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The Insulin-Like Activity of Human Serum

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THE INSULIN-LIKE ACTIVITY OF HUMAN SERUM

by

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FORWARD

I am deeply grateful to Loyola University of Chicago for allowing me to earn the PhD degree on a part-time basis while engaged in full-time employment, which was necessary in order to support my family. This arrangement, however, necessitated an extended period of time to complete the requirements for the degree. Preparation of this dissertation, for instance, required more than four years, most of which was devoted to the literature review. Chapter II was prepared during 1979, Chapter III 1979-1980, Chapter IV 1980-1981, and Chapter V 1981-1982.

Certain recent, dramatic developments in the field of insulin research, therefore, were not included in this review. Most important are the findings that the insulin receptor consists of two 125,000 M.W. α subunits and two 90,000 M.W. β subunits (M. Czech, Amer. J. Med. 70: 142, 1981). The α subunit presumably binds insulin, while the β subunit, which is highly susceptible to proteolytic cleavage, possesses kinase activity and phosphorylates itself at a tyrosine residue in response to binding of the hormone (M. Kasuga et al., Science 215: 185, 1982; Roth and Cassell, Science 219: 299, 1983). Binding of insulin activates a membrane protease which cleaves a glycopeptide mediator from the receptor. This mediator stimulates glycogen synthetase and inhibits glycogen phosphorylase by activating glycogen synthetase phosphatase and inactivating glycogen synthetase kinase (phosphorylase b kinase kinase, cAMP-dependent protein kinase), stimulates mitochondrial pyruvate dehydrogenase by activating the phosphatase that dephosphorylates the inactive dehydrogenase's

α subunit, inhibits the (Ca^{++} + Mg^{++})-ATPase of adipocyte plasma membrane by decreasing enzyme phosphorylation, and activates the endo-plasmic reticulum phosphodiesterase (Czech, 1981; Chan and McDonald, J. Biol. Chem. 257: 7443, 1982; J. Larner et al., Rec. Prog. Horm. Res. 38: 511, 1982).

Insertion of the human insulin gene into the genome of the bacterium Escherichia coli, resulting in microbial production of human insulin on an industrial scale, also was not reviewed. This achievement represents the first commercial health care product derived from recombinant DNA technology (I. S. Johnson, Science 219: 632, 1983). I believe, however, that these developments do not appreciably affect justification of the research described or evaluation of the results reported in this dissertation.

M.E.K.

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VITA

Melvin E. Klegerman is the son of Hyman and Esther Klegerman. He was born August 30, 1945, in Chicago, Illinois.

He received his primary education in the public schools of Chicago, San Antonio, Texas, and Morocco, Indiana, and secondary education at the Nicholas Senn and Albert G. Lane High Schools in Chicago. He graduated from the latter school in June 1963. In September of that year, he entered the University of Illinois, Chicago, on a Cook County Scholarship and in January 1968 received from that institution the degree of Bachelor of Arts with a major in Chemistry. During 1964, he was a James Scholar and from 1965 to 1967 he was editor and columnist for two student newspapers.

He was employed by Dr. Ernest Page, Department of Physiology, University of Chicago, as a research technician, in which capacity he measured ion fluxes across membranes of the working rat heart in vitro, from December 1967 to October 1969, when he became a graduate student in the Department of Biochemistry at the University of Chicago. He left that institution one year later to become an editor for the Encyclopaedia Britannica in Chicago, where he helped supervise completion of short biographies for the fifteenth edition of the Encyclopaedia until March 1973, when the project was finished.

During 1973-1978, he was employed as a research associate at Loyola University Medical Center, Maywood, Illinois. In this capacity, he developed and carried out clinical radioimmunoassays for insulin, somatotropin, thyrotropin, triiodothyronine and testosterone for Dr. Marion Brooks, Section of

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During 1979, he carried out research concerning the immunochemistry of the Thompson-Friedenreich Antigen for Dr. Georg Springer at Evanston Hospital, Evanston, Illinois, and from January 1980 he has investigated the immunochemistry of Group B Streptococcus for Dr. Samuel P. Gotoff, chairman of the Department of Pediatrics at Michael Reese Hospital and Medical Center, Chicago.

Melvin E. Klegerman is a member of the American Association for the Advancement of Science and is coauthor of the following papers:

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ABBREVIATIONS

ACD	acid-citrate-dextrose
ACTH	adrenocorticotrophic hormone, corticotropin
ADH	alloxan-diabetic, hypophysectomized
ADHA	alloxan-diabetic, hypophysectomized, adrenalectomized
AI	atypical insulin
AIB	α -aminoisobutyric acid
AIS	anti-insulin serum
ala	alanine
AMP	adenosine monophosphate
app.	apparent
arg	arginine
asn	asparagine
asp	aspartic acid
ATE	adipose tissue extract
ATP	adenosine triphosphate
B	bound
b	slope
B_0	percent tracer bound to antibody in absence of unlabeled antigen
BHK	baby hamster kidney
BI	"bound insulin"
biol. act.	biological activity

BME	Eagle's Basal Medium with Earle's salts
BSA	bovine serum albumin
cAMP	cyclic AMP, adenosine 3',5'-monophosphate
CD	circular dichroism
cGMP	cyclic GMP, Guanosine 3',5'-monophosphate
Ci	Curie (3.7×10^{10} disintigrations per second)
circul.	circulating
CM	complete medium (with FBS)
CM-	carboxymethyl-
CNBr	cyanogen bromide
CoA	coenzyme A
CPB	carboxypeptidase B
CPD	citrate-phosphate-dextrose
C-peptide	proinsulin connecting peptide
CPM	counts per minute
C.V.	coefficient of variation = $(\bar{x}/SD) \times 100$
cys	cysteine
$D_{20,w}$	diffusion coefficient at 20°C in water
DAA	desalanine-desasparagine
DEAE-	diethylaminoethyl-
DNA	deoxyribonucleic acid
DNP	dinitrophenyl
DOP	desoctapeptide
dpm	disintigrations per minute
EDTA	ethylenediaminetetraacetate

EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EOP	efficiency of plating
F	free
FBS	fetal bovine serum
FDNB	1,2,4-fluorodinitrobenzene
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GIT	glutathione-insulin transhydrogenase
gln	glutamine
glu	glutamic acid
gly	glycine
GMP	guanosine monophosphate
GR	glutathione reductase
GSH	glutathione
HGH	human growth hormone, somatotropin
his	histidine
HSA	human serum albumin
IGF	insulin-like growth factor
IgG	immunoglobulin G
ILA	insulin-like activity
ILA_S	specific ILA
ile	isoleucine
inc.	incubation
IRI	immunoreactive insulin

$K_{assoc.}$	equilibrium constant of association
K_D	equilibrium constant of dissociation
kd	Kilodalton
K_i	inhibition constant: concentration of inhibitor required for half-maximal inhibition of an enzyme-catalyzed reaction
K_m	Michaelis constant: concentration of substrate required for half-maximal velocity of an enzyme-catalyzed reaction
L	long-incubated (5 days at 4^oC) stripped plasma
leu	leucine
LH	luteinizing hormone
lys	lysine
MEM	minimal essential medium
met	methionine
mRNA	messenger RNA
MSA	multiplication stimulating activity
MTE	muscle tissue extract
M.W.	molecular weight
N	number of viable cells
n	number of determinations
N_g	geometric mean of viable cell number
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
NGF	nerve growth factor

NSB	nonspecific binding
NSILA	nonsuppressible insulin-like activity (ILA that cannot be suppressed by AIS)
NSILA-P	acid ethanol-precipitable NSILA
NSILA-S	acid ethanol-soluble NSILA
NSILP	nonsuppressible insulin-like protein
OGTT	oral glucose tolerance test
PAGE	polyacrylamide gel electrophoresis
PAP	peroxidase/anti-peroxidase
PbK	phosphorylase b kinase
PbKK	phosphorylase b kinase kinase
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
phe	phenylalanine
PMSF	phenylmethylsulfonyl fluoride
pro	proline
R	specific glucose uptake parameter = r_t/r_c, where r_c = control r and r_t = sample r
r	rate of glucose uptake (-mg glucose/10^6 cells/day)
R_f	relative electrophoretic mobility = (distance of band from top of gel)/(distance of bromphenol blue band from top of gel)
RER	rough endoplasmic reticulum
RIA	radioimmunoassay
RNA	ribonucleic acid
RRA	radioreceptor assay

$S_{20,w}$	sedimentation coefficient at 20 ^{o} C in water
S_b	standard error of the slope
SC	spinner culture medium
S.D.	standard deviation
SDS	sodium dodecyl sulfate
S.E.M.	standard error of the mean
ser	serine
SIA	synalbumin insulin antagonist
SILA	AIS-suppressible ILA
SM	statopmaru ,edoi, (BME with 0.35% BSA, no serum)
SMA	somatiomedin A
SMB	somatomedin B
SMC	somatomedin C
SP	stripped plasma (treated with anion exchanger)
SS	stripped serum (treated with anion exhanger)
sub.	subunit
T	total
t	time or t-statistic
t_D	doubling time
TCA	trichloroacetic acid
TGF	transforming growth factor
thr	threonine
TI	typical insulin
TSH	thyroid stimulating hormone, thyrotropin
tyr	tyrosine

U	unit, or total glucose uptake parameter = u_t/u_c, where u_c = control u and u_t = sample u
u	glucose uptake (mg)
V_e	elution volume
V_{max}	maximal enzyme-catalyzed reaction velocity
V_o	exclusion volume
val	valine
$\bar{x}$	mean
3OMG	3-O-methylglucose
β-ME	βmercaptoethanol
ν	proliferative index = (number viable cells in test well)/(number of viable cells in control well)

CHAPTER I

INTRODUCTION

STATEMENT OF THE PROBLEM

When Frederick Sanger completed the elucidation of the primary structure of bovine insulin in 1955, all appeared right with the world of insulin. The hormone had been purified to a high degree, making possible the effective treatment of diabetes mellitus. The physiologic effects of insulin were well understood and its site of biosynthesis and secretion was known. Many of the hormone's physicochemical properties, including its minimum molecular weight and aggregative behavior, were known. Sanger's accomplishment had verified several fundamental tenets of protein chemistry and opened new areas of investigation. Insulin had been crystallized in several different forms and the zinc atoms coordinately bonded in the hexamer were easily replaced by heavy metal atoms, promising another seminal accomplishment of protein chemistry: the elucidation of a protein's tertiary structure by x-ray diffraction. In vivo bioassays of increased sensitivity and a new in vitro rat diaphragm bioassy made possible the measurement of insulin in blood for the first time. A thorough understanding of the cause of diabetes as well as a possible cure for the disease appeared imminent. Yet, within five years, the question of circulating insulin and its relation to normal and diabetic physiology was characterized by a state of utter confusion.

With the introduction of *in vitro* bioassays for insulin, which measure what was termed *insulin-like activity* (ILA), a plethora of substances that mimic insulin effects measured by the assays (*e.g.*, stimulation of glucose uptake, glycogen synthesis or carbon dioxide production), but do not exhibit certain physicochemical properties of the hormone, were discovered in human serum and plasma. In 1958, an *in vitro* bioassay for insulin using rat epididymal fat pads was introduced (Martin *et al.*, 1958; Ref. 393). Although this assay was at least ten times more sensitive than the diaphragm assay, it was immediately noted that serum insulin levels measured with the fat pad assay were 5-20 times higher than those found using the latter assay.

Antoniades *et al.* (1965; Ref. 511, 512) then found that Dowex cation exchange resin used to prepare human blood plasma, when eluted with strong acid or alkali, yielded significant quantities of ILA detectable by the fat pad assay, but not by the diaphragm assay. When this cationic serum protein fraction was treated with acid, alkali, acid-ethanol or an aqueous extract of adipose tissue, the ILA was detectable by the diaphragm assay, was found to reside in a soluble, anionic fraction, and was neutralized by anti-insulin serum. This treatment also generated an insoluble, cationic protein fraction, suggesting a serum insulin-binding protein dissociated from the hormone by a factor produced by adipose tissue (Antoniades, 1961; Antoniades and Gundersen, 1961; Ref. 509, 514).

The existence of an insulin-binding protein in normal human serum was further supported by the work of Shaw and Shuey (1963; Ref. 390), who demonstrated that adipose tissue extract causes a 12-fold increase of normal fasting human serum ILA measured by the diaphragm assay and that anti-insulin

serum prevents this ILA increment. Gundersen and Lin (1965; Ref. 391) then found that heparin also increases diaphragm-assayable ILA in cationic serum protein fractions and in normal human sera to levels found with the fat pad assay and that this ILA increment is neutralized by anti-insulin serum. The existence of a human serum insulin-binding protein appeared virtually assured with the appearance of several reports of the electrophoretic migration of radioactivity with β-globulins after incubation of exogenous radiolabeled insulin with normal human serum (Clausen et al., 1963; Mitchell, 1960; Ref. 520, 521).

Berson and Yalow, however, disputed the concept of "bound insulin" on several grounds, contending that the only physiologically significant insulin-binding proteins are the anti-insulin antibodies found in the blood of insulin-treated diabetics. They observed no increase in insulin measured by radioimmunoassay (RIA) after treatment of human sera with adipose tissue extract (1961, Ref. 523) and they demonstrated that apparent association of radiolabeled insulin with normal serum proteins represents irreversible binding of damaged tracer by these proteins (1965, Ref. 515). They also pointed out that the behavior of "bound insulin" is inconsistent with established physiologic insulin response, since total plasma insulin levels ("bound" plus "free") reported by Antoniades decrease after intravenous glucose administration (1965,Ref.515).

Berson and Yalow also refuted the concept of "bound insulin" on theoretical grounds. They argued that rapid insulin kinetics in vivo, resulting in quick onset of diabetic symptoms after pancreatectomy, are inconsistent with the slow kinetics of hormones such as thyroxine that are known to be bound to serum carrier proteins. They pointed out that symptoms of thyroid hormone deficiency appears only weeks after thyroidectomy and that in cases of proven

insulin binding by serum proteins—formation of insulin-antibody complexes in insulin-resistant diabetics—insulin-dependent carbohydrate homeostasis is frankly deranged (1965,Ref.528).

The concept of "bound insulin" is also inconsistent with the observations of Froesch et al., who found that about 90% of human serum ILA measured by the rat fat pad assay is not suppressible by anti-insulin serum (nonsuppressible insulin-like activity, NSILA), that levels of antiserum-suppressible ILA are very similar to those found with RIA, and that only about 10% of serum NSILA is extractable with acid-ethanol (designated NSILA-S) (Froesch et al., 1963; Labhart et al., 1972; Jakob et al., 1968; Ref. 527, 546, 547). Furthermore, the remaining serum NSILA (NSILA-P) was found to be a protein of molecular weight 100,000-150,000 daltons that is not dissociable by acid or urea (Ref. 546, 547).

Subsequently, NSILA-S was found to be structurally similar to insulin, but to exhibit the function of a growth factor in vivo (Rinderknecht and Humbel, 1976; Zapf et al., 1975; Ref. 549, 550, 557), while the ILA exhibited by supraphysiologic concentrations of NSILA-S was demonstrated to be due to a 2% cross-reactivity of the factor with insulin receptors (Gavin et al., 1973; Ref. 565). In addition, other plasma growth factors (i.e., somatomedins A and C and multiplication stimulating activity) were also found to exhibit ILA at supraphysiologic concentrations, suggesting a family of insulin-like growth factors (IGFs) that, with insulin, derive from a common ancestral gene and diverged functionally (Shields, 1977; Ref. 728). The arguments of Berson and Yalow and characterization of the IGFs therefore caused the concept of "bound insulin" to fall into disfavor.

Two developments, however, have again made the question of "bound insulin" a worthwhile object of study. 1) Guenther (1974, Ref. 387) reported that heparin treatment of cationic serum protein fractions causes an increase of insulin measured by the RIA and implicated biotin as the ILA-potentiating factor in adipose tissue extract. 2) IGFs have been found to have serum binding proteins (Zapf et al., 1975; Van Wyk et al., 1974; Ref. 557, 598), making an insulin-binding protein a plausible expectation.

SPECIFIC OBJECTIVES

Zapf et al. (1975, Ref. 557) elucidated the serum carrier protein for NSILA-S by gel chromatography and charcoal adsorption analysis of radiolabeled NSILA-S incubated with human serum. They found that about 40% of ^{125}I-NSILA-S radioactivity added to normal human serum eluted from Sephadex G-200 at positions corresponding to proteins of significantly higher molecular weights than free NSILA-S (apprx. 7,000 daltons). Addition of excess unlabeled NSILA-S to serum-tracer mixtures resulted in nearly complete abolition of two upfield peaks of radioactivity with a concomitant increase of free NSILA-S peak activity. Acid treatment of serum, followed by dialysis and gel chromatography yielded fractions that exhibited reversible binding of NSILA-S.

This kind of study, however, has not been applied to the question of serum insulin-binding protein. Benson and Yalow utilized electrophoretic and ultracentrifugation techniques, but no gel chromatography, in their experiments, nor did they investigate the effect of heparin on serum immunoreactive insulin (IRI). Therefore, I endeavored to investigate both the question of a normal

human serum insulin-binding protein using the approach of Zapf et al. and the contribution of serum growth factors to ILA using a cultured cell bioassay capable of discriminating between specific insulin-like activity and the effects of growth factors.

