumes and hence useless for separating samples labelled with zinc-65 *in vivo*, where the low activities of the plasma samples necessitate separation of relatively large volumes.

2.9 Conclusions

From the results of the work described above it can be concluded that:-

(a) Zinc-65 added to plasma *in vitro* is bound almost entirely by two protein fractions, albumin and the α_2-globulins, and this is true over a wide range of levels of added zinc, from 0.5 to 1000% of the normal plasma zinc level.

(b) The ratio of amount of added zinc bound to albumin to amount bound to α_2-globulins varied with the amount of zinc added, a larger proportion of the zinc being bound by albumin for large quantities of added zinc, though these values are affected by gross variations in the concentrations of the particular proteins.

(c) The proportions of added zinc-65 binding to the two fractions are similar to those found for the partition of endogenous zinc between the two fractions, found by Parisi and Vallée, though complementary studies using another method of protein separation are necessary before the significance of this finding can be assessed.

CHAPTER 3

CHAPTER 3

INVESTIGATION INTO THE SUITABILITY OF CROSS-LINKED DEXTRAN GELS FOR THE STUDY OF PLASMA ZINC BINDING

Page

		Page
3.1	Requirements for Separation of Plasma Samples Labelled with Zinc-65 *in Vivo*	44
3.2	Chromatography Using Sephadex Gel	45
3.3	Measurement of Total Protein Concentration	47
3.4	Distribution of the Proteins among the Column Fractions	48
3.5	Quantitative Estimation of Individual Protein Concentrations	50
3.6	Measurement of Albumin Concentration	53
3.7	Determination of Optimum Sample Size	55
3.8	Addition of Zinc-65 to Plasma and Serum *in Vitro*	56
3.9	Discussion	59
3.10	Conclusions	61

3.1 Requirements for Separation of Plasma Samples Labelled with Zinc in Vivo

While the addition of zinc to plasma *in vitro* can give useful preliminary information on plasma protein binding the ultimate aim of any study on the transport of a particular element by plasma must be to examine the binding pattern obtained when it is introduced into the plasma *in vivo*, either by studying the binding of the endogenous stable element in plasma or the binding of a trace quantity of a radioisotope of the element administered *in vivo*. However since the levels of both stable and radioactive zinc will be low it is essential to be able to separate samples of several millilitres in volume to obtain a sufficient concentration of zinc in the protein fractions. Electrophoresis using cellulose acetate strips is therefore unsuitable for either study. The purpose of the work described below was therefore to develop a technique which would provide reliable separation into known fractions of sufficiently high concentration whose protein content could be accurately assessed. The technique was then used to study the binding of zinc-65 added *in vitro* to the plasma proteins and the results obtained have been compared with those previously reported using electrophoresis on cellulose acetate strips.

There are several methods which can be used to fractionate the required volume of plasma, notably preparative electrophoresis, chromatography using

ion-exchange cellulose and molecular sieve chromatography using cross-linked dextran gels. However the first two methods present particular difficulties in the measurement of the stable zinc levels in the protein fractions. The possible redistribution of the zinc caused by electrophoresis has been discussed in Chapter 2. Chromatography on ion-exchange cellulose requires elution of the proteins by a solution of gradually increasing pH and concentration. While this may not affect the binding of zinc to the plasma components these fractions are eluted in a solution of varying composition and concentration, which creates problems in the accurate assessment of the background level in the fractions when measuring the stable zinc concentration. The most suitable method therefore appeared to be chromatography using cross-linked dextran gels.

3.2 Chromatography Using Sephadex Gels

Water-soaked cross-linked dextrans form gels which act as molecular sieves for molecules within a certain size range, whose upper and lower limit depends on the degree of cross-linkage of the dextran monomers. Although strictly the separation is in order of molecular size, it is generally true that this is also the order of molecular weights, and it is found that the elution volume for a particular molecule is approximately proportional to the logarithm of its molecular weight.

Chromatography on cross-linked dextrans is

particularly suitable for separation of the zinc-binding plasma proteins for the following reasons:-

(a) The separation is a passive procedure depending only on size and not on reaction of the sample molecules with the gel. The possibility of the zinc binding being altered by the separation procedure is therefore minimised.

(b) Almost complete sample recovery is obtained and hence quantitation and standardisation of the procedure is readily achieved.

(c) Since the separation is a function of molecular weight, good separation of the two proteins known to bind zinc, namely albumin (molecular weight 69,000) and α_2-macroglobulin (molecular weight approximately 800,000) can be achieved using this method.

Sephadex G200, which fractionates in the molecular weight range 800,000 - 5,000 is particularly suitable for separation of the plasma proteins and this grade was used in the preliminary studies described in this chapter. A column 46 cm x 2.6 cm diameter was used and elution carried out using a 0.1 M Tris(hydroxymethyl)-methylamine / hydrochloric acid (Tris-HCl) buffer, pH 8.0, made up in a 1M solution of sodium chloride. The method used to prepare the gel and the separation procedure are described in Appendix III.

By this technique the separated sample emerges as a continuous flow from the column and the fractions

consist of equal volumes of the eluted solution. Each fraction contains a number of proteins in varying concentration, the concentration of a particular protein in any fraction being a function of the elution volume of that fraction. The total protein content of the fractions was assessed using ultraviolet spectrophotometry.

3.3 Measurement of Total Protein Concentration

Since protein solutions absorb ultraviolet light strongly, with maximum absorption at wavelength 280 nm, measurement of the absorption of light of this wavelength by the sample gives a useful indication of the protein concentration of the column fractions. The measurements were carried out as described in Appendix IV. The method is not suitable for quantitative evaluation, however. When the variation in absorbance at 280 nm with concentration was measured for serum diluted with Tris-HCl buffer and for albumin and transferrin dissolved in the same buffer, it was found that albumin has a lower absorbance at this wavelength than the same concentration of either transferrin or whole serum. Since the relative absorbances of the proteins vary in a non-linear manner with concentration attempts to estimate total protein concentration from ultraviolet absorption measurements are of little value for solutions containing a number of different proteins. However these measurements do provide a useful qualitative estimate of

the separation, since the distribution shows three separate peaks, the first representing the high molecular weight fraction (around 800,000) the second being composed mainly of the 7S gamma globulins and the third consisting mainly of albumin. The three peaks act as useful markers and provide a guide for more detailed analyses. Fig. 6 shows the variation of absorbance with elution volume for plasma samples of 3 ml, 5 ml and 10 ml, when separated into 6 ml fractions.

3.4 Distribution of the Proteins among the Column Fractions

Cellulose acetate electrophoresis and immunoelectrophoresis of the column fractions were carried out to determine the distribution of the individual proteins among the fractions. A sample of pooled normal serum (6 ml) was separated into 6 ml fractions using Tris-HCl buffer and a flow rate of 21.6 ml / hr. The absorbance at 280 nm was measured for each fraction and Fraction 1 was taken to be the first containing measurable protein concentration. The maxima for the three protein peaks occurred at fractions 3, 8 and 13. 5 μl samples of fractions 1 - 17 were then separated by electrophoresis on cellulose acetate strips and 2 μl samples separated using immunoelectrophoresis in agar gel. The methods used are described in Appendix I. Where difficulties in identifying the major individual arcs in the immunoelectrophoresis slides arose they

were identified using monospecific antisera. If the proteins are considered as occurring in three groups, corresponding to the three peaks in the protein distribution, then the first group consists of fractions 1 - 5 and contain the proteins with molecular weight in excess of 500,000. The major proteins are immunoglobulin-M, α_2-macroglobulin and α_2-haptoglobin, which, although it has a molecular weight of 160,000, is atypical and appears to have a much greater molecular weight when separated using Sephadex gel. The second group (fractions 6 - 11) consists of the proteins in the molecular weight range 500,000 - 100,000 and the main constituents are immunoglobulin-G, caeruloplasmin and significant quantities of albumin. The third group contains mainly albumin and transferrin. The immunoelectrophoresis slides showed that group III and, to a lesser extent, group II, contain a considerable number of other proteins which are present in lower concentrations. Fig. 7 compares the stained strips from cellulose acetate electrophoresis and the immunoelectrophoresis slides for the two samples with maximum protein concentration in group I with those having maximum concentration in group III, together with serum samples. It can be seen that while those in group III contain a fairly large number of proteins, group I contains only significant amounts of α_2-globulins, identified as α_2-macroglobulin and α_2-haptoglobin by immunoelectrophoresis.

3.5 Quantitative Estimation of Individual Protein Concentrations

Immunoelectrophoresis can identify the individual proteins contained in each fraction but can give no information on their relative concentrations. Since it has been shown that all the fractions contain a number of proteins in varying concentration single radial diffusion in agar gel containing monospecific antisera to the major serum proteins was used to measure the concentration of these proteins. Although agar plates incorporating antiserum are available commercially these are expensive and not sufficiently flexible to cope with a wide range of concentrations. Accordingly, the plates were made up when required using the necessary specific antisera. The technique used is described in Appendix V.

The most reliable method for quantitation of radial immunodiffusion is that described by Mancini, Carbonara and Heremans (Mancini et al, 1965), where the antigen samples are left to diffuse in the gel until no further precipitation of antigen-antibody complex occurs. Mancini and his associates found that when diffusion is complete there is a linear relationship between the area of the precipitin rings and the concentration of the antigen in the sample. In the present study it was found that the concentration could be described sufficiently accurately by an exponential function of the diameter, for varying times of diffusion.

A sample of serum was diluted to half strength with Tris-HCl buffer. Half of this sample (called 100% concentration) was further diluted with an equal volume of buffer and the process repeated six times, giving concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.13%, 1.56% and 0.78%. Three plates, area 8 cm x 16 cm, were made up incorporating antiserum to human albumin in the gel and 2 μl samples of serum at the required dilution placed in the wells. The diameters of the precipitin rings were measured at 17 hours, 41 hours and 65 hours. A least squares fit to both an exponential function of the diameter and to a linear function of the square of the diameter (i.e. to the area) was calculated for each plate. It was found that an exponential function gave a much better fit to the experimental points than a linear function for each of the time intervals chosen. The calculated exponential curves, together with the experimental points, are shown in Fig. 8. An exponential function of this form was therefore used for all subsequent estimations of protein concentration. Four standards consisting of known dilutions of serum or plasma were included in each plate and the equation of the standard curve plotted using a least squares fit to an exponential function. One sample containing a known concentration of the particular protein was included when absolute measurements of protein concentration were required.

The concentrations of several of the major proteins in the column fractions were then measured. The proteins were chosen such that their molecular weights covered the range of the plasma proteins and also so that the separation achieved of proteins of similar molecular weights could be assessed (for example, of albumin and transferrin.

The proteins studied were:-

	Molecular Weight
Albumin	69,000
Transferrin	90,000
Caeruloplasmin	160,000
Immunoglobulin-G	160,000
Haptoglobin	160,000 (separates as if of molecular weight 500,000)
α_2-macroglobulin	820,000
Immunoglobulin-M	1,000,000

Plates were made up using the appropriate specific antisera and the concentrations measured in those fractions shown to contain measurable quantities of the particular protein from the immunoelectrophoresis slides. The results are shown in Fig. 9, together with the absorbance at 280 nm of the fractions. To enable the distribution of the other proteins to be included only the lower half of the albumin peak has been shown. The protein distributions show the wide range of

concentrations of the individual proteins and particularly that even a small percentage of the total albumin content, which is present in many of the fractions, accounts for a significant proportion of the protein content of that fraction. However the fractions containing the proteins of high molecular weight, and, in particular, those containing α_2-macroglobulin, contain a negligible amount of albumin. If the relative positions of the peaks of the individual proteins are considered, rather than the amounts of protein present, it can be seen that separation of the protein peaks is achieved, even for proteins of similar molecular weight such as albumin and transferrin.

3.6 Measurement of Albumin Concentration

Since there is substantial evidence that the major part of the plasma zinc is bound to albumin it is particularly important in a study of this kind to be able to assess accurately the concentration of albumin in each of the fractions, especially since it is present in such excess over any of the other proteins. Also separation using gel chromatography has the disadvantage that albumin is poorly separated from a large number of other proteins and any binding of zinc to these proteins in fractions containing appreciable amounts of albumin will tend to be masked by it and will be detectable by its distortion of the shape of the albumin-bound zinc distribution rather than as a

separate peak. Separation from the greater binding to albumin is only possible if the albumin concentration in each fraction can be estimated accurately.

As with the other proteins radial immunodiffusion is the most accurate method available for albumin estimation. However there are several disadvantages to this method, particularly for samples of high albumin concentration. If the concentration of antiserum in the gel is adjusted to allow measurement of a large range of concentrations, samples with high concentrations produce precipitin rings which are large and difficult to see and therefore difficult to measure.

A more rapid estimation of albumin concentration can be obtained by utilising the marked affinity of albumin for certain dyes. In particular, methyl orange is bound almost completely by the albumin in serum or plasma and produces graded colour changes depending on the amount of albumin present. These changes can be measured using a spectrophotometer and the amount of albumin calculated from a calibration curve. The technique used is described in Appendix IV. The variation of absorbance at 540 nm for a solution of pure albumin dissolved in buffer and for diluted serum containing a known amount of albumin was studied. It was found that each curve could be described accurately by an exponential function, but that the curves obtained for the two samples were not coincident.

This is due to the existence in serum and plasma of other proteins with a slight affinity for the dye and of pigments such as bilirubin which affect the absorbance in proportion to their concentration. Hence in measurements of albumin levels in fractions from serum and plasma samples a range of dilutions of the serum or plasma to be studied was used to derive the standard curve, the equation of which was calculated using a least squares fit to an exponential function. The presence of other proteins in the fractions with low albumin content gives a slightly overestimated result for the albumin level in these fractions but this was not found to be significant. When compared with the results obtained using immunoelectrophoresis this method was found to be of comparable accuracy for albumin measurements and was therefore used except where absolute measurements were required.

3.7 Determination of Optimum Sample Size

Since the ultimate aim of the separation procedure is to relate the zinc level in the separated fractions with their protein content, it is important to obtain good separation of the proteins while retaining maximum protein concentration. Accordingly, plasma samples of 2.5 ml, 5 ml and 10 ml were separated and the albumin concentrations measured using methyl orange reagent. The variation of concentration with eluted volume is plotted in Fig. 10 for each case. If the width

of the albumin curve at half its height is used as a measure of the sharpness of the peaks (and hence as a measurement of the separation obtainable) the values are 16.8, 15.0 and 16.8 for the curves for 2.5 ml, 5 ml and 10 ml respectively. Although the sharpness of the peaks also depends on the evenness of the application of the sample onto the drained gel bed it can be seen that there is no measurable loss of resolution with this size of column in using 10 ml rather than smaller samples and that cutting down the sample size to less than 5 ml only reduces the protein concentration without a corresponding gain in resolution.

3.8 Addition of Zinc-65 to Plasma and Serum in Vitro

High specific activity zinc-65 chloride solution (100 mCi / mg) was diluted using double-distilled deionised water to a concentration of 0.01 μCi / ml. This solution was added to serum or plasma in a concentration of 0.1 ml per ml plasma or serum. In the work described below this is referred to as "trace amounts" of zinc and added less than 1 μg zinc per 100 ml serum or plasma.

A trace quantity of zinc-65 was added to a 4 ml sample of plasma from a normal donor, together with a trace amount of iodine-131-labelled human serum albumin, and the sample incubated at 37°C for 30 minutes. The sample was then separated into 6 ml fractions using a column of Sephadex G200. The zinc-65 and iodine-131

activities in the fractions and the absorbance of light at 280 nm were measured. The zinc-65 and iodine-131 activities are plotted as a percentage of the maximum value in Fig. 11. It can be seen that, though there is slight binding to the higher molecular weight proteins, the major proportion of the zinc-65 activity is coincident with the albumin fraction, whose distribution is outlined by the iodine-131 distribution. In particular there is little binding to fractions 1 - 5, which contain the α_2-macroglobulin of the plasma.

Comparison of the binding patterns of varying quantities of added zinc was carried out as follows. Amounts of zinc, containing zinc-65, equivalent to addition of 0.1 μg / 100 ml, 50 μg / 100 ml and 200 μg / 100 ml plasma, were added to aliquots of 2.5 ml plasma from a single donor and each sample incubated at 37°C for 20 minutes. The samples were then separated into 3.7 ml fractions using a column of Sephadex G200. The zinc-65 activity and the albumin level (measured using the absorption at 540 nm in methyl orange reagent) were measured for each fraction for each of the three separations. The distribution for trace amounts of zinc-65 was as in Fig. 11 and the distributions for 50 μg and 200 μg / 100 ml are shown in Fig. 12. It can be seen that the zinc-65 and albumin distributions are coincident, within the limits of experimental error, in all three cases. These results

suggest that albumin binds virtually all the added zinc, even when the plasma level is raised to twice the endogenous level.

When a trace amount of zinc-65 was added to serum a similar binding pattern was obtained, with almost all the added zinc associated with the albumin fraction.

These results are apparently in disagreement with those previously discussed in Chapter 2, obtained using electrophoretic separation on cellulose acetate strips, where an appreciable proportion (about one third) of the zinc-65 activity was associated with the α_2-globulin fraction. These results could be reconciled, however, if the molecular weight of the α_2-globulin fraction which binds the added zinc was very similar to that of albumin, since it would then coincide with albumin on separation using gel chromatography. To investigate this possibility, 8 x 5 μl samples of plasma, labelled with 20 μCi zinc-65 per ml, were separated using electrophoresis on cellulose acetate. The α-globulin fractions were cut out of each strip and the proteins washed using Tris-HCl buffer into a plastic vial. This eluate was made up to 4 ml and separated into 6 ml fractions using a column of G200 and the zinc-65 activity measured over the molecular weight range of the plasma proteins. Since the protein level in the fractions was too low to be measured in the usual way the void volume of the

column was measured (Appendix III) and the molecular weight range of the plasma proteins estimated from this value. The activity in each of the fractions is plotted in Fig. 13. This shows measurable activity over the whole range of the plasma proteins, with a small peak in the high molecular weight region coincident with the exclusion limit of the column. As previous separation of whole plasma samples labelled with zinc-65 in vitro have shown a concentration of more than 90% of the activity in the albumin region, it can be seen that the separation of the α-globulin fraction produced by electrophoresis does not provide a second zinc-binding fraction with α_2-globulin mobility and of molecular weight similar to albumin, but rather zinc labelling throughout the proteins of higher molecular weight.

3.9 Discussion

It has been shown that a column of Sephadex G200, 46 cm x 2.6 cm diameter, can provide adequate separation of the major proteins of serum or plasma, for samples of up to 10 ml volume, providing separation of such closely spaced proteins as albumin and transferrin, with a dilution to about a quarter of the total plasma level in the fractions containing maximum concentration of the protein. The method therefore provides adequate separation without too great sample dilution, even for relatively large sample volumes.

Separation of plasma samples to which zinc-65 has been added *in vitro* gives a distribution of zinc-65 activity with a single large peak coincident with that of the albumin distribution. It is significant that there is no appreciable binding in the high molecular weight range (fractions 1 - 5), which contain the α_2-macroglobulin in the sample, since this protein is known to be a zinc metalloprotein. These results agree with those obtained by Parisi and Vallée using chromatography on Sephadex G100 (Parisi and Vallée, 1970). This binding pattern was obtained for amounts of added zinc from 1 - 200% of the average endogenous level of 100 μg / 100 ml plasma.

Cellulose acetate electrophoresis separation, on the other hand, gives two distinct peaks in the distribution of protein bound zinc-65, one coincident with albumin and the other with the α_2-globulins. When the α_2-globulin fraction thus labelled is separated using a column of Sephadex G200 the zinc-65 activity is found throughout the whole molecular weight range of the proteins, and not only in the albumin region. These results support the hypothesis put forward in Chapter 2 that the electric field applied during electrophoresis causes redistribution of the bound zinc among the protein fractions.

These results show the important part which can be played by the method of protein separation chosen in

studying the binding of trace elements to proteins. Hence the results obtained, from both in vitro and in vivo experiments, may only be valid within the frame of reference defined by the method of protein separation chosen. The usefulness of any particular method will therefore depend on its ability to differentiate between certain states, for example, the ability to detect differences in plasma zinc binding caused by particular pathological conditions which affect the plasma zinc concentration. It is also essential that the method gives similar and repeatable results on plasma samples from patients with normal zinc metabolism. The suitability of a particular method will therefore only be determined after study of both normal and abnormal plasma or serum labelled with zinc in vivo, either by studying the distribution of endogenous zinc or in vivo labelling by zinc-65.

3.10 Conclusions

(a) Chromatography using Sephadex G200 gives good separation of plasma or serum samples up to 10 ml, with acceptable sample dilution. The method is therefore suitable for use in the study of endogenous zinc binding and of zinc-65 introduced into plasma in vivo.

(b) Separation of plasma or serum containing zinc, with zinc-65, added to plasma in vitro in amounts from 1 - 200% of the average endogenous plasma zinc level gives a zinc-65 distribution with a single peak

coincident with the albumin distribution, in contrast with the distribution obtained after separation using electrophoresis on cellulose acetate, which gave a second peak coincident with the α_2-globulins, as well as that in the albumin region. It is therefore apparent that the zinc-containing α_2-macroglobulin described by Parisi and Vallée is produced only <u>in vivo</u>, since this protein is well separated from albumin using gel chromatography.

CHAPTER 4

CHAPTER 4

DISTRIBUTION OF THE ENDOGENOUS ZINC IN PLASMA AND SERUM

		Page
4.1	Introduction	64
4.2	Measurement of Trace Amounts of Stable Zinc	66
4.3	Removal of Contamination	69
4.4	Separation Using Sephadex G200	71
4.5	Separation Using Sephadex G75 and G100	74
4.6	Discussion	80
4.7	Conclusions	83

4.1 Introduction

Measurements on the binding of zinc to plasma *in vitro* have shown that the major protein involved in binding exogenous zinc is albumin and that this is probably a fairly weak bond, easily broken by external forces. Provided *in vivo* binding of zinc is a passive process uninfluenced by factors only present in the body, it can be assumed that the *in vitro* binding pattern will be the same as that for newly absorbed zinc, that is, zinc released from the dietary constituents in the gastro-intestinal tract and absorbed into the plasma for transport to the organs and tissues. The endogenous zinc in plasma, on the other hand, has several possible components:-

(a) Newly absorbed zinc.

(b) Intermediate metabolites containing zinc - substances such as zinc-containing proteins which have been manufactured by the organs for use elsewhere in the body and which are being transported there by plasma.

(c) Excretion products, produced by tissue breakdown, whose zinc is in a form which can be excreted via the kidneys.

The binding pattern of the stable zinc to the plasma constituents is therefore an equilibrium pattern and, in a homeostatically controlled system is probably fairly constant for a particular individual. It is a

purely static picture, giving no information about the fate of a particular zinc-containing fraction. However, it does present a composite pattern containing all the physiologically important zinc-containing components. This makes it particularly suitable as a means of comparing plasma or serum samples, both normal and abnormal (from patients with abnormal zinc metabolism. Also when studies on the binding of stable zinc in plasma are combined with studies using radioactive zinc, useful information can be obtained on the kinetics of the various fractions.

After the initial studies of Vikbladh (Vikbladh, 1951) showed that serum zinc exists as two fractions, one tightly bound and the other fairly loosely bound and identifiable with albumin, the problem has been studied in greater detail, particularly by Vallée and his associates (Himmelhoch et al, 1966; Parisi and Vallée, 1970). The protein fractions appear to contain several zinc-containing proteins, none of which can be identified with known zinc metalloenzymes. Parisi and Vallée showed that the zinc present in normal pooled human serum has two main components, one loosely bound to albumin and the other firmly attached to α_2-macroglobulin, which they showed to be a zinc metalloprotein, containing between 320 to 770 μg zinc per g protein, in a number of α_2-macroglobulin preparations.

While little work has been reported on the stable

zinc content of the low molecular weight components (probably because of the small quantity) it is generally recognised that a small percentage of the plasma zinc is present in this fraction, probably bound to amino acids.

It has been shown previously that chromatography using Sephadex gel allows separation of plasma samples into fractions which are sufficiently concentrated to make measurement of the stable zinc content of the fractions feasible. The method also allows clear separation of albumin from α_2-macroglobulin. The aim of the present work has been to study the stable zinc binding using gel chromatography in serum and plasma from normal subjects and to establish whether the pattern obtained by Parisi and Vallée is found in individual samples of plasma. The relative amounts of zinc bound to each of the protein fractions has been determined, in both normal controls and in patients with suspected zinc deficiency, and a method developed for measuring the relative amounts of zinc bound to the protein fractions in plasma which is rapid and simple to perform.

4.2 Measurement of Trace Amounts of Stable Zinc

Early colorimetric methods of zinc estimation using reagents such as dithizone, which form a coloured complex with the zinc in solution and whose colour can be used to estimate the amount of zinc present, have been

largely superseded by methods which make use of the fundamental properties of the zinc atom. Two methods of this type are in current use to measure trace quantities of stable zinc. The first, activation analysis, uses irradiation of the sample by thermal neutrons, converting a proportion of the stable zinc atoms to zinc-65. Measurement of the zinc-65 activity in the irradiated sample enables the amount of stable zinc originally present to be calculated. This method is particularly useful for samples in solid form. However, for liquid samples atomic absorption spectrometry is more suitable. This method measures the absorption of light by atoms in the ground state in a sample which has been vapourised in a flame. Conventional emission spectrometry does not provide a sufficient number of excited atoms to make the method sensitive enough for use in trace element analysis, but use of the absorption of light by the more numerous unexcited atoms increases the sensitivity to an extent which makes accurate measurement of the zinc levels in biological fluids possible. The sensitivity of the method is virtually the same as for activation analysis - about 0.01 ppm or, in the case of plasma or serum, approximately 1 μg / 100 ml. Atomic absorption spectrometry has therefore been used to measure the zinc content of the plasma and serum samples, and of the fractions from the Sephadex columns.

The normal range of values for plasma zinc levels is 80 - 120 μg / 100 ml. Normal serum values are reported to be about 16% higher (Halsted and Smith, 1970) due to release of zinc on platelet disintegration. Abnormal levels to date have been reported as usually lower than the normal range and low plasma zinc values have been found to be a feature of a variety of pathological conditions, from myocardial infarction to hepatic cirrhosis. To be applicable over the whole range of plasma zinc levels, the method used to measure the stable zinc binding should be sensitive enough to measure the zinc content of fractions from both normal and abnormal plasma. It has been shown previously that there is no advantage to be gained in sharpness of separation in using samples of less than 5 ml. Samples of this volume give a dilution to approximately a quarter of the plasma level for individual proteins in the fraction with maximum concentration of the protein. It should therefore be possible to measure at least the major protein-bound components, even in samples with very low plasma zinc levels, provided background contamination can be kept to a minimum.

A Perkin Elmer atomic absorption spectrometer (Model 403) using acetylene as fuel and air as oxidant was used for all the stable zinc measurements. Zinc was measured at a wavelength of 213.8 nm and samples were diluted to quarter strength using propanol, to

reduce variations in sample viscosity. The method is described in Appendix VI.

4.3 Removal of Contamination

One of the major problems involved in trace element analysis is that of sample contamination by small quantities of the element on glassware, in reagents, etc. The number of intermediate stages in the sample preparation was therefore reduced to a minimum and plastic disposable containers used where possible. Blood samples were withdrawn into plastic vials and the plasma or serum removed after centrifugation using a disposable plastic syringe. Because of the powerful chelating activity of lithium EDTA, heparin was used as an anticoagulant for all plasma samples where protein-bound zinc was to be studied. It has been shown previously (Chapter 2) that no significant increase in plasma zinc level resulted from the use of heparin.

Water supplies used for both rinsing glassware and for making up solutions were first double-distilled then deionised until the resistivity was greater than 3 MΩcm. No zinc could be detected in the water after this procedure. All reagents used were of the highest purity obtainable (Aristar* grade when obtainable and failing that AnalaR).

Measurements of the zinc level of the Tris-HCl buffer in 1M NaCl solution gave values of approximately 12 μg / 100 ml. When the zinc content of the individual

* British Drug Houses

components of the buffer were measured it was found that a 1M sodium chloride solution gave the same value. The sodium chloride solution was then passed through a 46 cm x 2.6 cm column of Chelex-100 chelating resin (Bio-Rad Laboratories, Ltd) to remove the contaminating zinc. However this resulted in only marginal improvement. As Chelex-100 had been found capable of removing even tightly bound zinc from compounds it was assumed that the high sodium content of the solution increased the background in the atomic absorption measurements. Accordingly, the amount of sodium chloride in the buffer was reduced to 0.1M, which gave a total zinc level in the buffer of 0 - 2 μg / 100 ml. This reduction was found to have no measurable effect on the in vitro binding of zinc-65 to plasma. However albumin is known to have an increased tendency to form polymers in a solution of low ionic strength and appreciable amounts of albumin dimer and higher polymers in the sample result in measurable amounts of albumin across the major part of the plasma protein distribution. The albumin distribution obtained using 0.1M NaCl-buffer solution as eluant was compared with that obtained from 1M NaCl-buffer solution. No measurable increase in tailing of the protein could be detected.

The Sephadex gel, which itself contains appreciable amounts of zinc, was washed using several volumes of

of the buffer until the zinc level in the eluant from the column was equal to that of the original buffer.

The glass columns were decontaminated by soaking them in a 2% solution of Decon-90 at elevated temperature for several hours and rinsing several times with double-distilled deionised water. The plastic tubing connecting the buffer reservoir, the column and the fraction collector were decontaminated by pumping a 2% solution of Decon-90 through the system for several hours, followed by several rinses of double-distilled deionised water. Disposable plastic vials were used to collect the column fractions.

4.4 Separation Using Sephadex G200

Volumes of serum and plasma were separated into 6 ml fractions using a 46 cm x 2.6 cm diameter column of Sephadex G200 using a Tris-HCl buffer, pH 8.0, in 0.1M sodium chloride solution as eluant. Approximately half of each sample was poured into a second similar vial, for protein measurement, etc. and the zinc level of the solution in the original vial measured, thus reducing the chance of contamination in the sample. The absorbance at 280 nm and the albumin level, calculated using the absorption of the solution in methyl orange reagent, were measured for each of the fractions from the column. Fig. 14 shows the distribution of stable zinc in the fractions obtained on separation of a 6 ml plasma sample from a normal

donor, together with the albumin distribution. It can be seen that the zinc distribution has two distinct peaks, one roughly coincident with albumin and the other in the region of the exclusion limit for the gel, that is, in the molecular weight range 1,000,000 - 800,000. The protein fractions in this region contain mainly immunoglobulin-M, haptoglobin and α_2-macroglobulin. Quantitative immunodiffusion measurements of the levels of the three proteins in the relevant fractions were carried out and the individual protein distributions plotted. Only the distribution for α_2-macroglobulin conformed to that of the second peak, as shown in Fig. 14. Since extensive work has been carried out to show that this protein does contain zinc it was concluded that the peak in the zinc distribution is due to that associated with this protein. Assuming that the zinc is associated with only the two components represented by the two peaks and calculating the areas under the two peaks, the amount bound to albumin was found to be 70% of the total endogenous zinc level of 90 μg / 100 ml plasma and that to α_2-macroglobulin 30%.

The separation was repeated using a second sample of plasma taken at a later date from the same donor. A similar binding pattern was obtained, with 75% of the plasma zinc bound to albumin and 25% to α_2-macroglobulin.

A 7.5 ml sample of serum, again from the same subject, was separated into 6 ml fractions and the stable zinc content, absorbance at 280 nm and albumin

levels measured. As for the plasma samples, the zinc distribution had two peaks coincident with the distributions of α_2-macroglobulin and albumin. No obvious difference between the distributions of zinc in serum and in plasma could be detected. The percentages bound to the two fractions for the serum sample were 23% bound to α_2-macroglobulin and 77% bound to albumin. A trace amount of zinc-65 was added to 2.5 ml of the same serum and the sample separated into 6 ml fractions under the same experimental conditions. The distributions of endogenous stable zinc and added zinc-65 for the two serum samples are shown in Fig. 15. It can be seen that the single peak in the distribution for the added zinc-65 is coincident with the larger of the two peaks in the endogenous distribution but no measurable zinc-65 activity was found in the α_2-macroglobulin region.

Plasma samples from two other normal subjects were separated in the same way. Both gave zinc distributions of the same general shape as that obtained for the first subject, with two peaks coincident with albumin and α_2-macroglobulin. The percentages of zinc bound to albumin and to α_2-macroglobulin were 75% and 25% for one subject and 67% and 33% for the other.

In all the samples studied the total plasma zinc concentration was found to be associated with the plasma proteins. Measurements made of the zinc levels

in the fractions containing the lower molecular weight components in plasma gave values which were indistinguishable from background levels.

4.5 Separation Using Sephadex G75 and G100

The separation procedure outlined above has been designed to give maximum separation of the plasma proteins throughout the whole molecular weight spectrum. However, it is not particularly suitable for comparing the results obtained from a number of samples, since the procedure is a relatively lengthy one (about 12 hours) and if excessive dilution of the protein fractions is to be avoided, requires large blood samples (about 12 ml). Also the use of large volumes of buffer and a large column inevitably increase the background zinc level and the chance of contamination in the fraction. Since the distribution of stable zinc among the plasma proteins is concentrated in two regions, widely separated in molecular weight, use of a grade of Sephadex gel whose exclusion limit occurs for a lower molecular weight should bring the two peaks closer together, but still separable, assuming that the zinc is entirely bound to α_2-macroglobulin and to albumin, or to groups of proteins whose molecular weight is very similar to these proteins.

In theory, adequate separation is achieved when one fraction is obtained which has measurable amounts of one of the proteins and none of the other. The zinc

in that fraction is therefore associated with only one protein and, if the amount of the protein in the fraction is expressed as a percentage of the total plasma level, the total amount of zinc associated with the protein can be calculated. The amount of zinc bound to the second protein can then be found by subtraction.

Sephadex G75 fractionates in the molecular weight range 80,000 - 3,000 and therefore most of the plasma proteins are excluded from the gel and are eluted together at a volume approximately equal to the void volume of the column. However albumin is within the fractionation range of the gel and is therefore separated from the proteins, including α_2-macroglobulin, which are excluded from the gel.

An initial trial was carried out using an 18.5 cm x 1.6 cm diameter column of Sephadex G75, packed and equilibrated with Tris-HCl buffer in 0.1M sodium chloride solution as described in Appendix III. Samples of serum were obtained from six normal donors, five male and one female, in the age range 20 - 35, and 2 ml aliquots were separated into 2.4 ml fractions at a flow rate of 14.6 ml / hour. It was found that 95% of the proteins were concentrated into four fractions, with only a small proportion of the heavier proteins, α_2-macroglobulin in particular, in the second two fractions, which contained most of the albumin. The total serum zinc level, the stable zinc content of the

fractions, the albumin distribution (measured using the absorption by the fraction of light at 540 nm in methyl orange reagent) and the absorbance at 280 nm of the fractions were measured in each case. A typical separation is given in Fig. 16, showing the absorbance at 280 nm, the stable zinc concentration and the albumin level in the fractions. Using the fraction containing maximum albumin concentration and assuming that the concentration of α_2-macroglobulin in this fraction was negligible in comparison with the albumin, the total stable zinc bound to the albumin was calculated, and the amount of zinc bound to the α_2-macroglobulin found by subtraction. The results are summarised in Table 4. The percentages bound to albumin varied between 54% and 81%, with an average value of 68.7%. Again, no measurable zinc levels could be detected in those fractions containing the lower molecular weight plasma constituents for any of the samples studied.

While these initial studies produced encouraging results, with concentrations of stable zinc in the appropriate fractions which were satisfactorily above background level, the close proximity of the albumin and α_2-macroglobulin peaks on separation using Sephadex G75 means that the α_2-macroglobulin-rich fractions always contain appreciable amounts of albumin and thus the calculation relies on the albumin fractions alone. Improved results would be obtained if

the separation was extended so that fractions containing α_2-macroglobulin, but no albumin, could be obtained. The amounts of zinc bound to each of the proteins could then be estimated independently, instead of using subtraction from the total plasma zinc level. This would provide a check on the results, since the sum of the amounts bound to each protein should be the total plasma zinc level, if only albumin and α_2-macroglobulin bind zinc.