Purified ^{125}I-insulin will be incubated with normal human serum and plasma, followed by Sephadex G-200 gel chromatography in order to determine whether radioactivity becomes associated with high molecular weight fractions. Reversibility of association will be tested by gel chromatography of plasma-tracer mixtures and upfield peak material incubated with excess unlabeled insulin and by dextran-coated charcoal analysis of upfield peak material in the presence of excess unlabeled insulin and 8 M urea. Initially, the verifiability of heparin-induced IRI in cationic serum protein fractions will be tested in order to determine whether heparin can also be used as a potential dissociative agent. The possibility of irreversible binding of damaged tracer to albumin will be examined by affinity chromatography using anti-albumin antibodies coupled to Sepharose 4B, followed by ultrafiltration of radioactivity retained by the immunoadsorbent.

The appropriateness of both diploid and malignant human cell lines for use in an insulin bioassay will be tested based on three criteria: 1) demonstration of cell-surface insulin receptors by peroxidase-antiperoxidase immunocytochemical staining and specific adsorption of ^{125}I-insulin by cells; 2) demonstration of a significant insulin-induced, dose-dependent increment in the rate of glucose uptake by cells, using replicate multiwell cultures and measurement of glucose concentration in the supernatant medium; and 3) prolonged stasis of cell proliferation without cell death in serum-free media (with and without insulin), with resumption of log-phase growth after addition of serum.

An insulin bioassay capable of discriminating the effects of specific ILA and growth factors will thus be developed to generate the following parameters relative to control cultures: cell number (representing stimulation of cell proliferation by growth factors), total glucose uptake (representing ILA irrespective of cell number), and rate of glucose uptake (representing specific ILA). This assay will be used to examine serum fractions generated by molecular size separations (ultrafiltration and gel chromatography) in order to determine the relationship of proliferative effects and specific ILA to total ILA.

The relationship of immunoreactive insulin to specific ILA will be determined by radioimmunoassay of all fractions and the role of biotin and heparin in stimulating ILA will be ascertained by also carrying out the bioassay of all fractions in the presence of these agents.

The range of subjects involved in this research is broad. Examination of insulin structure is important to considerations of aggregation, immunochemistry and biologic activity. Knowledge of cellular insulin receptor properties is basic to evaluation of differential hormone effects relative to hormone concentration and to choice of cells appropriate for an insulin bioassay. Evidence pertaining to mechanisms of insulin action bear upon interpretation of observations that contradict previous reports. A survey of insulin assays, requiring an understanding of the hormone's physiologic and metabolic effects, is necessary for an appreciation of ILA in its proper historical perspective and for evaluation of assays employed in this work. A discussion of atypical ILA must include insulin precursors and, of course, serum growth factors. I review all these topics in what I hope is sufficient depth to enable the reader to evaluate the observations reported and to judge the appropriateness of the conclusions drawn from them.

CHAPTER II

INSULIN: THE ANABOLIC HORMONE

INSULIN—OR LACK OF IT (DIABETES)—IN ANTIQUITY

At least oblique reference to the most striking symptoms of diabetes—excessive thirst (polydipsia) and urination (polyuria)—may be found among the oldest human documents. The Ebers papyrus, the 16th century B.C. Egyptian medical treatise, contains the phrase, "A medicine to drive away the passing of too much urine" (1).

Unequivocal discussions concerning diabetes mellitus appear in Hindu medical texts dating back to the first century B.C. The writers of these texts anticipated use of the term mellitus, a Latin word meaning "honey sweet," by nearly two millenia, when they referred to the disease as Iksumeha or Madhumea (sugar or honey urine). This designation probably derived, however, from the resemblance of the diabetic's almost colorless urine to the juices of the sugar cane or from the particular attraction it had for insects, rather than from its taste, since no mention is made of tasting the urine (2). The writers recognized that the diabetic "gradually loses strength, flesh, and healthy glow of complexion." The symptoms of thirst, fatigue, diminution of sexual capacity, confusion, abscesses and carbuncles are also listed.

The first century Roman medical writer Aulus Cornelius Celsus rendered a concise description of the disease he declined to name when he stated, "But

when the urine exceeds the fluid taken . . . it gives rise to wasting and danger"(3). Although the perceived fluid imbalance was in error, appreciation of the impressive polyuria is clear. During the following century, the Greek physician Aretaeus of Cappadocia rendered the best ancient clinical account of the disease, which not only contained the first mention of the term diabetes, a Greek word meaning "siphon" ("because the fluid does not remain in the body, but uses the man's body as a ladder, whereby to leave it."), but also is the first concise and accurate contribution to the knowledge of diabetes in the medical literature of the western world. He described in great detail this wasting disease as a "melting of the flesh," enumerating such symptoms as thirst and loss of body constituents via the urine (4).

In analyzing two cases of diabetes, Aretaeus' renowned contemporary colleague and compatriot, Galen, demonstrates the Greek gift for intuitive reasoning when he postulates a failure of the assimilatory functions and considers the discoloration of the urine as the result of a lack of "coction" (analogous to the modern concept of metabolism), which Galen describes as an alteration of nutrients in the body (5).

The classic tradition of astute diagnosis was ably continued in medieval times by the great 12th century Jewish Aristotelian scholar, philosopher, and physician, Moses Maimonides, who noted twenty cases of diabetes in Egypt, but only two in Spain and northwest Africa (6). Thus, diabetes mellitus was considered a rare disease until the 17th century, when the British physician Thomas Willis diagnosed a considerable number of cases. Although ancient physicians are widely credited with knowledge of diabetic glycosuria because of references to honey or sugar urine in their accounts of the disease, Willis rendered the first true documentation of the sweet taste of the diabetic's

urine (5). He did not demonstrate the actual presence of sugar in the urine, however, but believed—like the 16th century physician and alchemist Paracelsus—that the focus of the body's derangement is salts.

Willis did recognize that diabetes mellitus is a metabolic disease and in fact was the first to implicate the blood's role in the malady, stating "the serum and the blood are in fault, because they lend too hastily their contents." It was the experiments of the British physician Matthew Dobson a century later that indicated excessive "saccharine matter" in both the urine and serum of diabetics, leading him to conclude that the glycosuria results from hyperglycemia (7). In 1815, the French chemist Michel-Eugène Chevreul showed that this saccharine matter is glucose (5). The foundation thus was laid for the general acceptance of William Cullen's earlier delineation of diabetes mellitus and diabetes insipidus (8).

Several developments during the 19th century formed the real basis for the discovery of insulin. First, the renowned French physiologist Claude Bernard established the concept of homeostasis based on the effects of internal secretions (9). Second, Paul Langerhans, a pupil of the German father of pathology, Rudolph Virchow, described in 1869 a group of cells in the pancreas that were different from the enzyme-secreting acinar cells (10). Third—and most important—was the work of the physiologists Oskar Minkowski and Baron Joseph von Mering.

In 1884, Minkowski had implicated β-hydroxybutyric acid as a major cause of diabetic acidosis, demonstrated a decrease of blood carbon dioxide during diabetic coma, and introduced alkali therapy to counteract the condition (11). Five years later, at the University of Strassburg, Minkowski and Mering attempted to disrupt fat digestion in dogs by removing the pancreas, known

to secrete lipases. They found that the dogs greatly increased their urine output and died in ten to thirty days. The presence of large amounts of sugar in the urine confirmed the diagnosis of classic diabetes mellitus. Furthermore, replacement of the pancreas under the skin ameliorated the symptoms. Thus, these facts and the influence of Bernard's milieu interieur concept enabled Minkowski to postulate the secretion by the pancreas of a substance responsible for the maintenance of systemic carbohydrate homeostasis (12).

When in 1901 the U.S. pathologist Eugene L. Opie described degenerative changes in the Islets of Langerhans associated with diabetes (13,14), leading such physiologists as Sir Edward Sharpey-Schafer to conclude that Islet cells are the source of the postulated hypoglycemic substance (15), the fundamental mechanism of diabetes mellitus was clear. The challenge that remained for a series of investigators during the next twenty years was the extraction of this pancreatic principle in a form capable of reversing diabetic hyperglycemia and its deadly consequences.

Georg Ludwig Zuelzer of Germany in 1907-1909 (16), Israel Kleiner of the United States in 1915-1919 (17), and Nicholas Paulesco of Romania in 1921 (18) were able to reverse the acute symptoms of diabetes with relatively crude pancreatic extracts, but frequent febrile responses to these extracts precluded their usefulness as therapeutic agents.

Zuelzer extracted calf pancreas and, after observing the extract's hypoglycemic effects in animals, attempted to use it to treat human diabetics. He was able to show that the extract reversed diabetic glycosuria, ketonuria, and ketonemia, but consistent hyperpyrexia forced him to abandon these efforts. Zuelzer was also hampered by an inability to monitor blood glucose levels in

humans, since micromethods for determination of the sugar were not developed until 1913 (5).

Kleiner injected aqueous filtrates prepared from canine pancreases into depancreatized dogs and observed almost immediate hypoglycemic effects, consisting of decreases in blood glucose as great as 200 mg/100 ml. This hypoglycemic effect lasted about two hours and was accompanied by decreased glycosuria. Kleiner also demonstrated that extracts from organs other than the pancreas fail to have this effect. He was disturbed, however, not only by febrile reactions, but also by "tremor of the head and hind legs" (probably symptoms of hypoglycemia). Furthermore, the necessity of injecting 100-150 ml of his weak extract to obtain the observed effects presented an obstacle to its use in human subjects.

In a well-controlled, thorough study, Paulesco repeated Kleiner's observations on pancreatectomized dogs and also noted a specific pancreatic extract-induced decrease of blood and urine urea and acetone. He demonstrated dose-response behavior of the extract-induced decrease in blood glucose and urea concentrations, not only in diabetic, but also in normal animals.

Because they isolated it in a form sufficiently pure to be consistently effective in the treatment of human diabetes without serious side effects, the physiologists Frederick Grant Banting and Charles Herbert Best are generally credited with the discovery of the hormone they named insulin, an obvious reference to its origin in the islets of Langerhans. A struggling Canadian orthopedic surgeon, Banting accepted a lectureship at the University of Western Ontario, London, in 1920. It was there, while preparing a lecture, that he came across a paper by Moses Barron, detailing destruction of the acinar pancreas in dogs after ligation of the pancreatic duct (19). At that point,

Banting was struck by the possibility that the proteolytic enzymes secreted by the acinar pancreas could have precluded the preparation of a hormone extract sufficiently potent to treat diabetes and that the ligation described by Barron might provide a solution to the problem. Destruction of the acinar pancreas was later found to be unnecessary for the isolation of biologically active insulin, but the inspiration proved fateful.

Banting obtained permission from John Macleod, director of the physiology laboratories at the University of Toronto, to use one of the laboratories at that institution and there entered into a fortuitous collaboration with Best, a student of biochemistry. The two worked incessantly through the summer of 1921, attempting to induce atrophy of the canine acinar pancreas. Finally, they succeeded in obtaining a potent aqueous extract that had a hypoglycemic effect in pancreatectomized dogs, then obtained a preparation suitable for treatment of human diabetics using an improved acid-ethanol extraction procedure (20). Immediately, Macleod put Best and J. B. Collip to work on the development of a large-scale bovine pancreatic insulin purification procedure for the Connaught Laboratories in Canada and the Eli Lilly Company in the United States (21,22). A grateful scientific community and public were reflected in the award of the 1923 Nobel Prize for physiology or medicine to Banting and Macleod.

Thus, one era ended and another began. The horrifying spectacle of the "melting of the flesh" gave way to the first consistent reports of diabetic neuropathy and angiopathy in those now able to live long enough—60 or more years past their former life expectancy—to manifest these pathologies. The history of diabetes as Aretaeus' "wonderful" and hopeless disease ended; the history of insulin as an illustrious influence on the development of endocrinology,

protein chemistry, and clinical chemistry began. As will be seen, although not the first proven instance of hormone action, insulin was the first protein whose primary structure was elucidated, the first complete protein to be synthesized in the laboratory, the object of the first radioimmunoassay, and the first hormone for which a prohormone was found. Insulin research continues more vigorously than ever, yet the ultimate cause and eradication of diabetes mellitus remain as elusive as ever. Perhaps insulin will be the focus of another discovery that will not only open a new field of endeavour, but will finally eradicate one of the oldest and most perplexing diseases to plague the human species.

PHYSIOLOGIC EFFECTS

Islet tissue containing insulin-secreting β-cells is found in all vertebrates, from cyclostomes to man (23). Insulin-producing cells have also been reported among vertebrate precursors, in the entodermal epithelium of certain starfishes and tunicates (24). Glucagon-secreting α-cells are found in vertebrates only beyond the cyclostomes, with the appearance of the organized proximity of exocrine and endocrine pancreatic tissues (25,26).

In mammals, the islets are scattered throughout the acinar pancreas and consist of cords of intermingled α-cells, β-cells, and D (α_1)-cells, which probably secrete gastrin (27) and/or somatostatin (28). It is in this class of vertebrates that insulin appears to have its greatest integrative influence on systemic anabolism, governing biosynthetic processes involving carbohydrates, proteins, fats, and electrolytes in liver, muscle, adipose tissue, connective tissue, leukocytes, endothelium, and epithelium. Insulin has not been found to

have an effect on nervous tissue or erythrocytes, nor does it promote intestinal absorption or renal tubule reabsorption of glucose.

In mammalian studies, concerning especially rats, dogs, and humans, insulin has been found to increase the uptake of glucose by muscle and adipose tissue (29-32). Although glucose uptake by liver is insulin-independent (33), insulin stimulates glucose utilization in that organ, as it does in muscle and adipose tissue, promoting glycogenesis in liver and muscle (34,35) and glucose oxidation in target tissues (36). At the same time, the hormone inhibits glycogenolysis in liver and muscle and gluconeogenesis in liver (34). The systemic result of insulin effects on target tissue carbohydrate metabolism makes it the most potent hypoglycemic agent of mammalian physiology.

Insulin promotes amino acid uptake and protein synthesis by muscle and liver (37-44), while decreasing tissue proteolysis (45,46). Thus, the hormone creates a positive nitrogen balance. Only recently has the profound impact of insulin on lipid metabolism in adipose tissue and liver been appreciated. The hormone induces the synthesis and esterification of fatty acids while inhibiting the hydrolysis of triglycerides in adipocytes and liver, serving as a potent antagonist of such fatty acid mobilizing hormones as epinephrine, thyrotropin, adrenocorticotropin, glucagon, somatotropin, and glucocorticoids (47-52). This anti-lipolytic effect prevents the formation and appearance in blood of the "ketone bodies," acetoacetate, β -hydroxybutyrate, and acetone. Insulin-induced uptake of K^+ also occurs in the absence of glucose (53) and, therefore, insulin is a hypokalemic, as well as a hypophosphoremic, agent (54).

The enormous impact of insulin on mammalian metabolism is profoundly appreciated by observing the course of "brittle" diabetes mellitus, whether induced by total pancreatectomy or selective destruction of the β -cells by

such agents as alloxan and streptozotocin, or appearing spontaneously, usually in the immature animal (juvenile onset).

Immediately after complete termination of β-cell function, the unopposed stimulation of liver gluconeogenesis by glucocorticoids and glucagon (if loss of islet function is subtotal), liver and muscle glycogenolysis by epinephrine and glucagon, and suppression of tissue glucose uptake by somatotropin, as well as a marked reduction of insulin-dependent glucose uptake and glycogenesis, results in general glucose mobilization and a hyperglycemic state (55,56). Within hours, the concentration of plasma glucose exceeds 300 mg/100 ml, twice the maximum attained in normal individuals (57).

The renal tubules normally cannot reabsorb glucose in excess of 160 mg/100 ml plasma (58) and, as this renal threshold is passed, the sugar "spills over" into the urine, creating glycosuria. Water is lost with the excess glucose, leading to polyuria. The need to replace this water causes polydipsia and, if replacement is insufficient, dehydration ensues.

Thyroid hormone-induced glucose oxidation and the energy requirements of tissues dependent on insulin for glucose uptake quickly result in exhaustion of these tissues' carbohydrate reserves. Epinephrine stimulation of proteolysis results in muscle wasting and mobilization of amino acids, which feed gluconeogenesis in the liver, exacerbating the hyperglycemia. Requisite deamination of these metabolized amino acids drives the plasma urea concentration to excessively high levels (55).

The virtual shutdown of carbohydrate utilization and the potent free fatty acid mobilizing, lipolytic, and anti-lipogenic effects of epinephrine, glucagon, somatotropin, corticotropin (ACTH), and glucocorticoids (48,49,51,59) force the diabetic individual to switch to the products of fatty acid catabol-

ism—the oxybutyrates—as his primary energy currency. Thus, the plasma concentration of free fatty acids as well as of acetoacetate, its reduction product β-hydroxybutyrate, and its spontaneous decarboxylation product acetone-the ketone bodies-rises precipitously, producing a striking lipemia, ketonemia, and a consequent ketonuria. Because two of the three ketone bodies are acids, a dangerous acidosis ensues, leaching K^+ from tissues and causing a further deterioration in the insulin-dependent electrolyte balance.

Acidosis and electrolyte imbalance have a disastrous effect on the central nervous system, leading to coma and death, despite the individual's attempts to alleviate the acidosis by conserving carbon dioxide through a deep Kussmaul respiration (60). Even with insulin control and non-ketogenic maturity-onset diabetes, the induction of normally minor pathways of carbohydrate metabolism by frequent hyperglycemia eventually results in thickening of vascular basement membranes with deposited mucopolysaccharides and abnormal intracellular osmolality in Schwann cells due to high concentrations of sorbitol. These derangements are major factors in diabetic angiopathy (including retinopathy) and neuropathy (61-64).

Because of the potent insulin antagonism of such pituitary hormones as corticotropin, somatotropin, and thyrotropin, such hormones of the adrenal medulla as epinephrine, and such hormones of the adrenal cortex as glucocorticoids as well as the pancreatic hormone glucagon, total pancreatectomy accompanied by hypophysectomy (Moussay animals) and/or adrenalectomy greatly alleviates the consequences of insulin loss, preventing or greatly delaying the onset of ketogenic diabetes by throwing the animal into a precarious balance between hyperglycemia and hypoglycemia (65).

METABOLIC EFFECTS

Most of the observed physiologic effects of insulin are necessarily based on some impact on the quantity or function of the enzymes governing intermediary metabolism. In this respect, the broad anabolic influence that insulin exerts on mammalian physiology can be seen to derive from the various ways in which the hormone affects "key" enzymes* of the major metabolic pathways.

Insulin has been found to influence intermediary metabolism in at least three ways: 1) a delayed influence on enzyme biosynthesis, requiring several hours to several days; 2) an immediate effect on preformed enzyme activity within minutes by affecting the intracellular concentration of one or more "messengers" (see Chapter III); and 3) an indirect feedback inhibition-based modulation of enzyme activity through changes in the concentration of insulin-affected key enzyme reaction products.

Insulin has been found to induce the key glycolytic enzymes glucokinase, phosphofructokinase, and pyruvate kinase (67-69) while suppressing the biosynthesis of the key gluconeogenic enzymes phosphoenolypyruvate carboxykinase, pyruvate carboxylase, fructose diphosphatase, and glucose-6-phosphatase (70-72).

Weber found that in liver the activities and quantities of the key glycolytic enzymes decrease and of the key gluconeogenic enzymes increase after induction of alloxan diabetes in rats (73-74). These trends, including a precipitous rise in the ratio of key gluconeogenic enzymes to key glycolytic

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*The use of the term "key enzyme" here is that of George Weber (66) in that it signifies an enzyme catalyzing an essentially irreversible reaction, which thus is not utilized in an opposing metabolic pathway; e.g., glycogen synthetase for glycogenesis and glycogen phosphorylase for glycogenolysis.

enzymes, continue over a period of several days (66). After insulin is administered to these diabetic rats, the trends reverse themselves and return to normal levels within two days.

The insulin-induced increase in key glycolytic enzyme activities is blocked by such inhibitors of protein synthesis as actinomycin, puromycin, and ethionine (73), while a glucocorticoid (triamcinolone)-induced increase of key gluconeogenic enzyme activities is prevented or reversed by inhibitors of protein synthesis and insulin (75). The same reciprocal relationship of the key hepatic enzymes was seen in starved rats that were subsequently re-fed. Increases of glycolytic enzyme activities and decreases of gluconeogenic enzyme activities upon re-feeding were blocked by administration of anti-insulin serum, indicating that this phenomenon is insulin-dependent. Hexokinase activity, however, remained unchanged during starvation and re-feeding, indicating insulin independence, although Krahl's group have reported insulin restoral of diabetes- and fasting-diminished hexokinase activity in rats (76-77).

Insulin promotes glucose oxidation while providing NADPH for fatty acid synthesis by inducing the key enzymes of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (78-80), and malic enzyme (NADP-malate dehydrogenase) (66,71), providing a critical link in the conversion of glucose to fatty acids. The hormone also induces key enzymes of lipid synthesis.

Like the key glycolytic enzymes, an insulin-induced increase of starvation- and diabetes-depleted citrate cleavage enzyme and fatty acid synthetase was seen in rat liver (81). Evidence for an insulin-induced increase in liver microsomal fatty acid desaturase and glycerol kinase, leading to triglyceride formation, has also been found (82-83). Meanwhile, insulin suppresses the

synthesis of hydroxymethyl glutaryl CoA synthetase, the key enzyme of acetoacetate formation in liver (84).

Insulin helps conserve a pool of amino acids for protein synthesis, thus promoting a positive nitrogen balance, by suppressing the biosynthesis of transaminases and other enzymes of amino acid degradation, as well as the key urea cycle enzymes, ornithine carbamyl transferase (ornithine transcarbamylase), arginine synthetase, and arginosuccinase in liver (85-88). Insulin suppression of urea cycle enzymes not only accounts for the marked increase of urea excretion by diabetics, but also increases the quantity of aspartate available for protein biosynthesis by decreasing urea cycle conversion of the amino acid to fumarate, a substrate for gluconeogenesis in liver cell cytoplasm. The ammonium ion normally converted to urea may be utilized in the glutamine synthetase-catalyzed conversion of glutamate to glutamine, increasing the availability of the latter amino acid for protein synthesis.

Increased glutamine and the carbon dioxide otherwise converted to urea may also serve as substrate for cytoplasmic formation of carbamyl phosphate, which can then be utilized, with aspartate (also a substrate of purine synthesis), in pyrimidine biosynthesis, leading to speculation that insulin causes a substrate-driven increase of nucleic acid synthesis (89). Insulin has also been found, however, to induce rat liver thymidine phosphorylase, the first enzyme in the degradative pathway for the nucleotide (66).

A more direct basis for insulin enhancement of nucleic acid synthesis may be found in evidence that the hormone induces RNA polymerase in rat liver (90), increases DNA polymerase activity in rat liver and mammary epithelium (91,92), and causes cell proliferation in severely diabetic rat liver (91).

The best example of a rapid effect of insulin on enzyme activity is found in hepatic glycogen metabolism, consisting of a simultaneous activation of glycogen synthetase and inactivation of glycogen phosphorylase within a few minutes (93). The hormone also causes an immediate inactivation of triglyceride lipase in adipose tissue (94). Evidence basing these effects on a reversal of the Sutherland cyclic AMP-mediated mechanism for ephinephrine and glucagon action is reviewed in Chapter III.

The most profound immediate effect of insulin on target tissues other than liver-an increase in glucose transport across the cell membrane, followed by a rise in the intracellular concentration of glucose-6-phosphate-remains an enigma. Neither the mechanism nor the structures responsible for this facilitated sugar transport has yet been elucidated, and there exists no clear evidence that insulin has any effect on hexokinase biosynthesis. However, several investigators have reported an insulin-dependent association of type II hexokinase with mitochondria in rat muscle, adipose tissue, and mammary epithelium (95-97), suggesting a rapid enhancement by the hormone of anabolic processes in these tissues through an increase of cellular energy charge, resulting from an accelerated rate of oxidative phosphorylation.

Weber (98) has proposed a system of insulin integration of hepatic intermediary metabolism, resulting from immediate insulin effects and occurring before insulin-induced influence on enzyme biosynthesis. This integrative effect is based on immediate insulin effects on the concentration of metabolites that inhibit key enzymes of pathways involved in their biosynthesis.

Free fatty acids, their oxidation product, acetyl CoA, and the substrates of gluconeogenesis-all decreased by insulin-have been found to inhibit the key enzymes of glycolysis. In addition, free fatty acids, which in high concentrations

nonspecifically inhibit many enzymes, are particularly inhibitory in high physiologic concentrations toward the key enzymes of glycolysis, the pentose phosphate pathway and the tricarboxylic acid cycle.

Insulin decreases liver uptake of L-alanine (99), an inhibitor of pyruvate kinase, and decreases the availability of ATP (by increasing its utilization in glucose-6-phosphate formation and lipid biosynthesis, while decreasing liver adenylate kinase activity (100)), an inhibitor of phosphofructokinase and pyruvate kinase, free fatty acids (by inactivating lipases), and acetyl CoA (by increasing its utilization in fatty acid synthesis).

Insulin's immediate effects in adipose tissue—facilitation of glucose uptake, resulting in a substrate (glucose-6-phosphate)-induced increase in α-glycerophosphate—causes a rapid fall in plasma free fatty acids, which in turn alleviates the free fatty acid feedback inhibition of key enzymes of glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle. These pathways and, consequently, lipid biosynthesis are facilitated before insulin effects on enzyme biosynthesis become apparent.

Even a cursory examination of insulin's effects on mammalian intermediary metabolism thus reveals impressive evidence that it is indeed the master hormone of anabolism in this class of animals, providing all materials—enzymes, substrates, high-energy phosphate anhydrides, and electron donors—necessary for biosynthesis of complex carbohydrates, fats, proteins, and nucleic acids in liver, muscle, adipose and connective tissue, lymphoid cells, epithelium, and endothelium. The fact that insulin triggers an orchestrated chain of such events, commencing immediately after ingestion of a meal and continuing at different levels of metabolic regulation for several days thereafter, also argues for insulin's proposed pivotal role as the "feast or famine" hormone in animal

evolution, enabling an animal to make the most efficient use of ingested nutrients for further utilization in times of starvation that often require vigorous exercise in pursuit of another meal.

One may thus speculate that man-made prosperity has created an evolutionary trap, evidenced by the apparent correlation of maturity-onset diabetes with consumption of fats in industrialized western nations (101).

CHAPTER III

INSULIN ACTIVITY ON THE MOLECULAR LEVEL

THE INSULIN MOLECULE

Extraction and Purification

The first insulin used to treat a human diabetic (January 11, 1922) was extracted from bovine pancreases obtained from commercial sources by Banting and Best. They extracted the pancreatic tissue with cold acidified saline, followed by ethanol to a final concentration of 60%. The filtrate was evaporated to dryness in a stream of warm air, the residue was washed with toluene and then was dissolved in saline. This material possessed an insulin activity of slightly more than one international unit per ml (102).

During the previous year, Banting and Best had also found this method to be the most effective in producing potent insulin extracts from canine and fetal calf pancreas, the latter being the source of a preparation possessing ten times the potency of the adult bovine extract, probably because fetal calf pancreas of under five months' development does not contain pancreatic enzymes, while producing a large quantity of insulin.

Soon after commencement of large-scale commercial production of insulin in 1922, acetone was substituted for ethanol and the wind tunnel was replaced by a vacuum still. After the U.S. chemist John Jacob Abel first crys-

tallized insulin around 1925 (103), it was found that crystallization of the hormone requires zinc or another divalent metal cation, such as cobalt, nickel or cadmium (104). Due in large part to D.A. Scott's delineation of the role of zinc in the crystallization of insulin (105), protamine zinc insulin was introduced in 1936 as a long-acting agent that abrogated the diabetic's requirement for frequent injections of the hormone (106).

As the requirement for pure insulin from a variety of animal species for physicochemical studies of the hormone became felt, ever more sophisticated biochemical separation techniques were brought to bear on the problem of insulin isolation and purification. Techniques used include fractional precipitation with ethanol or salt, isoelectric precipitation, ion exchange chromatography, gel filtration, and partition chromatography using paper and adsorbent columns (104).

For example, rat insulin has been isolated from frozen pancreas extracted in 80% ethanol adjusted to pH 3 with phosphoric acid, followed by addition of ammonium hydroxide to pH 8. After clarification of the solution, insulin is precipitated first by adding ethanol and ether and again with picric acid. The picrate is dissolved in acid acetone, precipitated with excess acetone, washed with ether and dried (104). Bovine insulin has been purified from pancreatic extracts by gel filtration on Sephadex G-50 equilibrated with 0.2 M ammonium bicarbonate, pH 7.8, at 4°C, followed by crystallization from zinc acetate, pH 5.9 (107). Sephadex G-50 has also been used to purify feline (108) and human (109) insulins, using 1 M acetic acid for elution. After crystallization from citrate buffer, the human insulin was found to be relatively free of glucagon. Cellulose acetate electrophoresis has also proven to be an effective means of separating the two hormones (110). The proinsulin (as much as 2%)

contaminating commercial preparations of insulin may also be removed by Sephadex G-50 chromatography with 1 M acetic acid (111).

Insulin has been extracted from human plasma with ethanol, normal butanol and toluene acidified with hydrochloric acid, resulting in recovery of 80-90% exogenous (^{35}S) insulin and a forty-fold increase of endogenous insulin activity (112).

Physical Properties

The zinc-complexed insulin first crystallized by Abel was in the rhombohedral form, consisting of insulin hexamers each containing 2 or 4 zinc atoms (113). Later, the zinc-insulin hexamer was crystallized in monoclinic form in the presence of phenol and a zinc-free orthorhombic form has been crystallized at acid pH (104).

It was the prevalence of the zinc-containing insulin hexamer (M.W. 35,000-36,000) in preparations subjected to physical studies before 1948 that led early investigators to believe that the molecular weight of the hormone lay somewhere between 36,000 and 48,000. In 1948, Gutfreund concluded that these species were aggregates of a molecular weight 12,000 subunit (114). Finally, in 1952, Harfenist and Craig showed that the ultimate insulin subunit, or monomer, was a molecule of approximately 6,000 daltons molecular weight (115), quickly confirmed by elucidation of the hormone's primary structure, which assigned to bovine insulin a molecular weight of 5,734 daltons. Based on its amino acid composition, the molecular weight of human insulin is 5,808 daltons.

The insulin dimer of molecular weight 10,000-12,000 has been shown to have a sedimentation coefficient ($S_{20,w}$) of 1.85×10^{-13} sec., a diffusion

coefficient ($D_{20,w}$) of 13.5 x 10^{-7} cm^2/sec., and a frictional ratio (f/fo) of 1.1, while the monomer of 6,000-7,500 molecular weight shows $S_{20,w}$ = 1.2 x 10^{-13} sec., $D_{20,w}$ = 16 x 10^{-7} cm^2/sec. and f/fo=1.12 (104), indicating that the molecule is nearly spherical in shape.

Both bovine pancreatic insulin and human plasma insulin derived from portal vein blood have been found to have an electrophoretic mobility between albumin and α_1-globulins (116), but a substantial portion of human endogenous plasma insulin of the peripheral circulation has been shown to migrate with β -globulins (see Chapter IV).

Primary Structure

The complete elucidation of the primary structure of bovine pancreatic insulin, an outstanding tour de force achieved by the biochemist Frederick Sanger and co-workers at Oxford University, reported in 1955 (117), was a watershed of protein chemistry, not only because it represented the first amino acid sequence determination of a protein, but also because it conclusively demonstrated the uniqueness and consistency of protein structure (i.e., the amino acid sequence of every molecule of a given protein is identical, but different from every other protein) and proved the veracity of the peptide bond hypothesis advanced by Hofmeister and Fischer in 1906 (118).

The chain of events leading to the elucidation of the amino acid sequence of bovine insulin, shown in figure 1, began in 1945 when Sanger introduced 1,2,4-fluorodinitrobenzene (FDNB, since called the Sanger reagent) for dinitrophenyl (DNP) labelling of amino-terminal amino acids according to the following reaction:

FIGURE 1. AMINO ACID SEQUENCES OF INSULINS AND CUMULATIVE AMINO ACID REPLACEMENTS AT POSITIONS WHERE VARIATIONS OCCUR.

B chain insulin:

[a] pig, dog, cattle, sheep, goat, horse; sperm, fin and sei whales

[b] human, African elephant

[c] rabbit

[d], [e] rat, mouse

[f] chicken

[g] guinea pig

[h] coypu

[i] bonito‡

[j] cod fish

[k] angler fish

* Either B 24 or B 25 phe deleted.

† Deletion.

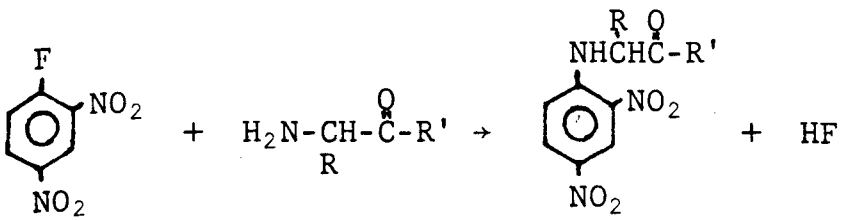
‡ Single component from mixture of bonito, tuna and swordfish insulins.