Sephadex G100 has a fractionation range from 100,000 to 4,000 and therefore α_2-macroglobulin is again eluted at approximately the void volume for the column, together with other heavier proteins such as the γ-globulins which are not thought to contain significant amounts of zinc. Albumin is within the fractionation range of the gel but because of the higher exclusion limit of G100, is better separated from the excluded proteins than is possible using G75. It was therefore decided to use the same size of column with G100 as the separating medium. Again, 2 ml samples were used, separated into 2.4 ml fractions using Tris-HCl buffer in 0.1M sodium chloride as eluant. An initial trial was carried out, using two samples of pooled serum from six normal subjects (each sample coming from a different group of donors) and called Serum I and Serum II. These samples were separated as above and the levels of α_2-macroglobulin measured using radial immunodiffusion

and of albumin, measured using the binding to methyl orange, and the total serum zinc and the zinc level in each of the fractions. The distributions obtained for each of the samples were similar and the distributions for Serum I are shown in Fig. 17. The proteins were concentrated in six fractions in this case and it can be seen that the first two contain virtually no albumin, while the last two contain no measurable levels of α_2-macroglobulin. Using these pairs of fractions to calculate the percentage of zinc bound to α_2-macroglobulin and to albumin gave values of 32% bound to α_2-macroglobulin and 63% to albumin and of 33% to α_2-macroglobulin and 70% to albumin for Serum I and Serum II respectively. Hence the results obtained from each of the protein fractions are reasonably consistent, adding up to approximately 100% in each case, and also the percentages obtained for the two sera are very similar - as would be expected from pooled samples of normal serum.

Plasma samples were then obtained from six normal subjects and 2 ml aliquots separated using G100 into 2.4 ml fractions. For each fraction measurements were made of α_2-macroglobulin and albumin concentrations, as well as the zinc content. The endogenous zinc level of the plasma was also measured in each case. The results are summarised in Table 5. The percentages of the total plasma zinc bound to α_2-macroglobulin were in

the range 25 - 32% and to albumin in the range 68 - 75%. The average values were 29% bound to α_2-macroglobulin and 71% bound to albumin.

Plasma samples were obtained from two patients suffering from pustular psoriasis, who were found to have plasma zinc levels which were lower than those of any of the normal controls (78 and 84 μg / 100 ml). Aliquots of 2 ml were separated into fractions as above, using Sephadex G100, and the percentage of zinc bound to the two proteins calculated. Values of 42% and 45% bound to α_2-macroglobulin and 58% and 55% bound to albumin were obtained and therefore in both cases the proportion of the zinc bound to albumin was lower than for any of the normal controls.

A single sample was obtained from a patient found to have the nephrotic syndrome, with a plasma albumin level of approximately 50% of the normal value. The plasma zinc level was found to be significantly lower than normal (64 μg / 100 ml) and, on separation of a 2 ml aliquot of plasma using G100, the percentages bound to α_2-macroglobulin and to albumin were found to be 39% and 61% respectively.

A 6 ml sample of pooled normal serum was shaken briefly with an equal volume of Chelex-100 chelating resin, which had been previously adjusted to pH 7.4. The sample was centrifuged and the supernatant separated using a 46 cm x 2.6 cm column of Sephadex G200. The

distribution of stable zinc in the protein fractions showed that most of the zinc was removed from the albumin region but that the peak in the α_2-macroglobulin region was little reduced, giving strong support to the hypothesis that the binding to albumin is much weaker than that to α_2-macroglobulin. However it was found that when the sample was passed through a small column of Chelex-100, the zinc could be removed almost entirely from both fractions.

4.6 Discussion

In all samples studied the pattern of the endogenous stable zinc binding showed that almost all the zinc is bound to two proteins, or groups of proteins, one having a molecular weight in the region of 800,000 where the zinc has the same distribution as the α_2-macroglobulin in the sample, and the other with a molecular weight of approximately 70,000, which is almost certainly albumin. These findings agree with the work of Parisi and Vallée who showed that in pooled normal serum the major part of the serum zinc was bound to two proteins, albumin and a zinc metalloprotein which combined with trypsin to produce a compound with esterase activity. As α_2-macroglobulin is the only protein in serum found to react in this way it was concluded that the zinc metalloprotein was identical with α_2-macroglobulin, a finding which was confirmed using immunological methods. The purified

α_2-macroglobulin preparations obtained were found to contain amounts of zinc in the range 320 - 770 μg / g protein (Parisi and Vallée, 1970). In view of the agreement obtained with their work in the present studies it has not been considered necessary to attempt further purification of the high molecular weight fraction in an attempt to obtain pure α_2-macroglobulin, especially since the distribution of zinc in these fractions was coincident with the distribution of α_2-macroglobulin.

Values for the proportions of zinc bound to each protein in normal pooled serum were found to be about one third of the total serum zinc bound to α_2-macroglobulin and two thirds to albumin, which agrees with the zinc content of the protein given by Parisi and Vallée. No significant difference could be detected between the binding patterns for zinc in serum and in plasma. The amounts of zinc bound to the two proteins in samples of plasma from normal individual subjects fell within a fairly narrow range, with between 25% and 32% of the total plasma zinc bound to α_2-macroglobulin and between 68% and 75% bound to albumin. No measurable amounts of zinc were found in the low molecular weight plasma components. Two plasma samples from patients suffering from pustular psoriasis, with low plasma zinc levels, gave percentages of 55% and 58% of the plasma zinc bound to albumin, values which are lower than any of those obtained from normal subjects, though

the plasma albumin levels in these samples were within normal limits.

Comparison of the binding pattern for trace amounts of zinc-65 added to serum *in vitro* with the pattern of the endogenous zinc in the same serum showed that while a zinc-containing α_2-macroglobulin fraction is clearly demonstrated in all distributions of the endogenous zinc, in both normal and abnormal samples, this protein show little tendency to bind zinc *in vitro*. The albumin peak, on the other hand, appears in both the endogenous binding pattern and in the pattern for zinc added to serum *in vitro*. This binding to albumin is almost certainly a weaker bond than that to α_2-macroglobulin, since chelation removes the zinc in the albumin fraction in preference to the zinc bound to α_2-macroglobulin.

It would therefore appear that zinc newly absorbed into the blood via the gastro-intestinal tract is bound initially to albumin and that the zinc in the α_2-macroglobulin fraction is a product of a later metabolic reaction. This does not exclude the possibility that the α_2-macroglobulin is a transport protein for zinc, since the protein could bind zinc at a specific site within the body, rather than immediately in the plasma, but later give up its zinc at some other site. Alternatively, the protein might be an enzyme whose function is as yet undiscovered.

If this is the case it would be expected that the ratio of protein-bound zinc to protein concentration would be constant, since the protein will only exist in a form containing a given number of zinc atoms per molecule. In a transport protein sites must be available in the molecule for binding absorbed zinc and hence the ratio of weight of bound zinc to weight of protein is not necessarily constant. It has been found that about one third of the total zinc in plasma is bound to α_2-macroglobulin. Taking an average normal plasma zinc level of 100 μg / 100 ml and an average α_2-macroglobulin concentration of 300 mg / 100 ml (Schultze and Heremans, 1966) gives a very approximate value of 1 atom of zinc per molecule of α_2-macroglobulin. However further studies on the fate of the zinc bound to each of the proteins are necessary before this point can be elucidated.

4.7 Conclusions

(a) In all samples of serum and plasma studied, the zinc content was found to be associated with two protein fractions, one of which was identified as α_2-macroglobulin and the other as albumin. In samples from normal subjects no measurable levels of zinc were found in the low molecular weight fractions.

(b) The average values for the proportions bound to each of the two fractions, in normal plasma or serum, were found to lie within a narrow range, with about

one third of the total plasma zinc bound to α_2-macroglobulin and two thirds bound to albumin, but the percentage bound to albumin was reduced in plasma samples from two patients suffering from psoriasis.

(c) Since the α_2-macroglobulin fraction does not appear to bind zinc added to the sample <u>in vitro</u> it is probable that zinc binds to the protein either at the site of protein production or by metabolic reaction in the body.

CHAPTER 5

CHAPTER 5

STUDY OF THE VARIATION IN THE ZINC BINDING PATTERN IN PLASMA WITH TIME AFTER AN ORAL DOSE OF ZINC-65

		Page
5.1	Introduction	86
5.2	Materials and Methods	87
5.3	Uptake and Clearance of Zinc-65 in Plasma of Normal Subjects	89
5.4	Uptake and Clearance of Zinc-65 by Plasma in Patients Suffering from Varicose Ulcers	94
5.5	Discussion	97
5.6	Conclusions	100

5.1 Introduction

It has been established that there are two major protein components which bind zinc in serum or plasma. One of these components binds zinc added to plasma *in vitro*, and the other more tightly bound fraction is only found in the pattern of endogenous protein-bound zinc. This suggests that the first fraction, almost certainly mainly albumin, is the transport medium for zinc initially absorbed into the system through the gastro-intestinal tract. Since the other fraction, a zinc metalloprotein which has been identified as α_2-macroglobulin, does not bind zinc *in vitro*. All that can be inferred from its presence in the endogenous pattern is that the protein binds zinc only *in vivo* and the previous studies provide no information on the clearance of zinc from either fraction.

To obtain this information, *in vivo* labelling of the proteins is necessary and zinc-65 provides a convenient tracer for this purpose. The isotope can be administered either intravenously or orally. While intravenous administration has the advantage that all of the administered dose is absorbed, the rate of clearance from the plasma is very rapid and there is the possibility that, since the zinc-65 has not passed through the gastro-intestinal wall it may not parallel the physiological process of zinc absorption. An oral dose of zinc-65 as the chloride in 100 ml

water was therefore used in this study.

Since zinc is known to have a long biological half-life and the radioisotope itself has a half-life of several hundred days, zinc-65 is a considerable radiation hazard in large doses and must be used with extreme care in clinical trials. Permission was obtained from the Isotope Advisory Panel of the Medical Research Council for the administration of 5 μCi zinc-65 to normal subjects as an oral dose.

The aim of the work described below was to study the uptake and clearance of zinc-65, particularly in normal subjects, but also in patients suffering from varicose ulcers, and to study the variation with time of the amounts of zinc bound to albumin and to α_2-macroglobulin. Gel chromatography on Sephadex G200 was used to separate the proteins into fractions whose activity could then be measured.

5.2 Materials and Methods

The dose administered was 5 μCi zinc-65 as the chloride in 100 ml water, obtained by dilution of a solution of zinc-65 chloride in 0.1N hydrochloric acid (Radiochemical Centre, Amersham). The dose was equivalent to about 20 μg zinc.

20 ml blood samples were withdrawn using a disposable polypropylene syringe and stainless steel needle. These were taken before administration of the dose and at intervals of approximately half an hour for the

first few hours, then at progressively longer intervals during the day. After the first day blood samples were taken at intervals of several days, then at longer intervals for a period of about 12 months. The blood samples were centrifuged immediately after withdrawal and the plasma drawn off using a polypropylene syringe. The red blood cells were washed three times in isotonic saline and retained for counting. After counting, the samples were separated using a 46 cm x 2.6 cm column of Sephadex G200 using a Tris-HCl buffer, pH 8.0, in 1M sodium chloride solution. To avoid possible contamination of the later samples by activity from the earlier more active samples the Sephadex gel was rinsed with a volume of the buffer solution at least equal to that of the column after each separation and the gel replaced after at most four separations had been carried out. Where the total plasma activity was low the fractions were pooled and concentrated either by evaporation in a refrigerator or by freeze-drying the pooled fractions and reconstitution to a suitable volume with isotonic saline.

The samples were counted using a Tracerlab Gammaguard 150 automatic gamma counter. Each of the plasma samples and plasma protein fractions was counted for several hours. To ensure that no drift in the counter settings occurred during counting each sample in the group to be counted was counted for 500 second

intervals and a standard, consisting of 2% of the dose administered, and several background samples, were included in each group. The counts were repeated until a sufficient time had elapsed. The mean and standard deviations for the background and standard counts were then calculated. Provided that the standard deviation did not differ significantly from the theoretical value of the square root of the mean counts in each case, constant conditions for counting were assumed and the mean values taken as the background and standard counts. This was found to be true in all cases in practice. The average sample counts were calculated and the counts expressed as a percentage of the dose administered per litre of plasma.

5.3 Uptake and Clearance of Zinc-65 in Plasma of Normal Subjects

Samples from two normal subjects (female) were studied. These subjects had plasma zinc levels of 86 and 88 μg / 100 ml, with normal values for total protein, haemoglobin and albumin, and α_2-macroglobulin levels. The dose was administered in the morning, several hours after a light breakfast.

Fig.18 shows the uptake and clearance of the zinc-65 in plasma for the two subjects. It can be seen that both curves have a similar shape, with very rapid uptake in the first half hour, reaching a maximum after about $1\frac{1}{2}$ hours, then declining steadily.

The level appears to stabilise to an almost constant level after about six days, with a very slow decline for a period of about 30 days. After 3 months the zinc-65 level has dropped below background. The curves show that the maximum plasma level for subject C is significantly greater than for subject E, with a more rapid clearance in the first few hours - the difference between the plasma levels for the two subjects is approximately 0.80% of the dose at the maxima, but only about 0.15% after 24 hours.

Plasma samples taken at times 1, 1.5, 2, 3, 4, 7 and 25 hours and 3 and 6 days from subject C were separated into 6 ml fractions using volumes of 7 - 12 ml. The activity of the later samples was found to be too low to give significant results for most of the separated fractions. The plasma samples taken at 2 hours and 4 hours were counted as 6 ml fractions. The fractions from samples at 1 hour, 1.5 hours and 3 hours were pooled in groups of two, into 12 ml fractions, taking the first fraction as the first which contained a measurable quantity of protein, measured using the absorption of light at 280 nm. The pooled fractions were then concentrated by freeze drying. The fractions from the later samples were pooled into larger volumes, depending on the activity of the fractions. The absorbance at 280 nm was measured for each of the column fractions and the albumin content of the pooled and concentrated fractions measured by absorbance in

methyl orange reagent at 540 nm.

Sample volumes of 7.5 - 10 ml from subject E were used and the samples for the times 35 minutes - 3 days were separated. Those samples having maximum activity ($1\frac{1}{2}$ - 2 hours) were separated into 6 ml fractions and those with less activity, though well above background (up to 7 hours) were separated into 9 ml fractions. Each of the 6 and 9 ml fractions were then counted without further concentration. The samples taken after 1 day and 3 days were separated into 9 ml fractions, which were then pooled in pairs, again taking as the first that sample first showing a measurable amount of protein. The pooled samples were then concentrated by freeze-drying and reconstituted in 6 ml isotonic saline. The albumin content of each unconcentrated fraction was found by measuring the absorbance at 540 nm in methyl orange reagent. The absorbance at 280 nm was also measured to give an estimate of the total protein level in each fraction.

Figs. 19 and 20 show the distribution of zinc-65 activity among the protein fractions at 2 hours and 25 hours after ingestion of the tracer dose for subject C and Figs. 21 and 22 show the distribution of activity at 1.5 and 4.4 hours for subject E. All the distributions obtained from plasma samples obtained from both subjects show a fairly similar pattern. In the early plasma samples (those with maximum activity) there is a

large peak in the albumin region, but in each case there is also measurable activity in the high molecular weight region, though initially this is only a small proportion of the total activity. As the activity contained in this region was only slightly above the background level it was difficult to measure and therefore estimates of the percentage of the total activity which appears in this high molecular weight fraction are inevitably subject to a high error. However all of the earlier samples from both subjects show a slight but definite peak in this region, which coincides with the α_2-macroglobulin distribution.

This finding can be contrasted with the binding of zinc added to plasma _in vitro_, where no significant binding in the α_2-macroglobulin region could be demonstrated. However, comparing the distribution of the earlier plasma samples with that for the stable zinc distribution for the same subject it is found that the distributions have similar shapes, with two peaks, one coincident with albumin and the other with α_2-macroglobulin. The distributions of stable zinc and zinc-65 (4 hours) in plasma are shown in Fig 23, for subject C, expressed as a percentage of the maximum value in each case. It can be seen that, allowing for differences in the separation, the two sets of peaks are coincident, but that the zinc-65 bound to α_2-macroglobulin is a smaller percentage of

the total level than the stable zinc bound to that protein. As the two peaks are distinct in the earlier, more active samples the total activity bound to each protein can be calculated from the area under the peak as a percentage of the total dose administered. In the later samples where the reduction in total plasma activity has necessitated pooling several of the protein fractions no obvious peaks can be detected but, knowing the distribution of α_2-macroglobulin and of albumin in the samples the activity associated with each protein can be calculated. The variation of the zinc-65 activity of both protein fractions with time is compared with the total plasma activity over a period of about 24 hours, in Fig. 24 for subject C and in Fig. 25 for subject E. It can be seen that the uptake and clearance of zinc-65 by albumin is fairly rapid and follows the shape of the plasma curve. However, after a rapid initial uptake, the amount of the dose bound to α_2-macroglobulin remains virtually constant over this period in both cases. At maximum zinc-65 level the percentages of the total plasma activity bound to albumin and to α_2-macroglobulin were 89% and 11% for subject C and 89% and 11% for subject E. After 24 hours the percentages were 72% and 28% and 78% and 22% respectively. The latter values are similar to the percentages of the endogenous zinc levels associated with the two proteins, which were 75% bound to albumin

and 25% bound to α_2-macroglobulin for subject C and also 75% bound to albumin and 25% bound to α_2-macroglobulin for subject E.

The percentages bound to each protein are therefore similar in each case, even though the total plasma zinc-65 levels at the maximum vary by approximately a factor of two. The levels of albumin and α_2-macroglobulin present in the plasma from each subject were found to differ by less than 20% of the mean, suggesting that the amounts of zinc bound by each fraction do not apparently depend on the amounts of protein present and that the α_2-macroglobulin fraction binds a definite percentage of the absorbed zinc, rather than a constant amount. As the amount of zinc bound to the protein increases with increasing absorbed zinc and the amount of protein present is fairly constant this implies a variable ratio of zinc to protein.

5.4 Uptake and Clearance of Zinc-65 by Plasma in Patients suffering from Varicose Ulcers

An oral dose of 5 μCi zinc-65 chloride was administered to two patients suffering from varicose ulcers (one male, one female). The dose was given just before the patients started on a course of zinc sulphate therapy. The zinc-65 chloride was added to 200 ml water in which 220 ml zinc sulphate had been dissolved. Plasma samples were taken at intervals of $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, 3, 4, and 6 hours and then at 48 hour

intervals for a week. The zinc-65 activity and the stable zinc level were measured for each sample. The clearance curves for zinc-65 (expressed as percentage dose per litre plasma) and for stable zinc are shown in Figs. 26 and 27*. It can be seen that the maximum levels for zinc-65 activity in plasma are of the same order as those obtained for subject E, but much less than those of subject C, though the maximum level occurs at approximately the same time after ingestion of the dose in all four cases. Also the clearance of the stable zinc follows the zinc-65 fairly closely, as expected.

Because of the low activity and the small sample volume it was not usually possible to separate individual samples. Hence the samples having maximum activity were pooled and separated into 6 ml fractions using a 46 cm x 2.6 cm column of Sephadex G200. In one case (patient S.G.) the samples from days 2 - 6 were pooled and separated in the same way. The 6 ml fractions from each separation were each pooled into three groups. Group 1 contained the high molecular weight proteins (with molecular weights greater than approximately 200,000), Group 2 the fractions for molecular weights approximately 200,000 - 100,00 and Group 3 the proteins in the molecular weight range 100,000 - 50,000. Hence

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Group 1 contained most of the α_2-macroglobulin and Group 3 most of the albumin. The grouped fractions were concentrated by evaporation then freeze-dried and reconstituted in 6 ml isotonic saline, then counted for several hours as described. Since the middle group (Group 2) contains appreciable amounts of albumin and α_2-macroglobulin the levels of each protein were measured for each grouped fraction. Relative albumin levels were estimated by adding trace amounts of human serum albumin labelled with iodine-131 to the serum samples before separation and the iodine-131 activity in each of the grouped fractions measured. The concentrations of α_2-macroglobulin were estimated by radial immunodiffusion. Since Group 1 contains negligible amounts of albumin and Group 3 no α_2-macroglobulin, the total amounts bound to each protein could be calculated. This was found to account for all the activity in Group 2.

It was found that in the three plasma samples separated the percentage of the total activity found in the α_2-macroglobulin region was greater than for the normal controls at similar times. At maximum plasma activity (1 - 2 hours) the percentage of the total zinc-65 activity bound to α_2-macroglobulin was 46% for patient S.G. (male) and 73% for patient A.R. (female), while that obtained for both normal subjects was 11%. For patient S.G. the aggregated samples for days 2,4

and 6 gave a much higher percentage of the zinc-65 activity bound to α_2-macroglobulin, an increase of from 46% to 80%. However, since both patients were give 220 mg zinc sulphate (equivalent to 50 mg Zn^{++}) the zinc-65 acts as a tracer for a therapeutic dose of zinc rather than as a tracer for dietary zinc intake.

5.5 Discussion

When zinc-65 is absorbed from a tracer dose given orally it is found that, in plasma samples separated using gel chromatography, zinc is bound to both the α_2-macroglobulin and albumin fractions in all samples. For samples taken up to 2 hours after ingestion of the dose from subjects with apparently normal zinc metabolism the percentage bound to α_2-macroglobulin was approximately 11% of the total but this gradually increased to about 25% of the total zinc-65 level after 24 hours. After an initial rapid uptake the amount of zinc bound by this protein remained constant throughout this period. The level of the albumin-bound zinc-65, however, dropped rapidly during this time, the clearance of zinc having roughly the same shape as the plasma curve.

While the finding of a constant level of zinc bound to α_2-macroglobulin would support the hypothesis that this is a metalloprotein which only exists in a form containing a constant number of zinc atoms per molecule of protein, it was found that, in two cases having virtually equal amounts of the protein present,

as measured by radial immunodiffusion, that the amounts of zinc-65 binding to the protein were different in each case, one having more than double the amount of zinc bound to this protein than was found in the second case (subjects C and E). However some caution is necessary in the interpretation of these results since although the same dose of zinc-65 was administered in each case it is virtually impossible to ensure that the same amount of total zinc (largely stable zinc) is administered. If the specific activity of the dose is reduced due to contamination of the sample by stable zinc, either externally or by zinc-containing compounds already present in the gastro-intestinal tract, the total amount of zinc bound to a particular fraction may only appear to have been reduced when the zinc-65 binding alone is used as a measurement of total zinc binding.

When zinc-65 was administered with a 220 mg dose of zinc sulphate to two patients with suspected zinc deficiency the percentage of the absorbed zinc in plasma which was bound to α_2-macroglobulin was found to be greater than in either of the two normal subjects. Since the total zinc administered and absorbed in these patients was much greater than the trace amounts given to the normal subjects the differences in the binding ratios could be due to either a difference in the binding pattern of large amounts of zinc (in these cases

the plasma zinc level increased to more than twice the original level) or to abnormalities in zinc metabolism. However experiments on the absorption of therapeutic doses of zinc by normal subjects (to be described in a later chapter) will allow comparison of normals with these patients with suspected zinc deficiency.

The binding of newly absorbed zinc-65 _in vivo_ to α_2-macroglobulin differs from the results obtained when zinc-65 is added to plasma _in vitro_, when only binding to albumin could be detected. It would appear, therefore, that there is some factor present in the _in vivo_ situation which acts as a catalyst for the incorporation of zinc into the protein. It is perhaps significant that the plasma, obtained from samples of peripheral venous blood, has already passed through the liver, known to be a site of α_2-macroglobulin synthesis (Prunier et al, 1964).

The varying ratio with time of zinc bound to albumin to zinc bound to α_2-macroglobulin is an indication of the more rapid clearance of zinc from albumin, a result which is not surprising in view of the much weaker binding of zinc to the protein compared with that to the metalloprotein, α_2-macroglobulin. This rapid clearance, together with the virtually constant level of the zinc-65 bound to α_2-macroglobulin, supports the hypothesis that albumin is the primary transport

protein for absorbed zinc. If this is the case it would be expected that the clearance of zinc from albumin would be related to the uptake of zinc by the organs and tissues. These results are described in the following chapter.

5.6 Conclusions

(a) When zinc-65 is absorbed into plasma from an oral dose the maximum plasma level occurs after one to two hours, declining rapidly during the first 24 hours then more slowly over a period of several weeks.

(b) Newly absorbed zinc-65 in plasma binds to both the albumin and α_2-macroglobulin fractions, in contrast to zinc added to plasma *in vitro* where only binding to albumin could be detected. In normal subjects the percentage of the total plasma zinc-65 level bound to the α_2-macroglobulin fraction in samples obtained during the first 2 hours after administration of the dose was less than half that obtained for the endogenous zinc bound to this protein. However after 24 hours the percentage of the zinc-65 bound to the protein had risen to that of the endogenous zinc.

(c) The amount of zinc bound to α_2-macroglobulin was found to be fairly constant over the first 24 hours. The level of zinc bound to albumin decreased much more rapidly, having the same shape as the plasma clearance curve.

(d) In patients suffering from varicose ulcers who were given a dose of 5 μCi zinc-65 in a solution

containing 220 mg zinc sulphate the peak plasma level was found to be of the same order as those obtained for the normal subjects given only trace amounts of zinc. However the percentage of zinc bound to α_2-macroglobulin at comparable times was found to be much greater than for the normal controls, and further experiments on the absorption of therapeutic doses of zinc in normal subjects are necessary to determine whether this is caused by absorption of large amounts of zinc or is due to abnormalities in zinc metabolism in these patients.

(e) The results of the experiments described above suggest that albumin is the primary transport protein for newly absorbed zinc. If α_2-macroglobulin has a role in zinc transport it would appear to release zinc only slowly to the tissues.

CHAPTER 6

CHAPTER 6

RELATIONSHIP BETWEEN THE CLEARANCE OF ZINC FROM THE PLASMA PROTEINS AND THE UPTAKE AND CLEARANCE BY THE TISSUES

		Page
6.1	Introduction	103
6.2	Plasma Clearance from an Intravenous Tracer Dose of Zinc-65	105
6.3	The Role of Albumin in Zinc Transport	108
6.4	Comparison of the Intravenous and Oral Clearance Curves	109
6.5	Relationship between Plasma Clearance and Tissue Uptake of Zinc	111
6.6	Mathematical Analysis of the Clearance Curves	115
6.7	Calculation of Zinc Content Using the Occupancy Principle	118
6.8	Discussion	122
6.9	Conclusions	128

6.1 Introduction

It has been shown that zinc absorbed from an oral dose into plasma is bound to both fractions, albumin and α_2-macroglobulin, even in the first hour after administration of the zinc. During the first 24 hours the amount of zinc bound to α_2-macroglobulin remains virtually constant, while the albumin level drops rapidly. As plasma is the medium which transports absorbed zinc to the organs and tissues this would suggest that the zinc which is weakly bound to albumin is easily removed by the tissues and is therefore the primary transport protein for newly absorbed zinc.

A transport protein can be defined as a protein which combines with the absorbed substance and carries it in combination to the tissues, where it then gives up the substance and itself returns to its original form, free to once more act as a transport medium for the substance. It therefore follows that, for a true transport protein, the half-life of the transported substance bound to the protein is much shorter than that of the protein itself. If albumin is indeed the main zinc transport protein the half-life of the zinc bound to albumin will be less than the biological half-life of the protein itself. However this information cannot be obtained from the oral clearance curve alone.

Although oral absorption is the most physiological

means of administering the element this advantage which absorption via the gastro-intestinal tract gives also causes problems in the interpretation of the plasma clearance curves. With oral absorption the clearance curve is complicated by the existence of two components, one the clearance of the zinc from plasma to the tissues and the other the gradual passage of the element through the gastro-intestinal tract into plasma. If the form of the intravenous clearance curve is known it is possible to compare the half-life of albumin-bound zinc with that of albumin alone. Since the plasma clearance curve from an oral dose is the product of the two functions, the clearance of zinc from plasma to the tissues and the transit function for zinc passing through the gastro-intestinal mucosa, if the oral and intravenous plasma clearance curves are known for the same subject it is possible to derive the shape of the transit function.

As zinc is lost from a transport protein a corresponding increase in the tissue levels should occur. By comparing the uptake of the tissues with the clearance from the plasma protein fractions it should be possible to gain some insight into the functions of the two fractions.

The aims of the work described below were, therefore:-

(a) To study the clearance of an intravenous dose of zinc-65 from plasma and to use this and the oral

clearance curves already obtained to derive the transit function for the passage of zinc through the gut wall.

(b) To compare the uptake of the tissues with the clearance of zinc from the two protein fractions and hence to obtain information about the possible functions of the two fractions.

6.2 Plasma Clearance from an Intravenous Tracer Dose of Zinc-65

Injection of a single bolus of tracer for the measurement of plasma clearance suffers from an important disadvantage. After the injection diffusion of the bolus occurs until the plasma is uniformly labelled with the tracer. However this diffusion requires a finite time to reach equilibrium and, with substances which are known to be cleared very rapidly from plasma, such as zinc, a significant fraction of the tracer may have disappeared before equilibrium has been reached. A clearance curve containing a significant number of samples withdrawn before equilibrium has been attained will therefore not be truly representative of the physiological disappearance. This difficulty can be largely overcome by using intravenous infusion of the substance over a period of time, since a uniform equilibrium concentration of the substance can then be built up in the plasma. When the infusion is stopped the clearance from plasma can be measured as usual, but since uniform labelling has been achieved, the

problems associated with instantaneous administration are avoided. This method has therefore been used in the measurement of the zinc-65 clearance from plasma. The total dose administered in each case was 0.4 μCi zinc-65 as the chloride. The radioactive solution was autoclaved and added to bottles containing standard solutions of sterile isotonic saline. The total stable zinc content of the active solution was measured and found to be 20 μg / 100 ml. A second infusion of isotonic saline was used in conjunction with a two-way tap to allow blood samples to be withdrawn easily as required. Disposable transfusion sets were used to connect each reservoir to a polythene cannula inserted into the vein. To obtain a constant rate of infusion for the radioactive solution a peristaltic pump was used to control the rate of flow. The flow rate was chosen so that the required volume was administered over a period of from one to three hours. The flow rate of the non-active saline infusion was controlled using the valve of the transfusion set and was kept to the minimum level necessary to maintain a steady flow.

Blood samples were withdrawn by stopping the infusion, clearing the tap of saline and withdrawing 10 ml blood samples using a polypropylene syringe into disposable plastic vials. These were then centrifuged immediately and the plasma removed into vials for counting. Samples were taken at intervals

during the infusion of zinc and for a period of 4 hours afterwards.

The samples were counted using a Wallac DECEM GTL 300 automatic gamma counter. Since the plasma activity was very low each sample was counted for one hour intervals, the counts being repeated several times. Known volumes of the infusion solution, made up to the same volume as the samples, used as standards, and backgrounds were counted each time and the stability of the counter settings assessed as described in Chapter 5.

The clearance curves for administration of zinc-65 were obtained for three subjects, one male (subject K) and two female (subjects M and C). In the first two cases (subjects M and K) 0.4 μCi zinc-65 in 100 ml solution was infused for a period of one hour at a flow rate of 100 ml / hour. In the third case (subject C) 0.4 μCi zinc-65 in 120 ml saline was infused for a period of three hours, at a flow rate of 40 ml / hour.

Figs. 28, 29 and 30 show the uptake and clearance of zinc-65 from plasma for the three subjects, expressed as a percentage of the maximum level for subjects M and K and as a percentage of the total dose administered for subject C. It can be seen that when infusion is stopped in each case the zinc-65 level drops very rapidly to an almost constant level and remains virtually unchanged over a period of several hours. The half-lives for the initial disappearance of

zinc-65 were found to be 18 minutes, 14 minutes and 18 minutes for subjects M, K and C respectively.

6.3 The Role of Albumin in Zinc Transport.

It has been shown previously that the initial rapid clearance of an oral dose of zinc-65 from plasma is due almost entirely to loss from the albumin fraction. If the oral clearance from plasma is considered as the product of the two functions, the transit function which describes the passage of the tracer through the walls of the gastro-intestinal tract and the intravenous plasma clearance, the first of these causes a rise in plasma tracer level while the disappearance of the tracer from plasma must result solely from the second function. Therefore the same conditions for disappearance of the tracer can be assumed for both intravenous and oral clearance, and it follows that since zinc has been shown to be lost mainly from the albumin fraction for oral clearance this is also true for the disappearance from an intravenous dose. Hence the initial clearance from albumin occurs very rapidly, with a half-life of less than 20 minutes in the three cases studied.

There are two factors which influence the disappearance of albumin itself from plasma. The first is the gradual breakdown of the protein with time, that is, the actual disappearance of albumin itself from the body, and various estimates of its biological half-life have been made. These have given values in

the range 14 - 20 days (Steinfeld, 1960; Cohen et al, 1961; Takeda and Reeve, 1963). However a more rapid clearance from plasma occurs due to the gradual mixing of the intravascular protein with that contained in the extravascular tissue spaces. While this diffusion through vessel walls appears to take place for most proteins the rate of diffusion has been studied in detail for albumin using iodine-131-labelled albumin. It has been shown that the half-life for this process is approximately 8 hours (Cohen et al, 1961; Takeda and Reeve, 1963). However since the half-life of zinc bound to albumin is, in its initial clearance, less than 20 minutes, this value is much less than either the biological half-life of the protein or the half-life for mixing of intravascular and extravascular protein. It would appear, therefore, that albumin is a true transport protein for zinc.

6.4 Comparison of the Intravenous and Oral Clearance Curves

Comparison of the intravenous disappearance curves of Figs 28 - 30 with those obtained for the clearance of an oral dose of zinc-65, shown in Fig. 18, shows that the clearance from an oral dose occurs over a much longer time interval than the intravenous disappearance. Both curves (oral and intravenous) are made up of a more rapidly decaying initial disappearance, followed by a much slower decline. However the initial disappearance

of the intravenous clearance curve takes place in approximately an hour but this stage occupies almost 24 hours for the oral clearance curve. Because of this rapid clearance of the intravenous dose the shape of the oral clearance is defined mainly by that of the transfer through the gut wall.

When any two of the three functions, the oral clearance curve, the intravenous curve and the transit function through the gut wall are known the third can be derived, since the three functions are related mathematically. The oral clearance curve is the convolution of the transit function and the intravenous curve. Hence:-

At time t,

$$c(t) = \int_0^T g(t-T).f(t)\ dT$$

where

$c(t)$ = oral clearance function

$g(t)$ = intravenous clearance function

$f(t)$ = transit function through gut

In one subject (subject C) both $c(t)$ and $g(t)$ have been determined. By using a series expansion for $c(t)$ in terms of $g(t)$ and $f(t)$ and a small, chosen finite time interval, Δt, successive values of $f(t)$ have been determined using a digital computer. The method is described in detail in Appendix VII. The method can only give an approximation for $f(t)$, since it relies on the choice of finite time intervals and

the form of the function f(t) depends on the interval chosen for the calculation. The results for time intervals Δt = 0.125, 0.25 and 0.5 hour are shown in Fig. 31 for the derived transit function. All three curves are similar in shape to the oral plasma clearance curve, with an initial sharp rise to a peak value and a slower decline. The oral plasma curve and the transit function for Δt = 0.125 hour are compared in Fig. 32. It can be seen that the rapid disappearance of the absorbed zinc from plasma has the effect that the sharp peak in the gut transfer function is considerably reduced in height in the oral plasma clearance curve.

6.5 Relationship between Plasma Clearance and Tissue Uptake of Zinc

Absorbed zinc appears first in plasma, by which it is transported throughout the body. Disappearance of zinc from plasma must imply either excretion or uptake by the tissues. Previous studies have shown that the major part of the absorbed zinc is retained in the body for long periods and excreted only slowly, the whole body half-life being of the order of several hundred days (Spencer et al, 1965; Newton and Holmes, 1966; Husain and Bessent, 1971). Zinc is therefore absorbed by the body tissues and retained there for some time.

On the whole, uptake by the tissues of a radioactive tracer is less easily measured than that for plasma since

it is impossible to count the area of interest isolated from the surrounding tissues. Because of their own activity the surrounding areas produce a high background count whose exact value cannot be measured, and this makes quantitation of the results difficult. Exceptions to this are the red cell activity, since the cells can be extracted for counting, and the whole body count, which is a composite measurement from all the tissues and therefore not subject to interference of this type. The absorption and clearance of zinc-65 from erythrocytes, whole body, liver and muscle were measured for the two normal subjects given an oral dose of 5 μCi zinc-65. Red cell activity was measured using a Tracerlab "Gammaguard 150" automatic gamma counter. The cells were washed three times in isotonic saline and the washed cells counted for 500 second intervals, for a total time of several hours, as described for the plasma samples. Red cells were counted from all the blood samples, with the exception of a few samples taken during the first few hours.

Whole body counts were measured using a shadow shield whole body monitor (Warner-Oliver type), using two 12.5 cm diameter sodium iodide crystals. Scans of 1000 seconds were carried out in each case and the results compared with a 5 μCi zinc-65 standard each time. Counts were made a few hours after administration of the dose (to enable the counts equivalent to 100%

absorption to be measured) and thereafter at intervals of several weeks.

Tissue counts were measured using a Selo scanner with 12.5 cm diameter sodium iodide crystal. Liver counts were carried out with the subject angled in such a way that as little as possible of the surrounding organs contributed to the count. The position of the scanner is shown in Fig. 33. At the position chosen only a small fraction of the stomach and the upper part of the small intestine is seen by the scanner, which is of particular importance for counts made in the first few hours after administration of the dose. Counts were also made of muscle, in areas (thigh and forearm) where contribution from adjacent tissues would be minimal. Liver counts were made as shown in Fig. 33 at a position centred 5 cm above the lower costal margin, at a set distance from the body. Counts of the thigh were made at a point halfway between hip and knee. Counts of right forearm were made approximately 8 cm down from the elbow. Calculation of the variation of liver activity expressed as a percentage of the administered dose was carried out using a phantom. The phantom consisted of a liver-shaped thin polythene mould filled with a 2% agar solution into which a known amount of zinc-65 had been mixed. The liquid agar was poured into the mould and allowed to set before use. Agar gel has the advantage that it is of a similar consistency to

tissue and therefore absorption of the activity by the mass of the gel should be of the same order as that occurring in the liver. The muscle counts were calibrated in an approximate way using a 1 litre polythene bottle filled with water and containing 5 μCi zinc-65.

The clearance curves for red cells and whole body are shown in Figs. 34 and 35 for subjects C and E, expressed as a percentage of the total dose administered in each case. It can be seen that the curves for the two subjects are similar in general shape in each case. Figs. 36 and 37 show the curves for liver and for muscle for subject E. A collimator was used in the earlier tissue measurements for subject C, but due to a fault the measurements were not sufficiently accurate for quantitation. Later measurements were calibrated using the liver and muscle phantoms. However the shape of the curves was approximately the same as for subject E, with maximum values attained at about the same times in each case.

It can be seen that uptake by liver is rapid, with a peak in the first 24 hours, followed by a much slower decline. The uptake by red cells is slower, with a maximum after about 10 days and a slow decline thereafter over a period of several hundred days. The muscle activity, however, increases only slowly, reaching a maximum about 50 days after administration

of the tracer, long after the maxima in liver, red cell and plasma curves. The whole body counts show a gradual decline over a period of several hundred days.

6.6 Mathematical Analysis of the Clearance Curves

The curves for uptake and clearance of zinc-65 in plasma, red cells, whole body, liver and muscle can be analysed into a series of exponential functions using an exponential stripping technique, where the final points on the curve are used to derive the equation of an exponential function using a least squares fit to the points. This equation is then used to derive the contribution made to earlier values by the exponential function and, by subtraction, the points on the next exponential function derived. This process can be continued to derive a series of exponentials, depending on the data available.

(i) Plasma Curve

This can be analysed into two exponential functions, with reasonably good fit.

If the plasma activity, A_t is given by

$$A_t = A_o . e^{-bt} + A_o' . e^{-b't} \quad \ldots . \quad (1)$$

where A_o and b are constants and the half-life, $T_{\frac{1}{2}}$, is given by:-

$$b = \frac{\ln 0.5}{T_{\frac{1}{2}}}^{*} \quad \ldots \ldots \ldots \quad (2)$$

Analysis of the curves obtained for subjects C and

*ln = natural logarithm

E gives values:-

	C	E	
A_o	0.92	0.50	% dose / 1
b	1.4	1.2	
$T_{\frac{1}{2}}$	12	14	hours
A'_o	0.24	0.18	% dose / 1
b'	0.033	0.040	
$T'_{\frac{1}{2}}$	21	17	days

(ii) Red Cell Curve

After the maximum, the clearance curve decays as a two exponential function of the form of equation (1). The values obtained from the curves for subjects C and E were:-

	C	E	
A_o	2.6	1.2	% dose / 1 packed cells
b	0.041	0.058	
$T_{\frac{1}{2}}$	17	12	days
A'_o	0.50	0.27	% dose / 1 packed cells
b'	0.0024	0.0020	
$T'_{\frac{1}{2}}$	286	347	days

(iii) Whole body curve

In each case the data gives a good fit to a single exponential, given by the equation:-

$$A_t = A_o . e^{-bt} \qquad \text{.............(3)}$$

The values obtained were:-

	C	E	
A_o	86	36	% dose
b	0.0022	0.0021	
$T_{\frac{1}{2}}$	316	331	days

(iv) Liver curve

Accurate results were obtained only for subject E. The values obtained gave a good fit to a double exponential of the form of equation (1).

	E	
A_o	8.1	% dose
b	0.091	
$T_{\frac{1}{2}}$	7.6	days
A_o'	5.7	% dose
b'	0.0064	
$T_{\frac{1}{2}}'$	108	days

(v) Muscle curves

This can be analysed into two exponential functions, an initial gain of zinc-65 activity of the form:-

$$A_t'' = A_o''.(1 - e^{-b''t}) \quad .... \quad (4)$$

and a single exponential of the form of equation (1) after the maximum. If $T_{\frac{1}{2}}$ and $T_{\frac{1}{2}}''$ are the half-lives for these functions the values obtained for subject E were:-

	Thigh	Forearm	
$T_{\frac{1}{2}}''$	15	12	days
$T_{\frac{1}{2}}$	198	101	days

6.7 Calculation of Zinc Content Using the Occupancy Principle

The Occupancy Principle, derived by Orr and Gillespie from Bergner's analysis of tracer dynamics (Orr and Gillespie, 1968; Bergner, 1964), gives, for a steady state system, a relationship between the amount of a particular substance in any defined part of the system (the capacity), the flow rate of the substance through the system, and the occupancy of a tracer for the substance administered at the input to the system. The occupancy, θ, is defined as the total integral with respect to time, of the tracer fraction f(t) present in that part of the system at any time, that is:-

$$\theta = \int_0^\infty f(t)\, dt \quad \ldots\ldots\ldots\ldots\ldots (5)$$

The Occupancy Principle states that the occupancy, θ, divided by the capacity, C, is equal to the reciprocal of the flow rate, F:-

$$\frac{\theta}{C} = \frac{1}{F} \quad \ldots\ldots\ldots\ldots (6)$$

Also, since the flow rate is a constant for the system the occupancies and capacities for the various components of the system are related by the equation:-

$$\frac{\theta_1}{C_1} = \frac{\theta_2}{C_2} = \ldots = \frac{\theta_n}{C_n} \quad \ldots\ldots\ldots (7)$$

The body approximates to such a steady state system, with a constant intake of essential nutrients and trace elements orally from the diet. Hence if the occupancy of any chosen part of the system for an orally administered tracer for a substance can be measured the Occupancy Principle can be used to calculate the total stable content of the substance on that part of the system, provided either the flow rate or the occupancy and capacity of any other part of the system are known.

Both plasma and red blood cells provide conveniently accessible components of the system for occupancy calculations, as samples can be withdrawn for counting, uninfluenced by other parts of the system. Also the stable content of the substance of interest can often be measured accurately in both plasma and red cells. If the occupancy is known for any other defined part of the system the capacity can therefore be calculated using either the plasma or red cell values.

In the present series of experiments the variation of the level of the orally administered tracer dose of zinc-65 has been measured for whole body, liver and muscle, as well as for plasma and red blood cells. The variation of the plasma zinc-65 activity with time can be described by an equation of the form of equation (1). The plasma occupancy is, therefore:-

$$\theta_{plasma} = \int_0^\infty (A_o . e^{-bt} + A'_o . e^{-b't}) \, dt$$

$$\therefore \quad \theta_{plasma} = \frac{A_o}{b} + \frac{A_o'}{b'} \quad \ldots\ldots\ldots\ldots (8)$$

For subjects C and E, therefore:-

	C	E	
θ_{plasma} (for 1 litre)	7.9×10^{-2}	4.8×10^{-2}	days

Similarly, the variation of red cell zinc-65 activity with time is also described by equation (1) and hence the red cell occupancy is also given by equation (8), together with a small fraction contributed by the area under the curve in the first 10 days, which can be neglected compared with the other contribution.

The values obtained for the two subjects, C and E were:-

For 1 litre packed cells

	C	E	
θ_{RBC}	2.7	1.5	days

Since the disappearance of zinc from plasma is very rapid and the levels in the samples used for the calculation of the second exponential function very near background, measurement of any plasma occupancy remaining after about 50 days is impossible with the counting system used. However, with the exception of only the first few hours, the zinc-65 activity of the erythrocytes is also greater than that of the plasma and this activity is measurable over a period of several hundred days. The red cell occupancy can therefore be estimated with greater accuracy. Knowing the plasma and red cell

stable zinc concentrations the equation

$$\frac{\theta_{plasma}}{C_{plasma}} = \frac{\theta_{RBC}}{C_{RBC}} \quad \ldots\ldots\ldots \quad (9)$$

can be used to predict the plasma occupancy.

For subjects C and E,

	C	E	
Plasma zinc	8.6×10^{-4}	8.8×10^{-4}	g / l
Red cell zinc	1.34×10^{-2}	1.24×10^{-2}	g / l
θ_{RBC}	2.7	1.5	days
$\therefore \theta_{plasma}$	0.172	0.110	days

In each case the value for the plasma occupancy is smaller than that predicted from the red cell curve. This suggests that there is a significant part of the plasma clearance curve which is below the limits of measurement. If the plasma activity at time t = 35 days is taken as the first point on a final exponential decay then an approximate estimate of the half-life of this curve can be made, since the remaining occupancy

$$\theta_{predicted} - \theta_{measured} = 1.44.A_o.T_{\frac{1}{2}} \quad \ldots.(10)$$

This gives values of $T_{\frac{1}{2}}$ of 86 days for subject C and 97 days for subject E, suggesting that there is a third slowly decaying component in the plasma zinc-65 clearance curve with a half-life of approximately 100 days.

The red cell occupancy and capacity can also be used to calculate the whole body and liver capacities

using equation (7).

The whole body occupancy,

$$\theta_{\text{whole body}} = A_o/b \quad \ldots\ldots\ (11)$$

For subjects C and E,

	C	E	
$\theta_{\text{whole body}}$	390	172	days
$\therefore C_{\text{whole body}}$	2.0	1.4	g zinc

The liver occupancy is given by an equation of the form of equation (8). Hence for subject E,

$$\theta_{\text{liver}} = 9.8 \text{ days}$$

$$\text{and} \quad C_{\text{liver}} = 0.079 \text{ g zinc}$$

Using the final values of the zinc-65 liver clearance curve, which were the only usable data obtained for subject C, and assuming the same values as subject E for the half-lives of the two components of the clearance curve gave an approximate value for the liver occupancy for subject C

$$\theta_{\text{liver}} = 24.1 \text{ days}$$

$$\text{and} \quad C_{\text{liver}} = 0.123 \text{ g zinc}$$

6.8 Discussion

The clearance curves obtained on administering an intravenous tracer dose of zinc to three normal subjects have shown that about 75% of the absorbed dose disappears into tissue very rapidly, the half-life for initial disappearance being of the order of 16 minutes. These results agree with those obtained by other workers (Prasad et al, 1963; Spencer et al, 1965; Sullivan and

Heaney, 1970), though of these only Prasad and his colleagues studied zinc clearance in normal controls. The rapidity of the initial clearance from plasma means that though as much as 86% of an oral dose of zinc-65 can be absorbed (subject C) there is never more than 4% of the dose in the total plasma volume. Using the oral and intravenous plasma clearance curves obtained at different times for one normal subject the shape of the transfer function for zinc through the gastro-intestinal wall was determined. This function was found to rise rapidly to a peak, then decay more slowly, suggesting that while maximum uptake occurs almost immediately after administration of the dose zinc uptake is not limited to a small section of the gut but can occur over a considerable length. While it is obviously impossible to locate the exact section of the gut involved the rapid rise to a maximum within a short time would suggest uptake from the upper part of the small intestine, possibly in the duodenum or even in the stomach.

The initial rapid clearance of zinc from plasma is paralleled by a similar rise in the zinc-65 activity in the liver. In subject E it was found by extrapolation of the calculated clearance curve that at maximum about 25% of the absorbed dose was concentrated in the liver. After 24 hours the activity starts to decline, initially with a half-life of about 8 days. This is

accompanied by a rise in the activity of muscle, the rise having a half-life of the same order as the decline in the liver. This strongly suggests transfer of zinc stored in the liver to the muscle mass. Comparing the half-lives for the initial decline in the liver activity and the rise in muscle activity with that of the decline in activity in plasma during this period it is found that all three half-lives are of the same order of magnitude, allowing for the considerable experimental error which is unavoidable in these results. This would suggest equilibrium between liver, plasma and muscle during this phase and represents rapid transfer of zinc from its initial store in the liver to the muscle mass by plasma.

These results agree with the whole body profile scan curves of Newton and Holmes for a subject who had accidentally absorbed an amount of zinc-65 into the lungs (Newton and Holmes, 1966). They showed that there was an initial large concentration of zinc-65 in the liver region and that this steadily declined during a period of about 100 days to the level of the surrounding soft tissue. There is agreement also with the post-mortem measurements of Spencer and her colleagues (Spencer et al, 1965) who showed that the zinc-65 concentration in the liver was highest in the first few days after administration of the dose, with a gradual decline over 70 days and that the zinc content of muscle, initially much lower than liver, rose to about the same value as

in the liver after 70 days.

The existence of a second component in the liver clearance curve, with a half-life of the order of 100 days, shows that the total zinc-65 absorbed into the liver is not transferred in this way. This value for the half-life is similar to the decline in muscle activity and the final decay in plasma activity predicted from the occupancy values, suggesting that the zinc in muscle, liver and plasma are in equilibrium at this stage and form an exchangeable pool of zinc. Since these half-lives are not the same as the whole body values this would imply that the zinc is being deposited elsewhere in the body, though it is possible that if the final measurements in liver and muscle (which are very near the limits of measurement after about 200 days) could be continued for longer periods and the final exponential calculated more accurately, that values of the same order as the whole body half-life would be obtained.

In red blood cells the zinc-65 level rose to a peak 10 days after administration of the dose, falling thereafter as a double exponential clearance, the first part having a half-life of about 15 days and the second part a half-life which in each case is similar to the values obtained for the whole body curve (about 300 days), suggesting that the greater part of the zinc-65 uptake by the erythrocytes is not exchangeable except within

the red cell pool.

The whole body curves, plotted over a period of about 400 days, show a steady monoexponential clearance of the zinc-65 from the body, with half-lives of 316 and 331 days in the two subjects studied. These values are in agreement with those of other workers (Graig and Seigel, 1960; Spencer et al, 1965; Newton and Holmes, 1966) all of whom reported values for whole body half-lives of several hundred days.

The rapid transfer of a large part of the zinc-65 absorbed into plasma to the liver is in agreement with the change in the relative concentrations of zinc-65 bound to the two plasma protein fractions discussed in the previous chapter. In the first few hours after swallowing the dose the plasma zinc-65 level is largely due to zinc newly absorbed from the gut, but this zinc is removed very rapidly, probably to the liver, once in plasma. It was found that in the first few hours the greater part of the plasma zinc-65 was bound to albumin though there was a measurable quantity (about 11%) bound to α_2-macroglobulin, and that the level of the albumin-bound zinc fell until after 24 hours the equilibrium partition obtained for the endogenous zinc had been attained for the zinc-65. These findings suggest that newly absorbed zinc is bound to albumin and that a large proportion of the albumin-bound zinc is removed by the liver which then gradually releases a part of the

absorbed zinc firmly bound to α_2-macroglobulin. This interpretation is reinforced by the results obtained when zinc-65 was added to plasma *in vitro*, when binding only to the albumin fraction could be detected. The liver is also known to be a site of α_2-macroglobulin production. That the zinc-65 remains associated with these two proteins in plasma and does not exist for an appreciable period in another form is shown by the Occupancy Principle. Rearrangement of equation (7) shows that the ratio of the occupancy of any other zinc-containing plasma component to that of the total plasma zinc is equal to the ratio of their capacities. Hence since measurable zinc could only be detected in the albumin and α_2-macroglobulin fractions the capacity of any other component in plasma is very small compared with the total plasma value and therefore the occupancy is also small.

Calculation of the stable zinc content of the body for each of the two normal subjects using the Occupancy Principle gave values of 2.0 g and 1.4 g. These results give excellent agreement with those of Widdowson, McCance and Spray (Widdowson et al, 1961) who measured the total body zinc content of whole cadavers using conventional chemical methods and who found whole body zinc values in the range 1.4 - 2.2 g for adults. The values calculated for the total zinc content of the liver also agree well with the results of these workers.

The Occupancy Principle, therefore, which to date offers the only method for estimating total body zinc <u>in vivo</u>, gives results which are very similar to those obtained by detailed analytical techniques. Assuming that the liver mass is approximately 3% of the total body mass the stable zinc values for the liver (0.12 g and 0.08 g) are approximately 6% of the whole body zinc levels, suggesting that while there is an initial concentration of at least 25% of the absorbed zinc in the liver there is no long-term concentration of zinc by the organ. These results agree with the stable zinc values obtained for samples of normal liver by activation analysis which show that the stable zinc concentration in liver and muscle are similar.

6.9 Conclusions

It has been shown that:-

(a) The greater part of a tracer dose of zinc-65 administered intravenously to normal controls is cleared very rapidly from plasma, with a half-life of less than 20 minutes. The derived transit function which describes the passage of zinc through the wall of the gastro-intestinal tract rises sharply to a peak within half an hour after administration of the zinc then declines more slowly, indicating early absorption of zinc through the gut, possibly in the stomach or duodenum.

(b) The initial plasma clearance, shown previously to be due to loss of zinc from the albumin-bound

fraction, coincides with uptake of zinc by the liver, suggesting that newly absorbed zinc is bound to albumin and transported to the liver. There a fraction of the zinc is firmly bound to α_2-macroglobulin and this process continues until equilibrium binding of zinc to albumin and to α_2-macroglobulin is achieved.

(c) Comparison of the clearance curves for liver, plasma and muscle shows that, in the first 50 days after ingestion of the dose the zinc-65 activity in muscle rises gradually, the half-lives for the decline in liver and plasma zinc-65 activity being similar to that of the corresponding increase in muscle, suggesting rapid transfer of zinc from liver to muscle by plasma during this phase. This is followed by a much slower decline in activity in all three compartments, suggesting exchange of zinc among them.

(d) Calculation of the amount of zinc contained in the body using the Occupancy Principle gave values of 1.4 and 2.0 g for the two normal subjects studied, equivalent to an average concentration of 0.028 g / kg body weight. The amount of zinc contained in the liver was about 6% of the whole body value.

CHAPTER 7

CHAPTER 7

EFFECTS OF INGESTION OF THERAPEUTIC AMOUNTS OF ZINC ON PLASMA ZINC LEVELS AND ON THE DISTRIBUTION OF ZINC AMONG THE PLASMA PROTEINS

		Page
7.1	Introduction and Purpose of the Study	131
7.2	Methods	133
7.3	Variation of Plasma Zinc Level with Time in Normal Subjects	135
7.4	Comparison of Distribution of Protein-Bound before and after Ingestion of Zinc	135
7.5	Discussion	139
7.6	Conclusions	145

7.1 Introduction and Purpose of the Study

The work described in the previous chapters has shown that the endogenous protein-bound zinc in plasma is associated with two protein fractions, which have been identified as albumin and α_2-macroglobulin. A trace quantity of zinc newly absorbed into plasma from the gastro-intestinal tract is bound mainly by albumin but this level drops rapidly until after 24 hours the equilibrium binding ratio of the endogenous distribution has been achieved. Thus albumin is a transport protein for zinc. The results given in the last chapter suggest that zinc is bound to α_2-macroglobulin in the liver and the virtually constant level of zinc-65 associated with the protein during the first 24 hours after administration of the dose indicates that any clearance of zinc from the protein to the tissues is much slower than the initial clearance of the zinc from albumin. However it has been shown that during the phase when transfer of zinc from liver to muscle occurs an appreciable percentage of the total plasma zinc-65 is bound to α_2-macroglobulin, in contrast to the first initial rapid transfer from plasma to liver, which apparently involves only the albumin fraction. It would therefore seem that the zinc bound to α_2-macroglobulin is in some way involved in the gradual transfer of zinc stored in the liver to the muscle mass but the precise nature of its role in this process is not clear. In

particular it is not known whether the protein actually transports zinc, that is, whether the zinc bound to α_2-macroglobulin is released and incorporated into the tissues or whether it is in some way involved in control of the transport of the element by albumin.

As discussed in Chapter 4 it is not possible to obtain such information from a study of the endogenous distribution of zinc among the plasma proteins, since this is a steady state distribution. However it is known that after ingestion of 220 mg zinc sulphate the plasma zinc level rises in some cases to as much as twice the endogenous level and that this level returns to the normal value within a few hours (Chapter 5). Study of the change in distribution of the zinc between the protein fractions should show whether the α_2-macroglobulin - bound zinc is significantly increased in cases where the plasma zinc level shows a pronounced rise and whether the variation in the amount of zinc bound to the protein follows the rapid rise and fall of the plasma zinc level, without an accompanying change in the plasma α_2-macroglobulin concentration.

The aim of the work described below was therefore to study the variation of total plasma zinc level with time after ingestion of a single therapeutic dose of zinc sulphate, mainly in normal controls, but also in patients with suspected disorders of zinc metabolism, and also to study the effect of continued administration

of the zinc sulphate over a period of several days on the total plasma zinc level, the distribution of zinc among the proteins and on the urinary output of zinc.

7.1 Methods

A single gelatine capsule containing 220 mg zinc sulphate B.P. was swallowed by six normal volunteers, four male and two female, aged between 25 and 35 years, none of whom were suffering from any pathological condition known to be associated with altered zinc metabolism. The capsules were taken approximately 1½ hours after a light meal (since the subjects all took part in the study during a normal working day it was not thought advisable for them to take the capsules in the fasted state because of the possibility of gastric upset). In one case capsules were taken for a period of 5 days and after the first capsule, were taken immediately after meals. In this case 24 hour urine collections were made before and during these five days and then at intervals afterwards until the urine zinc level had returned to normal. Two patients suffering from pustular psoriasis also participated in the study.

Blood samples (10 ml) were obtained from all subjects before swallowing the capsules and in most cases at intervals of ¾, 1½, 3 and 6 hours afterwards. Samples were withdrawn into heparinised plastic containers, centrifuged immediately and the plasma removed into

untreated plastic vials. An aliquot (1 ml) was reserved for plasma zinc estimation and, in the samples chosen for separation, 2 ml was passed through a column of Sephadex G100, 18.5 cm x 1.6 cm, using Tris-HCl buffer in 0.1M sodium chloride solution as eluant and collected in 2.4 ml fractions as described in Appendix III. In the single case studied for several days 20 ml blood samples were withdrawn at 1½ hours and after 5 days and plasma samples of approximately 6 ml were separated into 6 ml fractions using a 46 cm x 2.6 cm column of Sephadex G200, to enable more detailed analysis of the protein distribution to be made. Blood samples were taken from the two patients suffering from psoriasis before and 2 hours after taking the capsule.

Plasma zinc levels were measured by atomic absorption spectrometry, albumin levels by measurement of the absorption of light at 540 nm by the sample in methyl orange reagent and α_2-macroglobulin levels by single radial diffusion. For both albumin and α_2-macroglobulin measurements dilutions of the original plasma were used as standards in each case and the protein level in the plasma samples measured by comparison with a reference serum containing a known amount of both proteins.

Urine samples were collected in 2.5 l polythene bottles containing thymol. The volume of each 24 hour collection was measured and the zinc level estimated by atomic absorption spectrometry.

7.3 Variation of Plasma Zinc Level with Time in Normal Subjects

Fig. 38 shows the variation of plasma zinc level with time for the normal volunteers studied. It can be seen that there is a wide range of variation of zinc levels in the six subjects, both initially and at various times after ingestion of the capsule. The largest rise was found in the two female subjects (C and E), where the plasma level rose to approximately double the original level after about 1½ hours. Maximum increases in plasma zinc level in the range 0 - 61% were found in the other four male subjects and in the case of subject D no significant increase in zinc level was found in any of the samples measured. In subject J the initial plasma zinc level was found to be higher than the final value six hours later, though there was a significant rise in plasma zinc level after 1½ hours. Maximum values were usually obtained about 1½ hours after administration of the capsule, though this value occurred somewhat earlier for subject K.

7.4 Comparison of Distribution of Protein-Bound Zinc before and after Ingestion of Zinc

Fig. 39 compares the distribution of endogenous zinc among the plasma proteins (T = 0) with that obtained from plasma at the time of maximum increase in plasma zinc level (T = 1½ hours), separated using Sephadex G200. It can be seen that both distributions

have exactly the same shape, with peaks in the albumin and α_2-macroglobulin regions, but that both albumin- and α_2-macroglobulin-bound zinc levels are increased when the zinc level is raised. If the area under each of the peaks is measured in each case the ratios of zinc bound to albumin to zinc bound to α_2-macroglobulin are 3.0:1 and 3.2:1 for the endogenous zinc and for the zinc absorbed from the capsule respectively, showing an increase in the zinc bound to both protein fractions to approximately double the normal level.

Plasma samples (2 ml) taken from each of the normal volunteers before swallowing the capsule and 1½ hours after were separated using a column of Sephadex G100. From measurements of the zinc concentration in each fraction and of the albumin and α_2-macroglobulin concentrations of the fractions the total amounts of zinc bound to the two proteins were calculated, as discussed in Chapter 4. Table 6 compares the percentages of zinc bound to albumin and to α_2-macroglobulin for plasma samples from the six normal volunteers at time T = 0 and T = 1½ hours. Also included are the values obtained from the two patients with psoriasis before and two hours after swallowing the zinc capsule, separated in the same manner. It can be seen that both protein fractions are increased in approximately the same proportion in all cases, though there is a slightly larger proportion of the zinc bound to albumin

after two hours in the two patients, J.G. and R.G.

Plasma samples, taken from those subjects where the plasma zinc level of the last sample taken had dropped to almost the original level, were also separated in this way and the results (from subjects C, K, R and J) are given in Table 6. In two subjects (C and J) the percentage of the total zinc bound to the α_2-macroglobulin fraction is significantly higher at this time but, in the case of subject R, where the plasma zinc level had returned to the original value after six hours, the same percentage of the total plasma zinc was found associated with each of the proteins for the samples taken at times T = 0 and 6 hours.

To determine whether the increase in α_2-macroglobulin-bound zinc was accompanied by an increase in the concentration of the protein the α_2-macroglobulin levels were measured for all the plasma samples obtained from subjects C and E during the first 5 hours. No change in α_2-macroglobulin level with time could be detected in either case. The α_2-macroglobulin levels in the plasma samples from all eight subjects were measured by radial immunodiffusion. The values obtained are given in Table 7. It can be seen that, while the results are too few to be statistically significant, the α_2-macroglobulin levels tend to be higher in those whose zinc level showed the greatest increase on taking the capsule.

A plasma sample was obtained from subject C after taking three zinc sulphate capsules daily for five days. The sample was taken one hour after swallowing the last capsule. The plasma zinc level was found to be 134 μg / 100 ml, a value intermediate to the value obtained $1\frac{1}{2}$ hours after taking the first capsule and the initial plasma zinc level (200 μg and 94 μg / 100 ml respectively). 6 ml of the sample were separated into 6 ml fractions using a 46 cm x 2.6 cm column of Sephadex G200 and the zinc level in each of the fractions measured. The distribution was found to be similar in shape to both the endogenous zinc distribution and to that obtained for the sample taken $1\frac{1}{2}$ hours after swallowing the first capsule of the fifteen, shown in Fig. 39. Measurement of the areas under each of the peaks gave values for the percentages of the total plasma zinc bound to albumin and to α_2-macroglobulin of 70% and 30% respectively.

A plasma sample was taken 24 hours after swallowing the last capsule and the zinc level measured. It was found that the value was 106 μg / 100 ml, which is significantly greater than the initial plasma level of 94 μg / 100 ml. A 2 ml sample was separated into 2.4 ml fraction using the small column of G100 and the percentage of the total zinc bound to each of the two proteins calculated, giving values of 34% bound to α_2-macroglobulin and 66% bound to albumin.

Fig. 40 shows the variation in urine zinc levels with time for subject C during the five days of the experiment and for several days afterwards. The urine zinc level rose during the first day and continued to rise in the following period until the urine level was about double the initial value. On stopping the capsules the zinc level fell gradually to the original value after a time of about 11 days from the beginning of the experiment.

In the present work the variation of plasma zinc level with time after an oral dose of 50 mg zinc has been measured for subjects C and E. In both cases the response to an oral tracer dose of zinc-65, equivalent to approximately 20 μg zinc has already been described. Fig. 41 compares the variation of plasma zinc and zinc-65 levels with time for the two cases. The graphs show that, while both stable and radioactive zinc levels reach a peak at between $1\frac{1}{2}$ and 2 hours after administration of the zinc there is a more rapid clearance of the extra stable zinc from plasma, with a a return to the original stable level in a few hours. The curves also show that both stable and radioactive zinc are cleared more slowly from plasma in subject E than in subject C.

7.5 Discussion

The graphs of variation of plasma zinc level with time after ingestion of a single 220 mg capsule of zinc

obtained from six normal volunteers show that, in the majority of cases, a significant rise in the plasma zinc level occurs, with a maximum at between ½ and 2 hours after taking the capsule and with a rapid decline to almost the normal level within a few hours. A rise in plasma zinc level to more than double the original level was found in two cases, but in one case no significant increase in the plasma level over a period of 6 hours could be detected. It is therefore evident that there is a wide variation in the amount of zinc absorbed even in subjects with no known disorders associated with altered zinc metabolism. Also, when the capsules were given orally three times daily for 5 days it was found that the plasma zinc level did not increase continually during this time. Measurement of the plasma level 1 hour after taking a capsule on the fifth day gave a zinc level (134 μg / 100 ml) between the initial value obtained before the first capsule was taken and the maximum value obtained 1½ hours after taking the first capsule (94 and 200 μg / 100 ml respectively). This result is consistent with the rapid return of the plasma zinc level to almost the original level a few hours after swallowing a single capsule. If there is a six hour interval on average between swallowing the capsules taken three times daily the plasma zinc level will have returned to almost the original level before swallowing the next

capsule causes a further rise in the plasma level. These results agree with those obtained by Hallböök and Lanner (Hallböök and Lanner, 1972) who found that the plasma level of patients on 220 mg zinc sulphate three times daily, measured after overnight fasting and before taking a capsule rose only very slowly to a maximum level of approximately 150 μg / 100 ml and that this rise was significant only in cases where the initial plasma zinc was lower than 110 μg / 100 ml. Abdulla et al (Abdulla et al, 1973), who obtained a similar result, interpreted this finding to mean that only small amounts of zinc were absorbed into the body during this time. However considerable absorption of zinc could have occurred in these cases, since the plasma zinc would have returned to the equilibrium value after taking the capsule at the time when the measurements were made.

Administration of a tracer dose of zinc-65 to two normal volunteers gave maximum levels in plasma of about 2% of the absorbed dose per litre of plasma, calculated from the zinc-65 levels in plasma and whole body. If, to give a rough guide, this is taken to be the case for the absorption of stable zinc, then an increase of 100 μg zinc per 100 ml plasma at maximum is equivalent to absorption of 50 mg zinc, that is, virtually the whole of the administered dose. After 5 days i.e. 15 capsules the total absorption is

therefore 750 mg. The total urinary excretion from these 15 capsules was calculated to be about 10 mg for subject C and assuming that the faecal excretion of absorbed zinc is roughly five times the urinary level (Husain and Bessent, 1971) this gives a total excretion of about 60 mg during this time, an amount equivalent to less than 10% of the amount absorbed. If it is assumed that the tracer and therapeutic absorbed dose follow the same metabolic path, this quantity would be retained for long periods in the body. Hence, if therapy is carried out for several weeks, as is the case for patients on oral zinc sulphate supplements for conditions such as varicose ulcers the amount of zinc deposited in the body could be increased to several times the pre-therapy level.

Greaves and Skillen have shown that prolonged zinc therapy has no effect on parameters such as haemoglobin level, platelet and white cell counts, plasma urea and bilirubin concentrations and on serum L.D.H. isoenzyme patterns, which have been found to be a sensitive index of liver function, and in particular, on the levels of the main zinc metalloenzymes in plasma, alkaline phosphatase and lactic dehydrogenase, even after four months on zinc therapy and though significant increases in plasma zinc level were found in 67% of the patients studied (Greaves and Skillen, 1970). This lack of change in the levels of the zinc metalloenzymes even after prolonged absorption of significant amounts

of zinc suggests that though zinc is retained in the body the greater part of it is stored.

It has been shown that, when the plasma zinc level rises following ingestion of a single dose of zinc sulphate, the distribution of zinc among the plasma proteins has a similar shape to that obtained for endogenous zinc, with two peaks, one coincident with α_2-macroglobulin and the other with albumin. Measurement of the percentages of the total zinc bound to each fraction in the sample having maximum zinc level shows that zinc absorbed into plasma is shared between the α_2-macroglobulin and the albumin fractions in the same ratio as the endogenous zinc (approximately 1:3). This has been shown to be true for a group of normal subjects but in two patients suffering from psoriasis, a condition thought to be associated with disordered zinc metabolism, there was a slight increase in the proportion of the total plasma zinc bound to albumin in the plasma samples obtained 2 hours after swallowing a capsule of zinc sulphate. After about 6 hours, when the plasma level has dropped to almost the original level the amount of zinc bound to both albumin and α_2-macroglobulin falls again. In some cases a slightly higher concentration of zinc is found in the α_2-macroglobulin fraction but in others the original level is regained and no difference could be detected between the distribution patterns for zinc for the pre-dose plasma sample and that obtained

6 hours after taking the capsule. As no change could be detected in the concentration of α_2-macroglobulin in plasma during this period it can be seen that α_2-macroglobulin acts as a true carrier for zinc, since it loses zinc to the body tissues without itself being broken down. α_2-macroglobulin is therefore a transport protein for zinc.

After taking three zinc capsules daily for 5 days the distribution of zinc among the plasma proteins was found to be the same as in the earlier samples and the proportions bound to the two proteins were 70% to albumin and 30% to α_2-macroglobulin. A further sample obtained on the sixth day, 24 hours after taking the final capsule, gave percentages of 66% bound to albumin and 34% bound to α_2-macroglobulin. There is thus a gradual slight increase in the proportion of the plasma zinc bound to α_2-macroglobulin during this period in this subject but further work is necessary before the significance of this finding can be assessed.