A chain insulin

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Human, rabbit, pig, dog, sperm & fin whale	Gly	Ile	Val	Glu	Gln	Cys	Cys	Thr	Ser	Ile	Cys	Ser	Leu	Tyr	Gln	Leu	Glu	Asn	Tyr	Cys	Asn	
Cattle								Ala		Val												
Sheep, goat								Ala	Gly	Val												
Horse									Gly													
Sei whale								Ala		Thr												
Rat				Asp																		
Chicken								His	Asn	Thr												
Guinea pig				Asp					Gly	Thr		Thr	Arg	His			Gln	Ser				
Coypu				Asp					Asn				Arg	Asn			Met	Ser				Asp
"Bonito" ‡								His	Lys	Pro		Asn	Ile	Phe	Asp		Gln					
Cod fish				Asp				His	Arg	Pro		Asp	Ile	Phe	Asp		Gln					
Angler fish								His	Arg	Pro		Asn	Ile	Phe	Asp		Gln					
African elephant									Gly	Val												
				Glu				Thr	Ser	Ile		Ser	Leu	Tyr	Gln		Glu	Asn				
				Asp				Ala	Gly	Val		Thr	Arg	His	Asp		Gln	Ser				Asp
								His	Asn	Thr		Asn	Ile	Asn			Met					
									Lys	Pro		Asp		Phe								
									Arg													

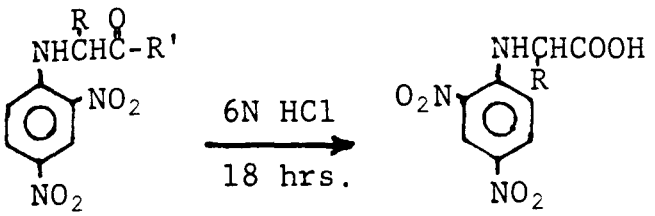
B chain insulin

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
[a]	Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Ala
[b]																														Thr
[c]																														Ser
[d]			Lys						Pro																					Ser
[e]			Lys																											Met-Ser
[f]	Ala-Ala																										Ser			
[g]			Ser-Arg							Asn				Thr			Ser			Gln-Asp-Asp							Ile		Asp	
[h]	Tyr		Ser		Arg					Gln			Asp-Thr				Ser			Arg-His				•			Arg	Asn-Asp	
[i]	Val-Ala-Pro-Pro												Asp							Asp							Asn	†	
[j]	Met-Ala-Pro-Pro												Asp							Asp							Asn	†	
[k]	Val-Ala-Pro-Pro												Asp							Asp							Asn	†	
	Phe-Val-Asn-Gln-His								Ser	His			Glu	Ala			Leu			Gly	Glu	Arg		Phe	Phe		Thr		Lys	Ala
	Val-Ala-Ala-Lys-Arg-Arg								Pro	Asn			Asp	Thr			Ser			Gln	Asp	Asp		•			Ser		Met	Thr
	Met-Tyr-Pro-Ser									Gln										Arg	His						Ile		Asn	Ser
			Pro																								Arg			Asp
			Ala																								Asn			†



(119)

The labelled protein was then subjected to acid hydrolysis as follows:



+ amino acids.

The bright yellow DNP-N-terminal amino acid was separated from other amino acids by extraction with ether and identified by partition chromatography with reference to standard DNP-amino acids. Thus, FDNB labelling of insulin followed by acid hydrolysis yielded the expected ε-DNP derivative of lysine and α-DNP derivatives of phenylalanine and glycine. Based on the molecular weight of 12,000 assigned to insulin at that time, Sanger deduced that the hormone consists of four amino acid chains, two with N-terminal phenylalanine and two with N-terminal glycine (120).

By 1947, Sanger was able to obtain the separated chains of insulin intact by oxidation of the hormone with performic acid, yielding the cysteic acid derivatives (121). Because an accurate amino acid analysis of insulin was available to Sanger from the beginning of his endeavour, he knew that complications resulting from oxidation of tryptophan and methionine would be avoided since these amino acids are not present in bovine insulin. He found two kinds of polypeptide chains: one, designated A-chain, was acidic and had a simpler amino acid composition than the complete insulin molecule, lacking lysine, arginine, histidine, phenylalanine, threonine, and proline; the other, designated B-chain, was basic, containing all the basic amino acids of the insulin molecule.

The A-chain, with N-terminal glycine, was found to consist of 21 residues, four of which were cysteic acid, while the B-chain, with N-terminal phenylalanine, possessed 30 residues, two of which were cysteic acid.

The B-chain, having a more distinctive composition than the A-chain, proved easier to study, and was sequenced first. Mild acid hydrolysis yielded about 45 peptides of varying size, which were separated and recovered by electrophoresis, ion-exchange chromatography, and adsorption on charcoal, followed by two-dimensional paper chromatography. Such treatment of the DNP derivatives of the separated chains enabled the first four N-terminal amino acids of the B-chain and the first five N-terminal amino acids of the A-chain to be deduced. Preliminary sequencing and amino acid analysis of the separated chains demonstrated that insulin consists of only two kinds of polypeptide chains. Thus, it was concluded that insulin either consists of two identical halves or is a molecule of molecular weight 6,000. The latter alternative was later found to be the case.

Peptide fragments enabled all but four B-chain amino acids to be assigned their proper sequence. The complete B-chain amino acid sequence was determined in 1950 after additional, longer peptides were obtained by proteolytic cleavage with pepsin, trypsin, and chymotrypsin (122), which also enabled assignments of positions for glutamine and asparagine (123), otherwise converted to their corresponding acids by acid hydrolysis.

The major problem encountered in sequencing the A-chain arose from the juxtaposition of two cysteine residues at positions 6 and 7, resulting in the formation of peptides that could not be separated adequately by paper chromatography. This problem was solved by paper electrophoresis at pH 2.75, at which point carboxylic acids are uncharged, cysteic acid is negatively

charged, and amines are positively charged. Thus, the sequence of the A-chain was known by 1953 (124,125).

At this time, a molecular weight of approximately 6,000 daltons had already been assigned to insulin. Thus, the hormone had to consist of one copy of each of the two polypeptide chains, A and B, linked by cystine disulfide bonds. The disparity between the number of cysteines found in the A and B chains indicated that they were linked by two interchain disulfide bonds and that the remaining two cysteines in the A-chain formed an intrachain disulfide bond. Because of rearrangement of disulfide bonds that occurs in acid solution, however, it wasn't until 1955 that the correct linkages could be determined after it was learned that enzymic proteolysis at neutral pH and acid hydrolysis in the presence of sulfhydryl compounds such as thioglycollic acid proceeds without disulfide rearrangement (126).

Implications of Variations in Primary Structure

More than twenty distinct amino acid sequences for insulin from the various species studied, the majority of which are depicted in figure 1, are now known (127). It may be seen that the insulin molecule consists of certain amino acids that are invariant among the different species: all the cysteines, A1, A2, A16, A19, B6, B8, B11, B12, B15, B16, B18, B23, B24, and B26 hydrophobic residues, and A5 and A21 amides; other amino acids that are conservatively variant: A3, B1, and B2 apolar residues, A18 polar residue, and A4, B5, and B13 charged residues; and the remaining amino acids, that are freely variant, especially at positions A8, A9, A10, B20, B21, B22, B29, and B30. In addition, coypu (or nutria, Myocastor coypus, a large South American rodent) insulin A-chain has been found to have an additional amino acid, a

C-terminal aspartate, and B-chain one less residue at position 25, while certain fish insulin B-chains have an additional amino acid at the N-terminus, but are lacking an amino acid at the C-terminus. Because even guinea pig, coypu, and fish insulins exhibiting differences of 19 to 22 residues compared to bovine insulin have been shown to have biological activity among different species (128), one may conclude that the invariant residues are important to the biological activity of the hormone, either by insuring a molecular conformation that preserves the shape of the active site or by functioning in the active site itself.

The results of chemical modification of the insulin molecule, summarized in Table I, tend to confirm that invariant amino acids play a critical role in the hormone's biological activity. Specifically, oxidative or reductive cleavage of the disulfide bonds and removal of the A1 glycine, A2 isoleucine, A21 asparagine, and the last eight amino acids of the B-chain, which include B23 glycine, B24 phenylalanine, and B26 tyrosine, result in serious losses of activity. On the other hand, removal or modification of the variable B1, B29, and B30 residues causes no significant reduction of insulin activity. Further confirmation of the modification studies is provided by proinsulin, which possesses 17% insulin bioactivity (141) and 0.06-28% insulin immunoreactivity (136). The proinsulin connecting peptide, which joins the amino terminus of the A-chain to the carboxy terminus of the B-chain, not only obscures the A-chain amino terminal residues, but also is known to cover the A14 and A19 tyrosines (143), which appear to be important to insulin immunoreactivity.

TABLE I. EFFECTS OF CHEMICAL MODIFICATIONS ON INSULIN BIOLOGICAL ACTIVITY AND IMMUNOREACTIVITY

Altered Residues	Type of Residue*	Method of Alteration	% Biol. Act. of Native Insulin	% Immunoreactivity of Native Insulin	Ref.
Free amine-bearing	1 I, 1 C, 1 V	acetylation	78-98[a]	——	129
Free carboxy-bearing	1 I, 2 C, 3 V	esterification	<0.5[g]	——	130
Cystine S_2	I	reduction	45-63[a]	——	131
		oxidation	0[a,f]	——	132
A7, B7 cystine	I	S-carboxy-methylation	40-100[a,f]	——	133
		S-sulfonation	15[a]	——	133
A6, A11 cystine	I	S-thionylation	1[f]	†	134
Tyrosine-OH	3 I, 1 V	acetylation	<3[a]	——	129
A1 glycine	I	removal	2-10[f,h]	20-30[j]	133
		amine removal	15[h]	40[j]	133
		arginine addition	59-68[a,h]	40[j]	133
		lys-arg addition	20[h]	34[j]	133
		acetylation	40-100[f,h]	47[j]	133
			35-40[h]	——	135

TABLE I (CONTINUED)

Altered Residues	Type of Residue*	Method of Alteration	% Biol. Act. of Native Insulin	% Immunoreactivity of Native Insulin	Ref.
A1 glycine	I	t-butoxy-carbonylation	17[h]	——	135
		2-dimethyl-3-formyl-D-thiazolidine-4-carbonyl conjugation	13[h]	——	135
A14 tyrosine	V	nitration	60-70[a,c]	6-38[j,k]	136
		iodination	100[i]	93[j]	137
A14, A19 tyrosines	V, I	iodination	70[a,c]	4-59[j,k]	136
A21 asparagine	I	removal	0-2[g]	——	138
A-chain	10 I, 3 C, 8 V	removal	0[a,b]	——	139
B1 phenylalanine	C	removal	90[f]	103[j]	133
		p-iodination	60-70[h]	85[j]	133
		fluorescein isothiocyanate conjugation (FITC)	40[a]	24-56[j]	136
B29 lysine	V	ε -acetylation	75-100[f,h]	85[j]	133
B30 alanine	V	removal	98-100[a,g]	——	138,140
		arg_2 addition	61[a]	——	141

TABLE I (CONTINUED)

Altered Residues	Type of Residue*	Method of Alteration	% Biol. Act. of Native Insulin	% Immunoreactivity of Native Insulin	Ref.
B1 phe/B29 lys	C, V	gly subst./ε -acetylation	35[a]	25[j]	133
B1 phe,B29 lys	C, V	acetylation	85-100[f,h]	30[j]	133
B1-B6	1 I, 3 C, 2 V	removal	>44[a]	——	142
B23-B30	3 I, 5 V	removal	15[d,g]	——	138
			<0.2	——	143
			0[a]	0.08-2.0[j,k]	136
			<4[a,g]	——	148,149
B-chain	12 I, 4 C, 14 V	removal	0[a,b]	——	139
A1 gly, B1 phe	I, C	removal	1.6-7[f,h]	15[j]	133
		FITC	4[a]	3-32[j]	136
A1 gly, B1 phe, B29 lys	I, C, V	FITC	0[a]	0-4[j]	136
A1-A2, B1-B2	2 I, 2 C	removal	0.2[h]	2[j]	133
A21 asn, B30 ala	I, V	removal	±[a]	0.17-3.5[j,k]	136

FOOTNOTES TO TABLE I.

a. Mouse convulsion assay
b. Mouse hemidiaphragm assay
c. Mouse blood sugar assay
d. Rat hemidiaphragm assay
e. Rat epididymal fat pad assay
f. Rat blood sugar assay
g. Rabbit blood sugar assay
h. Fat cell assay (incorporation of 3-^{3}H glucose into adipocyte lipids; 144,145)
i. Fat cell assay (glucose oxidation)
j. Radioimmunoassay
k. Immunohemolysis

* I = invariant
C = conservatively variant
V = variant

Secondary Structure

Calculations based on low ultraviolet optical rotatory dispersion (ORD) and circular dichroism (CD) spectra have led investigators to conclude that 30 to 40% of insulin residues are confined to tight right-handed α-helical conformations (146,147). This figure is in good agreement with the 35% α-helix found in x-ray diffraction studies, which also indicate that nearly 50% of the insulin residues contribute to right-handed helices and none to left-handed helices (104,143). All the α-helical residues are found in two stretches of α-helix, one in the A-chain, between A2 isoleucine and A8 alanine, and the other in the B-chain, between B9 serine and B19 cystine. A stretch of non-α-helix lies between A13 leucine and A21 asparagine. In addition, hydrogen bonds are formed between the A19 carbonyl oxygen and the B25 α-amido hydrogen, the A21 α-carboxy oxygen and the B22 arginine guanidinium amino hydrogen, and the A4 glutamate carboxy oxygen and the B29 α-amido hydrogen, while the C-terminal residues of the B-chain, from B21 glutamate to the B30 terminus, form half of an antiparallel β-pleated sheet important in insulin dimer formation (143) (See Chapter IV, "Insulin Aggregates").

Tertiary Structure

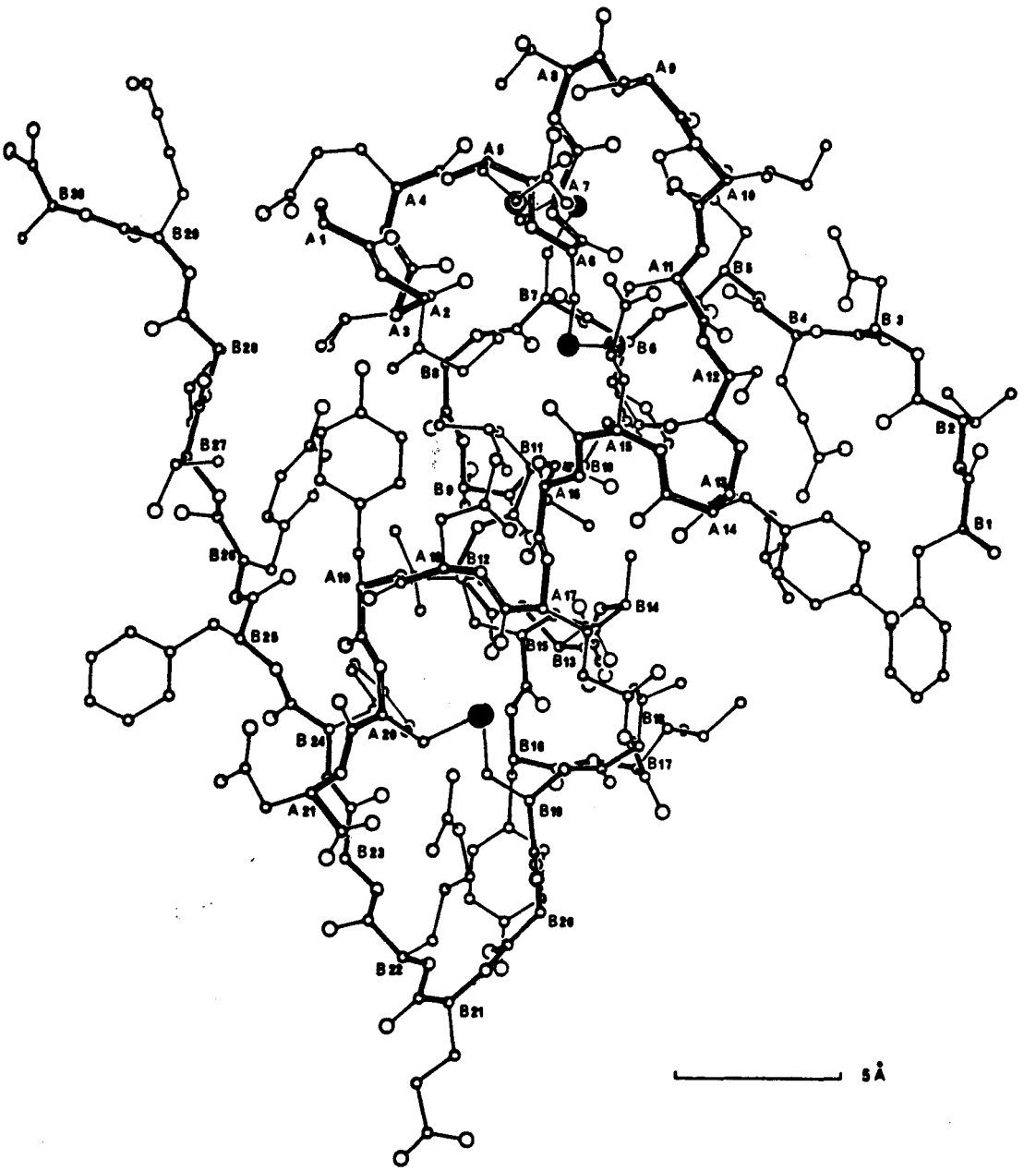
The elucidation of insulin's tertiary structure began in 1935, when the British chemist and crystallographer Dorothy Hodgkin crystallized insulin in the rhombohedral form, as Abel had done, and performed single-crystal x-ray analysis on the preparation (150). She found that the protein molecular weight of the unit cell of these crystals was about 36,000, the same value Svedberg had deduced from sedimentation velocity studies of insulin in solution (151), and that the crystals had trigonal symmetry. This finding meant that the unit

could be divided into three equivalent parts, each with a molecular weight of 12,000, suggesting that the predominant insulin species being studied at the time consisted of at least three equal subunits. It is now known that this species is actually the insulin hexamer.