The results obtained in the work described above agree with those obtained from separation of plasma samples from patients given an oral dose of 220 mg zinc sulphate, together with 5 μCi zinc-65, described in Chapter 5. It was found that, in the plasma samples with maximum zinc-65 level, a much higher proportion of the plasma zinc-65 activity was associated with the α_2-macroglobulin fraction (about 50%) than that found in

plasma samples obtained when a tracer dose of zinc-65 alone was administered, when the value was only 11% of the total. Hence loading the plasma with zinc appears to cause a more rapid rise in the amount of zinc bound to the α_2-macroglobulin fraction. It has been suggested (Chapter 6) that this fraction is connected in some way with the uptake of zinc by muscle and it is possible that this increase in the zinc bound to the protein causes the greater part of the zinc absorbed from therapeutic doses to be transported to muscle and stored there. In this connection it is of interest that evidence has been produced that α_2-macroglobulin is one of the few plasma proteins to be synthesised by muscle (Prunier et al, 1964).

The concentration of α_2-macroglobulin in plasma is therefore not an indication of the amount of zinc bound to this protein, since this level can vary according to the amount of zinc ingested. This finding agrees with the results of McBean and his colleagues (McBean et al, 1972) who found that patients with suspected zinc deficiency had plasma α_2-macroglobulin levels which did not differ significantly from those of normal controls.

7.6 <u>Conclusions</u>

The experiments described above have shown that:-

(a) When a single dose of zinc (50 mg) was administered orally to six normal subjects and to two patients suffering from psoriasis the plasma zinc level rose

significantly in all but one case, the increases being in the range 0 - 110% of the original level, but fell to almost the initial value after approximately six hours. After taking 150 mg zinc per day for a period of five days the plasma level showed the same response to a single dose of zinc, with only an increase of 10% of the original level found in a plasma sample taken 24 hours after administration of the last capsule of zinc.

(b) When the distribution of zinc among the plasma proteins was studied for the plasma samples with maximum zinc level it was found that the excess zinc was associated with the albumin and α_2-macroglobulin fractions, distributed between the fractions in the ratio of albumin / α_2-macroglobulin zinc of 3:1, the same ratio obtained for the endogenous plasma distribution. When the amount of zinc bound to the two fractions was determined for the plasma samples taken six hours after swallowing the capsule, the level of the zinc bound to α_2-macroglobulin had fallen, in some cases to the level obtained for the initial plasma sample. Since no change in the concentration of α_2-macroglobulin could be found the protein must be capable of binding zinc and later releasing it to the tissues without itself being destroyed. α_2-macroglobulin is therefore a true transport protein for zinc.

CHAPTER 8

CHAPTER 8

REVIEW AND DISCUSSION OF RESULTS

		Page
8.1	Review of Results Obtained	148
8.2	Relationship between the Zinc-Binding Proteins	152
8.3	Relationship between α_2-macroglobulin and Tissue Uptake of Zinc	155
8.4	Effectiveness of Zinc Therapy	156
8.5	Conclusions	158

8.1 Review of Results Obtained

Initial studies were carried out on the binding of trace amounts of zinc-65 added to plasma *in vitro*, using protein separation by electrophoresis on cellulose acetate strips. It was found, both by counting transverse sections of the strips and by autoradiography of whole strips, that all the zinc-65 was associated with two protein fractions, albumin and the α_2-globulins. This was found to be true for a range of amounts of added zinc, from 0.5 - 1000% of the endogenous plasma level, though there was a slightly larger proportion of the added zinc associated with the α_2-globulins at the lower end of the range. As this method was only capable of analysing small quantities of plasma it was not suitable for use in the major part of the work, on the study of the binding pattern of both endogenous zinc in plasma and the variation of the binding pattern with time of zinc-65 absorbed into plasma from tracer doses administered *in vivo*.

Chromatography on Sephadex cross-linked dextran gels was chosen as the most suitable method, one reason being that it is particularly suitable for separation of the two main proteins thought to bind most of the endogenous zinc in plasma, identified by Parisi and Vallee as albumin and α_2-macroglobulin which are well separated using the higher grades of Sephadex gels (G100 - G200). It was found that there was no

significant binding to α_2-macroglobulin of zinc-65 added to plasma *in vitro*. Zinc-65, added with amounts of stable zinc in the range 0.1 - 200% of the endogenous plasma zinc level was found almost totally concentrated in a peak coincident with the albumin distribution, confirming the results of Parisi and Vallee that zinc is bound to α_2-macroglobulin only *in vivo*.

When samples of plasma and serum were separated using a column of Sephadex G200 the distribution of stable zinc among the protein fractions was found to have two distinct peaks coincident with the distributions of albumin and α_2-macroglobulin. At least 95% of the total plasma level was contained in these fractions, with about a quarter of this bound to α_2-macroglobulin and the other three quarters to albumin. Analysis of several plasma samples from six normal volunteers gave values for the percentage of the total plasma zinc bound to α_2-macroglobulin in the range 25 - 32%. In two samples from patients suffering from psoriasis the percentages were slightly higher, 45% and 48%.

When the binding pattern of zinc-65 in plasma samples taken at varying times after administration of an oral tracer dose was studied in two normal subjects it was found that in the first few hours after administration of the dose about 90% of the zinc-65 in plasma was bound to albumin. The zinc-65 level bound to α_2-macroglobulin remained constant over a period of

24 hours but the albumin-bound zinc-65 level dropped until the ratio of binding to the two proteins had reached the equilibrium value found for the endogenous zinc.

Intravenous clearance curves were measured in normal controls using tracer doses of zinc-65 and it was found that clearance of zinc from plasma was very rapid, with about 75% of the plasma zinc-65 being cleared into tissue with a half-life of about 20 minutes. When the curves showing the variation of absorbed zinc-65 with time for both oral and intravenous administration of zinc-65 were compared in one subject the shape of the transfer function for zinc crossing the gastro-intestinal tract was found to rise to a sharp peak within half an hour after administration of the zinc and then decline more slowly, showing that the major part of the zinc is incorporated in the upper part of the gastro-intestinal tract, possibly in the stomach or duodenum.

When the uptake and clearance curves from the orally administered dose of zinc-65 were measured for erythrocytes, whole body, liver and muscle it was found that the whole body curves showed a single exponential decline with a half-life of about 300 days in the two subjects studied. While the zinc-65 activity of the liver rose rapidly to a maximum after about 24 hours the activity in erythrocyes and in muscle rose more

slowly to a maximum, the erythrocyte peak occurring about 10 days after administration of the tracer and that of muscle after about 50 days. Analysis of the curves as a series of exponential functions showed that, after the initial 24 hour period, the fall in the zinc-65 level in plasma occurs at roughly the same rate as both the fall in activity of the liver and the corresponding rise in the activity in muscle, a correlation strongly suggestive of rapid transfer of zinc from liver to muscle by plasma during this period.

When 50 mg doses of stable zinc were given orally to six normal volunteers it was found that the plasma zinc rose to a maximum value between $\frac{1}{2}$ and 2 hours after administration of the zinc in all but one case but that this level had fallen again to almost the normal value within six hours. In one case, when 150 mg zinc was taken for five days, a similar response to a single capsule was found on the fifth day as on the first and the plasma zinc level, measured 24 hours after the final capsule, showed an increase of about 10% above the initial level.

On separation of plasma samples at the maximum zinc level using Sephadex gel it was found that the excess zinc was bound with a distribution similar to that of the endogenous zinc in plasma, with two peaks in the α_2-macroglobulin and albumin regions. Measurement of the percentage of the total plasma zinc bound to each protein fraction showed that the zinc is shared between

them in the same ratio as the endogenous zinc, a rise in the amount of zinc bound to α_2-macroglobulin of as much as 100% of the original level, with no accompanying change in the concentration of the protein in plasma. As the α_2-macroglobulin-bound zinc level had dropped significantly within a few hours, in some cases back to the original level, α_2-macroglobulin has the properties of a zinc transport protein.

Calculation of the zinc content of the total body mass using the Occupancy Principle gave values of 2.0 g and 1.4 g zinc, which agree well with the results of previous chemical studies using cadavers. Similarly, calculation of the total zinc content of the liver gave values of 0.12 g and 0.08 g zinc, results which also agree very well with those from chemical analyses.

8.2 Relationship between the Zinc-Binding Proteins

While there are apparent differences in the binding pattern obtained when zinc-65 is added to plasma or serum depending on the method of protein separation used, and thought to be due to redistribution of the added zinc during electrophoretic separation, separation of these samples using Sephadex gel produces a zinc-65 distribution in which almost all the activity is concentrated in the area of the albumin peak. On the other hand, the distribution of the endogenous zinc in plasma or serum obtained using the same separation technique has two peaks, one coincident with that obtained for the

in vitro zinc-65 distribution and a second peak which coincides with that of the α_2-macroglobulin. While there is considerable evidence that albumin and α_2-macroglobulin are the two proteins which bind the major part of the zinc in plasma the possibility that other proteins are also involved which are indistinguishable from either of these proteins on separation using cross-linked dextran gels does not affect the results of the present work. This has been concerned with an analysis of the change in the levels of zinc bound to the high and low molecular weight protein fractions and does not depend on properties of either protein, apart from in the identification of their position. Again, while it is of interest that the distribution obtained on separation of plasma samples labelled with zinc-65 *in vitro* using electrophoresis on cellulose acetate resembles the endogenous distribution obtained using Sephadex gel rather than that obtained when zinc is added to plasma *in vitro*, these differences do not affect the results of later *in vivo* studies, since these have all been carried out using the same method of separation using Sephadex gel and the results compared accordingly.

The finding that only one fraction binds zinc *in vitro* suggests that newly absorbed zinc is initially bound by this fraction since, in the absence of factors present in plasma only *in vivo*, zinc ions added to plasma

in vitro should bind to the proteins in the same way as zinc entering from the gastro-intestinal tract. Comparisons of the binding obtained using increasing amounts of zinc have shown that, on addition of amounts equivalent to several times the endogenous plasma zinc level, all the extra zinc binds to the same protein fraction as trace amounts of zinc. It is therefore probable that even large amounts of zinc absorbed from orally ingested doses of zinc can be cleared by plasma to the tissues.

Clearance curves obtained for tracer doses of zinc-65 administered intravenously have shown that, once absorbed, the major part of the zinc in plasma is cleared very rapidly. However the oral curves are modified by the slower process whereby the orally ingested zinc crosses the gastro-intestinal tract. This means that during the first few hours after swallowing a tracer dose of zinc-65 a large proportion of the zinc-65 in plasma has just entered the plasma, but that because of the rapid clearance there has been a considerable amount of the zinc-65 previously removed from plasma into the tissues. On separation of plasma samples taken during this time it is found that even only an hour after swallowing the dose there is appreciable binding to the α_2-macroglobulin fraction, though the major part of the zinc is bound to albumin, suggesting that the zinc cleared from plasma is rapidly incorporated into the

α_2-macroglobulin fraction and that the larger amount bound to albumin is mainly newly absorbed zinc-65. This interpretation is reinforced by the finding that a considerable percentage of the newly absorbed zinc is taken up by the liver, which is known to be capable of producing the protein. Absorption into the liver from plasma stops when the ratio of zinc-65 bound to albumin to that bound to α_2-macroglobulin has reached the equilibrium value found for the endogenous zinc.

8.3 Relationship between α_2-macroglobulin and Tissue Uptake of Zinc

For the first 24 hours after administration zinc-65 is cleared preferentially into the liver and though during this time measurable amounts of zinc-65 can be detected in muscle there is a gradual increase in activity for about 50 days while after 24 hours the zinc-65 activity in the liver starts to decline. This increase in muscle activity is too great to be provided solely from the plasma zinc pool, since during this time the average total zinc-65 content is only about 0.5% of the administered dose. The liver, on the other hand, contains about 16% of the dose after 24 hours but only 4% at the time of peak zinc-65 activity in muscle. It therefore seems fairly certain that the increase in zinc-65 level in muscle is derived largely from the liver. Corroborative evidence is provided by the finding that the half-lives for the decline in zinc-65 activity of

liver and plasma are the same as that for the gain in activity of muscle during this period.

Uptake of zinc-65 by liver occurs only while the percentage of the total plasma zinc-65 bound to albumin is greater than the equilibrium value. Uptake by muscle, however, is appreciable only when there is measurable binding of zinc-65 to the α_2-macroglobulin fraction. This would suggest that α_2-macroglobulin-bound zinc is particularly important in this transfer.

8.4 Effectiveness of Zinc Therapy

It is apparent that large amounts of zinc can be absorbed from therapeutic doses but the rapid clearance of absorbed zinc into the tissues makes study of the resultant change in plasma level difficult if only stable zinc is given. In particular it is not possible to study the uptake and clearance by liver and muscle of zinc absorbed from therapeutic doses unless zinc-65 is administered with the dose. It has been shown that relatively large amounts of zinc added to plasma *in vitro* are bound in the same way as trace amounts, suggesting that most of this zinc will be transported to the tissues in the same way as the much smaller quantities absorbed from the diet. However the preliminary studies described in the present work have shown some evidence of more rapid clearance of zinc from an oral dose, since approximately 30% of the peak zinc-65 level was found in plasma 24 hours after oral administration of a tracer

dose of zinc-65, but only 10% of the peak increase in stable zinc level was found in plasma 5 hours after a therapeutic dose. This more rapid clearance of the absorbed zinc is accompanied by a much faster rise in the proportion of the total plasma zinc bound to α_2-macroglobulin. While if the tracer excretion pattern is maintained, very little of the absorbed zinc from a therapeutic dose is excreted during the first few days it is not clear at present whether the absorbed zinc is used immediately by the tissues or whether it is merely stored within the body.

These results are obviously of importance when the use of zinc therapy in routine clinical practice is considered, particularly in cases where the regime is continued for long periods. While the considerable number of clinical trials of zinc sulphate therapy carried out to date have in the main shown no adverse effects on the patients to whom the zinc was administered, the present work has highlighted the need for more basic information on the uptake and clearance of therapeutic zinc by the organs and tissues and of the excretion of these amounts both in normal controls and in patients suffering from conditions thought to bear some relationship to zinc deficiency. These can then be compared with the results obtained from tracer doses of zinc and any differences assessed. A study of the levels of some of the more readily estimated zinc metalloenzymes

(such as the carbonic anhydrase level in erythrocytes) would give valuable information about the utilisation of the absorbed zinc.

8.5 Conclusions

In the introduction to this work the main aims were stated as being the study of the distribution of zinc among the components of plasma both in the steady state, as given by the binding of the endogenous zinc, and as a function of time, studied by measuring the variation in the binding pattern of zinc-65 in plasma from a tracer dose given orally, and to correlate these results with the uptake and clearance of zinc by the tissues. It has been found that zinc, like many other trace elements, possesses a sensitive and sophisticated mechanism for its uptake and control in plasma and that one of the zinc transport proteins, α_2-macroglobulin, is apparently specific for the element. Both the transport proteins, albumin and α_2-macroglobulin, transport zinc to the tissues but it seems that the relative levels of zinc bound to these two proteins act as a control for the destination of the zinc in plasma, excess zinc on albumin being deposited in the liver and this zinc then transferred to the muscle tissue. Whlie these results are necessarily of a preliminary nature they suggest future areas for research into the functions of an element which is not only essential for the maintenance and repair of the body tissues but may also provide benefit in

therapeutic doses to patients with a wide variety of healing problems.

KINETIC AND THERAPEUTIC ASPECTS OF ZINC METABOLISM

VOLUME II

by

Cecilia Mary Feasey

(B.Sc. Honours)

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CONTENTS

VOLUME II

	Page
Appendix I	
Properties of Zinc	1
Appendix II	
Techniques for Protein Separation Using Electrophoresis	3
Appendix III	
Method for Protein Separation Using Sephadex Gels	7
Appendix IV	
Estimation of Total Protein and Albumin Levels using Spectrometric Methods	11
Appendix V	
Method for Measurement of Protein Concentration by Radial Immunodiffusion	14
Appendix VI	
Estimation of Stable Zinc Levels Using Atomic Absorption Spectrometry	18
Appendix VII	
Calculation of the Transfer Function for the Passage of Zinc through the Gut Wall	20
References	23
Tables	35
Figures	43

APPENDICES

APPENDIX I

PROPERTIES OF ZINC

I.1 Properties of Zinc

I.2 Principal Radioisotopes of Zinc

I.1 Properties of Zinc

Atomic Weight	65.38
Atomic Number	30
Periodic Classification	IIB
Electron Distribution	2 - 8 - 18 - 2
Density	7.14 g / ml at 20°C
Melting Point	419.5°C
Boiling Point	907°C
Electrode Potential	-0.762 V at 25°C

I.2 Principal Radioisotopes of Zinc

There are two radioisotopes of zinc which are readily available, zinc-65 and zinc-69m.

Isotope	Type of Decay	Half-life	Gamma Energy (MeV)
zinc-65	β^+	245 days	0.51
			1.11
zinc-69m	isomeric transition	13.8 hours	0.44
zinc-69	β^-	52 minutes	

APPENDIX II

TECHNIQUES FOR PROTEIN SEPARATION USING ELECTROPHORESIS

II. 1 Technique for Electrophoresis on Cellulose Acetate Strips

II.2 Technique for Immunoelectrophoresis

II.1 Technique for Electrophoresis on Cellulose Acetate Strips

Electrophoresis was carried out on 12 cm x 2.5 cm cellulose acetate strips (Oxoid, Ltd.) using a 0.05M barbitone / sodium barbitone buffer, pH 8.6, containing 5 ml / l of a solution of 0.1% thymol in isopropanol as antibacterial agent. The buffer was made up using double-distilled deionised water. 5 μl samples were applied in a uniform line at a position about one third of the strip length from the cathode. Separation was carried out using a constant current of 0.6 mA / cm strip width for one hour and usually of eight 5 μl samples per run. After separation the strips were either stained using a 0.5% solution of Ponceau-S in 3% trichloracetic acid and washed in 5% acetic acid, then in water, or, since the staining procedure removed the zinc from the strips, prepared for counting in either of the following ways:-

(a) The strips were dried and cut longitudinally into three sections in the ratio $\frac{1}{4}:\frac{1}{2}:\frac{1}{4}$ of the total width and the outer sections stained using Ponceau-S to locate the protein bands. The middle section was cut crosswise into 2 mm wide strips, each of which was then counted. The position of each strip was marked on the stained portions to enable its activity to be related to the protein fraction concerned.

(b) It was found that a solution of ninhydrin in

butanol did not remove the zinc-65 activity from the fractions as did the staining procedure using Ponceau-S and hence this solution could be used to locate the protein fractions, which could then be cut out and counted. The cellulose acetate strips were sprayed with a 0.05% solution of ninhydrin in butanol using an aerosol spray (British Drug Houses, Ltd.) and dried at 100°C to develop the stain. The strips were then cut into individual protein fractions through the midpoint of the unstained area between the fractions. The fractions were then counted.

Samples were counted in plastic containers using a well counter with a two inch thallium-activated sodium iodide crystal at an energy corresponding to the major γ-peak for the particular isotope. The time for which the samples were counted varied according to the activity of the samples and was the time required for at least 50 counts above background to be obtained. In practice this was in the range 100 - 800 seconds.

II.2 Technique for Immunoelectrophoresis

Samples were separated using a 0.1M sodium barbitone / sodium acetate buffer, pH 8.6, as electrolyte. The agar solution was made up using Ionagar No.2 (Oxoid, Ltd.) in quarter strength buffer at a concentration of 20 g / l. The agar solution was heated for one hour at 100°C in a water bath and then allowed to solidify. The gel was kept at 4°C until required, when it was reheated to

$60^{o}C$ before use. A layer of the gel was then poured on to eight 10 cm x 2.5 cm glass slides positioned in a holder and the gel allowed to solidify. Two wells and one trough were cut in each slide and the gel in them removed by suction. 2 μl samples were placed each well and electrophoresis carried out at a constant current of 36 mA for $1\frac{1}{2}$ - $1\frac{3}{4}$ hours.

The troughs were then filled with the required antiserum, either goat antiserum to human serum or the appropriate monospecific antiserum (Hyland). The slides were placed in a diffusion chamber and left for 16 hours to allow diffusion of the antiserum. They were then washed in isotonic saline for 48 hours to remove excess protein then in distilled water for 24 hours to remove the salt. A fresh layer of liquid agar was poured onto the slides and when this had solidified the slides were placed in an incubator at $37^{o}C$ to allow the gel to dry out. When dry the slides were stained using triple protein stain in 2% trichloracetic acid, washed in 2% acetic acid and covered.

APPENDIX III

METHOD FOR PROTEIN SEPARATION USING SEPHADEX GELS

III.1 Column Preparation

III.2 Sample Separation

III.1 Column Preparation

The required quantity of dry gel grains was added to a volume of the eluting buffer approximately twice the expected volume of the swollen gel. The gel was heated in a water bath for at least 5 hours to swell the grains and remove the air bubbles. The gel was then allowed to cool to room temperature. The column was filled about one third full of buffer and the swollen gel poured gradually into the column down a glass rod. The gel was then allowed to pack by allowing the buffer to run from the column, fresh gel being added continually from the top. When the column was full and no further packing observed a volume of buffer at least equal to the bed volume of the column was pumped through at the the rate of flow used in the protein fractionation experiments. Uniformity of packing was checked by visual inspection of the gel bed and in some cases by passing a sample of Blue Dextran (Pharmacia, Ltd., Uppsala) dissolved in buffer through the column. This procedure also enabled the void volume of the column to be measured.

III.2 Sample Separation

Three grades of Sephadex gel, G75, G100 and G200 (Pharmacia, Ltd.), were used in the work described. Two column sizes, 46 cm x 2.6 cm diameter and 18.5 cm x 1.6 cm diameter were used in the experiments (Pharmacia, Ltd.), depending on the grade of gel used and the degree of

separation required. Samples were separated by pumping the eluant buffer through the column at a constant flow rate using a peristaltic pump (Chromapump, Baird and Tatlock, Ltd.). The flow rates normally used were 14.4 and 21.6 ml / hour. The fractions from the column were collected automatically using a Chromafrac fraction collector set to deliver a constant volume. Fraction volumes were in the range 2.4 - 9 ml, depending in the sample volume and the size of column used.

The buffer used in all the experiments was Tris(hydroxymethyl)-methylamine - hydrochloric acid, 0.1M, pH 8.0. To ensure a sufficiently high ionic strength the buffer was made up in a solution of sodium chloride whose concentration was in the range 0.1 - 1.0M. This ensures that the ionic strength of the eluted protein fractions is constant and that possible adsorption of proteins onto the gel, which can occur at low ionic strength, is minimised.

The sample was applied to the drained gel bed using a disposable polypropylene syringe. To ensure evenness of application and to remove any precipitate some samples were applied to the gel surface using a plastic sample applicator faced with fine nylon mesh but where the stable zinc content of the fractions was to be measured the sample was applied directly on the drained gel bed to avoid contamination of the sample. The fractions from the column were collected in 10 ml

disposable plastic vials.

After each sample fractionation a volume of buffer at least equal to the bed volume of the column was pumped through before re-use and the gel was discarded after at most six separations had been performed and replaced with unused gel.

APPENDIX IV

ESTIMATION OF TOTAL PROTEIN AND ALBUMIN LEVELS USING SPECTROMETRIC METHODS

IV.1 Measurement of Protein Content by Absorption of Light at 280 nm

IV.2 Measurement of Albumin Concentration in Methyl Orange Reagent

A Pye-Unicam SP500 spectrophotometer was used for all measurements

IV.1 Measurement of Protein Content by Absorption of Light at 280 nm

0.1 ml of the sample to be measured was diluted with Tris-HCl buffer in a 1 cm x 1 cm x 5 cm quartz cuvette. A similar cuvette containing 2.1 ml of the buffer was used as a reference. The absorbance of the reference solution was adjusted to read zero at a wavelength of 280 nm and the absorbance of the samples measured as the increase in absorbance above this level.

IV.2 Measurement of Albumin Concentration in Methyl Orange Reagent

A stock solution was made up from 1 l 0.055M Citrate buffer, pH 3.5 and 0.1% methyl orange solution in water. Using each solution in the temperature range 24° - 29°C, the methyl orange solution was added to the buffer in 5 ml aliquots and stirred. The spectrometer was set to read zero for distilled water and after each addition the absorbance of 2.1 ml of the resultant solution was compared with that of the same volume of water at 540 nm. When the absorbance of the solution was in the range 0.79 - 0.82 the solution was ready for use and was stored at 4°C until required.

The albumin content of the samples was measured by adding 0.1 ml of the sample to 2 ml methyl orange / buffer solution at approximately 27°C in a

TYPICAL EXAMPLES OF ALBUMIN STANDARD CURVES

Absorbance = Absorbance of 0.1 ml standard solution in 2 ml methyl orange reagent

(i) Purified human serum albumin dissolved in Tris-HCl buffer

Albumin Concentration (g / 100 ml)	Absorbance at 540 nm
4.0	0.15
2.0	0.23
1.0	0.36
0.5	0.48
0.25	0.59

(ii) Normal plasma diluted using Tris-HCl buffer

Albumin Concentration (g / 100 ml)	Absorbance at 540 nm
5.10	0.090
2.55	0.125
1.02	0.205
0.25	0.470
0.06	0.630

1 cm x 1 cm x 5 cm quartz cuvette and the absorbance measured. Distilled water in a similar cuvette was used to define the reference zero point. Standard curves were derived using the absorbance of known amounts of human albumin (Behringwerke, Ltd.) dissolved in Tris-HCl buffer and of serum of known albumin concentration diluted with buffer. The two curves were found to be slightly different due to the presence of other substances with a slight affinity for methyl orange and of bilirubin in serum. However it was found that both sets of results gave a good fit to an exponential curve of the form:-

$$C = Ae^{bx}$$

where A, b = constants
C = concentration of albumin
x = absorbance at 540 nm

The standard curve was derived using a least squares fit to an exponential function and the albumin concentration of the sample estimated using the calculated values of A and b and the measured value of x.

APPENDIX V

METHOD FOR MEASUREMENT OF PROTEIN CONCENTRATION BY RADIAL IMMUNODIFFUSION

V.1 Preparation of Plates

V.2 Measurement of Protein Concentration

V.1 Preparation of Plates

A stock gel preparation was made up as follows:- 2 g Ionagar No. 2 was added to 100 ml sodium barbitone / hydrochloric acid buffer, pH 8.6, containing 0.1% thiomersal. The solution was heated in a water bath at 100°C to dissolve the agar and the gel allowed to solidify at room temperature and stored in 10 ml quantities at 4°C. When required the gel was melted and allowed to cool to 50°C. The required quantity of antiserum (see Table V below) was warmed in a sterile bijou to 48°C in a water bath. 2.5 ml agar solution was transferred to the bijou and, after thorough mixing, the agar / antiserum mixture was poured onto 5 cm x 5 cm glass slides and allowed to solidify at room temparature. 2 mm diameter wells were then punched out in the gel and the agar in the wells removed by suction. The optimum spacing of the wells depended on both the concentration of the protein in the sample and on the the protein concerned. This spacing was determined empirically. It was found necessary to dilute serum or plasma samples to a quarter of the normal strength for measurement of albumin or immunoglobulin-G, though undiluted samples could be used for all the other proteins. It was not found to be necessary to dilute any of the column fractions.

V.2 Measurement of Protein Concentration

The wells were filled with 2 μl of the sample

using an Oxford Ultramicro-pipetting system and disposable plastic tips. Four standards made up of dilutions of serum or plasma were included in each plate, at concentrations of 100%, 50%, 25% and 12.5%. The absolute value of the protein concentration was found when required by including a 2 μl sample of standard serum (Behringwerke, Ltd.) containing known amounts of the proteins. The plates were then placed in a moist atmosphere at 37°C and diffusion allowed to take place for from 16 - 48 hours. After the required time the slides were placed in 1% tannic acid for about 15 minutes to make the precipitin rings visible. The diameter of the rings was measured using a lens incorporating a millimetre scale. From the measured diameters and the known concentrations of the standards the equation of the standard curve was computed using a least squares fit to an exponential function of the diameter of the rings, i.e an equation of the form:-

$$C = Ae^{bd}$$

where A, b are constants

C = protein concentration

d = diameter

Using the computed values of A and b and the measured values of d the concentration of protein in the samples was found.

Table V gives the amounts of antiserum used for measurement of the various proteins.

TABLE V

Antiserum	Amount added to 2.5 ml agar (ml)
Anti-albumin	0.025
Anti-Immunoglobulin-G	0.05
Anti-Haptoglobin	0.10
Anti-α_2-macroglobulin	0.05
Anti-Caeruloplasmin	0.10
Anti-Transferrin	0.05
Anti-Immunoglobulin-M	0.10

APPENDIX VI

ESTIMATION OF STABLE ZINC LEVELS USING ATOMIC ABSORPTION SPECTROMETRY

All measurements were made using a Perkin-Elmer atomic absorption spectrometer Model 403 with a Boling triple slot burner. The fuel used was acetylene, with air as oxidant. A non-luminous acetylene flame was used and zinc levels were determined at a wavelength of 213.8 nm. Instrument adjustments were made according to the instruction manual.

All glassware was soaked in 5N nitric acid and rinsed in deionised double-distilled water before use.

A zinc reference standard solution * containing 1 mg zinc per ml was diluted with deionised double-distilled water to give standard solutions containing 0.25, 0.50, 1.00, 2.00, 2.50 μg zinc / ml. Both standard and protein sample solutions were diluted one in five with 10% analytical grade Propan-1-ol. The instrument was set to read zero for the Propan-1-ol diluent and samples and standard solutions were aspirated directly into the spectrometer. A calibration curve was then calculated using the standard solutions and the zinc content of the samples found using this curve.

It was found that the error in the method was ± 2 μg / 100 ml.

All stable zinc measurements were carried out in the Department of Biochemistry, Glasgow Royal Infirmary.

* British Drug Houses laboratory reagents products No. 14040

APPENDIX VII

CALCULATION OF THE TRANSFER FUNCTION FOR THE PASSAGE OF ZINC THROUGH THE GUT WALL

If the disappearance with time from plasma of a bolus of tracer injected intravenously and the uptake and clearance by plasma of an amount of the same tracer administered orally are known, then it is possible to derive the transit function which describes the passage of the tracer from the gut into plasma.

If

$c(t)$ = the amount of tracer in plasma at time t from an amount given orally at time $t = 0$

$g(T)$ = the amount of tracer remaining in plasma at time T from unit amount injected at time $T = 0$

$f(t)$ = the fraction of the tracer dose passing through the gastro-intestinal tract at time t.

Of the amount $f(t - T).\Delta T$ absorbed into plasma in the short time interval $t - T$ to $t - T + \Delta T$ an amount $g(t).f(t - T).\Delta T$ remains at time t. Then the amount of tracer absorbed into plasma at time t is the sum of these amounts.

Therefore as $T \rightarrow 0$,

$$c(t) = \int_0^t g(T).f(t - T)dT$$

Since the values but not the equations are known for $c(t)$ and $g(t)$ an approximate solution for $f(t)$ can be obtained by expanding $c(t)$ in terms of a small finite time interval, ΔT.

$$\therefore\ c(t) = c(n\Delta t) = g(0).f(n\Delta T).\Delta T + g(\Delta T).f\left[(n-1)\Delta T\right].\Delta T$$
$$+ \ldots + g(i-1)\Delta T.f\left[(n-i+1)\Delta T\right].\Delta T + \ldots$$

Hence for $n = 1$,

$$c(\Delta T) = g(0).f(\Delta T) = f(\Delta T).\Delta T \text{ since } g(0) = 1$$

$$\therefore\ f(\Delta T) = 1/\Delta T.c(\Delta T)$$

for $n = 2$,

$$c(2\Delta T) = f(2\Delta T).\Delta T + g(\Delta T).f(\Delta T).\Delta T$$
$$= f(2\Delta T).\Delta T + g(\Delta T).c(\Delta T)$$

$$\therefore\ f(2\Delta T) = 1/\Delta T.c(2\Delta T) - g(\Delta T).c(\Delta T)$$

and similarly for $n = 3$ . . . . . .

Hence by substituting the known numerical values for $c(t)$ . . . . $c(n\Delta T)$ and $g(\Delta T)$ . . . $g(n\Delta T)$ the values of $f(t)$ at times T, $2\Delta T$ . . . $n\Delta T$ can be calculated.

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TABLES

TABLES 1 - 7

Numbered consecutively as
mentioned in the text

TABLE 1

Comparison of the mean ratios of albumin- to α_2-globulin-bound zinc-65 on addition of 10 μg and 25 μg zinc / 100 ml plasma, from 14 sample separations in each case

Amount of zinc added (μg/100 ml)	Mean Ratio	Standard deviation of mean	Probability p
10	2.25	0.41	0.63
25	2.32	0.39	

p = probability that the distributions are the same, using the Student-t test

TABLE 2

Comparison of the mean ratios of albumin- to α_2-globulin-bound zinc-65 activity, obtained on addition of 0.5 µg, 10 µg, 100 µg and 1000 µg zinc / 100 ml plasma to aliquots of the same plasma sample, separated under the same experimental conditions and from 12 separations in each case

Amount of zinc added µg / 100 ml	Mean Ratio	Standard deviation of mean	Probability p
0.5	1.31	0.25	8×10^{-4}
10	1.88	0.44	
10	1.88	0.44	8×10^{-2}
100	2.16	0.28	
100	2.16	0.28	5×10^{-2}
1000	2.43	0.34	
10	1.88	0.44	2×10^{-3}
1000	2.43	0.34	

p = probability that the distributions of the results in the two groups are the same, using the Student-t test.

TABLE 3

Comparison of the mean ratios of albumin-to α_2-globulin-bound zinc-65 activity obtained on addition of 20 μg zinc / 100 ml to plasma samples from five normal subjects

Subject (Sex)	Number of C.A. Strips	Mean Ratio	Standard Deviation of Mean	p
1(M)	6	3.53	0.39	0.44
2(M)	6	3.31	0.55	
3(M)	4	2.96	0.45	0.57
4(M)	5	3.12	0.33	
5(F)	8	4.04	0.78	0.34
1(M)	8	4.46	0.92	

p = probability that the distributions are the same, using the Student-t test.

TABLE 4

Comparison of the percentages of the total serum zinc level bound to α_2-macroglobulin and to albumin in samples from 6 normal subjects

Subject (Sex)	Serum Zinc Level (μg/100 ml)	Protein-bound Zinc (%)	
		α_2-macroglobulin	Albumin
1(M)	80	26	74
2(M)	78	34	66
3(F)	90	37	63
4(M)	84	46	54
5(M)	100	28	72
6(M)	76	19	81

(Separation of 2 ml samples into 2.4 ml fractions using a small column of Sephadex G75)

TABLE 5

Comparison of the percentages of the total plasma zinc level bound to α_2-macroglobulin and to albumin in samples from 6 normal subjects

Subject (Sex)	Plasma Zinc Level (µg/100 ml)	Protein-bound Zinc (%)	
		α_2-macroglobulin	Albumin
C(F)	88	32	68
E(F)	86	31	69
K(M)	96	26	74
R(M)	88	31	69
J(M)	124	25	75
D(M)	114	32	68

(Separation of 2 ml samples into 2.4 ml fractions using a small column of Sephadex G100)

TABLE 6

Comparison of the percentage of the total plasma zinc bound to albumin in plasma samples obtained pre-dose, 1½ hours after swallowing the zinc sulphate capsule and approximately 6 hours after taking the capsule

Subject	Albumin-bound Zinc (% total)		
	pre-dose	1½ hours	6 hours
C	68	76	53
E	69	73	—
K	74	72	69
R	69	75	69
J	75	71	49
D	68	63	—
	pre-dose	2 hours	
J.G.	58	69	
R.G.	55	66	

Subjects C,E,K,R,J and D were normal volunteers, J.G and R.G. patients suffering from psoriasis.

TABLE 7

α_2-macroglobulin concentrations in plasma from 6 normal subjects and 2 patients measured using radial immunodiffusion

Subject	α_2-macroglobulin Concentration (mg / 100 ml)
C	300
E	258
K	191
R	145
J	134
D	98
J.G.	278
R.G.	190

FIGURES

Fig. 1

Variation of zinc-65 activity, added to plasma *in vitro*, with distance migrated along a cellulose acetate strip, measured by counting 2 mm transverse sections

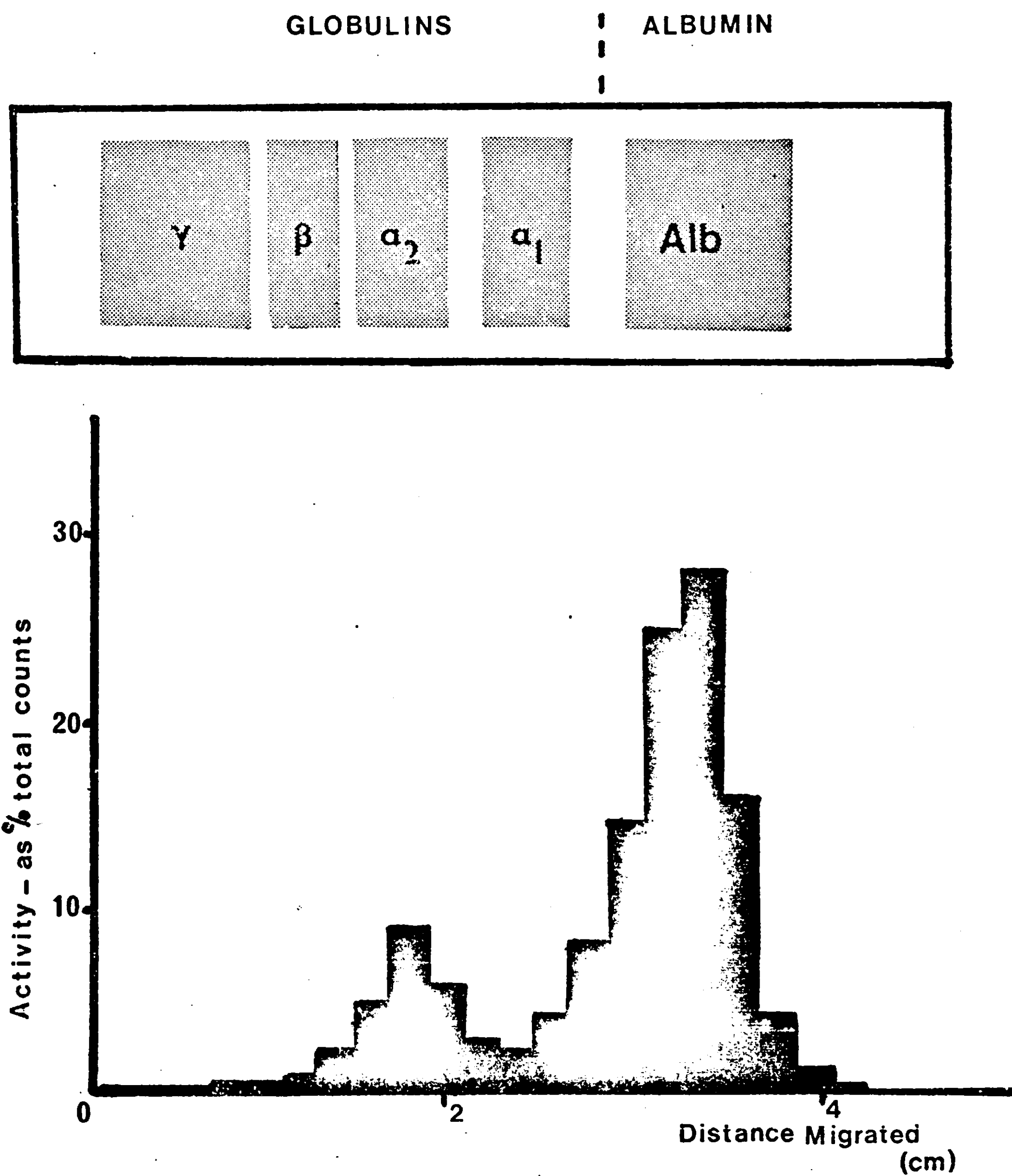


Fig. 2

Distributions of zinc-65 and iodine-131-labelled albumin, added in vitro to plasma and separated by electrophoresis on cellulose acetate strips.

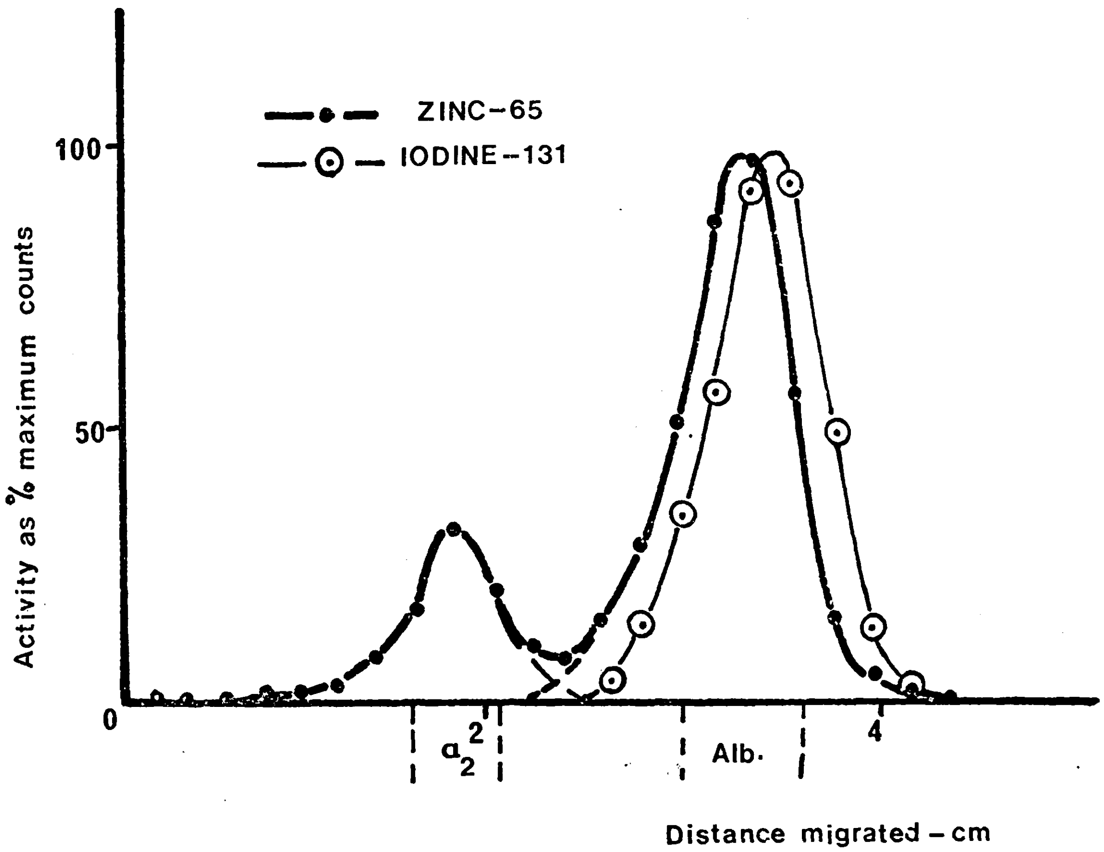


Fig. 3

Comparison of a stained cellulose acetate strip and an autoradiograph of a similar unstained strip from a plasma sample containing 20 μCi zinc-65 / ml, added *in vitro*.

Plasma sample stained using protein stain, showing all protein bands.

Autoradiograph of similar strip, showing zinc-65 associated with only the albumin and α_2-globulin bands.

Fig. 4

Comparison of the distributions obtained when 10 μCi zinc-65 and 5 μCi iron-59 were added to the same 1 ml sample of normal plasma.

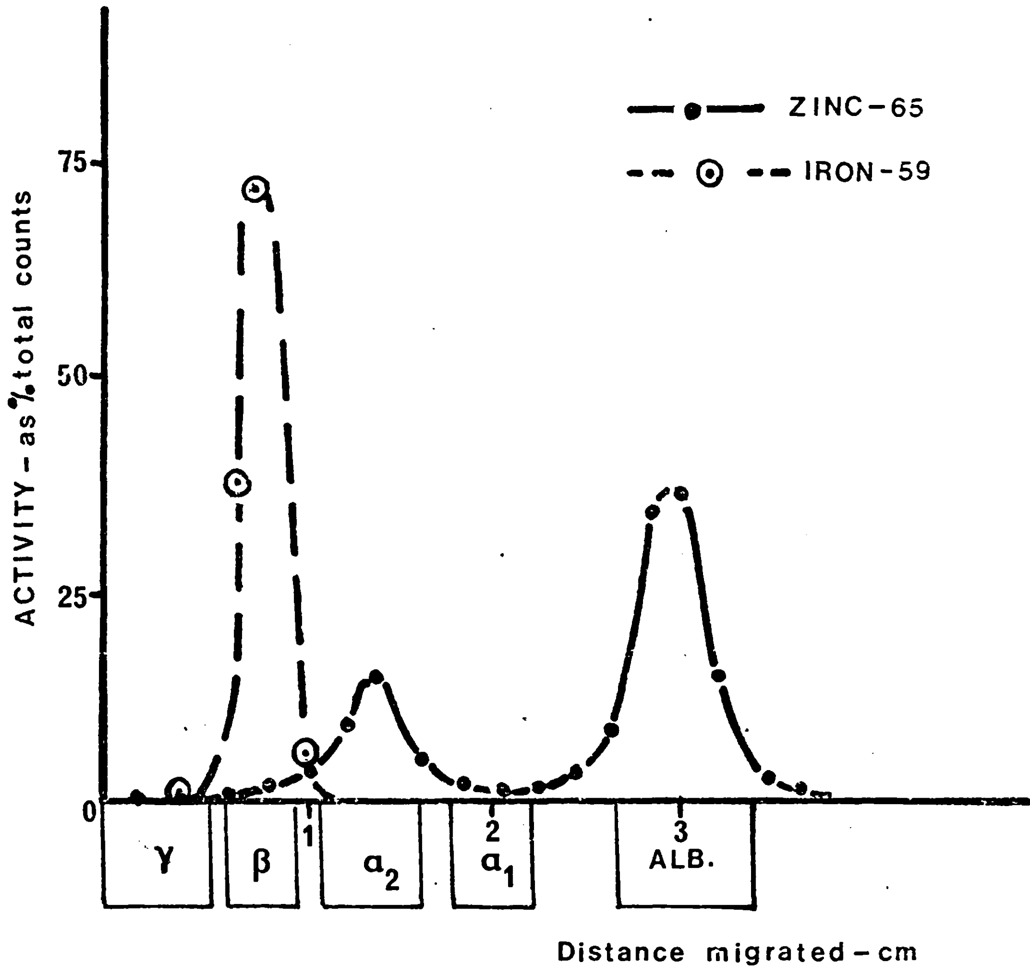


Fig. 5

Gain of activity with time of non-active serum on dialysis of serum samples containing trace amounts of zinc-65 and iron-59 against serum containing no radioactive material

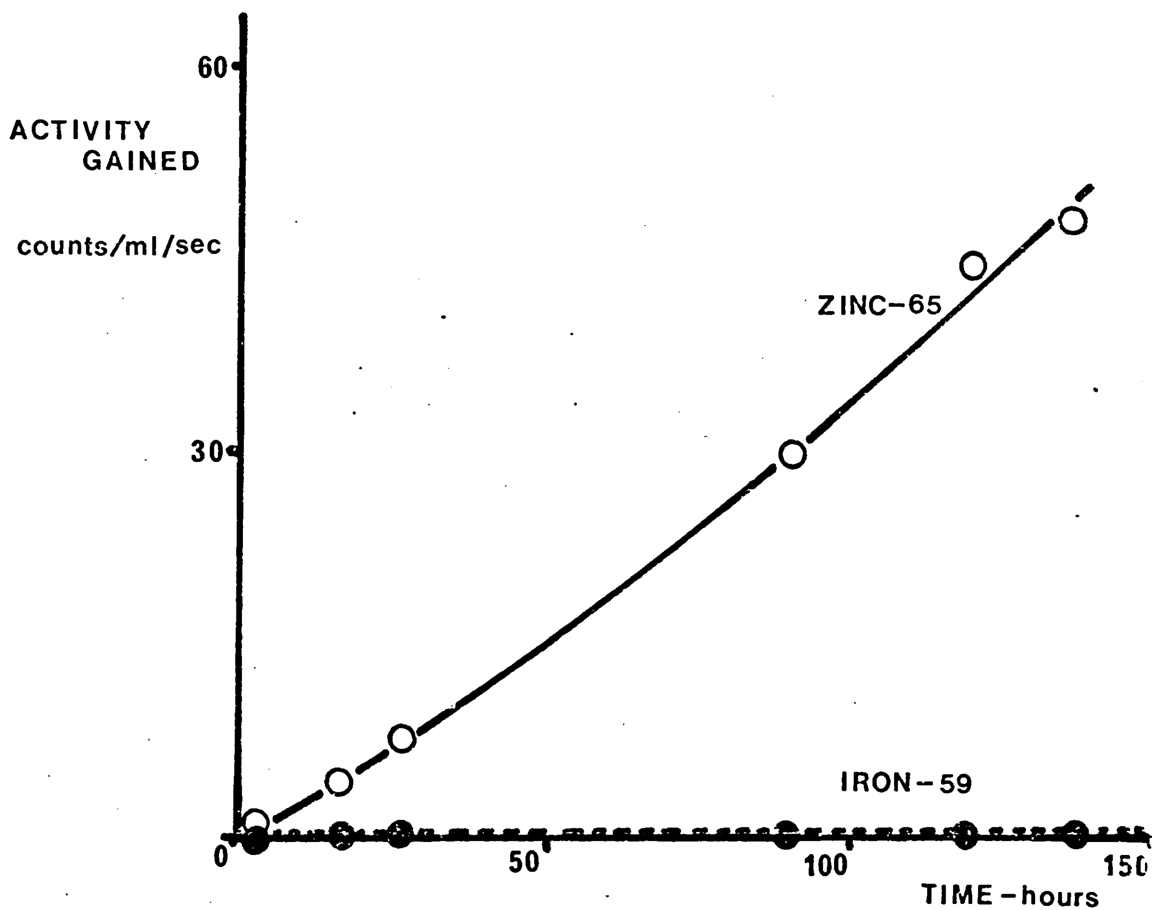


Fig. 6

Variation in absorbance at 280 nm among the protein fractions, obtained on separation of plasma samples of 3 ml, 5 ml and 10 ml using a column of Sephadex G200

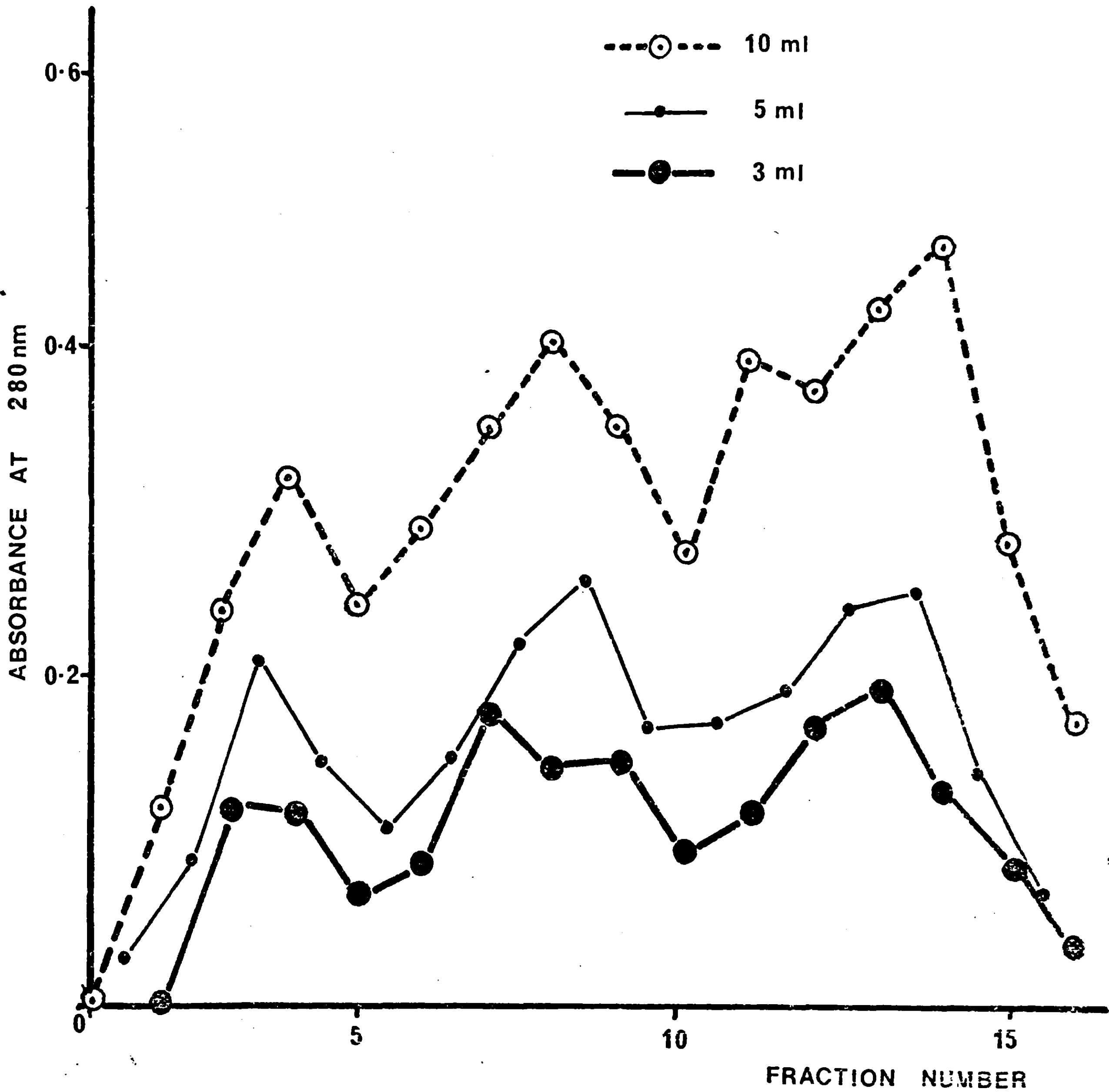
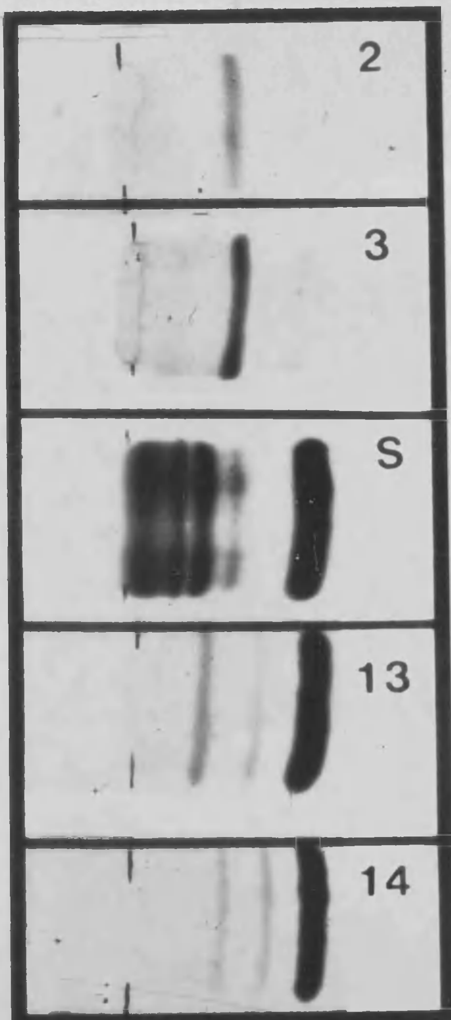
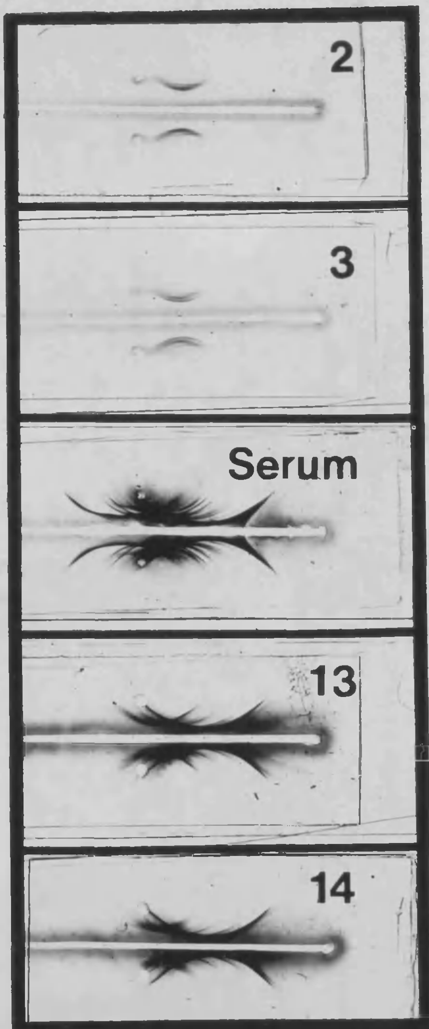


Fig. 7

Protein Content of Serum Fractions Containing Maximum α_2-macroglobulin and Albumin Concentrations

Separation using electrophoresis on cellulose acetate and immunoelectrophoresis of G200 column fractions 2 and 3, which contain maximum levels of α_2-macroglobulin and 13 and 14, which contain maximum levels of albumin, compared with serum in each case.





Electrophoresis on cellulose acetate

Immunoelectrophoresis

Fig. 8

Radial Immunodiffusion Calibration Curves

Graphs of concentration against diameter of the precipitin rings from a range of dilutions of albumin solutions, measured at times 17, 41 and 65 hours and showing the least squares exponential fits in each case.

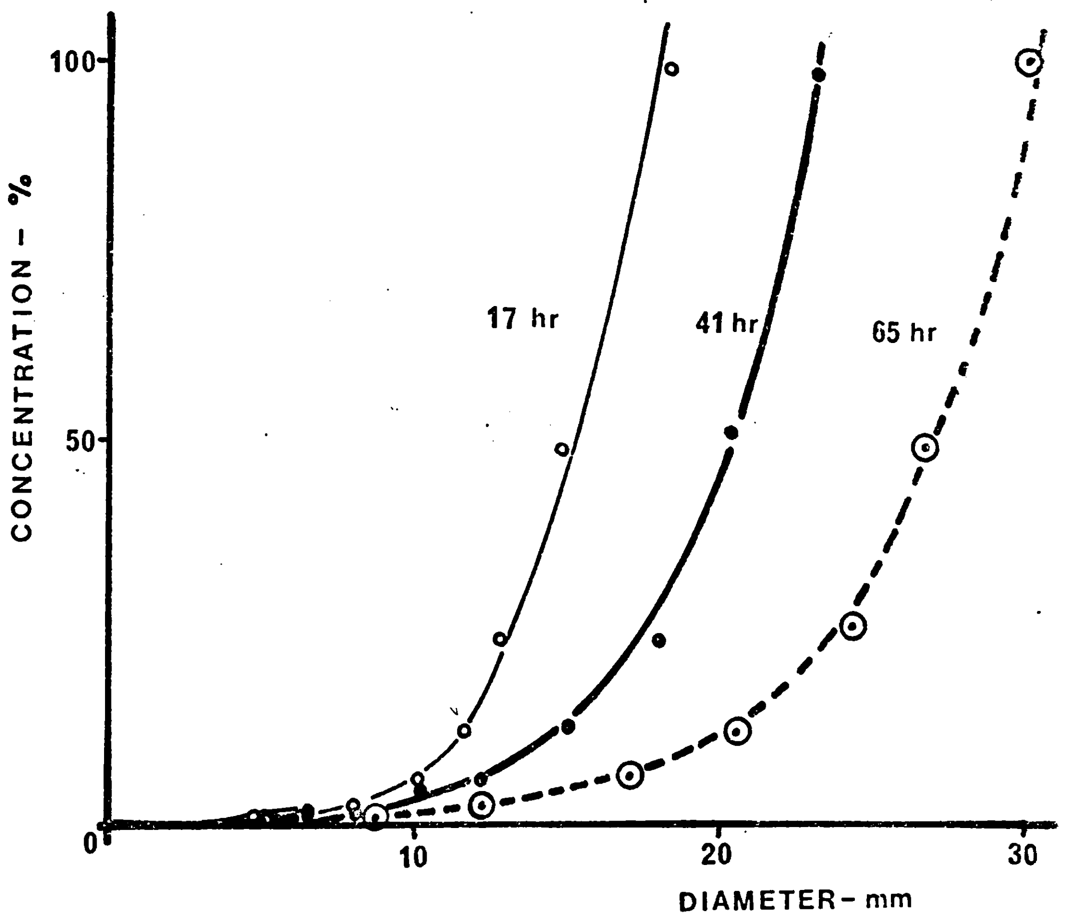
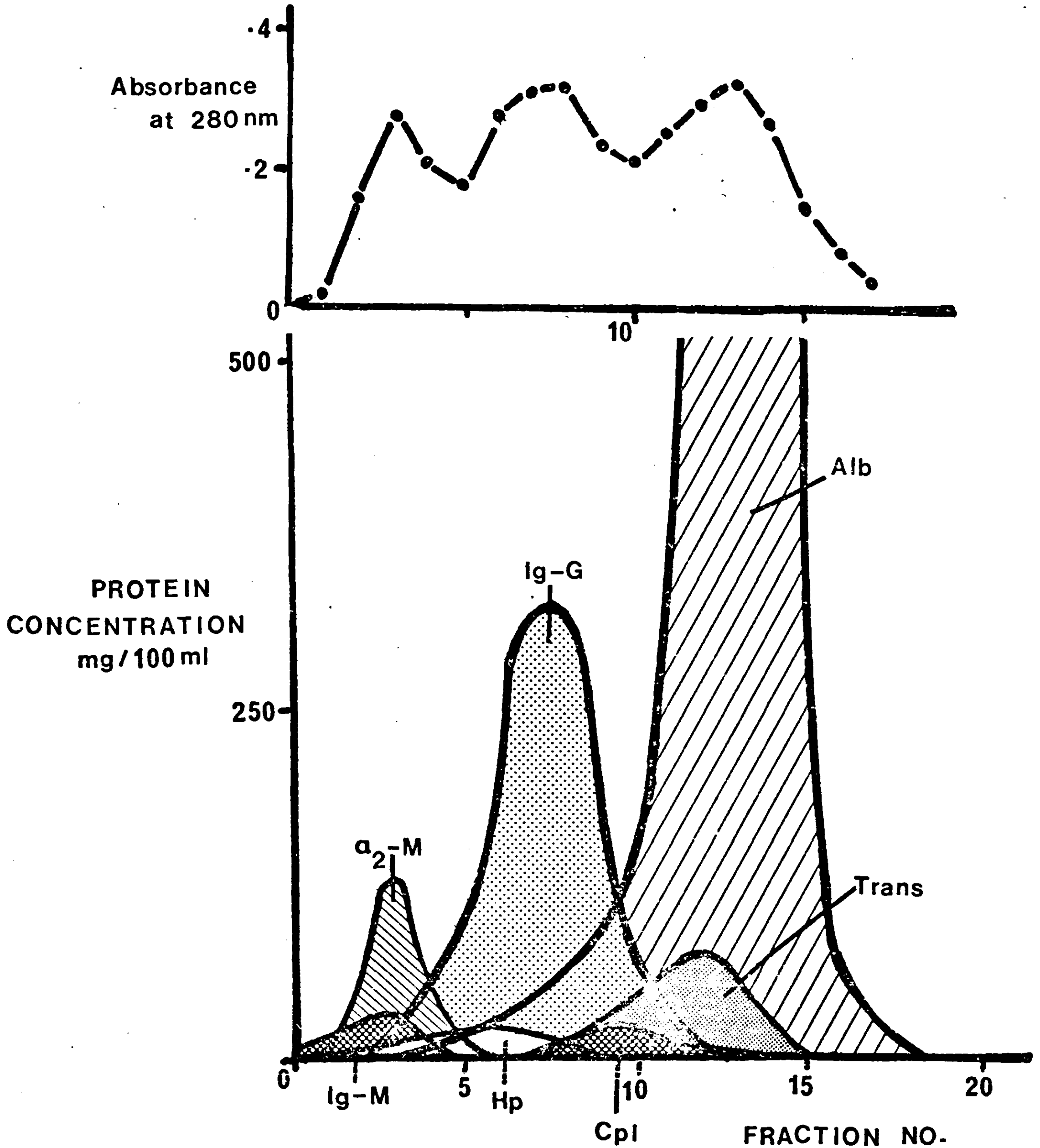


Fig. 9

Distribution of the serum proteins among the fractions obtained on separation of normal serum using a large column of Sephadex G200, measured using radial immunodiffusion



Ig-M - Immunoglobulin-M   Hp - Haptoglobin

α_2-M - α_2-macroglobulin   Ig-G - Immunoglobulin-G

Trans - Transferrin   Cpl - Caeruloplasmin

Alb - Albumin

Fig. 10

Comparison of the sharpness of the albumin distributions among the protein fractions obtained on separation of plasma samples of 10 ml, 5 ml and 2.5 ml using a large column of Sephadex G200

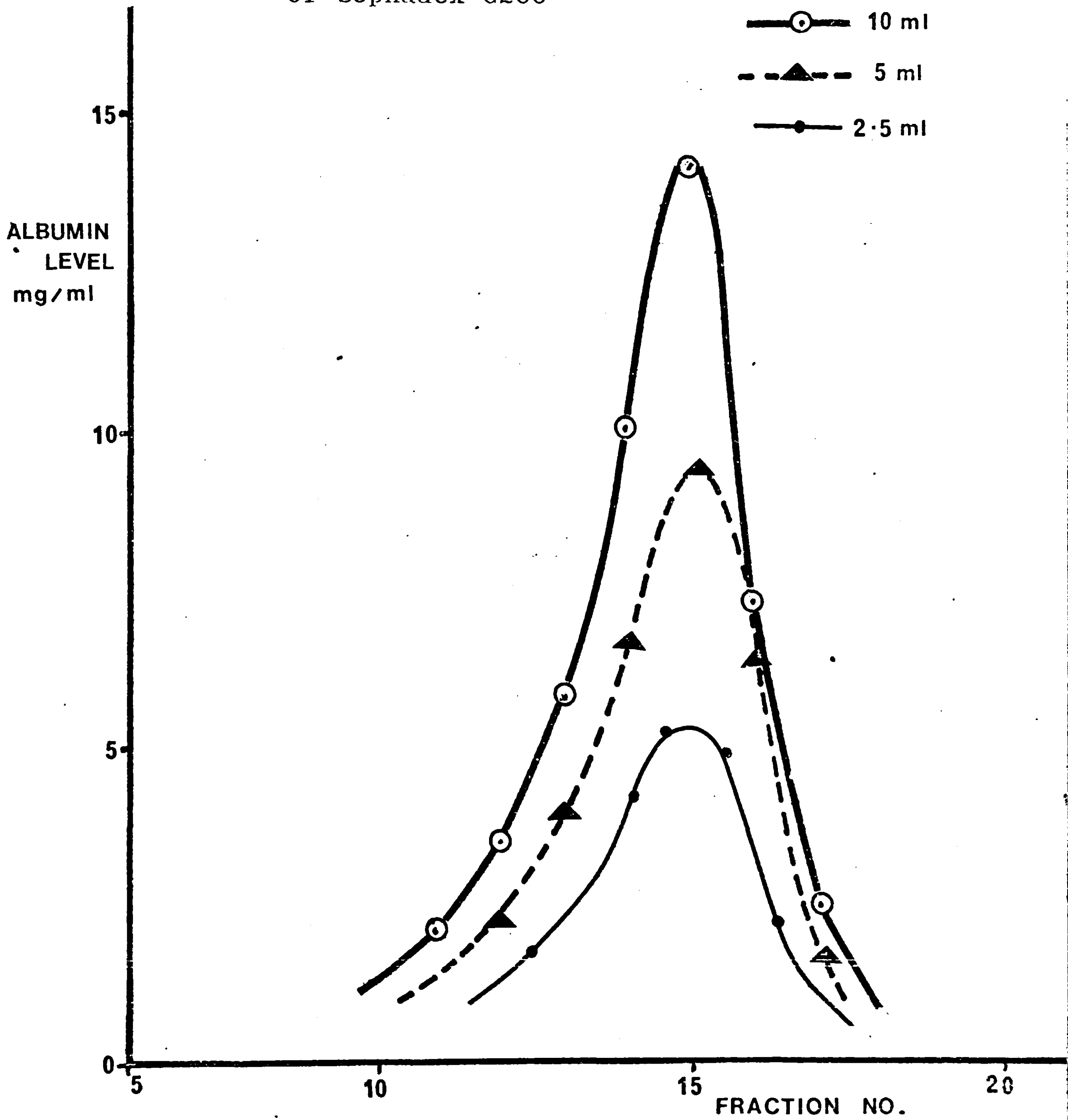


Fig. 11

Distribution of zinc-65 activity among the protein fractions, obtained on separation of 4 ml plasma containing a trace amount of zinc-65 added <u>in vitro</u>, together with a trace quantity of iodine-131-labelled albumin, into 6 ml fractions using Sephadex G200