It was not until 1960 that Barbara Low, using the acid orthorhombic crystal form of insulin, demonstrated that each asymmetric unit contained two molecules related by a twofold (xy-) axis perpendicular to the vertical threefold crystal (z-) axis (152). After Schlichtkrull's discovery that a minimum of two zinc ions per hexamer are necessary for crystallization of insulin in the rhombohedral form (153), it was concluded that these ions must lie along the threefold axis, equally spaced above and below the local twofold axes. After isomorphous replacements of these zinc atoms with heavy metal atoms were made, x-ray diffraction patterns were obtained that enabled the construction of electron density maps at 2.8 Å resolution. Based on the known primary structure of porcine insulin, a three-dimensional model of the porcine insulin monomer, with a diameter of 50 Å and a height of 35 Å (pictured in figure 2), was constructed (154,155).

Examination of the tertiary structure of the insulin monomer, based on x-ray diffraction studies, reveals that the B-chain termini emerge from the central stretch of α-helix, through sharp turns at B8 glycine and the tetrapeptide from B20 glycine to B23 glycine, in a fully extended conformation. The A-chain sits compactly between these arms, on the B-chain α-helix. The A2-A8 α-helix runs across the top of the molecule and the A13-A21 helix folds across the face of the molecule to bring the C-terminal residues of the A-chain back beneath those of the A-chain N-terminus. Eight of 14 invariant hydrophobic residues—B6, B11, B15 and A16 leucines, A2 isoleucine, B18 valine,

FIGURE 2. REPRESENTATION OF THE TERTIARY STRUCTURE OF THE INSULIN MONOMER, VIEWED PERPENDICULAR TO THE THREEFOLD AXIS


A 8
A 9
A 5
A 4
A 7
A 10
A 1
A 6
A 11
B 7
B 5
A 2
B 4
B 3
A 3
B 6
B 8
A 12
B 2
B 11
A 15
B 27
B 10
A 13
B 9
A 16
B 1
A 14
B 26
A 18
B 12
A 19
A 17
B 14
B 25
B 15
B 13
B 16
B 24
A 20
B 18
B 17
A 21
B 19
B 23
B 28
B 22
B 21
5 Å

B24 phenylalanine, and B26 tyrosine—form the molecule's apolar core, while many other nonpolar residues are located on the surface with their side chains directed inward toward the interior of the molecule. Thus, the invariant hydrophobic residues appear to be critically important to the maintenance of the molecule's tertiary structure, which is requisite for insulin's biological activity, as evidenced by the fact that desoctapeptide (B23-B30 deletion) insulin, which has not been found to possess more than 15% insulin activity, has been shown by CD studies to undergo extensive conformational changes compared to native insulin (156).

The B-chain α-helix is flanked by the two interchain disulfide bonds, the B7-A7 cystine, which lies on the surface of the molecule, and the B19-A20 cystine, which is partially concealed. The intrachain A6-A11 cystine is completely buried in the hydrophobic core. A number of invariant residues at the surface of the monomer, including several apolar amino acids with their side chains directed outward, appear to be important to the biological activity and aggregative properties of insulin. A1 glycine, A5 glutamine, A19 tyrosine, A21 asparagine, and B25 phenylalanine are especially likely to be involved in the active site (135), with an important contact being made between the A19 tyrosine phenolic hydroxy group and the A2 isoleucine side chain. Removal of A21 asparagine, which nearly abolishes biologic activity, has also been found to cause extensive conformational alterations and to reduce greatly the ability of the radiolabeled (^{125}I) insulin derivative to bind to adipocyte receptors (135).

The remainder of the insulin monomer surface consists of two hydrophobic faces, one consisting of B12 valine, B16 tyrosine, and B30 alanine and the other of B1 phenylalanine, B14 alanine, B17 leucine, B18 valine, A13 leucine, and A14 tyrosine, which are involved in formation of the insulin dimer and

hexamer. The quaternary structure of insulin is dealt with further in Chapter IV, where insulin aggregates are discussed.

Synthesis

During the 1960s, insulin was the object of yet another landmark in protein chemistry, the first laboratory synthesis of a complete protein, carried out by Katsoyannis and co-workers. The synthesis involved initial amino acid conjugation, peptide elongation, and joining of peptide fragments after reactive side chains and amino and carboxy termini had been masked with blocking groups easily removed under mild conditions with a minimum of racemization to yield a crystalline product readily separated from other reaction components. The A- and B-chains were synthesized separately as sulfocysteinyl derivatives and, after reduction of the A-chain to the cysteinyl form, they were combined in the presence of air, resulting in a 12-16% yield of biologically active, completely synthetic sheep insulin (157-158). Synthesis of insulin derivations has proven to be a valuable means of studying the effects on biological activity of the otherwise unattainable modification of specific amino acids.

THE INSULIN RECEPTOR

It is now widely recognized that the first event of cellular hormone action is the binding of the hormone to a cell "receptor." Indeed, one may apply equally well to any hormone receptor Pedro Cuatrecasas' operational definition of the insulin receptor (159), which "signifies those molecules of the cell which are uniquely capable of recognizing and interacting with insulin with a high degree of selectivity and affinity and which, in addition, possess the capability of conveying the occurrence of the interaction to biochemical

processes resulting in metabolically significant events. Thus, the receptor has at least two functions: 1) to specifically recognize insulin and 2) to somehow convey to other molecules the fact that insulin has been recognized."

In relation to the first function, it is pertinent to examine where in the cell recognition of insulin takes place, the specificity and affinity of the insulin-receptor association, the nature of the insulin receptor, the fate of both the hormone and receptor, and the means by which the cell modulates its insulin response at the receptor level; regarding the second function, the question obviously remains how the signal generated by insulin-receptor association is conveyed to the cell's metabolic machinery.

Location

Unlike steroid and thyroid hormones, which enter the cell before encountering specific receptors, protein hormones are now generally believed to trigger their effects at the cell surface by binding to receptors integrally associated with the plasma membrane. Several lines of evidence strongly suggest that insulin receptors are situated exclusively at the surface of the cell:

1. Specific binding of insulin to fat cell homogenate fractions is almost exclusively limited to particulate, or plasma membrane, fractions, which exhibit an insulin binding capacity comparable to that of intact adipocytes. Little binding of insulin to nuclear or mitochondrial fractions was found and the soluble cellular fraction did not compete effectively with insulin binding to membranes (160,161).

2. Trypsin treatment of intact adipocytes and isolated adipocyte plasma membranes abolishes the ability of these preparations to bind insulin (161). The same abolition of insulin binding to adipocytes was found after the cells

were exposed to trypsin covalently bound to agarose beads larger than the cells themselves, indicating that the insulin receptors are limited to the cell surface (162).

3. Insulin covalently coupled to dextran-40 (average molecular weight = 40,000) stimulates glycogen synthesis and sugar transport by intact rat diaphragm (163) and stimulates glucose metabolism in adipose tissue (164). Insulin covalently coupled to dextran-2000 (average molecular weight = 2,000,000) has been found to stimulate glucose metabolism in adipocytes (165).

4. Cuatrecasas attached insulin to agarose beads 60 to 300 μm in diameter via the B1 α-NH_2 or the B29 lysine ε-NH_2 group (159,166). As little as 1 to 3 μ U of this preparation per ml immediately stimulated glucose utilization and inhibited hormone-stimulated lipolysis in adipocytes 50 to 100 μm in diameter, increased RNA synthesis and α-aminoisobutyrate accumulation in isolated mammary cells, and activated glycogen synthetase in tadpole liver slices (159,166-169). The increased glucose utilization by adipocytes could be completely reversed by addition of anti-insulin serum, even after prolonged incubation with insulin-Sepharose (166).

Extensive washing of the beads with 8M urea and 6M guanidine did not result in liberation of free insulin (166), but Oka and Topper (170) have found that an insulin-like material can be extracted from insulin-Sepharose in the presence of 2.5% bovine serum albumin. This material elicits "super-insulin" responses from virgin and pregnant mouse mammary epithelial cells, consisting of greater than standard insulin stimulation of α-aminoisobutyrate uptake, DNA synthesis, and glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase activities. The increment in α-aminoisobutyrate uptake, greater

than 5 times that obtained with insulin, is completely abolished by anti-insulin serum.

5. Inverted, or "inside-out," adipocyte plasma membrane vesicles, or "ghosts," don't bind insulin. If such inside-out ghosts are formed in the presence of insulin, the vesicle-bound insulin exchanges with the medium much more slowly than when "right-side out" vesicles are used, but no discrepancy is seen after the inverted vesicles are broken (171). The observations indicate that insulin receptors are located on the outside of the plasma membrane.

Although there appears to be widespread concurrence that insulin receptors are confined to the plasma membrane, controversy, which is a common feature in many areas of insulin research, has arisen around this issue also.

Goldfine and co-workers are foremost among those who postulate that intracellular penetration and binding of insulin to receptors on organelle surfaces is the underlying mechanism of many insulin effects. They have reported a reversible, saturable binding of ^{125}I-insulin to rat liver nuclei which they determined to be free of plasma membranes by electron microscopy and the absence of Na^{-}-K^{+} ATPase activity, a plasma membrane marker (172). The binding, which amounts to about 10% of that found with plasma membranes, is abolished by trypsin treatment and inhibited by insulin analogues in proportion to their biologic activity and ability to inhibit binding of radiolabelled insulin to plasma membrane receptors.

These investigators obtained similar results with cultured human lymphocytes, finding 7% of total insulin bound to cells associated with purified nuclei (173). Using radioautographic analysis, they observed 54% of grains over the plasma membrane, 19% over the cytosol and 21% over the nucleus 5 minutes after addition of ^{125}I-insulin to lymphocytes at 37°C. After 120 minutes,

38% of grains were observed over the plasma membrane, 27% over the cytosol, and 28% over the nucleus. Goldfine's group have also demonstrated intracellular uptake of insulin by cultured lymphocytes using radioautographic electron microscopy (174). They noted increasing concentrations of grains over the cell interior, localized mainly over the cytoplasm and nucleus, from 30 seconds to 30 minutes after addition of ^{125}I-insulin at 37°C.

Several other investigators have used radioautography as a basis for claims of intracellular penetration of insulin. Carpentier and co-workers found a penetration of ^{125}I-insulin into cultured lymphocytes of 0.9 μm (175) and into rat hepatocytes of 1.6 μm at 37°C (176). In the latter case, it was found that I-125 activity became increasingly associated with lysosomes, possibly indicating a mechanism of insulin degradation following pinocytosis. Apparent support for this contention may be found in the results of Bergeron et al. (177), who found that in rat livers removed 3 minutes after injection of 68.2 μ Ci (426 ng) ^{125}I-insulin into the portal vein, radioautographic grains were confined to the plasmalemma, but that in livers removed 10 to 20 minutes after injection, a high proportion of grains were located over lysosomes, Golgi apparatus and other organelles.

Properties

The necessity of strong insulin binding to cells prior to the onset of the hormone's biological effects was first postulated by W.C. Stadie and co-workers in 1949 (178) after they observed that the action of insulin on glycogen synthesis in diaphragm muscle, initiated during a short exposure to insulin, persisted after the tissue was rinsed with and transferred to insulin-free medium. Subsequently they found that ^{131}I- and ^{35}S-labeled insulin was bound by muscle

in proportion to the hormone's effect on glycogen synthesis (179). Narahara (180) also found that an immediate binding of insulin to frog sartorius muscle precedes the insulin-dependent acceleration of glucose transport, with an appreciable interval observed between binding and the commencement of transport acceleration. Numerous investigators have demonstrated that the specific binding of insulin from various animal species, proinsulin, and several insulin analogues to isolated cells and cell membranes is directly proportional to the biologic activity of the insulin or insulin analogue, as determined by such criteria as glucose oxidation in adipocytes. Receptor preparations employed include rat liver membrane (181,182), isolated rat adipocytes (159), cultured human lymphocytes (183), and cultured human fibroblasts (184).

Although, generally, procedures that result in alterations of the insulin-binding properties of cell receptors also cause quantitatively similar impairments of insulin-induced metabolic responses in these cells (159), the insulin-receptor interaction and the hormone's physiologic effects can be uncoupled. Insulin binds to frog muscle at 0°C, but does not cause the increase of glucose transport seen at higher temperatures (180). Treatment of cells with neuraminidase, which removes terminal sialic acid residues from cell-surface glycoproteins, abolishes insulin-induced glucose uptake and inhibition of lipolysis, but doesn't alter insulin binding to the cells (159,171,183,185-187). Starvation of rats or prior treatment with prednisone or streptozotocin reduces the metabolic response of fat cells to insulin, but does not affect the quantity or affinity of the receptors for the hormone (188). Furthermore, the binding of insulin to fat cells obtained from hamsters, rabbits, mice and guinea pigs is similar to that of rat adipocytes, but cells from the former group of rodents are significantly less responsive to insulin than are rat cells (188). It has also

been reported that the insulin insensitivity of large fat cells is not due to a reduced insulin binding (189).

The properties of insulin-receptor interactions have been studied in a great variety of tissues, mainly from humans and rats. It may be seen from Table II that insulin receptors fall roughly into two categories: high affinity ($K_{assoc.} \simeq 10^9$-10^{10} M^{-1})/low concentration (40-11,000 receptors per cell) and low affinity ($K_{assoc.} \simeq 10^7$-10^9 M^{-1})/high concentration (1,300-250,000 receptors per cell). These categories of insulin receptors, represented by curvilinear Scatchard plots (201), have been found in cells isolated from animal tissues, circulating leukocytes, cultured cells, plasma membrane preparations and solubilized membrane proteins. Good correspondence of affinities for insulin of different preparations from the same tissue (e.g., rat adipocytes) have been reported.

Association of insulin with human erythrocytes, which are insensitive to the hormone, is of a decidedly lower order than the other tissues studied and may be due to nonspecific protein binding (202). Crofford et al. observed no significant binding of ^{125}I-insulin to erythrocyte plasma membranes (203). Likewise, no specific binding of insulin to circulating human lymphocytes purified on a nylon-fiber column was found by Krug et al. (195), contrary to reports of insulin binding to normal circulating lymphocytes by others (196,204). Subsequently, it was demonstrated that the specific insulin binding observed using preparations of normal human mononuclear leukocytes was due to monocytes, not lymphocytes (205). Lymphocytes induced to undergo blastogenesis in short-term culture by mitogens such as concanavalin A (195), immune stimulated lymphocytes (206), and malignantly transformed lymphocytes in continual culture, obtained from hosts suffering from lymphoproliferative dis-

TABLE II A. SOME PROPERTIES OF INSULIN RECEPTORS

Species	Tissue	Preparation		Temp. °C	pH optimum	$K_{assoc.}$ M^{-1}	$K_{dissoc.}$ M	k_1* $M^{-1}sec.^{-1}$	k_{-1}† $sec.^{-1}$
Human	Adipose	Isolated cells	1.			6.7×10^8	1.5×10^{-9}		
			2.			1.3×10^8	8.0×10^{-9}		
	Fibroblasts	Cultured cells	3.	15		4.9×10^9	2.0×10^{-10}	1.7×10^5	3.4×10^{-5}
	Placenta	Plasma membranes	5.	30		5.0×10^7	2.0×10^{-8}		
		Particulate receptors	6.	4		2.8×10^9	3.6×10^{-10}		
			7.	4		2.5×10^7	4.0×10^{-8}		
			8.	24		2.0×10^8	5.0×10^{-9}		
			9.	24		4.7×10^7	2.1×10^{-8}		
		Soluble receptors	10.	4	7.8	1.0×10^9	1.0×10^{-9}	6.6×10^3	6.6×10^{-6}
			11.	4		3.1×10^8	3.2×10^{-9}		
			12.	24		3.0×10^9	3.3×10^{-10}	3.8×10^4	1.3×10^{-5}
			13.	24		1.5×10^8	6.7×10^{-9}		

TABLE II A. (CONTINUED)

Species	Tissue	Preparation		Temp. °C	pH optimum	$K_{assoc.}$ M^{-1}	$K_{dissoc.}$ M	k_1* $M^{-1}sec.^{-1}$	k_{-1}† $sec.^{-1}$
Human	Placenta	Cultured cells	14.	15	7.6	4.8×10^{9}	2.1×10^{-10}	4.8×10^{6}	1.0×10^{-3}
			15.	15		9.8×10^{8}	1.0×10^{-9}		
	Granulo-cytes	Circul. cells	16.	22	8.0	6.0×10^{9}	1.7×10^{-10}	7.4×10^{6}	1.2×10^{-3}
			17.	22		2.0×10^{7}	5.0×10^{-8}		
	Lymphocytes	Cultured cells	18.	15	7.8	1.2×10^{10}	8.3×10^{-11}	1.0×10^{6}	8.3×10^{-5}
			19.	15		1.1×10^{9}	9.1×10^{-10}	9.2×10^{5}	8.3×10^{-4}
			20.	30		1.5×10^{9}	6.7×10^{-10}	1.2×10^{6}	8.1×10^{-4}
	Erythrocytes	Circul. cells	21.	15	8.0	2.1×10^{6}	4.8×10^{-7}	3.7×10^{2}	1.8×10^{-4}
			22.	15		5.8×10^{2}	1.7×10^{-3}		
Rat	Liver	Isolated cells	23.			1.4×10^{10}	7.1×10^{-11}		
		Membranes	24.	24		1.5×10^{10}	6.7×10^{-11}	3.5×10^{6}	2.7×10^{-4}
		Soluble receptors	25.	24		7.7×10^{9}	1.3×10^{-10}		