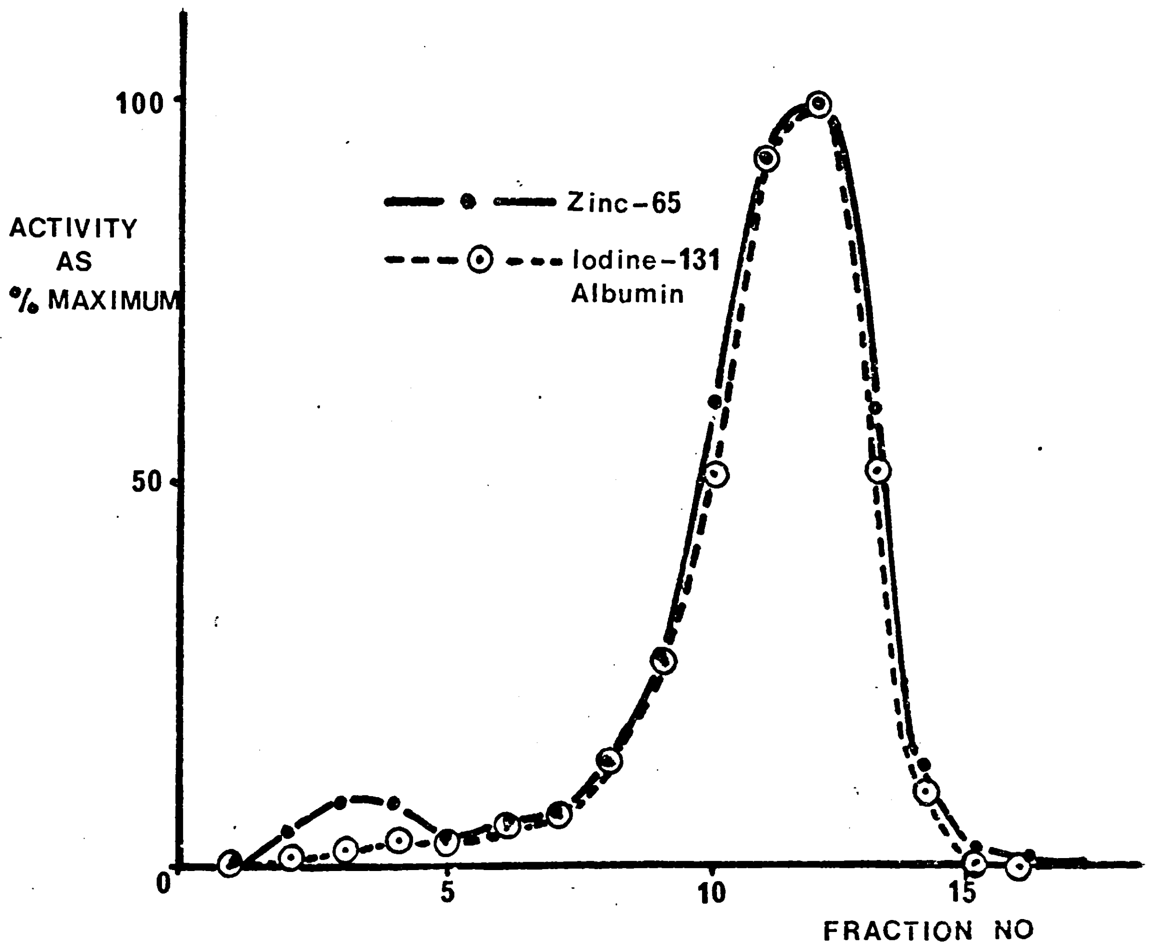
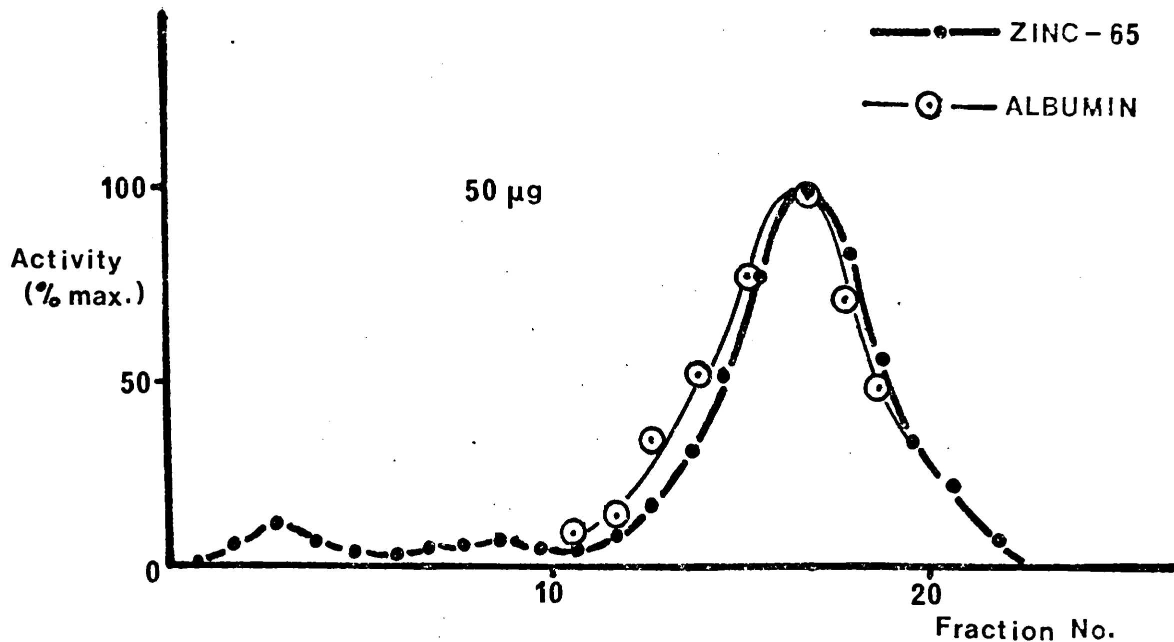
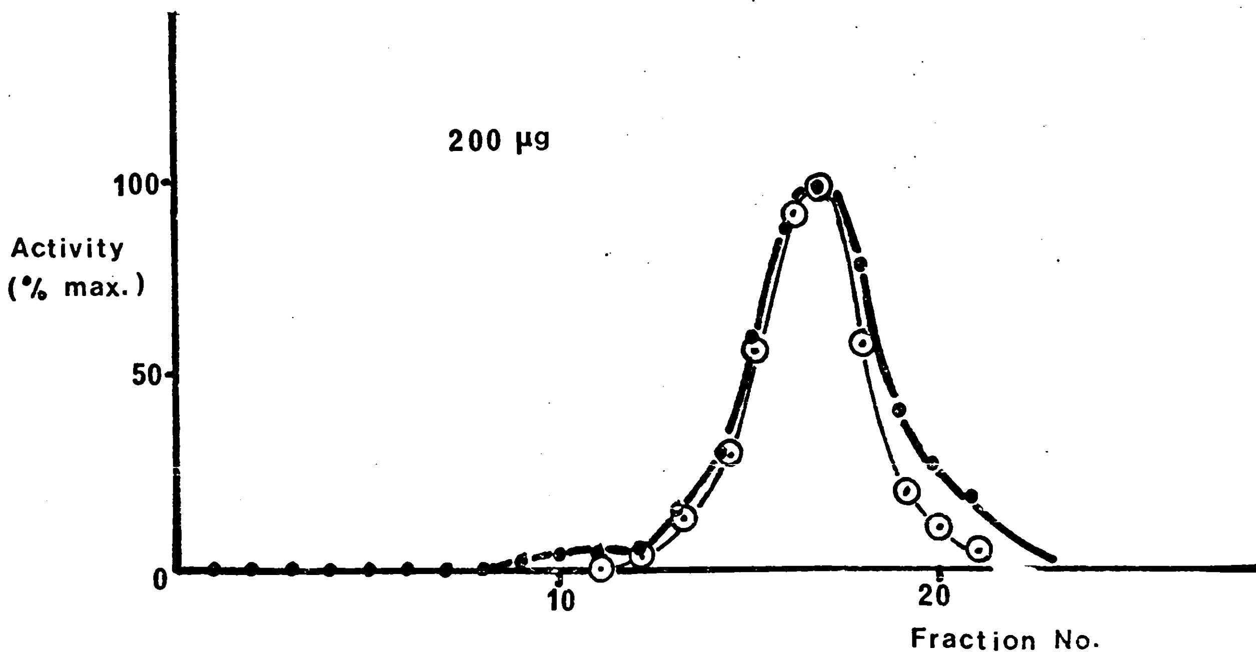


Fig. 12

Distributions of zinc-65 activity among the plasma protein fractions from separation of 2.5 ml plasma samples containing 50 μg and 200 μg added zinc / 100 ml





(Separation into 3.7 ml fractions using a 46 cm x 2.6 cm column of Sephadex G200)

Fig. 13

Distribution of zinc-65 activity among the α-globulins obtained after electrophoresis of plasma containing added zinc-65 and separated using Sephadex G200

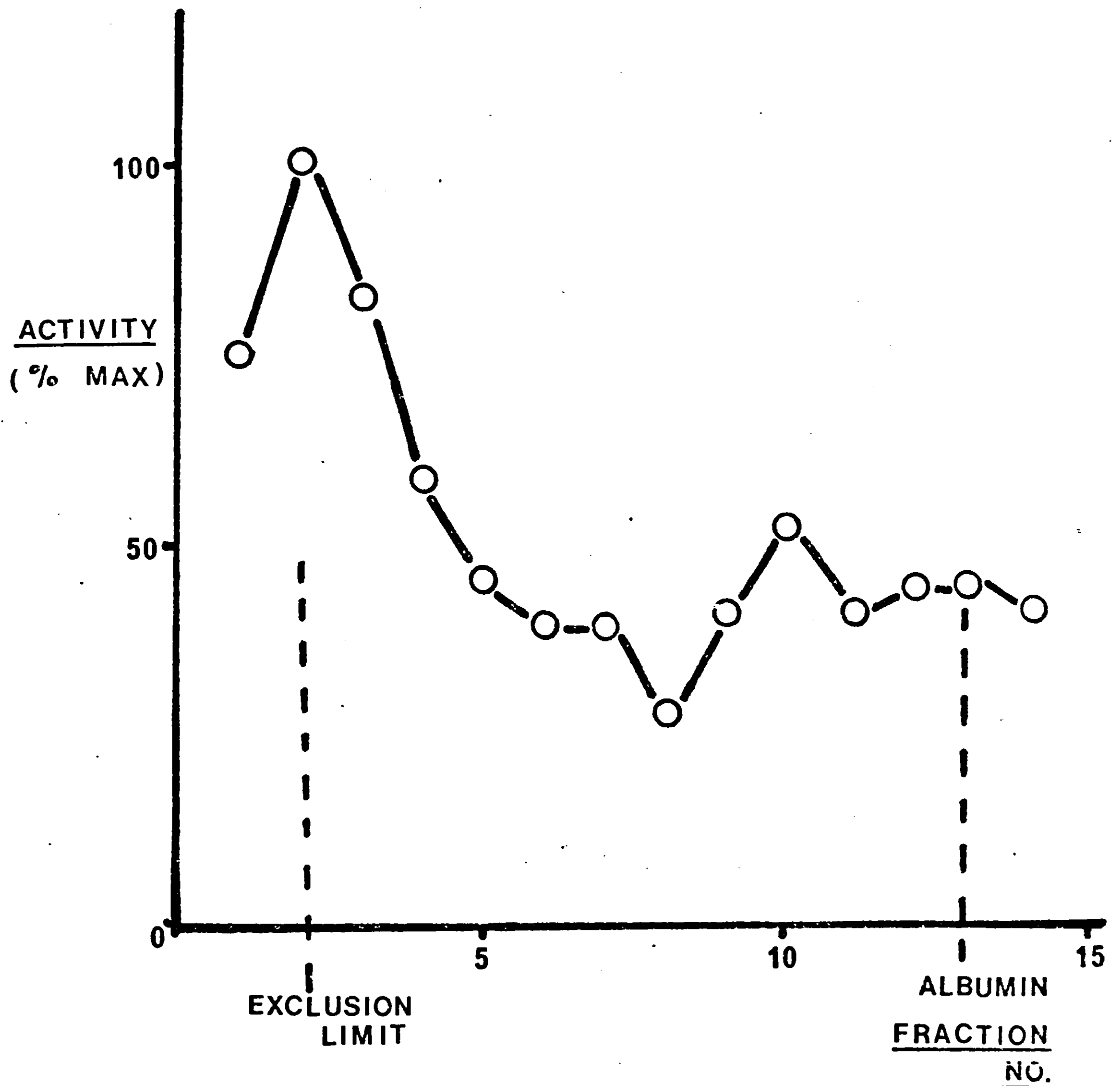
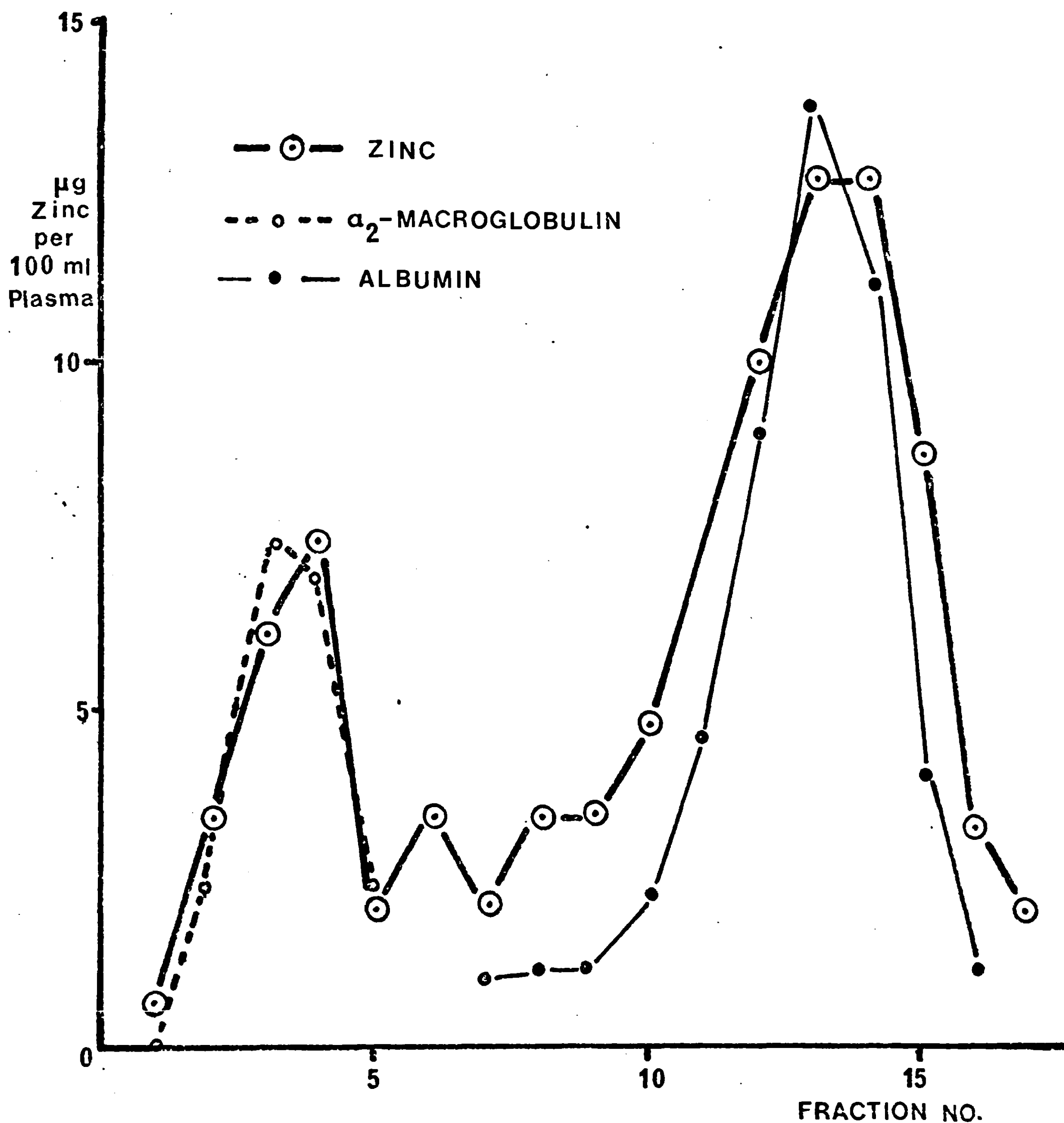


Fig. 14

Distributions of endogenous zinc, albumin and α_2-macroglobulin among the protein fractions obtained on separation of 6 ml normal plasma into 6 ml fractions using Sephadex G200



(The α_2-macroglobulin and albumin distributions are expressed as a fraction of the maximum value, adjusted to enable comparison of the distributions with the two zinc peaks to be made)

Fig. 15

Distribution of endogenous zinc among the serum proteins, together with the *in vitro* zinc-65 distribution, obtained on separation of 7.5 ml normal serum into 6 ml fractions using Sephadex G200

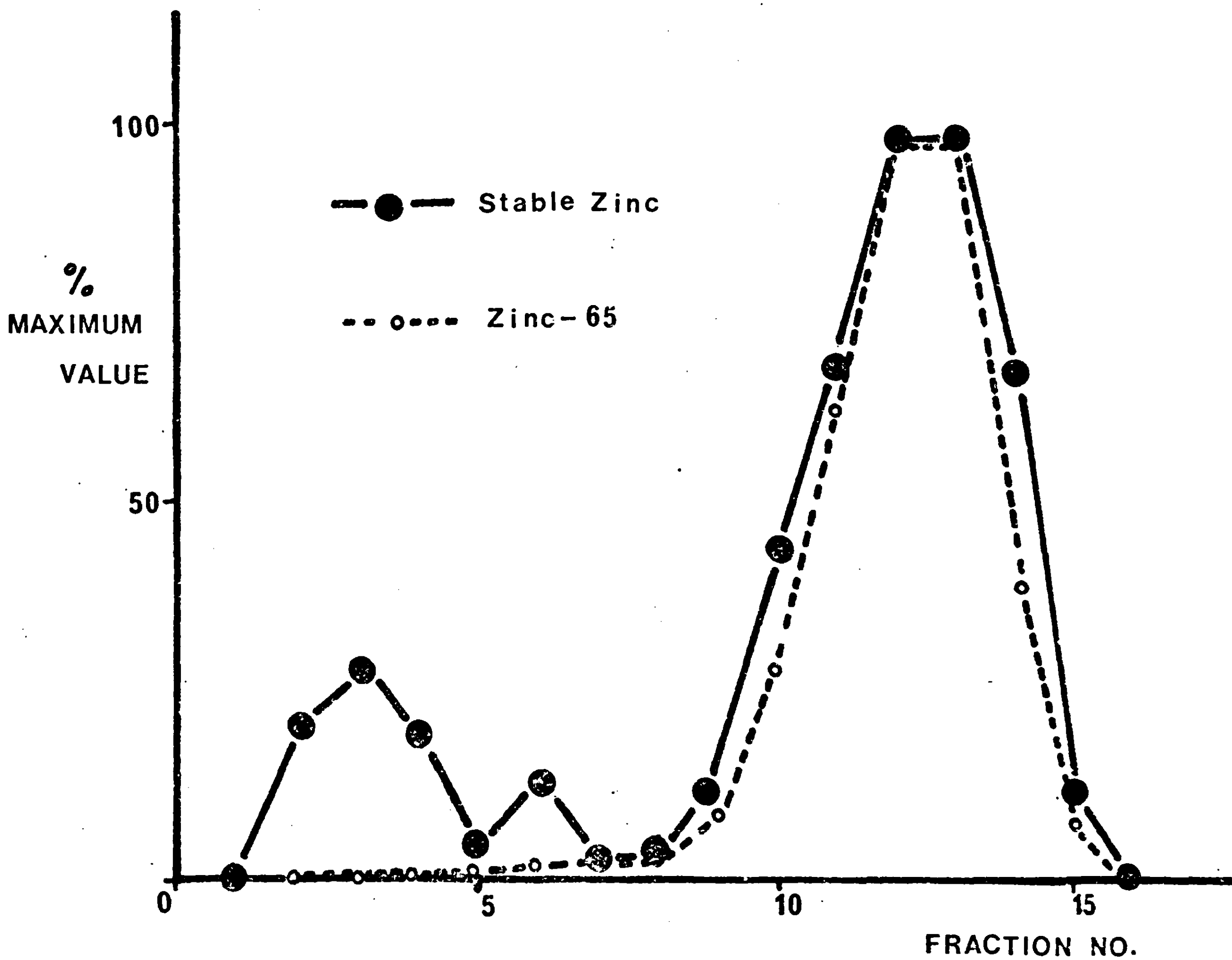


Fig. 16

Variation in the absorbance at 280 nm, the endogenous zinc level and the albumin level among the protein fractions from a 2 ml sample of normal serum separated into 2.4 ml fractions using Sephadex G75

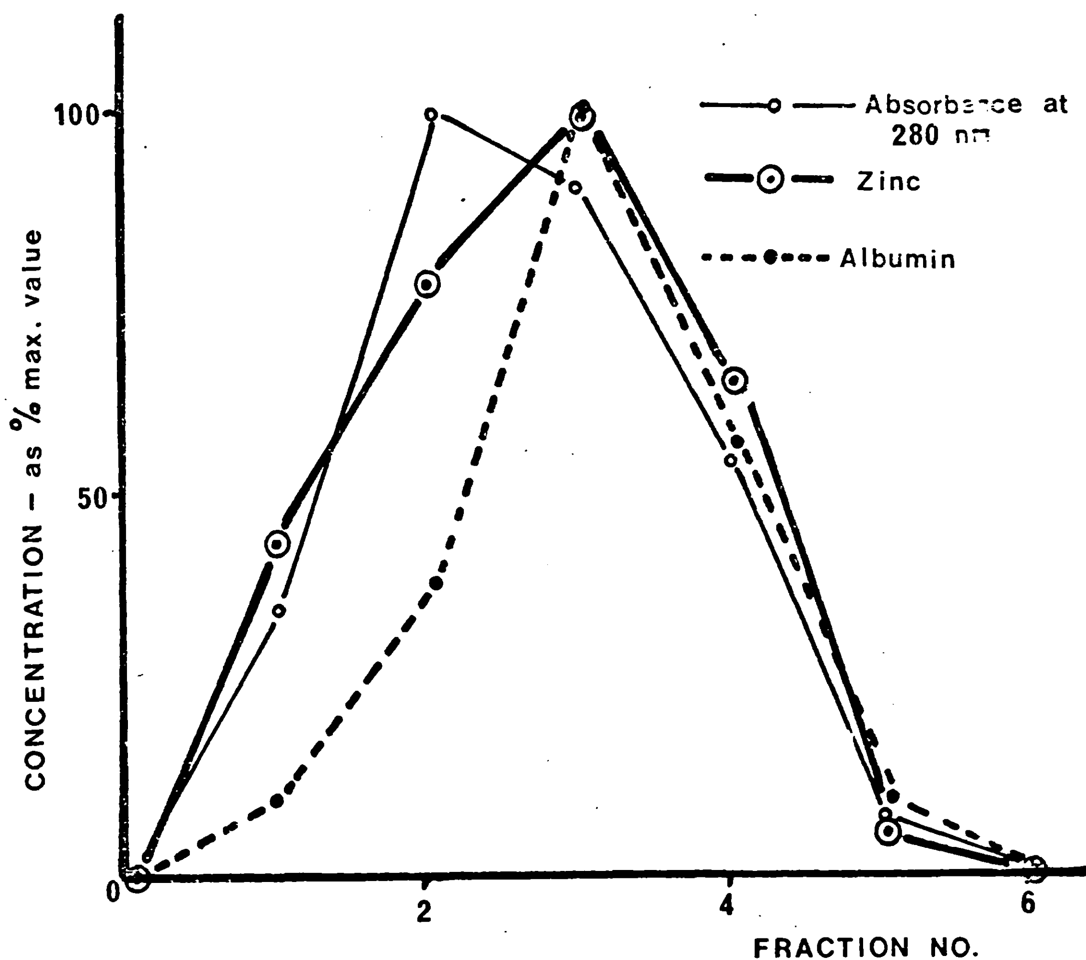


Fig. 17

Distributions of endogenous zinc, albumin and α_2-macroglobulin among the protein fractions from a 2 ml sample of pooled normal serum separated into 2.4 ml fractions using Sephadex G100

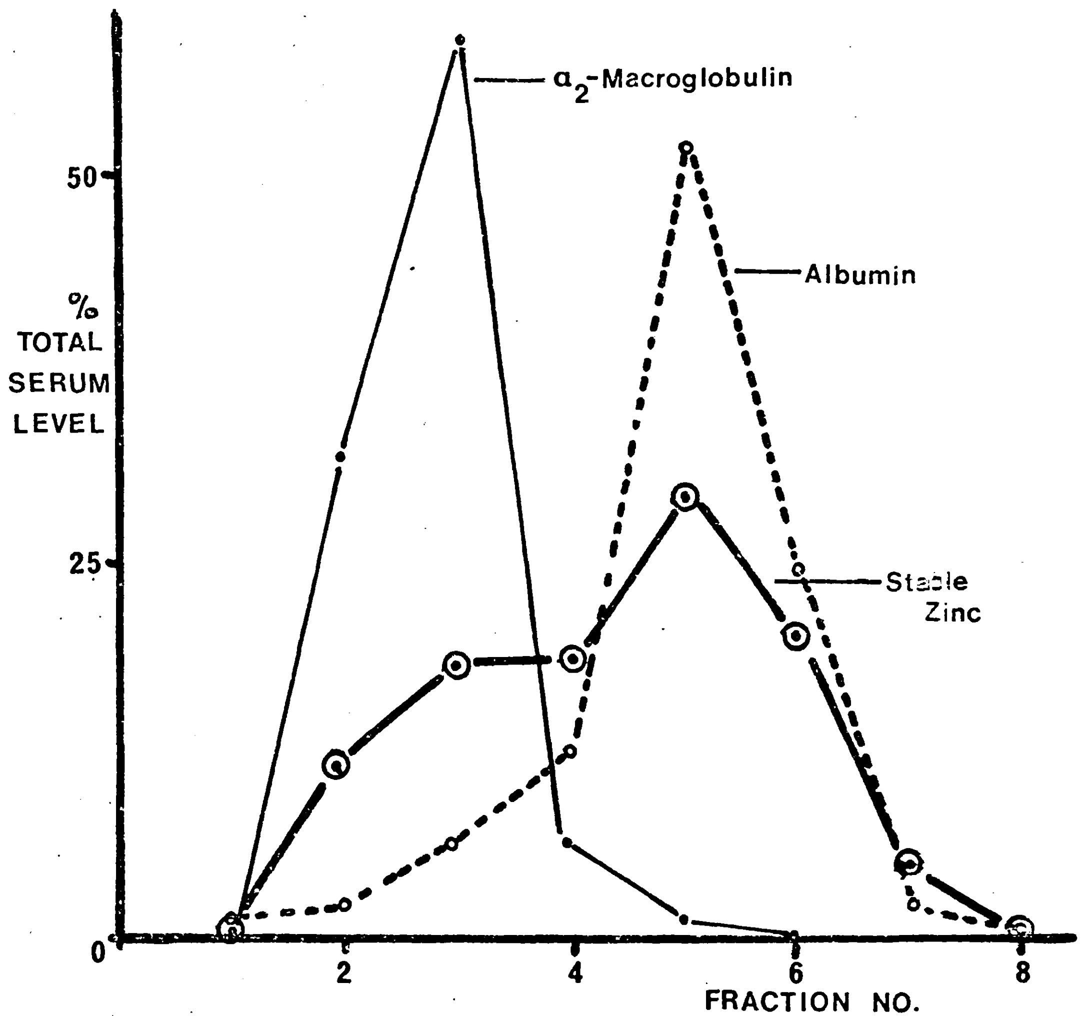
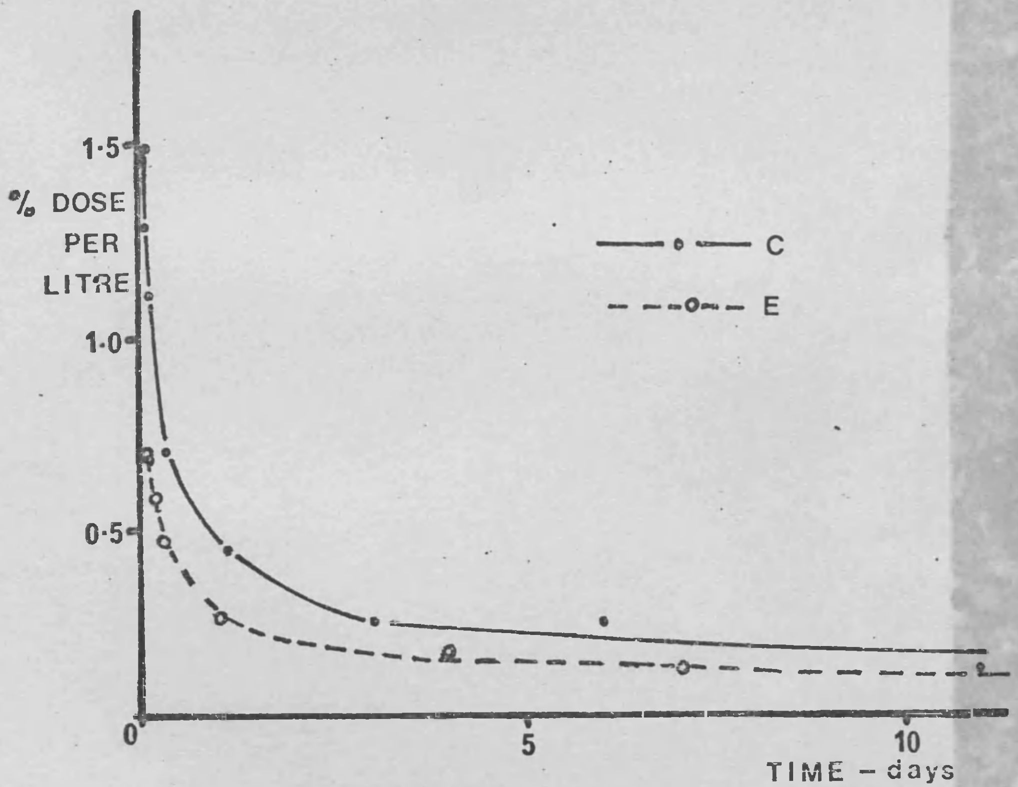


Fig. 18

Oral Plasma Zinc-65 Curve

Variation of plasma zinc-65 level with time for subjects C and E after an oral tracer dose of zinc-65 chloride



Percentage of Dose in 1 litre of

Plasma Bound to each Protein Fraction

2 hours

Fraction Number	% Dose / litre	Fraction Number	% Dose / litre
1	0.000	10	0.085
2	0.055	11	0.132
3	0.059	12	0.186
4	0.026	13	0.216
5	0.000	14	0.228
6	0.014	15	0.120
7	0.028	16	0.041
8	0.032	17	0.000
9	0.069		

25 hours

Fraction Number	% Dose / litre
2 - 4	0.101
5 - 7	0.047
8 - 10	0.095
11 - 13	0.179
14 - 15	0.028

(6 ml fractions in each case)

Distribution of plasma zinc-65 among the proteins in samples obtained from subject C 2 hours and 25 hours after ingestion of a tracer dose

Fig. 19

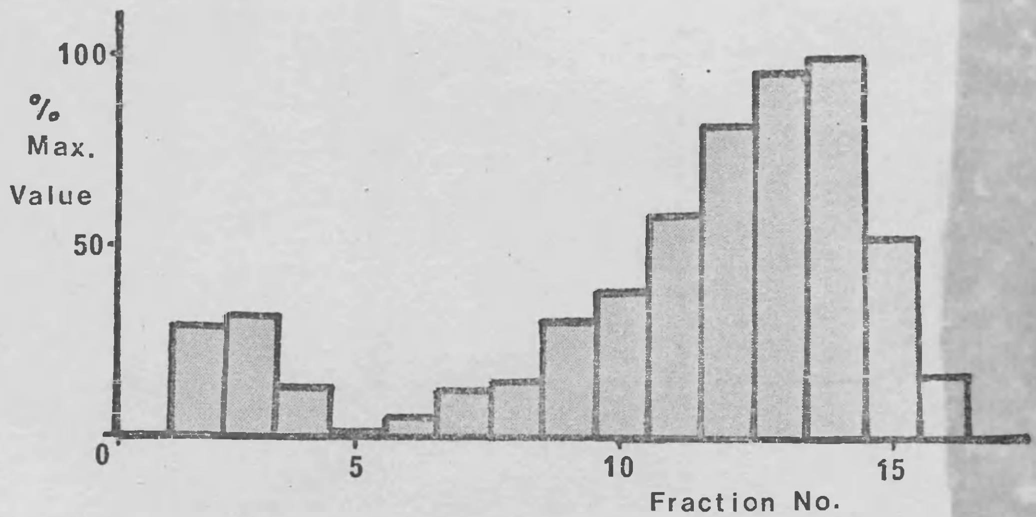
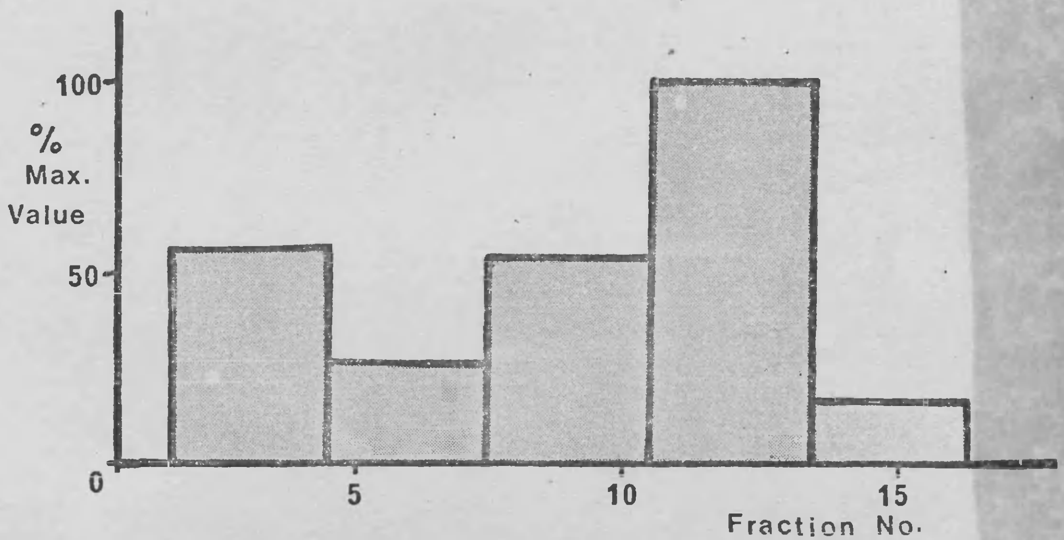


Fig. 20



Percentage of Dose in 1 litre of

Plasma Bound to each Protein Fraction

1.5 hours (6 ml fractions)

Fraction Number	% Dose / litre	Fraction Number	% Dose / litre
1	0.000	10	0.053
2	0.017	11	0.065
3	0.027	12	0.099
4	0.034	13	0.111
5	0.009	14	0.077
6	0.019	15	0.048
7	0.025	16	0.011
8	0.032	17	0.000
9	0.033		

4.4 hours (9 ml fractions)

Fraction Number	% Dose / litre	Fraction Number	% Dose / litre
1	0.032	7	0.077
2	0.051	8	0.098
3	0.010	9	0.133
4	0.014	10	0.082
5	0.026	11	0.004
6	0.049	12	0.000

Distribution of plasma zinc-65 among the protein fractions in samples obtained from subject E 1.5 hours and 4.4 hours after ingestion of a tracer dose

Fig. 21

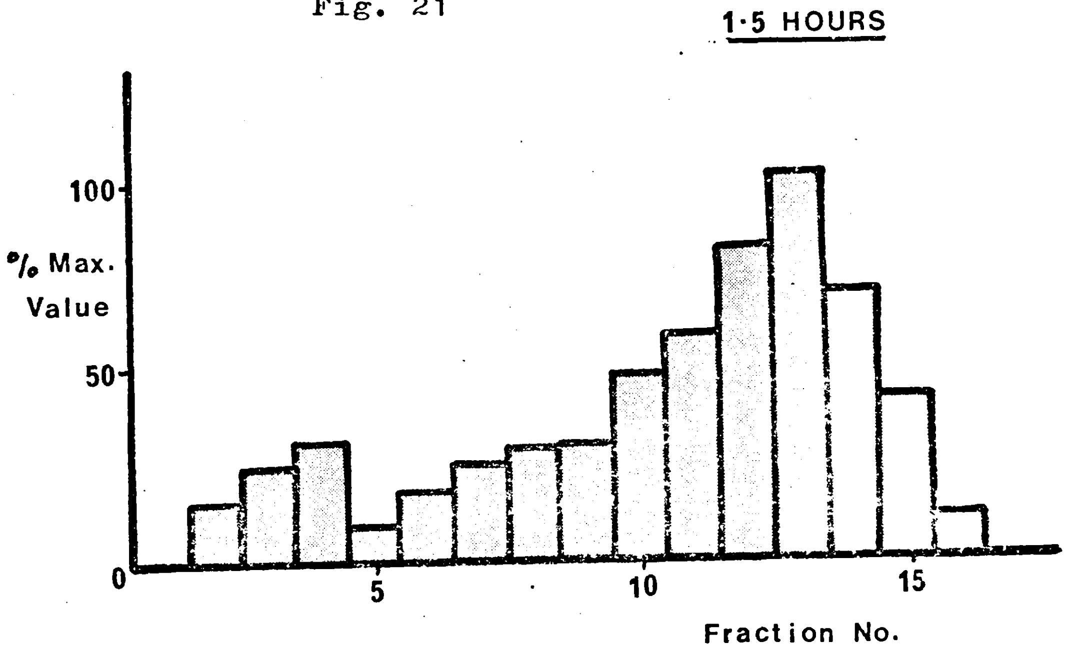


Fig. 22

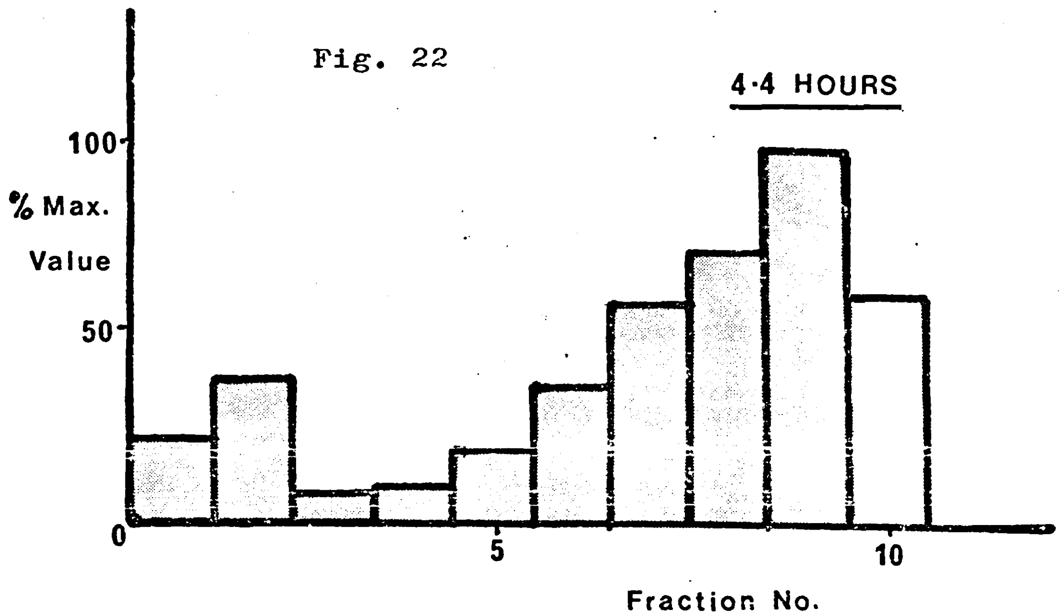


Fig. 23

Comparison of the distributions of endogenous zinc and plasma zinc-65 4 hours after an oral dose among the protein fractions for subject C