TABLE II A. (CONTINUED)

Species	Tissue	Preparation		Temp. °C	pH optimum	$K_{assoc.}$ M^{-1}	$K_{dissoc.}$ M	k_1* $M^{-1}sec.^{-1}$	k_{-1}† $sec.^{-1}$
Rat	Adipose	Isolated cells	26.	24		1.3×10^{10}	8.0×10^{-11}	1.5×10^{7}	7.4×10^{-4}
			27.	24		2.0×10^{9}	5.0×10^{-10}		
			28.	24		3.3×10^{8}	3.0×10^{-9}		
			29.	37		3.3×10^{8}	3.0×10^{-9}	4.2×10^{5}	1.2×10^{-3}
		Membranes	30.	24	7.5	1.3×10^{10}	7.5×10^{-11}	8.5×10^{6}	4.2×10^{-4}
			31.	24		2.0×10^{9}	5.0×10^{-10}		
			32.	24		3.3×10^{8}	3.0×10^{-9}		
		Soluble receptors	33.	24		7.7×10^{9}	1.3×10^{-10}		

*Association rate constant

†Dissociation rate constant

TABLE II B. SOME PROPERTIES OF INSULIN RECEPTORS

	Sites/Cell	Sites/ μ m^2	pmole/mg	Degradation fmole/10^6 cells or mg·hr. 4°	15°	24°	37°	Ref.
1.	50,000	2.3	—					190
2.	250,000	11.5	—					190
3.	3,300		—		0			184
4.	87,600		—					184
5.	—	—	0.9					191
6.	—	—	0.45					192
7.	—	—	18.1					192
8.	—	—	1.4					192
9.	—	—	4.6					192
10.	—	—	4.2	0		2.1	15	192
11.	—	—	6.9					192
12.	—	—	0.6					192
13.	—	—	6.3					192
14.	480	0.4	—		0		0.36	193
15.	50,000	36	—					193

TABLE II B. (CONTINUED)

	Sites/Cell	Sites/ μm^2	pmole/mg	Degradation fmole/10^6 cells or mg·hr. 4°	15°	24°	37°	Ref.
16.	40	0.07	—			0.01†	1.4	194
17.	1,300	2.3	—					194
18.	350	0.12	—		0		0.5**	183,195
19.	12,500	4.4	—					183
20.	12,000	4.2	—		0		0**	196
21.	10,500	74	—	0	0		0**	197
22.	2.79×10^6	2.0×10^4	—					197
23.								160
24.	—	—				0		181
25.	—	—	0.3					198
26.	11,000		—			0		159
27.	3,000		—					199
28.	30,000		—					199
29.	50,000		—				16	144
30	—	—	0.2			0		200

TABLE II B. (CONTINUED)

	Sites/Cell	Sites/ μm^2	pmole/mg	Degradation fmole/10^6 cells or mg·hr. 4°	15°	24°	37°	Ref.
31.	—	—						199
32.								199
33.	—	—	0.63					198

† 22°C
** 30°C

orders (195,196), however, do possess specific insulin receptors and are widely used in studies of receptor properties and development of radioreceptor assays for insulin.

Based on the expectation that the steady-state concentration of free insulin maintained in the tissues is approximately equal to the concentration of insulin in the peripheral circulation, the dissociation constant (K_D) exhibited by a given set of insulin receptors will be that concentration of circulating insulin at which the receptors are half saturated. Thus, comparison of insulin concentrations necessary for half maximal stimulation of insulin-dependent processes with dissociation constants observed for insulin-receptor interactions suggests the existence of several populations of receptors responsible for initiating various insulin-dependent functions. Insulin concentrations of 5-7 x 10^{-11} M (7-10 μU/ml), in the range of dissociation constants for the highest affinity insulin receptors, have been found to produce half-maximal stimulation of glucose oxidation (160,207) and lipogenesis (144), indicating that one or more rate-limiting steps of these processes are affected by high affinity insulin receptors at fasting insulin levels.

On the other hand, much higher insulin concentrations have been necessary to affect glucose transport in cells. For instance, Guidotti et al. (208) found a significant increase in glucose uptake by 10-day-old chick embryo hearts only at 10 mU insulin/ml (7 x 10^{-8} M). As will be seen, large doses of insulin have also been required to stimulate cell proliferation (Chapter 4).

Gammeltoft and Gliemann (144) postulated that about 2% of rat adipocyte receptors, for which they determined the K_D to be 3 x 10^{-9} M, were bound to insulin when lipogenesis was half-maximally stimulated in these cells at an insulin concentration of 7 x 10^{-11} M. Kono and Barham (207) found that

approximately 2.4% (corresponding to 4,000) rat adipocyte receptors of K_D = 7 x 10^{-9} M were bound to insulin when glucose oxidation was maximally stimulated at an insulin concentration of 40 μ U/ml (2.8 x 10^{-10} M) and that 1,200 receptors were bound at half-maximal stimulation (4.4 x 10^{-11} M insulin). Crofford et al. (209) estimated that both glucose and amino acid metabolism in rat adipocytes are stimulated maximally when approximately 3,000 insulin molecules are taken up by each cell. At about the same time that the above studies were published, Cuatrecasas reported the discovery of high affinity rat adipocyte insulin receptors with K_D = 8 x 10^{-11} M (159,160,200).

Thus, it may be seen from Table III that 50% of the high affinity receptors will be bound to insulin when the low affinity receptors are less than 10% saturated. In fact, 78% of the high affinity receptors ($K_{assoc.}$ = 1.25 x 10^{10} M^{-1}; K_D = 8 x 10^{-11} M) will be bound in the presence of 2.8 x 10^{-10} M (40 μU/ml) insulin. At 22 μU insulin/ml (1.6 x 10^{-10} M), the point at which maximal stimulation of glucose oxidation in adipocytes is just attained, 2.2% receptors of K_D = 7 x 10^{-9} M ($K_{assoc.}$ = 1.4 x 10^8 M^{-1}) are bound to insulin while the high affinity receptors would be two-thirds saturated. Based on 160,000 low affinity receptors per cell reported by Kono and Barham and 400 high affinity receptors per cell, 3,600 low affinity receptors and 270 high affinity receptors would be filled at this point. At 6 μ U insulin/ml (4.4 x 10^{-11} M), where half-maximal stimulation of glucose oxidation is attained, 0.6%, or 980, low affinity receptors and one-third, or about 140, high affinity receptors would be bound to insulin.

Until 1973, the non-linearity of the Scatchard plots describing insulin-receptor interactions (in contrast to linear plots obtained for other hormones, such as somatotropin binding to cells) was routinely ascribed to receptor hetero-

TABLE III. INSULIN-RECEPTOR BINDING AT PHYSIOLOGIC INSULIN CONCENTRATIONS

$K_{assoc.}$	No receptors/cell	[insulin] *		% receptors bound†	No. receptors bound/cell
M^{-1}		nM	μ U/ml		
1.25×10^{10}	300	0.01	1.4	11	33
		0.1	14	56	168
		0.5	70	86	258
		1.0	140	93	279
5.0×10^{9}	3,000	0.01	1.4	5	150
		0.1	14	33	990
		0.5	70	71	2,130
		1.0	140	83	2,490
1.0×10^{9}	50,000	0.01	1.4	1	500
		0.1	14	9	4,500
		0.5	70	33	16,500
		1.0	140	50	25,000

---

* [free insulin] = [total insulin] in physiologic steady state.

† Based on $\frac{[\text{bound receptor}]}{[\text{free receptor}]} = K_{assoc.} \times [\text{insulin}]$;

$$\% \text{ receptors bound} = \left\{1 - \left(\frac{1}{1 + \frac{[\text{bound receptor}]}{[\text{free receptor}]}}\right)\right\} \times 100.$$

geneity. At that time, however, De Meyts et al. (210) suggested that the curvilinear Scatchard plots characteristic of insulin-receptor interactions could be the result of site-site interactions among insulin receptors, or negative cooperativity; that is, that binding of insulin at one site causes a decrease in affinity for insulin of other receptors. Indeed, Hill plots (211) for insulin-receptor interactions yield Hill coefficients, n = 0.53 - 0.73 (193,194,212), indicative of negative cooperativity (n = 1 indicates no cooperativity and n>1 indicates positive cooperativity). However, the results of Hill plots appear to be contradictory. Although Podskalny et al. (193) report an average Hill coefficient of 0.73 for insulin binding to cultured placental cells, the first three points of their plot, covering the physiologic insulin range (0-100 μU/ml), fall on a line of slope, n = 1, while points above 10^{-8} M insulin tend toward a Hill coefficient less than 0.73. On the other hand, Fussganger et al. (194) have demonstrated a Hill coefficient of 0.7 for insulin binding to human granulocytes throughout the physiologic range with a sudden shift to n = 1 at insulin concentrations greater than 10^{-7} M.

The most compelling evidence for negative cooperativity among insulin receptors was introduced by DeMeyts et al. in 1973 (210): radiolabeled insulin is released from cells at a significantly higher rate when the cells are diluted with a large excess of unlabeled insulin-containing medium compared to dilution with insulin-free medium, indicating that the decrease in the apparent affinity of insulin receptors characteristic of negative cooperativity is due to an increase in the rate of dissociation. The insulin-induced increment in dissociation is unaffected by varying the dilution factor or by adding cells, and the effect is specific for insulin, since substitution of other hormones for unlabeled insulin does not accelerate dissociation. A number of insulin analogs also accelerate

dissociation of labeled insulin in proportion to their ability to inhibit binding of radiolabeled insulin to the receptors. Using this method, investigators have demonstrated cooperative effects among insulin receptors on cultured human lymphocytes (210), cultured human placental cells (193), circulating human granulocytes (194), cultured human fibroblasts (184), circulating human monocytes (213), mouse, rat, guinea pig and chicken liver membranes (214,215), and turkey, frog and trout erythrocytes (215).

Receptor binding and cooperativity can apparently also be uncoupled. Greatly decreased or no cooperative effect is seen with desalanine-desasparagine (DAA) and desoctapeptide (DOP) insulins (184,193,210), high concentrations of insulin ($\geq 10^{-4}$ M; 210), insulin dimers (possibly accounting for loss of cooperativity at high insulin concentrations; 210), the plant lectin concanavalin A (212), urea and acid pH (216). Recently, DeMeyts et al. (217) have implicated a surface region of the insulin molecule as responsible for the negative cooperativity of insulin-receptor interactions, based on the ability of insulin analogues to accelerate insulin-receptor dissociation. This "cooperative" site is situated on the hydrophobic face that includes the putative receptor-binding region, borders those structures necessary for insulin dimerization, and comprises the B23 glycine, B24-25 phenylalanines, B26 tyrosine, and A21 asparagine.

The finding of curvilinear Scatchard plots for and insulin-augmented dissociation of the hormone from solubilized insulin receptors (192,218) has called into question the concept of negative cooperativity as site-site interactions among membrane-bound receptors. The observations of Ginsberg et al. (218), however, may provide a basis for negative cooperativity as a modulation of the insulin-receptor association at the receptor itself, initiated by insulin binding and resulting in an alteration of the quaternary structure of the

receptor. They found that incubation of solubilized avian erythrocyte receptors with 25 ng insulin/ml (625 μU/ml) produced a shift of 35% receptors to the low affinity state. Gel chromatography revealed that about 25% of the receptors had shifted from molecular weight 300,000 to 75,000 daltons, consistent with a tetramer to monomer conversion. The fact that no more than one-third of the receptors are affected considerably above the physiologic range of insulin concentration may mean that the cooperative effect is physiologically unimportant and points to the need for caution in interpreting evidence for negative cooperativity. For example, the possibility that this evidence may also be produced by a receptor-associated insulinase has not been eliminated. Resolution of the debate regarding insulin receptor heterogeneity versus negative cooperativity awaits definitive demonstration of the existence or non-existence of discrete subpopulations of cell insulin receptors.

Binding of insulin to cell receptors is a specific, reversible, and saturable process that is dependent on time and temperature. Harrison et al. (192) found that insulin association with soluble placental receptors attained an apparent steady state within one hour at 37°C and 24°C, by 3 hours at 15°C and by 12 hours at 4°C. Gavin et al. (183) determined that maximum binding of insulin to cultured lymphocytes occurs at 15°C, at which temperature a steady state is reached by 60 minutes and is maintained for at least one hour. The inverse relationship between apparent association constant and temperature is probably due to the marked temperature-sensitivity of insulin-receptor dissociation, the rate of which increases with temperature to a half-life of about 7-10 minutes for adipocytes at 37°C (144,219).

The kinetics of insulin-receptor association are second-order, or bimolecular, while dissociation obeys first-order kinetics (159,181). Cuatrecasas (200) has determined that the free energy of association (ΔG) of insulin and adipocyte membranes of -14 kcal/mole, based on an equilibrium constant of 2×10^{10} M^{-1} at 24°C, is due to a very large decrease in enthalpy (ΔH = -28 kcal/mole) partially offset by a large decrease in entropy (ΔS = -45 cal $mole^{-1}$ $deg.^{-1}$).

Insulin-receptor association is also dependent on pH, but the role of divalent cations in the interaction is questionable. Maximum binding has been observed at pH 7.8 for lymphocytes (183), placental cell membranes and solubilized placental receptors (192), pH 7.6 for cultured placental cells (193), pH 7.5 for adipocyte membranes (200), and pH 8.0 for circulating granulocytes (194). Binding is totally absent below pH 5.0 (183,192). Gavin et al. (183) found that Ca^{++}, Mg^{++}, and ethylenediaminetetraacetate (EDTA) had no effect on ^{125}I-insulin binding to cultured lymphocytes at concentrations of 1 to 10 mM. Likewise, Harrison et al. (192) reported that 5 mM Ca^{++}, Mg^{++}, and EDTA were without effect on insulin binding to soluble placental receptors. However, Podskalny et al. (193) noted a 25% inhibition of ^{125}I-insulin binding to cultured placental cells in the presence of 1 to 5 mM $CaCl_2$.

The insulin receptor apparently is a highly asymmetric glycoprotein of molecular weight 300,000 (218,220,221), which sediments with a coefficient of 11 S and behaves like a particle with a Stokes radius of about 70 Å (220,222). The protein nature of the receptor is indicated by the fact that proteolytic digestion of cells or receptor preparations with such enzymes as trypsin, chymotrypsin and papain impairs or abolishes insulin binding and (in the case of cells) insulin-induced metabolic effects, while pretreatment with insulin protects the receptor from proteolytic agents (181). Cuatrecasas (162) has

found that exposure of fat cells to 10 μg trypsin per ml at 37°C for 15 minutes causes a 10-fold fall in receptor affinity for insulin, while affecting only slightly insulin-stimulated adipocyte glucose oxidation and the cell's insulin binding capacity. All these functions were completely abolished in the presence of higher trypsin concentrations. Very similar results were obtained with chymotrypsin and papain. Gavin et al. (183) found that treatment of cultured lymphocytes with more than 100 g trypsin/ml for 15 minutes at 37°C resulted in significant decreases in insulin binding. Indication that the insulin receptor is an integral membrane glycoprotein, largely immersed in membrane phospholipids, is afforded by the fact that low concentrations of trypsin even somewhat mimic the metabolic effects of insulin on adipocytes (223), that pretreatment of cells and membranes with phospholipases enable complete abolition of insulin binding at low protease concentrations that otherwise would have no significant effect on these preparations' ability to bind insulin (159), and that only 2 μg trypsin/ml (15 min., 37°C) is sufficient to abolish completely the ability of solubilized placental receptors to bind insulin (192). However, Cuatrecasas (220) has provided evidence that the insulin receptor is not a lipoprotein. The lack of functionally important nucleic acid in the insulin receptor is indicated by the fact that digestion of cultured human lymphocytes with ribonuclease and deoxyribonuclease has no effect on binding of ^{125}I-insulin to the cells (183).

As mentioned above, neuraminidase digestion of cells indicates that sialic acid is unimportant to the insulin recognition function of the receptor, but is critical to the transmission of the insulin-binding signal from the recognition site to the glucose transport site. Mild β-galactosidase digestion affects neither cellular insulin binding nor response, but β-galactosidase treatment subsequent to neuraminidase digestion, which itself abolishes insulin response,

results in complete abolition of cell insulin binding (222), suggesting that penultimate β-linked D-galactose, normally masked by terminal sialic acid (or terminal galactose occluded by proximal sialic acid termini), is important to insulin recognition. Further, Cuatrecasas (171,224) has found that the plant lectins concanavalin A, which is specific for terminal α-D-mannopyranosyl or α -D-glucopyranosyl and internal 2-O-linked-D-mannopyranosyl residues (225, 226), and wheat-germ agglutinin, which is specific for terminal N-acetylglucosamine or its dimer (di-N-acetylchitobiose; 227), in low concentrations produce insulin-like effects on fat cells, while at higher concentrations they inhibit insulin binding to the cells.