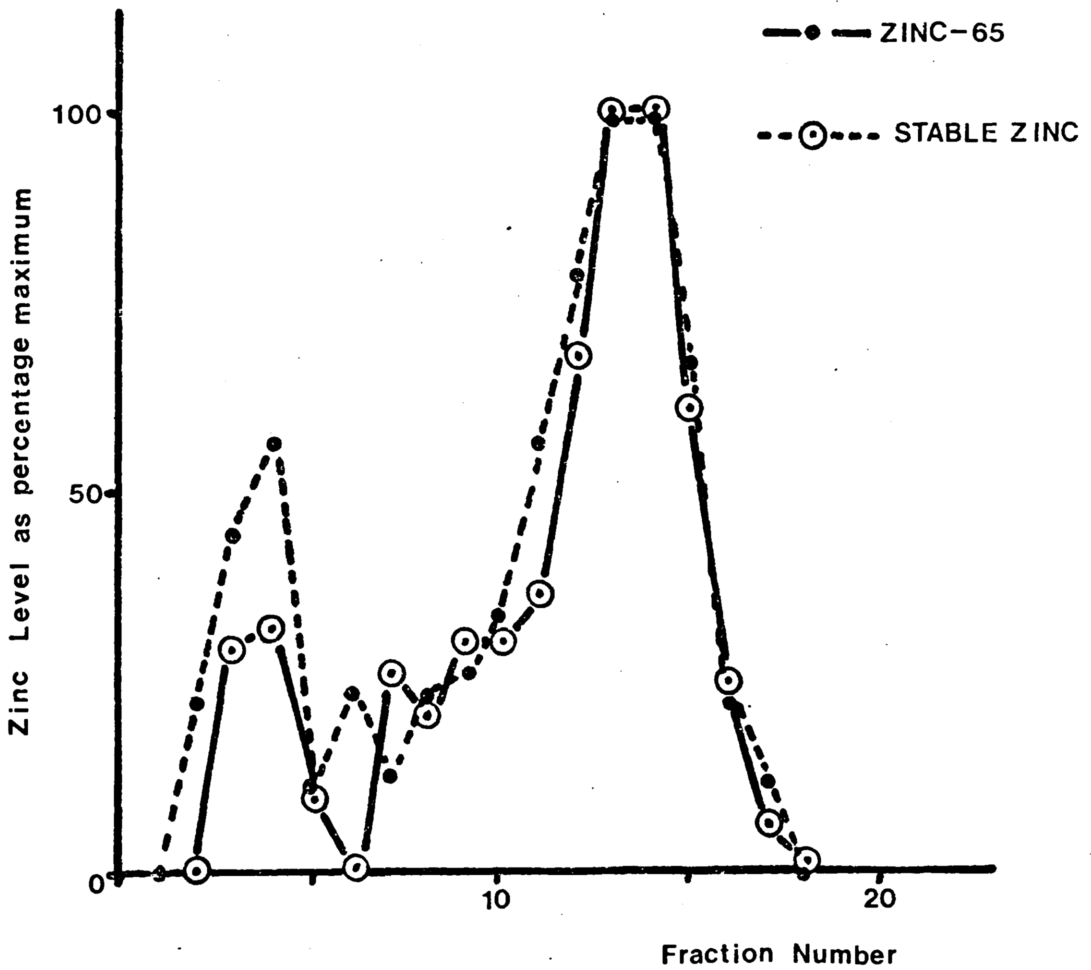


Fig. 24

Variation of zinc-65 level with time bound to plasma, albumin and α_2-macroglobulin during the first 24 hours after administration of an oral dose of zinc-65 to subject C

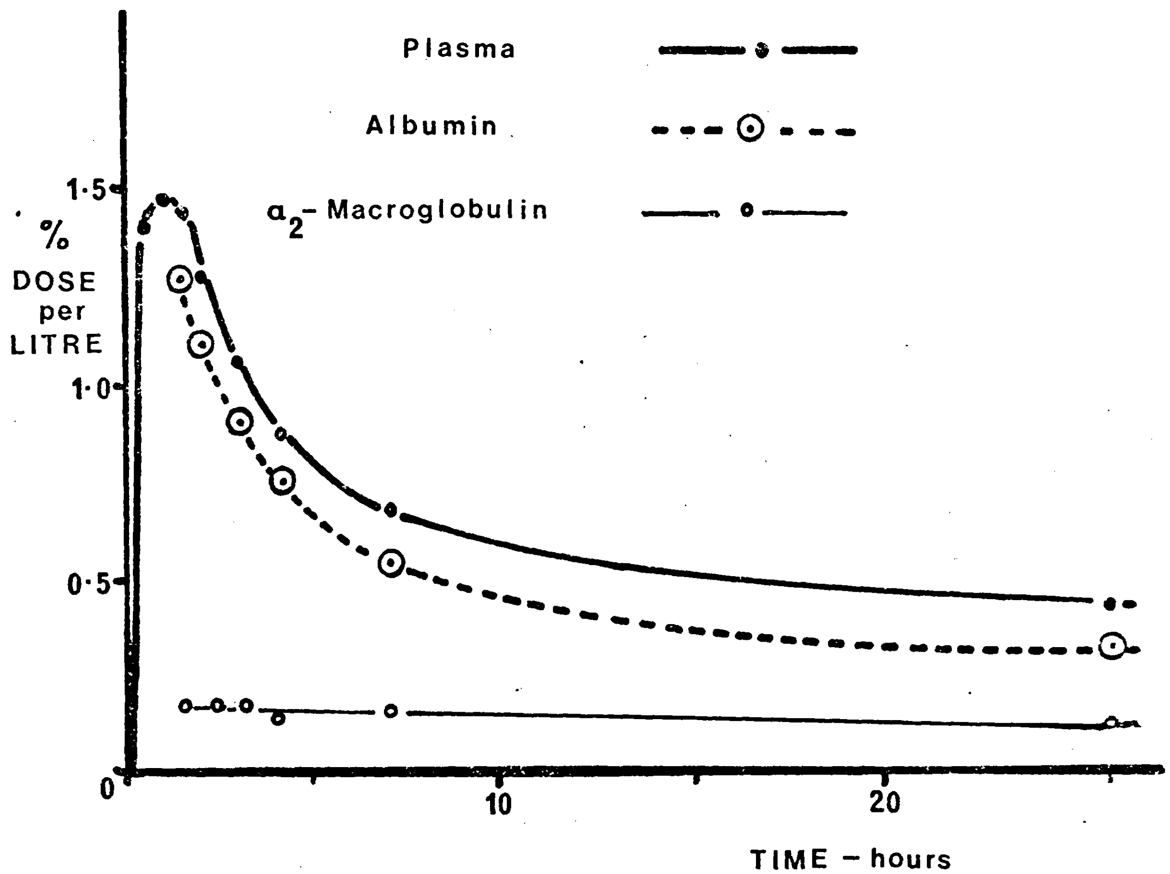


Fig. 25

Variation of zinc-65 level with time bound to plasma, albumin and α_2-macroglobulin during the first 24 hours after administration of an oral dose of zinc-65 to subject E

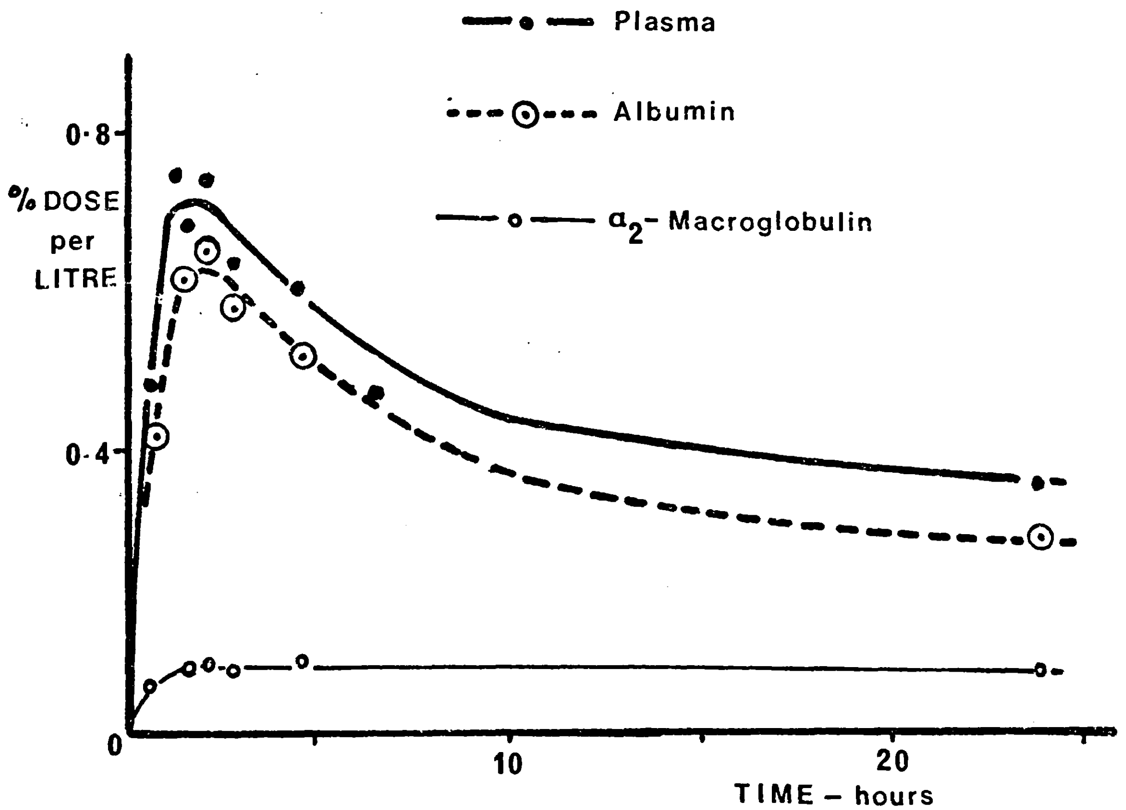


Fig. 26

Variation in stable zinc and zinc-65 levels in plasma with time after administration of an oral dose of 50 mg zinc together with 5 μCi zinc-65 to patient S.G.

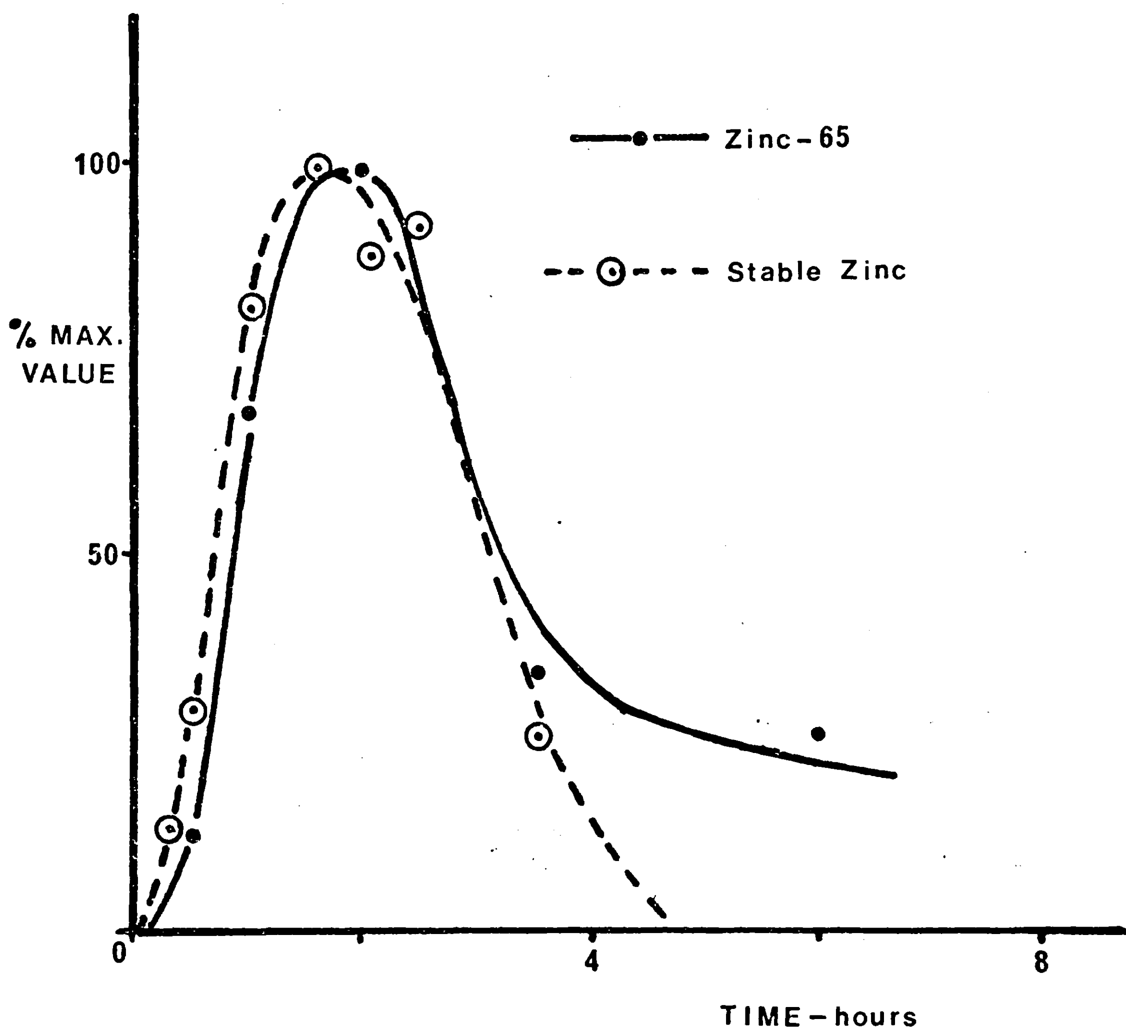
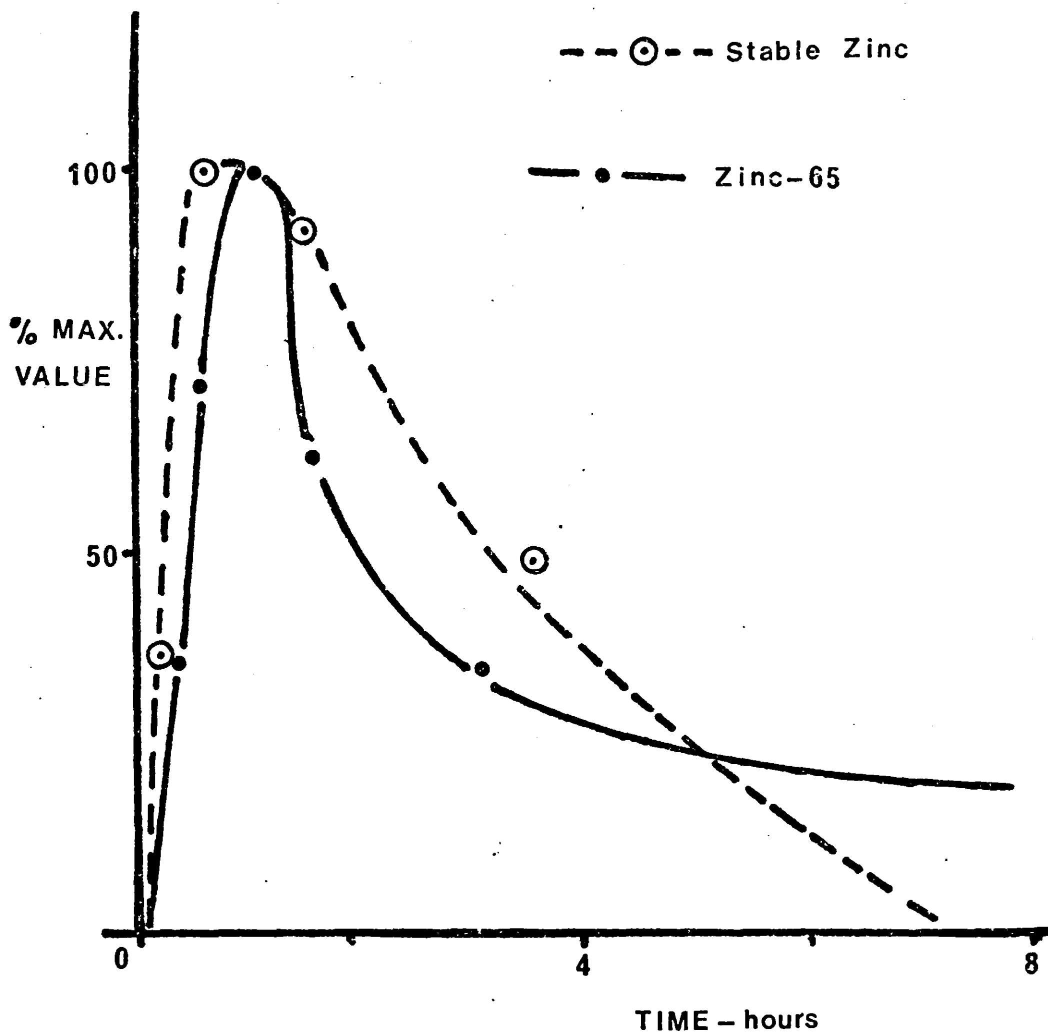


Fig. 27

Variation in stable zinc and zinc-65 levels in plasma with time after administration of an oral dose of 50 mg zinc together with 5 μCi zinc-65 to patient A.R.



Variation of plasma zinc-65 level with time on intravenous infusion of 0.4 μCi zinc-65 for subjects K and M, expressed as a percentage of the maximum level

Fig. 28

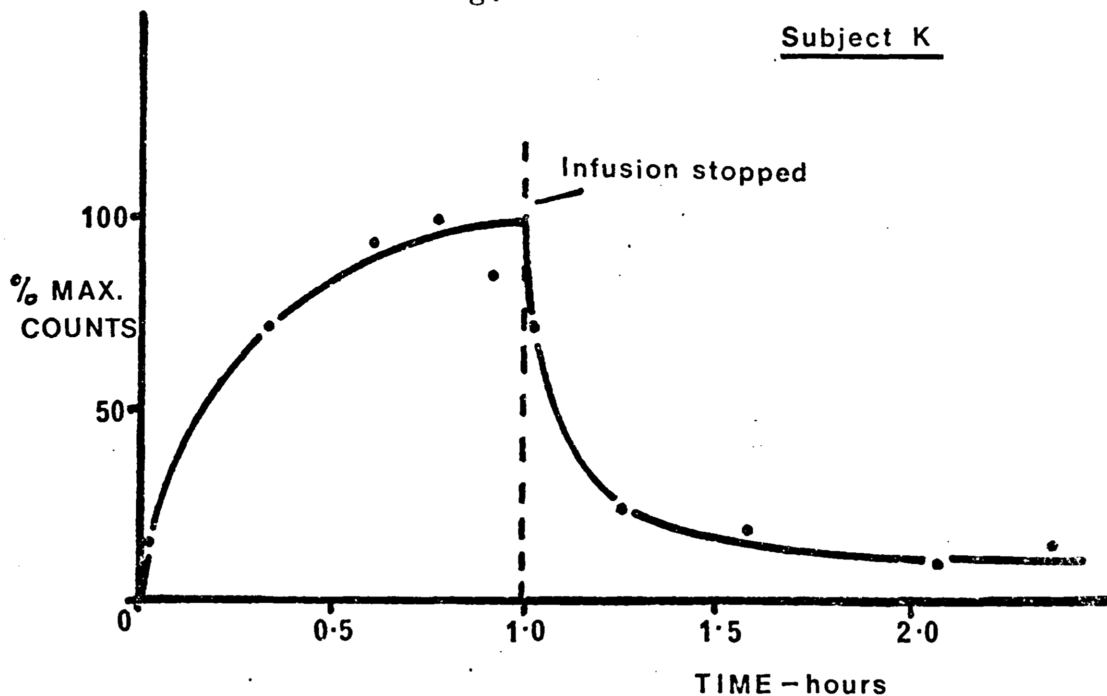


Fig. 29

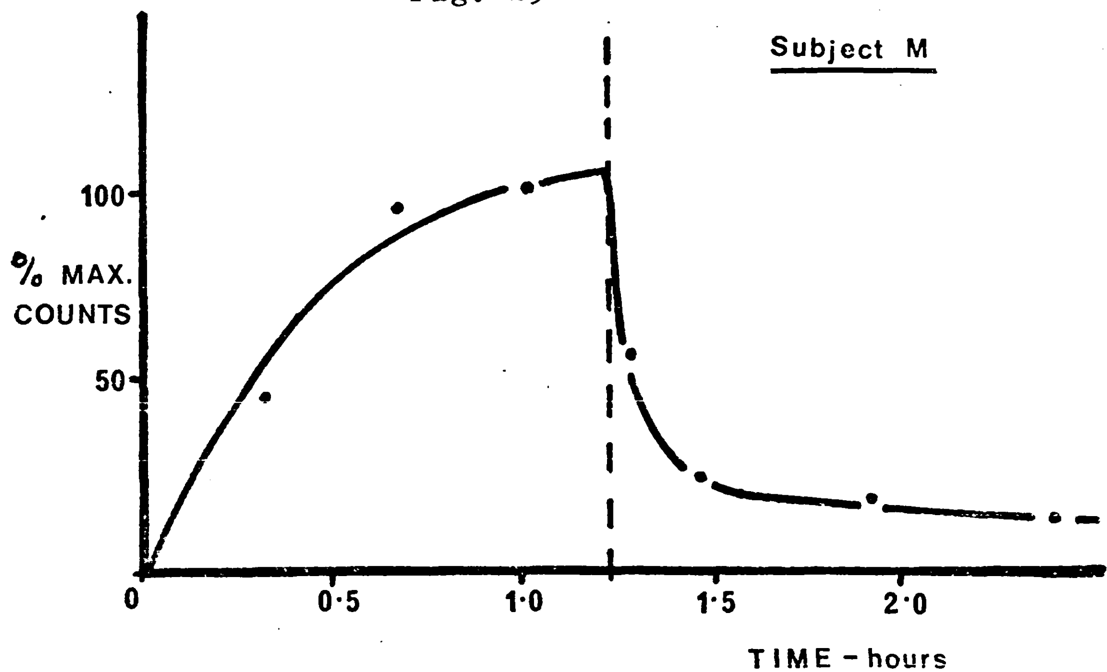


Fig. 30

Variation of plasma zinc-65 level with time on intravenous infusion of 0.4 μCi zinc-65 for subject C, expressed as a percentage of the total dose administered

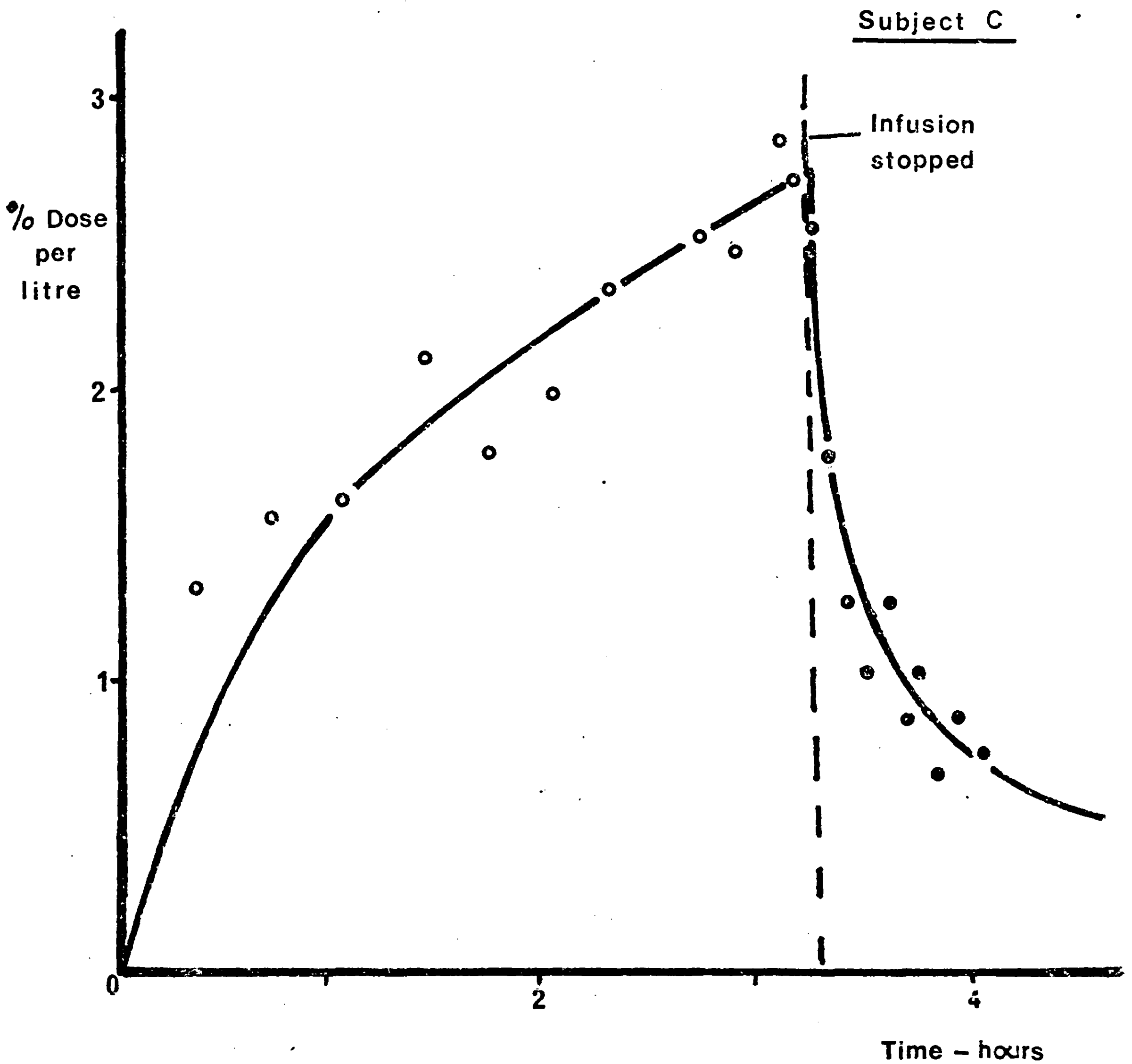


Fig. 31

Comparison of the curves obtained for the gut transfer function f(t) using values of Δt of 0.125 hour, 0.25 hour and 0.5 hour

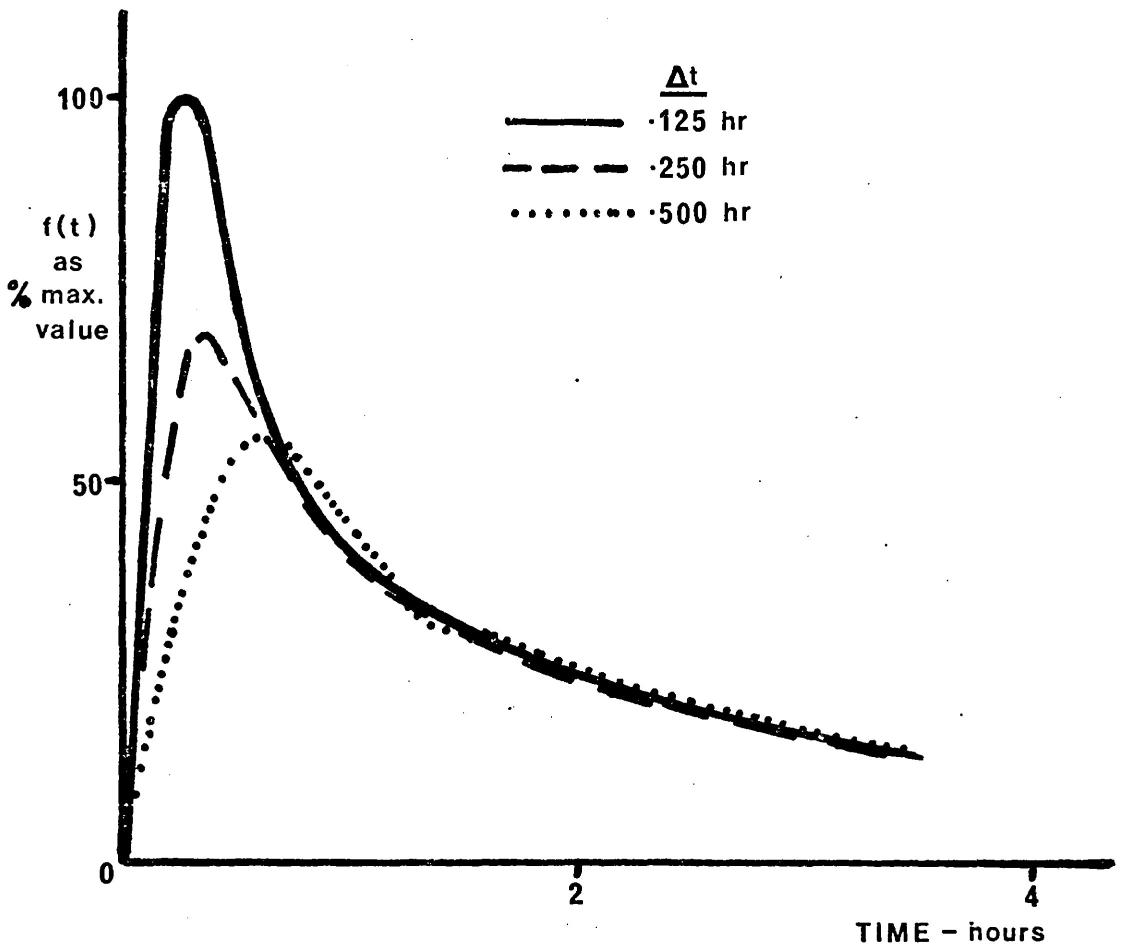


Fig. 32

Comparison of the oral plasma clearance curve and the gut transfer function describing the variation in the amount of zinc-65 crossing the gut wall into plasma, for Δt = 0.125 hour

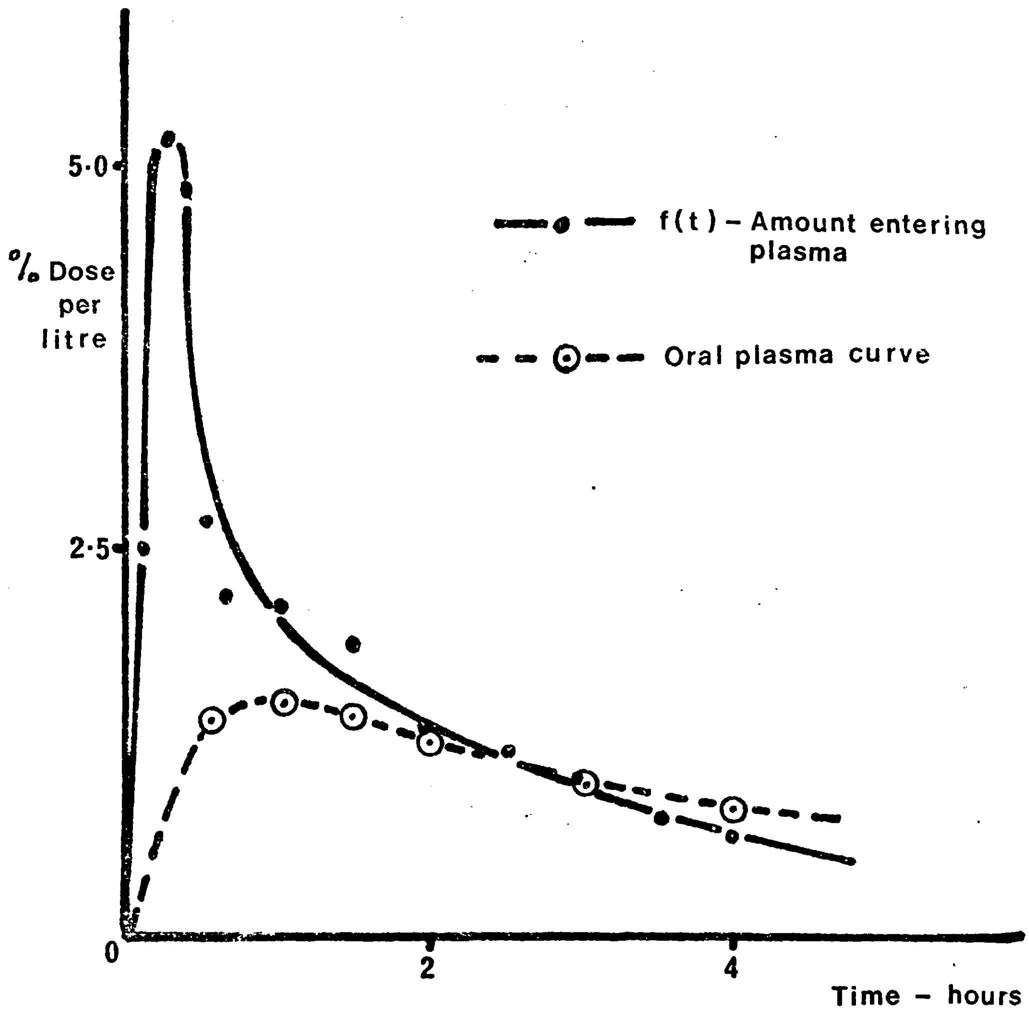
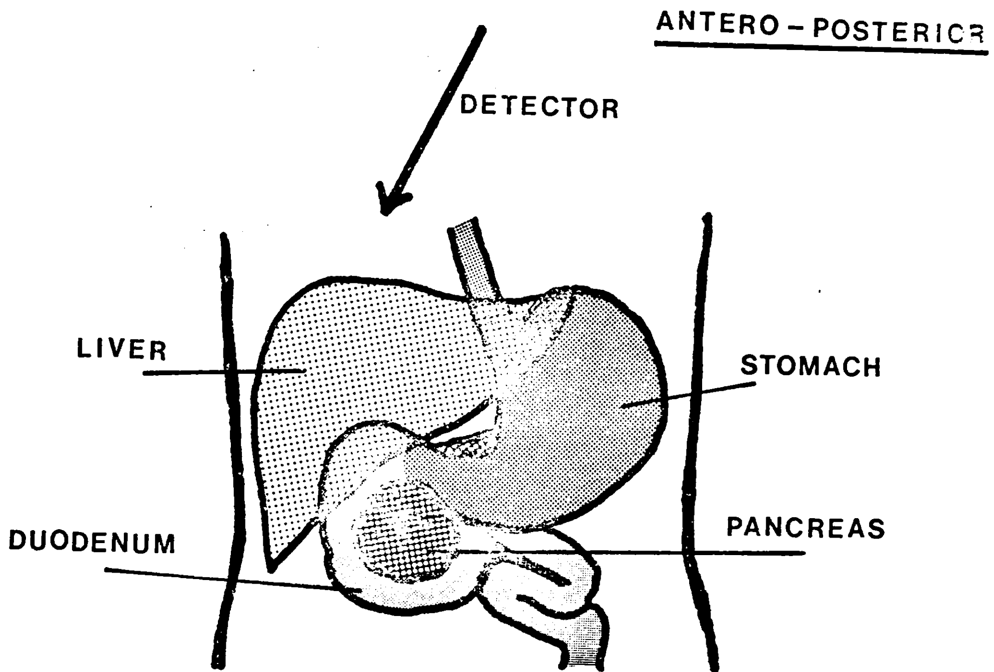