Early claims of inhibition or reversal of tissue insulin effects and binding in the presence of sulfhydryl-blocking or disulfide disruptive agents, respectively, led to postulation of a covalent bond between insulin and its receptors involving disulfide bonds (228-231). This notion has been discarded, however, due to the realization that insulin-independent metabolic effects are brought about by these agents, more recent studies showing no effect of disulfide inhibitors such as N-ethylmaleimide, iodoacetamide, iodoacetate, arsenite, dithiothreitol, and parachloromercuribenzoate on specific insulin-receptor association, demonstration that insulin dissociated from receptors retains full binding, biological and physical properties, and the non-covalent association kinetics of insulin-receptor interaction (192,198,200,219,232). Cuatrecasas (200) has also reported that the water-soluble carbodiimide reagent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, which forms peptide bonds via conjugation with free carboxy functions, does not modify insulin binding after reaction with adipocyte membranes in the absence or presence of the amino ligand glycine-O-methyl ester. Neither is insulin binding affected by treatment of

the membranes with the tryptophan-modifying agents 2-hydroxy-5-nitrobenzyl bromide and 2-methoxy-5-nitrobenzyl bromide. However, treatment of membranes with tetranitromethane, which reacts with tyrosyl as well as sulfhydryl and tryptophanyl residues, results in a dramatic reduction of insulin binding. Diazonium-1-H tetrazole, which reacts primarily with histidyl and tyrosyl and to a lesser extent with tryptophanyl residues, totally abolishes the ability of the membrane to bind insulin. Thus, tyrosyl and possibly histidyl, but not sulfhydryl, free carboxyl, or tryptophanyl groups are involved in the specific recognition of insulin.

As can be seen from Table II, the quantity and density of insulin receptors varies widely, from 40 per cell for granulocytes (194) to 250,000 per cell for human adipocytes (190), from less than 0.1 per μm^2 for granulocytes to 36 per μm^2 for cultured placental cells (193), and from 0.2 pmole per mg membrane protein for adipocytes to more than 18 pmole per mg membrane protein for placental cells (192). An enigmatic, but consistent observation has been the presence in membranes of "occult" insulin receptors unmasked at high ionic strength or by treatment with phopholipases. A two- to sixfold increase in insulin binding capacity was seen following treatment of adipocyte membranes (200), hepatocyte membranes (181), and cultured lymphocytes (195) with 5-100 μg phospholipase C/ml. A lesser increase in membrane insulin binding capacity can be brought about by treatment with phospholipase A in the presence of Ca^{++} (181), but not with phopholipase D (222), and the phospholipase-induced unmasking of occult insulin receptors can be partially reversed by addition of exogenous phospholipid (171). Although the occult receptors (approximately 65,000 per adipocyte; 162) are very similar to the normally exposed receptors

in their affinity for insulin, association-dissociation kinetics and sensitivity to trypsin, they are unable to trigger a biological response to the hormone.

A very similar increase in the insulin-binding capacity of adipocyte (200) and hepatocyte (181) membranes was found in the presence of hypertonic salt solutions, attaining a maximum in 2 M NaCl and also (for adipocytes) in 2 M KCl or 0.5 M LiCl. The salt-induced increment in binding capacity was observed to be much lower after treatment of adipocyte membranes with phospholipase C, indicating that occult insulin receptors are also exposed at high ionic strength. Harrison et al. (192) also found an increase in the insulin binding capacity—apart from an increase in affinity—of particulate placental membranes at low temperature.

Regulation

As was mentioned at the beginning of this section, it is now generally believed that protein hormones elicit their effects at the cell surface via membrane-bound receptors. Therefore, the cell must possess the means by which to adjust, or regulate, the magnitude of its metabolic response to a given external concentration of the hormone effector. Cellular regulation of hormone response can be envisioned to involve at least three critical aspects of cell function: 1) hormone recognition, at the level of the receptor; 2) amplitude of the signal generated by hormone-receptor association, at the level of one or more intracellular mediators; and 3) the magnitude of metabolic response to a given signal amplitude, at the level of key components of metabolic processes. The last two aspects will be discussed later in this section, under consideration of mechanism; the first aspect, that of regulation at the receptor level, will be dealt with here.

Any discussion of cellular regulation of hormone response must endeavor to explain how the response is initiated (cell sensitivity to the hormone), how the response is maintained (time-dependent magnitude of response at a given hormone concentration), and how the response is terminated. As was made evident in the foregoing discussion of receptor binding properties, similar hormone response at the receptor level (assuming constant signal amplitude and metabolic response per receptor bound) is dependent upon both the affinity of the receptor for the hormone and the number of receptors per cell. Thus, for a constant number of receptors per cell, the fraction bound, and therefore magnitude of response, is directly proportional to the affinity of the receptor for hormone; for a receptor population of given affinity, the absolute number bound, and therefore magnitude of response, is directly proportional to the number of receptors per cell. Both the sensitivity of a cell to a hormone and the magnitude of the cell's response to a given concentration of hormone may be modulated at the receptor level by varying receptor affinity for hormone and/or number of receptors per cell.

A built-in decay of response, and therefore a constant duration of signal maximum dependent upon the magnitude of pancreatic insulin release, is provided by the concept of negative cooperativity, in which the affinity of receptor for hormone is inversely proportional to hormone concentration. It is to be expected that such a system would require connection of each receptor to mediators for all insulin-affected cell functions, with different signal amplitudes and/or response/signal characteristics for different sets of functions, resulting in characteristic insulin sensitivities and insulin concentration-response profiles for each function. The same kind of variation of hormone sensitivity and response can be provided by discrete receptor subpopulations, each with

a characteristic affinity for hormone and each connected to mediators for a specific set of cell functions. This receptor heterogeneity model, however, does not provide by itself for decay of hormone response, which would have to be effected by other means. In fact, evidence does exist for such a separate mechanism of response decay via cell modulation of receptor concentration.

The fact that cells can increase the number of surface insulin receptors is indicated, not only by discovery of occult receptors, but also by the emergence of approximately 350 insulin receptors per cell when normal circulating lymphocytes, which exhibit no significant insulin-binding capacity, undergo blast transformation in the presence of concanavalin A, phytohemagglutinin, or periodate (195,233). The number of receptors increases sharply to reach a maximum between 24 and 46 hours after exposure of the cells to mitogen, coinciding with an increase in thymidine incorporation into nuclear DNA, but preceding the appearance of enlarged, morphologically transformed cells. A similar density of insulin receptors was found on giant, polynucleated cells resulting from transformation of circulating lymphocytes with cytochalasin B and on circulating leukemic lymphoblasts obtained from patients suffering from acute lymphoblastic leukemia, but not on chronic lymphocytic leukemia cells.

More important to cellular regulation of the physiologic insulin response is evidence for a decrease in the number of cell surface insulin receptors in response to extracellular conditions, known as "down-regulation." A 25% decrease in insulin-binding capacity was observed after cultured lymphocytes were preincubated with 4 ng (100 μ U) insulin/ml for 1 hour or 16 hours at 37°C, but 10 μ g insulin/ml (3 hours, 37°C preincubation) was required to produce the same decrease in insulin-binding capacity of adipocytes (234). A maximum decrease in binding capacity of 75% was seen after preincubating

lymphocytes 16 hours with 100 ng insulin/ml. A significant decrease in insulin-binding capacity was also found for rat hepatocytes obtained from 72 hour-fasted animals, parallel to a decrease in liver membrane phospholipids and despite a 3-fold increase in membrane protein (235).

Although it is tempting to postulate an internalization of the insulin-receptor complex followed by lysosomal degradation of both insulin and receptor, which would neatly explain evidence of intracellular insulin receptors, certain observations argue against receptor internalization prior to degradation. The down-regulation noted by Huang and Cuatrecasas (234) was not affected by the metabolic poisons sodium fluoride and dinitrophenol, inhibitors of RNA and protein synthesis, or agents that interfere with phagocytosis and microtubule and microfilament function. The insulin-dependent reduction of insulin binding also occurs with insulin analogs, such as reduced and carboxymethylated and synthetic B-chain, which possess no biologic activity, suggesting the triggering of a receptor-degrading system separate from the insulin recognition function. Using a radioimmunoassay for the human insulin receptor, Harrison et al. (236) have been able to study the fate of the receptor during down-regulation of cultured human lymphocytes. They found that cellular insulin binding decreased 88% during maximum down-regulation with 10^{-6} M insulin. When the receptors were solubilized with 1% Triton X-100, the decrement in insulin binding was reduced to 62%, suggesting dilution by intracellular binding sites. The decrease in immunoassayable solubilized receptors was almost identical, indicating that down-regulation of insulin binding is due to a decrease in the number of receptor molecules. Establishment of the phenomenon of down-regulation as a physiologic mechanism for response decay is problematic, however, since maximum down-regulation has been seen only with pharmacologic insulin

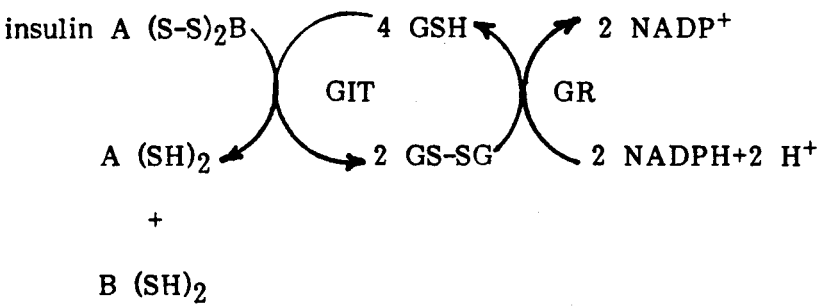
concentrations, where the possibility of artifactual observations due to contaminating proteases is most likely.

A separate mechanism is also required to account for termination of insulin response in both the negative cooperativity and receptor heterogeneity models. This mechanism is provided by the well-documented process of systemic and localized insulin degradation.

Insulin Degradation

The critical role of the liver in removal of insulin from systemic circulation was appreciated as early as 1927, when Kepinow and Dutaillis demonstrated that the hypoglycemic activity of the peripheral blood of a dog injected intravenously with 12 units of insulin was not detectable 3 to 20 minutes afterwards, but that hypoglycemic activity persisted beyond this time interval if the portal vein was anastomosed to the renal vein, bypassing the liver (237). Later, it was found that 8 passages through isolated bullfrog liver were necessary for complete removal of insulin activity (238), but that approximately half the insulin entering rat and human liver is removed in a single passage (239,240). The importance of hepatic uptake, or capture, of insulin in systemic clearance of the hormone was indicated by a rapid decrease of trichloroacetic acid (TCA)-precipitable I-131 activity compared to a slow increase of TCA-soluble radioactivity during cyclic perfusion of ^{131}I-insulin through isolated rat liver (239,241). Normally the half-life of crystalline insulin in human circulation is approximately 5 minutes (242). An increase in hepatic clearance rate of the hormone has been observed in fasted and diabetic animals (238,243-245).

By 1939, it was known that insulin degradation proceeded by two mechanisms: a reductive cleavage of disulfide bridges mediated by sulfhydryl compounds such as glutathione and cysteine and an enzymic proteolysis. The previous year Lehmann and Schlossmann (246) had reported a two-component system for reductive degradation of insulin in a cell-free muscle extract, consisting of a heat-stable, diffusible compound capable of catalyzing the reduction by a non-enzymatic mechanism and a heat-labile enzyme. The former component is now known to be glutathione (GSH) and the latter has been termed glutathione-insulin transhydrogenase (GIT), or protein-disulfide reductase (glutathione) (EC 1.8.4.2), in deference to the wide range of substrates it has been found to act upon. In liver, the enzyme is coupled to the oxidation of NADPH via glutathione reductase (GR) as follows:



(247).

Glutathione-insulin transhydrogenase is a glycoprotein of molecular weight 53,000-62,000 daltons and possesses amino terminal lysine, leucine, and valine, suggesting three polypeptide chains or subunits (248,249). The pH optimum for GIT is between 7.5 and 8.5 (250). Understandably, GIT is inhibited by thiol reagents (251). The enzyme is also inhibited competitively by insulin analogs, oxytocin and vasopressin, noncompetitively by glucagon, and both competitively and noncompetitively by somatotropin (252). The activity of GIT is enhanced by ethylenediaminetetraacetate (EDTA) (253).

In the rat, relative tissue concentrations of GIT have been found to be as follows: pancreas>liver>intestine>spleen>kidney = testis = thymus = fat = lung>brain>heart>diaphragm = skeletal muscle (254). The kinetics of the GIT-catalyzed separation of insulin A and B chains is second order (255), exhibiting a K_m of approximately 50 μM insulin (256). The velocity of GIT-catalyzed insulin degradation at 37°C using 8.4 μM insulin and 3 mg rat liver homogenate protein/ml was found to be 8.4 nmole per hour per mg wet weight homogenate and 5.8 pmole/hr./mg for 0.8 nM insulin and 0.5 mg liver homogenate protein (255,257).

In 1949, Mirsky and Broh-Kahn (258) described a specific enzymic proteolysis of insulin by liver, kidney, and muscle tissue preparations and termed the enzyme responsible insulinase. Later, Brush and Kitabchi (259) demonstrated the presence in both soluble and particulate fractions of rat diaphragm muscle homogenates of an insulin-specific protease that degrades the hormone to oligopeptides and amino acids and is inhibited by N-ethylmaleimide. The soluble fraction had a K_m (app.) for ^{131}I-insulin of 2×10^{-7} M, while the particulate fraction had a K_m apparently 15 to 20 times higher (249).

Probably the most physiologically significant insulinases described thus far are the A and B chain-degrading neutral peptidases, which are inhibited by EDTA, indicating a divalent metal cation requirement (255,260). Proteolytic A-chain degradation, which obeys first-order kinetics, also requires the presence of sulfhydryl compounds (255). The concentration of enzyme activity has been found to be greatest in kidney, followed by skeletal muscle, liver and heart (261). The velocity of A-chain degradation is 1.7×10^{-10} mole/hr./mg wet weight homogenate using 8.4 μM insulin and 3 mg rat liver homogenate protein

(255) and 2.0 x 10^{-13} mole/hr./mg homogenate with 0.8 nM insulin and 0.5 mg homogenate protein (257).

In contrast, the B chain-degrading enzyme recently described by Phelps et al. (260) is inhibited by dithiothreitol as well as potassium phosphate salts, but not by trypsin inhibitors, Trasylol, or phenylmethylsulfonyl fluoride (PMSF). Relative tissue concentrations of the enzyme in the rat were found to be as follows: kidney>>intestine>pancreas ≃ testis>liver>thymus>heart ≃ skeletal muscle ≃ diaphragm>lung ≃ spleen>fat. The B-chain degrading activity of intestine and pancreas was observed to be partly inhibitable by trypsin inhibitors, Trasylol and PMSF, suggesting contamination with pancreatic proteases. Optimum activity of B-chain degrading neutral peptidase was observed at pH 6.5-7.0. An insulin-reversible depression of the enzyme in the kidney, liver and adipose tissue of streptozotocin diabetic rats was also noted.

Varandani has presented much evidence indicating that GIT and the neutral peptidases constitute a stepwise mode of insulin degradation, not only in liver and kidney, but also generally in insulin target tissues, representing the primary means by which the hormone is degraded—first by reduction of interchain disulfide bonds and then by proteolysis—both systemically and locally (253,255,257,260,261). Although the ability of insulin analogs to inhibit insulin binding to and degradation by target cells is closely parallel (262), the insulin degradative function of cells, also a time- and temperature-dependent process (263), is distinct from receptor binding of the hormone (192,264). Both GIT and neutral peptidase activities have been found to reside in cell microsomal and plasma membrane fractions, near insulin receptors (265,266).

Insulin degradation rates found for placental, granulocyte and adipocyte cell and membrane preparations (Table II) at 37°C range from 3.6 x 10^{-16}

mole/10^6 cells/hr. to 1.6 x 10^{-14} mole/10^6 cells (mg)/hr., more than an order of magnitude lower than degradation rates observed for hepatic tissue. PMSF-inhibition of granulocyte insulin degradation (194) may indicate the nonspecific action of lysosomal proteases.

Mechanism of Insulin Action

While much detailed information has been obtained concerning the insulin receptor and the hormone's effect on its ultimate targets, such as key enzymes of intermediary metabolism, the means by which the two are connected remains elusive. Both the nature of the signal generated by insulin-receptor association and the mechanism by which the signal is propagated, modulated and interfaced with well-characterized molecular effectors of the cell's biological response have been particularly subject to the speculation and faddism to which insulin research is prone.

During the 1940s, when the pathways of intermediary metabolism were being elucidated, attempts were made to explain insulin's effects based on a putative modulation of the activity of critical enzymes involved in carbohydrate metabolism, most notably Carl and Gerty Cori's hexokinase theory (267). According to this view, insulin counteracts inhibition of hexokinase by pituitary, adrenal and other substances. The hexokinase theory was abandoned with the accumulation of evidence that insulin exerts no effect on the enzyme, that insulin's impact on carbohydrate metabolism actually is enhanced in hypophysectomized, adrenalectomized animals, and that the hormone profoundly influences metabolic events independent of glucose phosphorylation (see Chapter I).