Fig. 33

Position of the detector for measurements on liver uptake and clearance of zinc-65



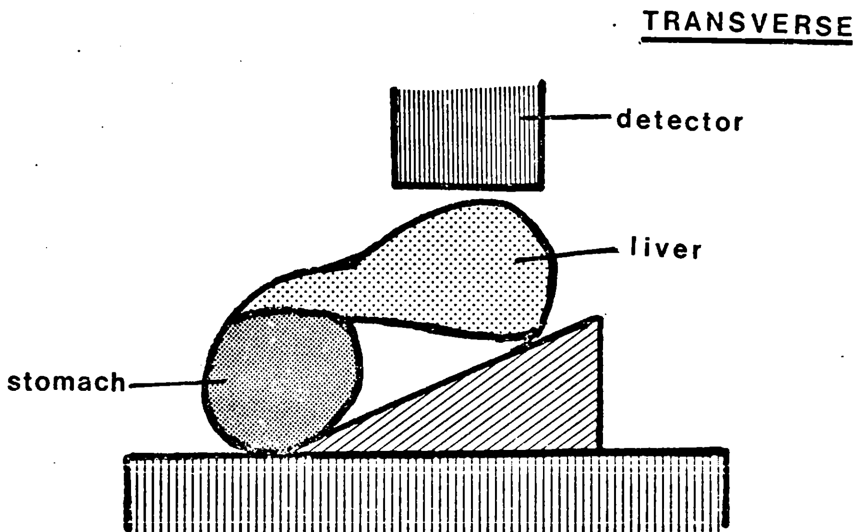


Fig. 34

Uptake and clearance of zinc-65 by erythrocytes after an oral dose of zinc-65 for subjects C and E, expressed as a percentage of the total dose administered

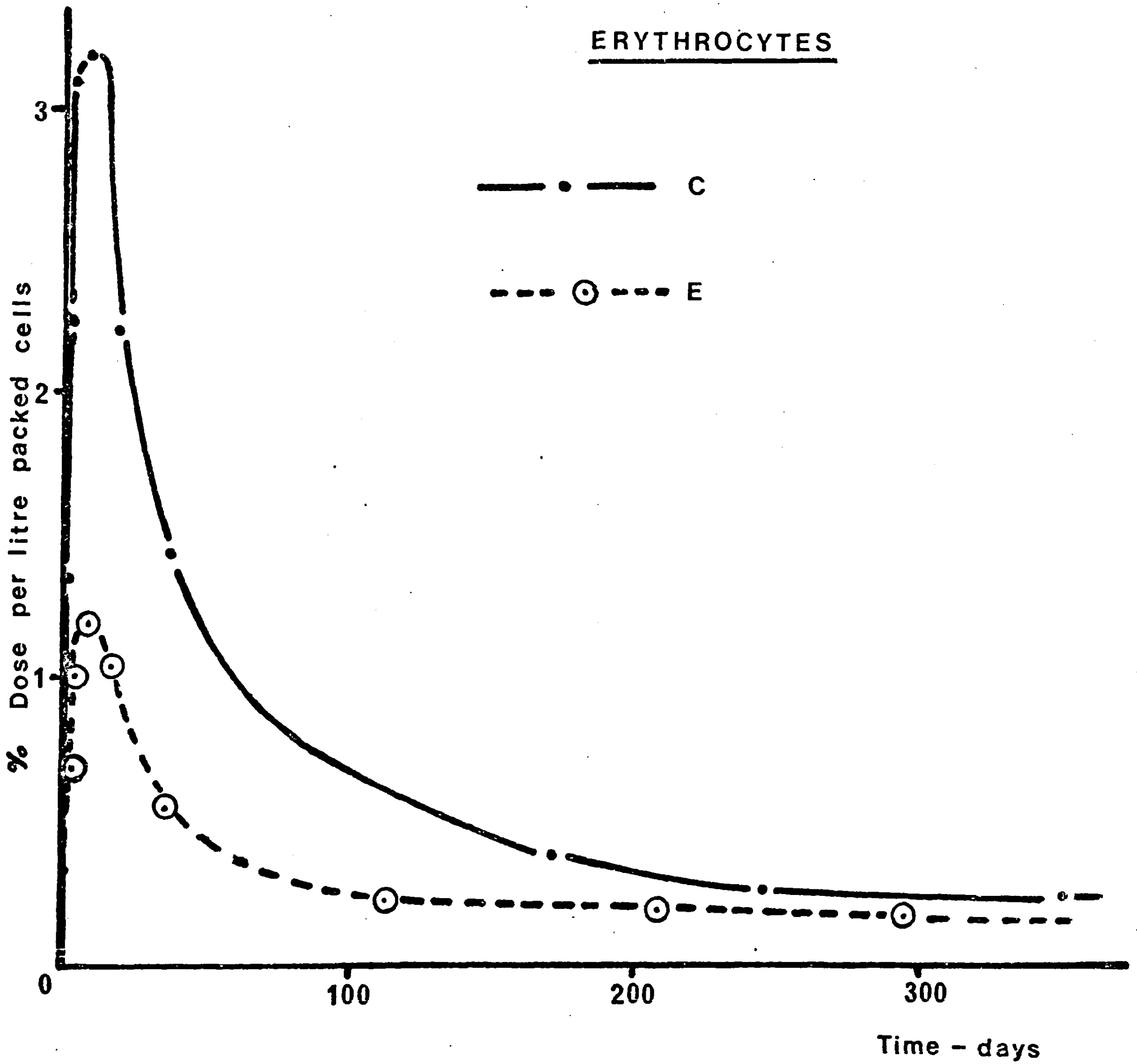


Fig. 35

Whole body clearance curves for subjects C and E after an oral dose of zinc-65, expressed as a percentage of the administered dose, showing the least squares exponential fit to the points in each case

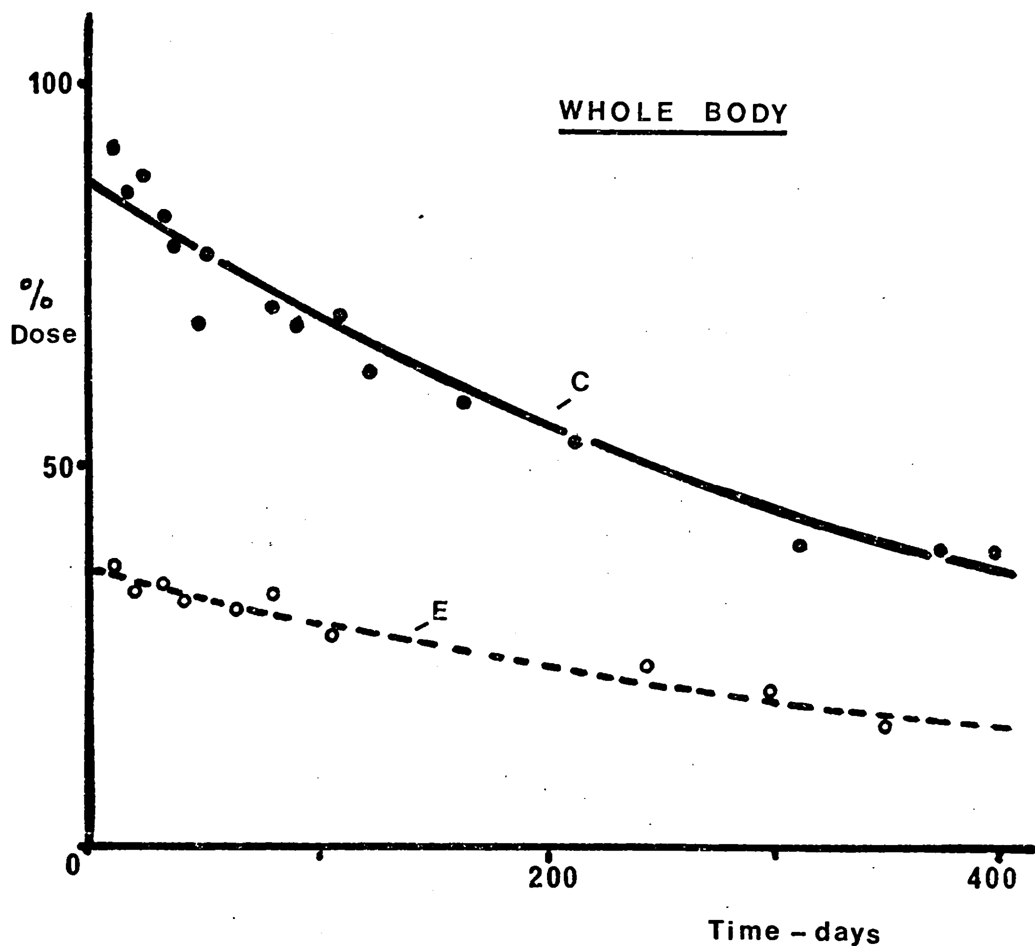


Fig. 36

Uptake and clearance of zinc-65 by the liver after an oral dose of zinc-65 for subject E, expressed as a percentage of the total dose administered

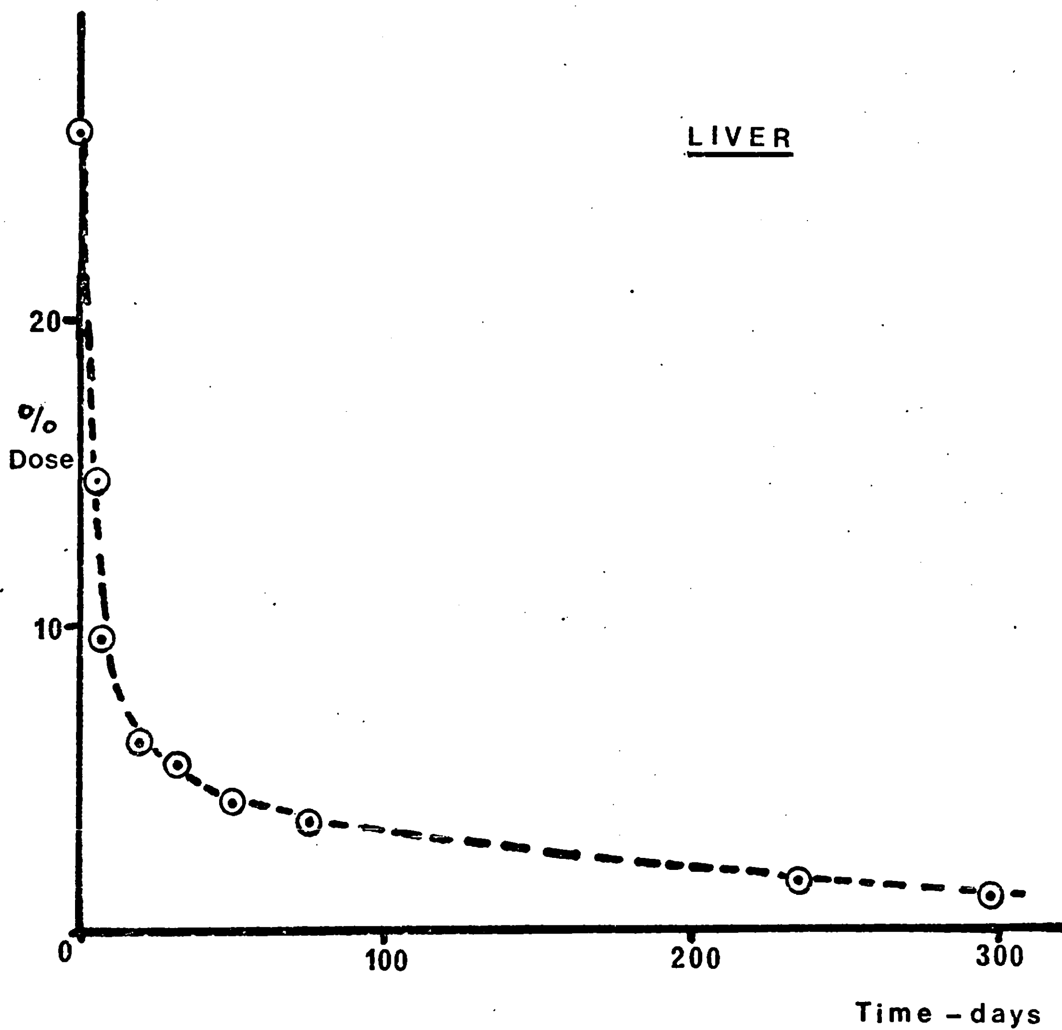


Fig. 37

Uptake and clearance of zinc-65 by muscle (thigh and forearm) after an oral dose of zinc-65 for subject E, expressed as an approximate percentage of the total dose administered

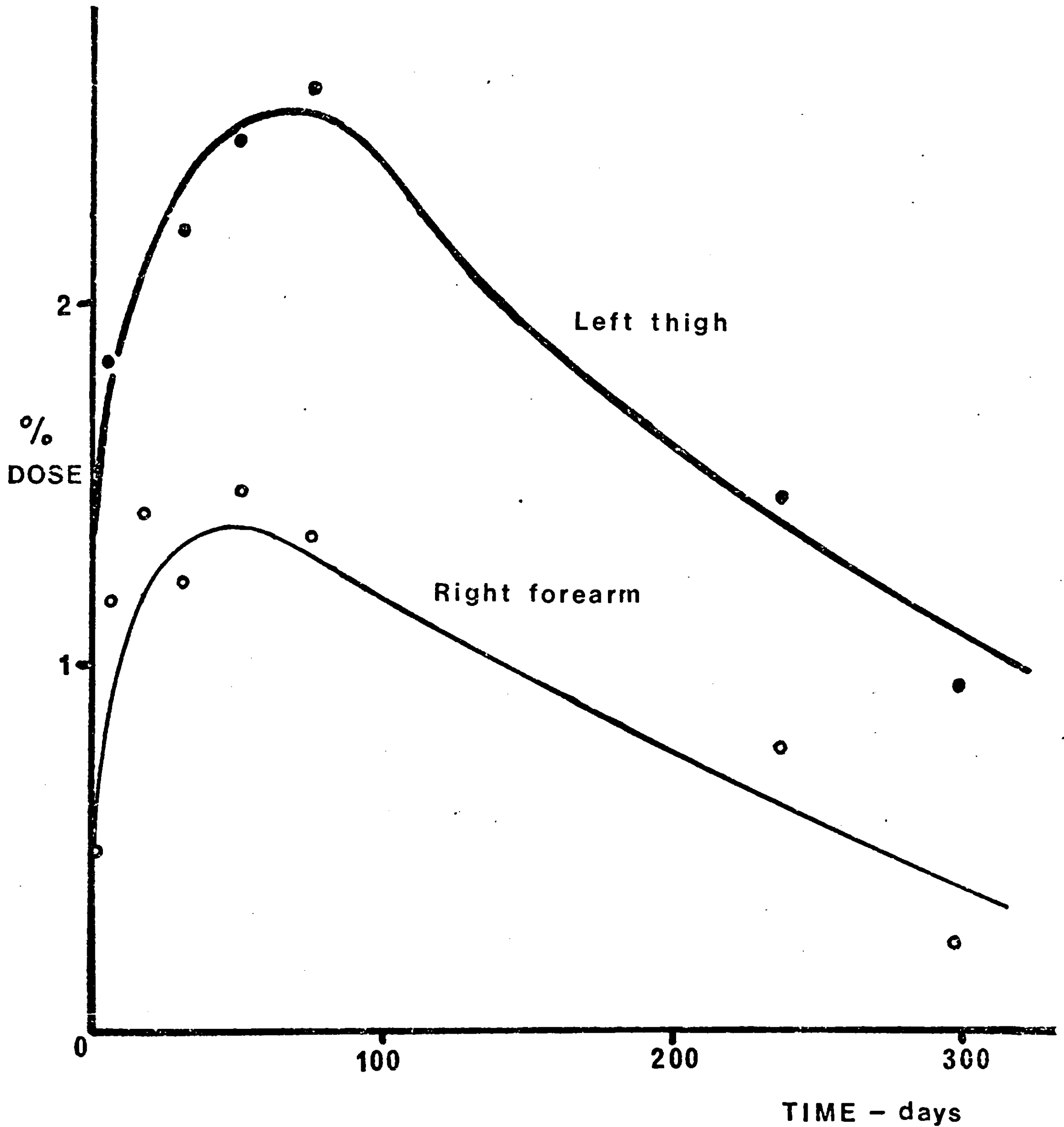


Fig. 38a

Variation in plasma zinc level with time after an oral dose of 220 mg zinc sulphate in 6 normal subjects

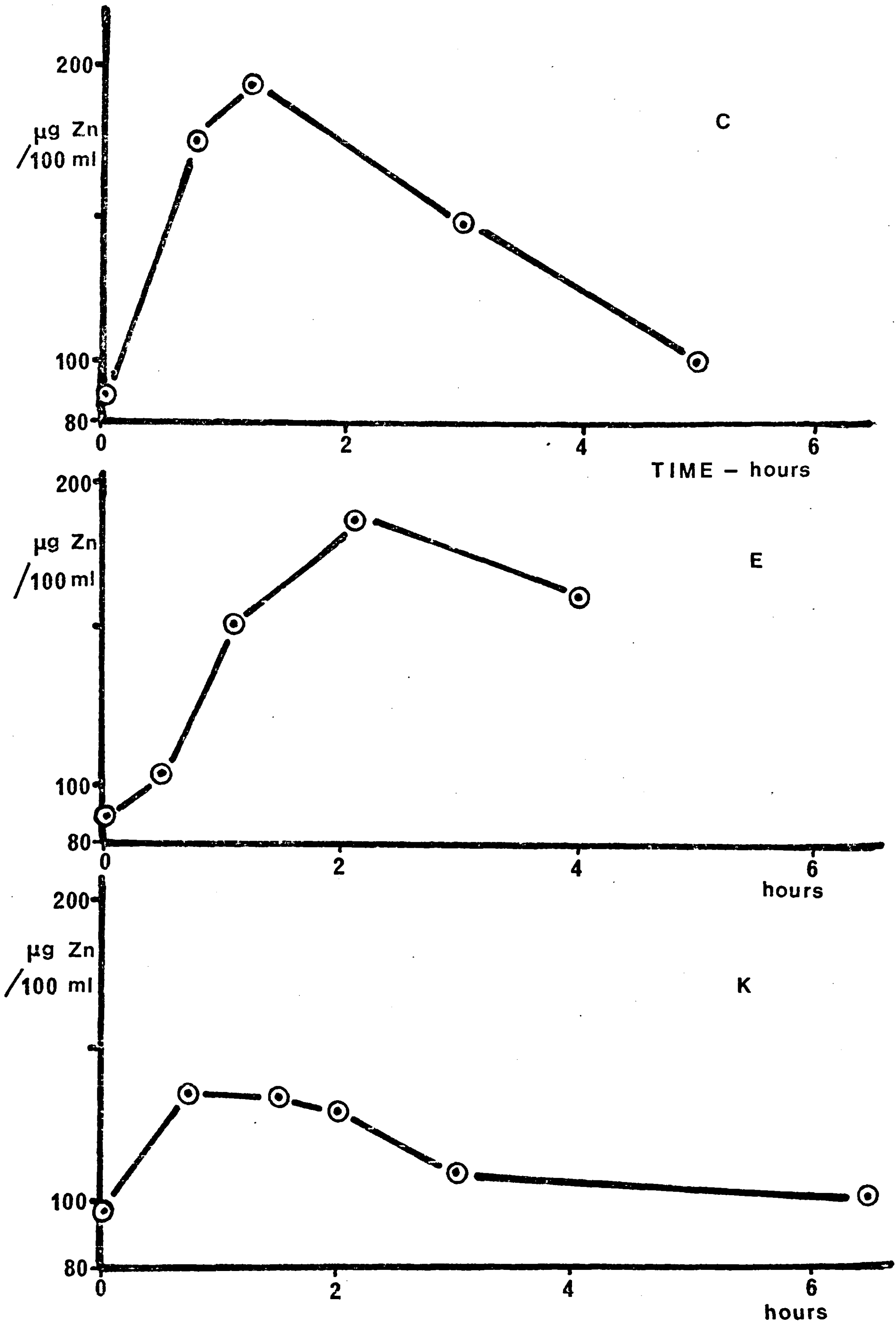


Fig. 38b
Variation in plasma zinc level with time after an oral dose of 220 mg zinc sulphate in 6 normal subjects

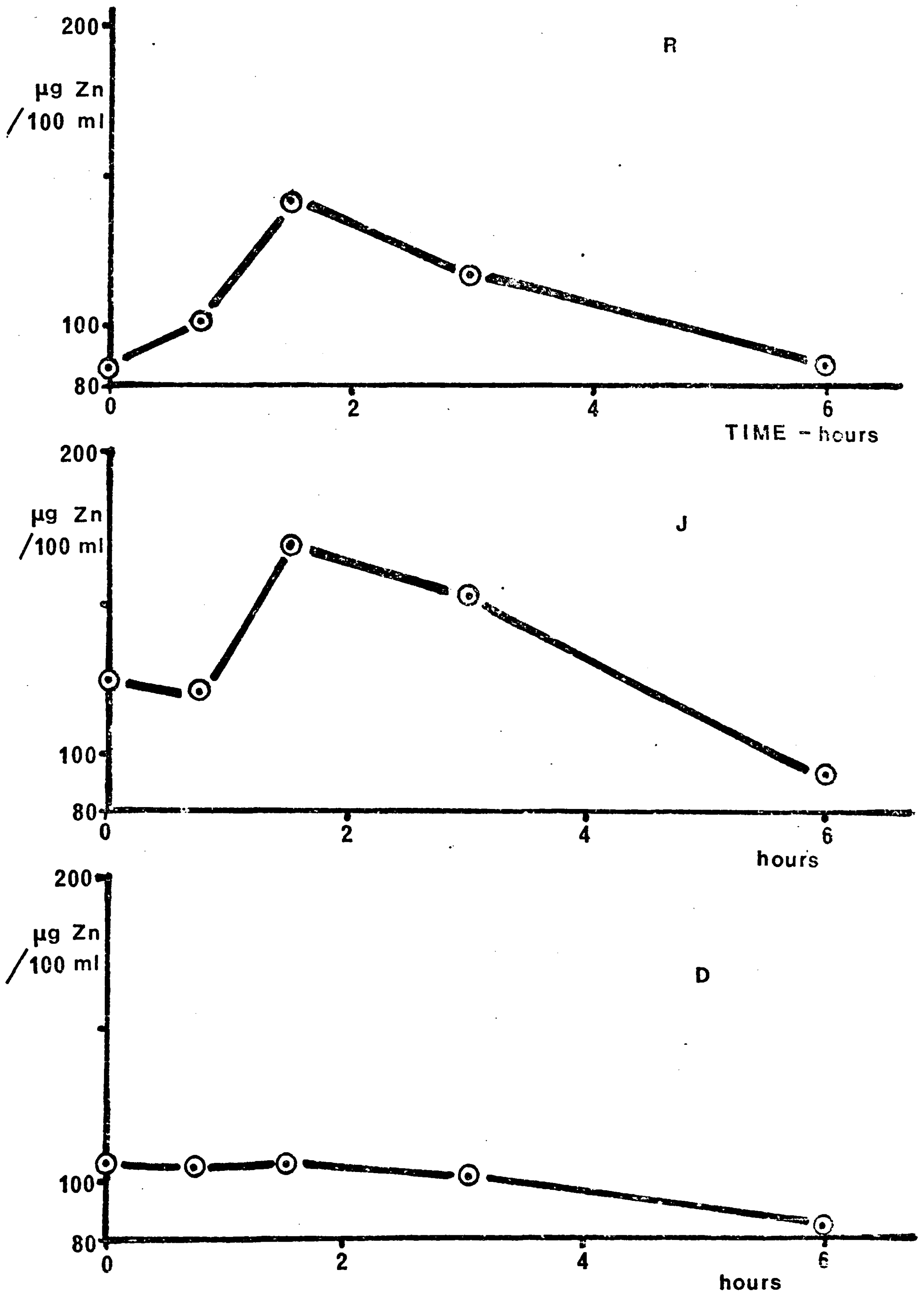


Fig. 39

Comparison of the distributions of the plasma zinc among the protein fractions for the endogenous zinc and in a plasma sample obtained $1\frac{1}{2}$ hours after ingestion of a 220 mg zinc sulphate capsule for subject C

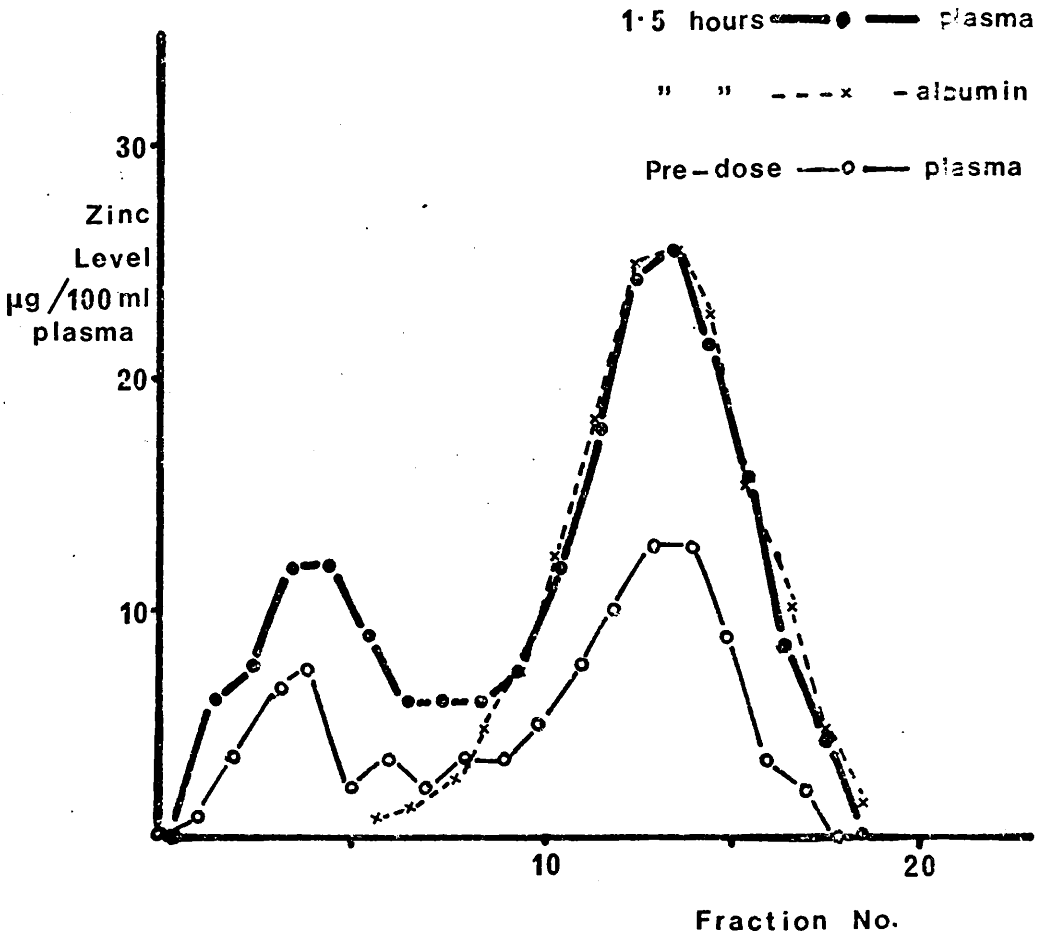


Fig. 40

Variation in urine zinc level with time on taking 3 capsules of zinc sulphate daily for 5 days (subject C)

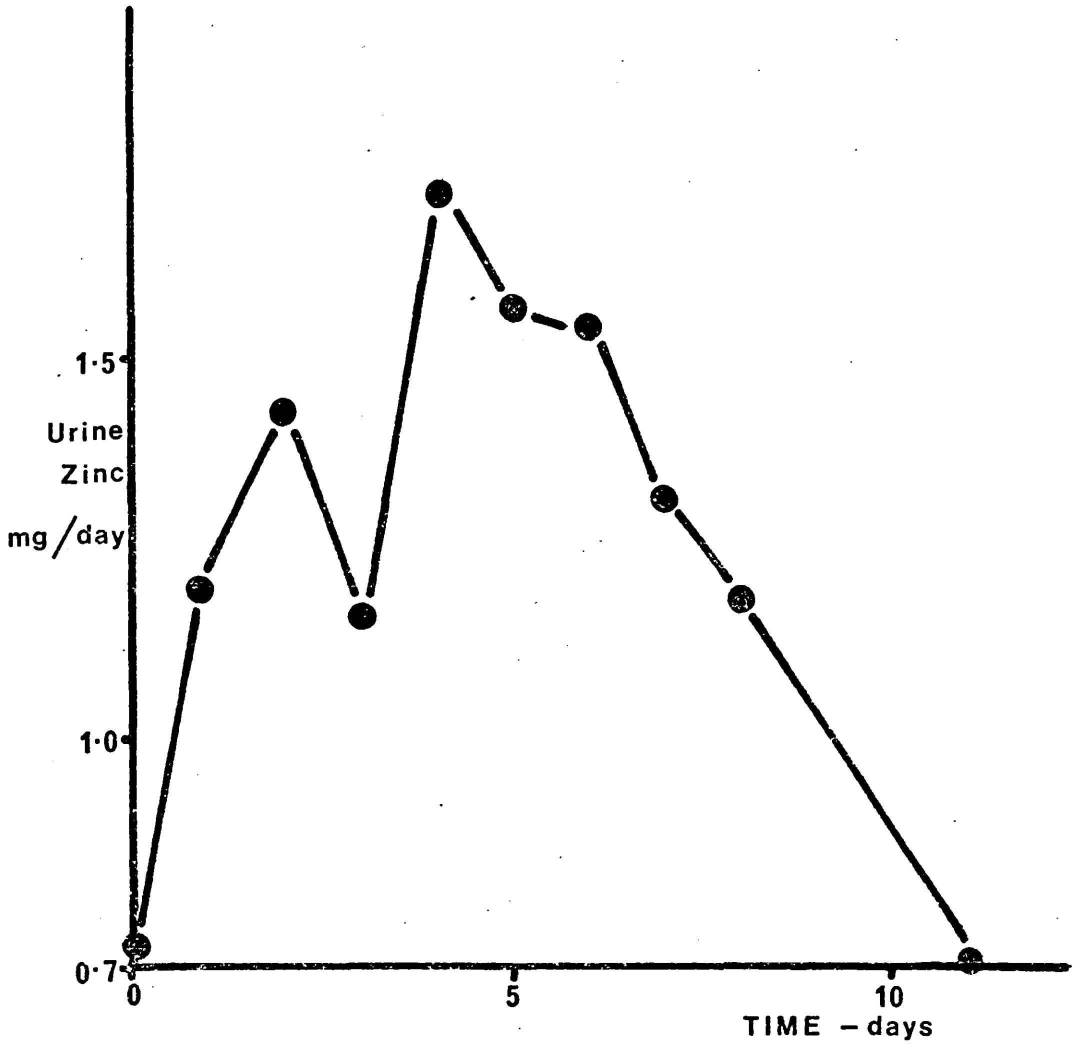
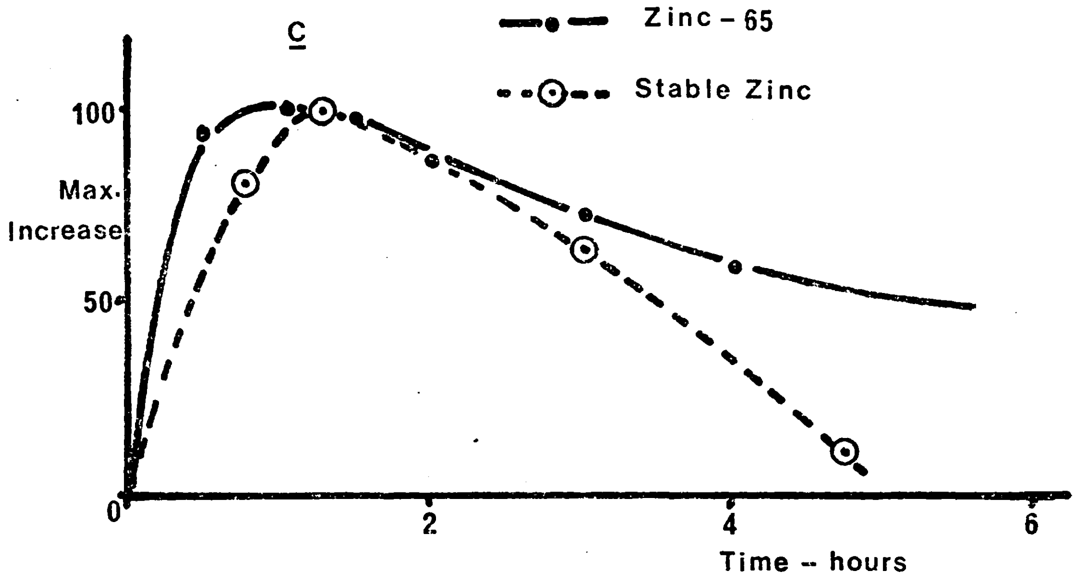
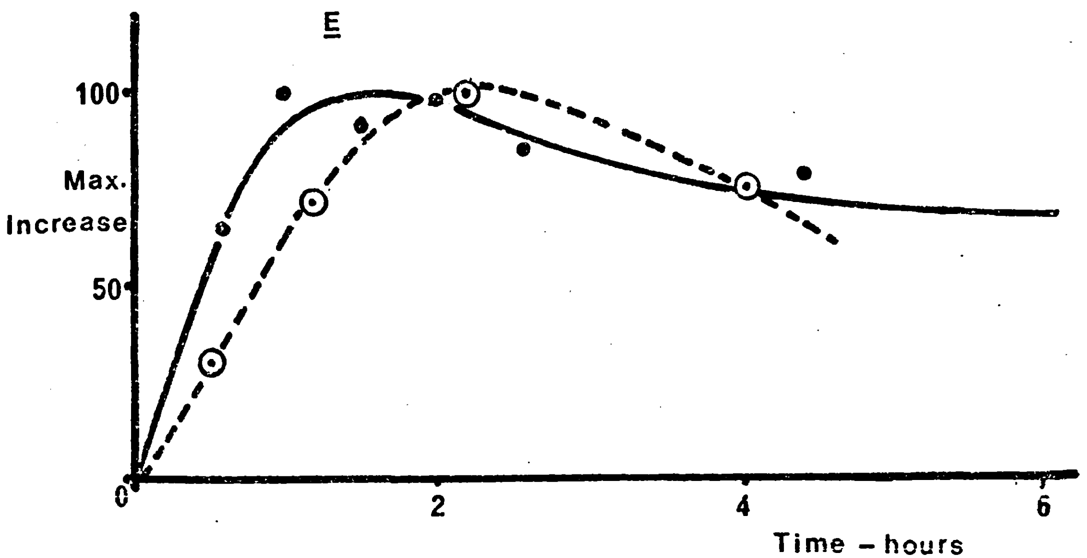


Fig. 41

Comparison of the variations in plasma level with time after oral administration of a therapeutic dose of stable zinc and a tracer dose of zinc-65 for subjects C and E





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