The efforts of physiologists during the following decade resulted in considerably greater understanding of sugar transport across plasma membranes

and insulin's effect on this process. Insulin's profound enhancement of glucose uptake by non-hepatic target tissues, independent of other metabolic events, is the hormone's signature, so to speak. At that time, therefore, the belief that all metabolic effects on the hormone were consequential to increased cellular glucose uptake became prominent. Although glucose transport continues to be recognized as a unique and fundamental target of insulin action and itself has served as a basis for abandoning otherwise universal theories of insulin action (e.g., cyclic AMP, below) several aspects of cellular metabolism profoundly affected by insulin, such as amino acid and electrolyte transport, are known to be independent of glucose uptake.

Perhaps the most significant development in endocrinology research during the 1960s was the elucidation of the role of adenosine 3',5'-monophosphate, or cyclic AMP, as an intracellular mediator of hormone action. After a somewhat complete picture had been obtained regarding the intracellular events commencing with binding of epinephrine or glucagon to plasma membrane receptors, resulting in an increase in the intracellular concentration of cyclic AMP, and culminating in a concomitant activation of glycogen phosphorylase b and inactivation of glycogen synthetase in muscle, it was logical to suppose that insulin, which exerts an influence on the cell largely antithetical to these two hormones, causes a decrease in the intracellular concentration of cyclic AMP. This hypothesis was largely discredited, however, by an inability to demonstrate a consistent insulin-induced decrement in intracellular cyclic AMP.

There was a brief flurry of excitement early in the 1970s following publication of evidence indicating a complementary antagonism between cyclic AMP and cyclic GMP due to their mediation of opposing cellular processes, with subsequent reports of insulin-induced increments in intracellular cyclic

GMP concentrations. Unfortunately, the results of cyclic GMP research have proven somewhat ephemeral, being plagued by poor reproducibility. Currently, the most promising hypothesis attributes a pivotal role to calcium, probably acting in concert with cyclic nucleotides, as an intracellular mediator of insulin action.

The major concepts involved in postulated mechanisms of insulin action, i.e., membrane sugar carriers, cyclic nucleotides, and calcium, will now be examined in more detail.

1. Sugar transport. Transport of monosaccharides across cell plasma membranes is temperature-dependent, selective, and bidirectional. The rate of D-glucose accumulation inside adipocyte ghosts at 25°C was found to be five times greater than at 4°C (268). Both hexoses and pentoses enter cells, but sorbitol, the alcohol analog of glucose, is excluded (269). Although many of the transported sugars, such as L-arabinose and 3-O-methylglucose (3OMG), are not metabolized by the cell, they inhibit the uptake of other, including metabolizable, sugars, indicating a common site of sugar entry (269). The affinity of this site for sugars has been found to be: glucose=3OMG> galactose>xylose>L-arabinose (270).

Transport of sugars across the cell membrane is also stereospecific. In erythrocytes, D-galactose enters the cell while L-galactose is excluded (271); rat epididymal adipose tissue is slightly permeable to L-glucose, but far more so to D-glucose (32); and the transport of D-glucose across rat fat cell membranes has been found to be ten times that of L-glucose (268). The cellular sugar transport mechanism also favors D-glucose in the chair conformation, with the greatest number of hydroxyl groups in the equatorial position

(272). That this mechanism is also capable of transporting sugars out of the cell is evidenced by the finding that after equilibration of cells with the nonmetabolizable sugars 3OMG and L-arabinose, addition to the medium of D-glucose, which is rapidly phosphorylated inside the cell, causes an efflux of the nonmetabolizable sugar, even against a concentration gradient (32,268-270,273). This phenomenon, known as countertransport, provides strong support for the concept that sugar transport across the cell membrane does not proceed by simple symmetrical diffusion, but is facilitated by a mobile membrane carrier. Facilitated diffusion obeys Michaelis-Menten kinetics, which are reflected in the equation for the steady-state ratio of internal ($[S_i]$) to external ($[S_o]$) nonmetabolized sugar concentrations during countertransport in the presence of glucose ([G]):

$$\frac{[S_i]}{[S_o]} = \frac{1 + [G_i]/K_g}{1 + [G_o]/K_g} \qquad (270),$$

where K_g is the Michaelis constant for glucose. Thus, countertransport has been very useful in determining the kinetic parameters of glucose transport across cell membranes.

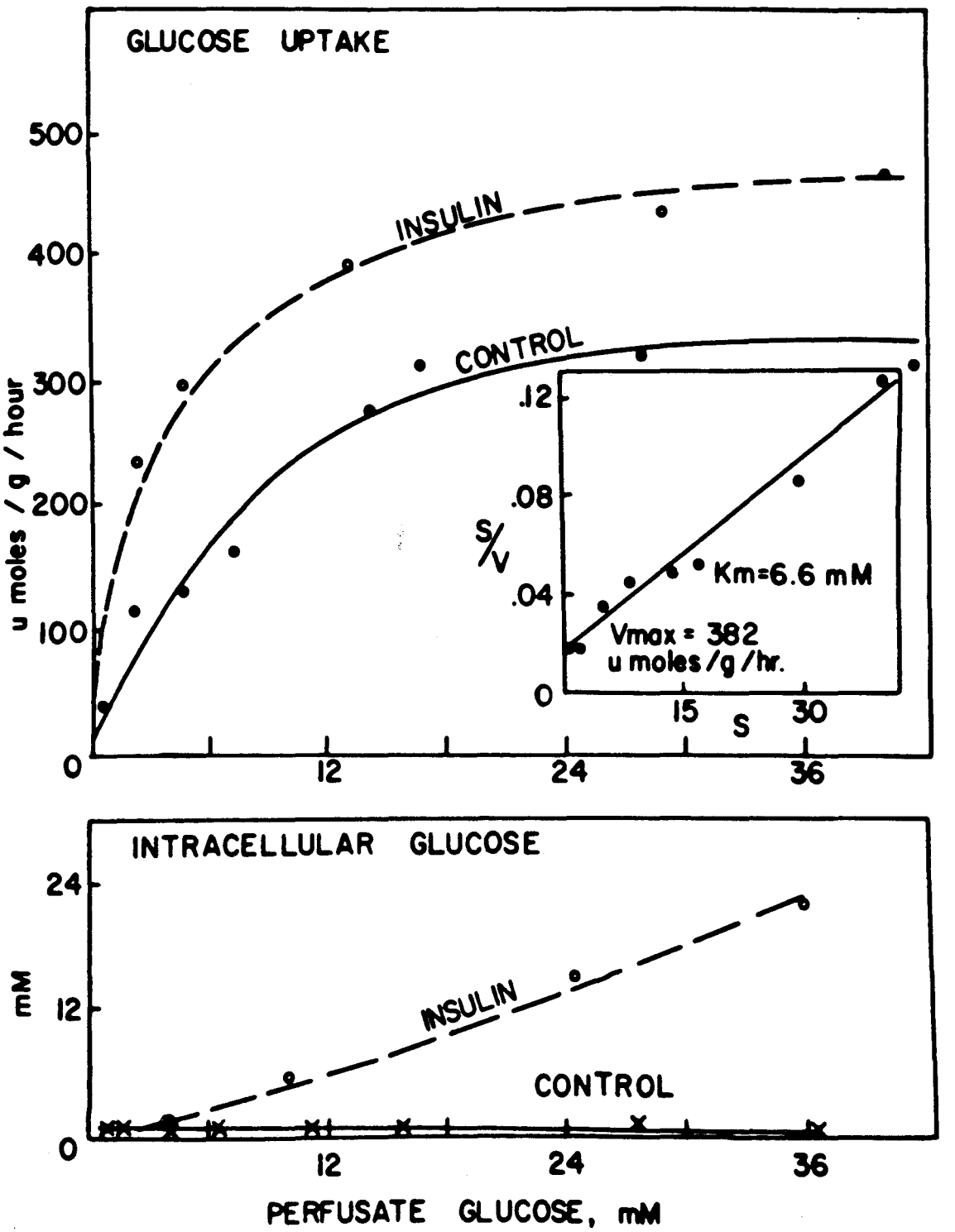
Although the glucose uptake of most tissues, like that of working rat heart depicted in figure 3, obeys Michaelis-Menten kinetics and does not require energy expenditure by cells, some cells accumulate the sugar by mechanisms of active transport (273). The passage of molecules from the capillary, through the extracellular spaces, to the cell surface generally takes place by a process of simple diffusion, resulting in a rapid equilibration of the extracellular space with the peripheral circulation. For instance, 80% of the equilibrium distribution

FIGURE 3. EFFECT OF GLUCOSE CONCENTRATION IN PERFUSATE AND INSULIN ON GLUCOSE UPTAKE AND INTRACELLULAR GLUCOSE LEVELS IN THE PERFUSED RAT HEART.

Results are expressed per gram of dry heart. Insert in upper panel is a Lineweaver-Burk plot of the glucose uptake of control hearts.

S sugar concentration (mM)

V rate of glucose uptake (μmoles/g·hr.)


GLUCOSE UPTAKE
500
400
300
200
100
0
u moles / g / hour
INSULIN
CONTROL
12
24
36
.12
.08
.04
0
S/V
Km=6.6 mM
Vmax = 382
u moles/g/hr.
15
S
30
INTRACELLULAR GLUCOSE
24
12
0
mM
INSULIN
CONTROL
12
24
36
PERFUSATE GLUCOSE, mM

of sorbitol in the extracellular volume of the perfused rat heart is attained within 30 seconds after the compound is added to the perfusate (269). In such insulin target tissues as muscle, however, simple diffusion plays no significant role in sugar transport into the cell (270). The expression for glucose transport into the cell is

$$T_{inward} = T_{max} \left(\frac{G_o}{G_o + K_t} \right) ,$$

where T_{max} is the maximal transport rate, G_o is the glucose concentration outside the cell and K_t is the concentration of glucose at which the transport rate is half maximal. This expression is completely analogous to the Michaelis-Menten equation for the enzyme-catalyzed reaction velocity,

$$v = V_{max} \left(\frac{S_o}{S_o + K_m} \right) ,$$

where V_{max} is the maximum velocity, S_o is the initial substrate concentration, and K_m is the Michaelis constant, the initial substrate concentration required for half maximal velocity. For bidirectional transport,

$$T_{net} = T_{max} \left(\frac{G_o}{G_o + K_t} \right) - T_{max} \left(\frac{G_i}{G_i + K_t} \right) \quad (269).$$

The contention that glucose enters the cell by facilitated transport is further supported by the observation that the first five minutes of efflux of the sugar from adipocyte ghosts during countertransport conforms to zero-order kinetics and that glucose is not transported against a concentration gradient (268). As early as 1937 it was noted that glucose uptake by canine extra-hepatic tissues is dependent upon blood sugar concentration, to the point where large

extracellular glucose concentrations could compensate for the lack of insulin in diabetes (274).

The apparent Michaelis constant, K_m (app.), or the glucose transport constant, K_t, reported for muscle and erythrocytes falls into a narrow range of 4 to 11 mM (269,270,273,275). The reported V_{max}, or T_{max}, for perfused rat heart varies between 100 and 487 μmole glucose/g dry tissue/hr. (269,275). On the other hand, kinetic parameters of glucose transport in adipose tissue have been found to vary widely (K_m = 0.2-140 mM; V_{max} = 100-833 μ mole/g·hr.) (32,268).

In heart muscle, glucose transport is inhibited by free fatty acids (leading to preferential utilization of free fatty acids by myocardium), serum hypoosmolality, and high potassium concentrations, and is stimulated by ouabain (which inhibits Na^+/K^+ ATPase), serum hyperosmolality, increased muscle work, and metabolic poisons such as dinitrophenol and salicylate (269,276). In both muscle and adipose tissue, the process is inhibited by the polyphenol compound phloretin and its glycoside phlorhizin, and nonmetabolizable sugars such as 3OMG (K_i = 16.3 mM for fat cell membranes) and is stimulated by anoxia (27% increase in rat heart muscle) (268-270,272,276).

Intact transport of glucose into adipocyte ghosts (268) and intracellular accumulation of the sugar in the presence of inhibitors of glucose metabolism (277) indicate that sugar transport is not coupled to glucose metabolism. Furthermore, transport has been found to be the rate-limiting step in glucose utilization at physiologic plasma concentrations (<20 mM in adipose tissue and <11 mM in both normal and anoxic rat heart; 132,269) of the sugar. Consequently, intracellular glucose concentrations are normally low and glucose efflux from cells into which its transport is facilitated is negligible (269).

At greater than physiologic plasma concentrations of glucose, when sugar transport approaches maximum velocity, hexokinase-catalyzed glucose phosphorylation, which also obeys Michaelis-Menten kinetics, becomes limiting for cellular glucose utilization (269). In normal rat heart, glucose phosphorylation has been found to have a K_m of 0.6 mM and a V_{max} of 81 μmole/g·hr. Anoxia is also stimulatory for glucose phosphorylation, decreasing the K_m and increasing the V_{max} (269).

Since Levine et al. (278) outlined the transfer theory of insulin action in 1949, there have emerged five major lines of evidence for a discrete mobile membrane carrier mechanism for sugar transport: 1) the specificity and 2) kinetics of transport; 3) inhibition of transport by specific reactive compounds; 4) competitive inhibition of glucose transport by certain other sugars, suggesting a common transport site; and 5) countertransport (273). In addition, the rate of glucose uptake by adipocyte ghosts has been found to be directly proportional to the concentration of membrane protein in the preparation (268).

With general acceptance of the Singer-Nicholson fluid mosaic model of membrane structure (279), there has emerged a popular concept of the sugar carrier (pictured in figure 4) as an intrinsic membrane protein containing an interior hydrophilic channel through which sugar transport is facilitated and subject to allosteric regulation. Certain investigators, however, have advanced alternate theories of facilitated sugar transport, exemplified by Stein's dimerization concept (280), in which he considers evidence derived from erythrocyte studies suggesting a specific catalytic component of the membrane, termed a dimerizer, which could bring sugar molecules together in pairs and so orient them as to induce dimer formation. Since the dimers have fewer free hydrophilic groups they could cross the cell membrane more easily.

FIGURE 4. ACTIVE OR FACILITATED TRANSPORT OF MOLECULE THROUGH MEMBRANE PROTEIN CHANNEL VISUALIZED TWO-DIMENSIONALLY.

Molecule impinges (top) on active site (shaded) of protein, following which some energy-yielding enzyme reaction triggers shift in subunit configuration (bottom) that "squeezes" the molecule through the membrane.

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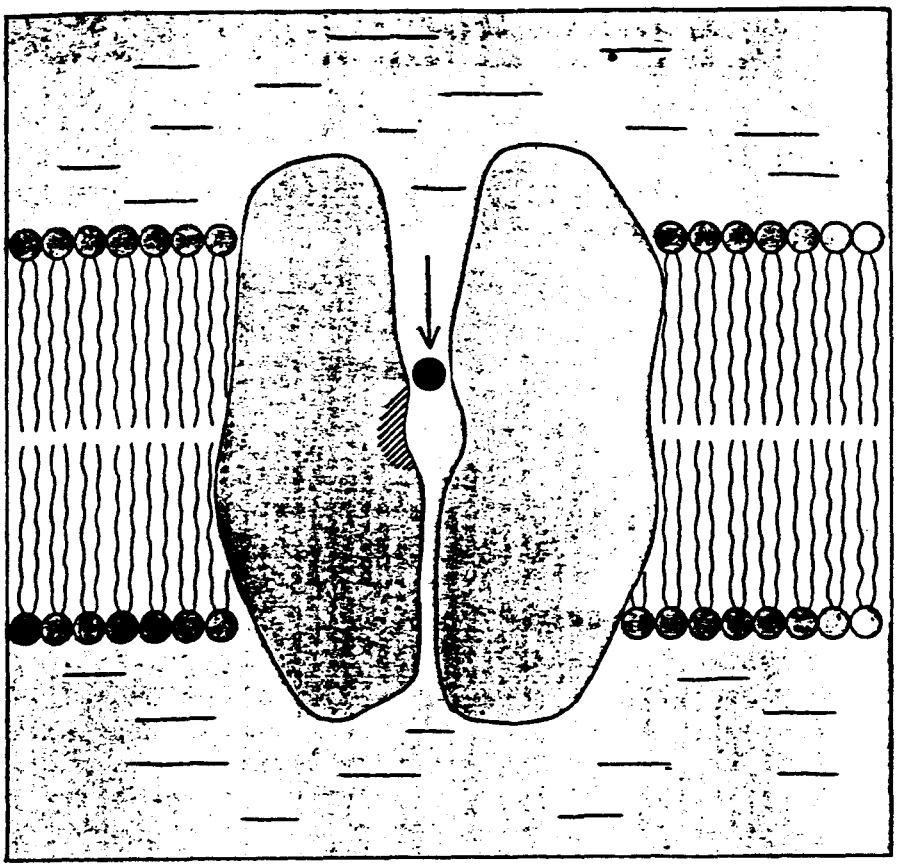
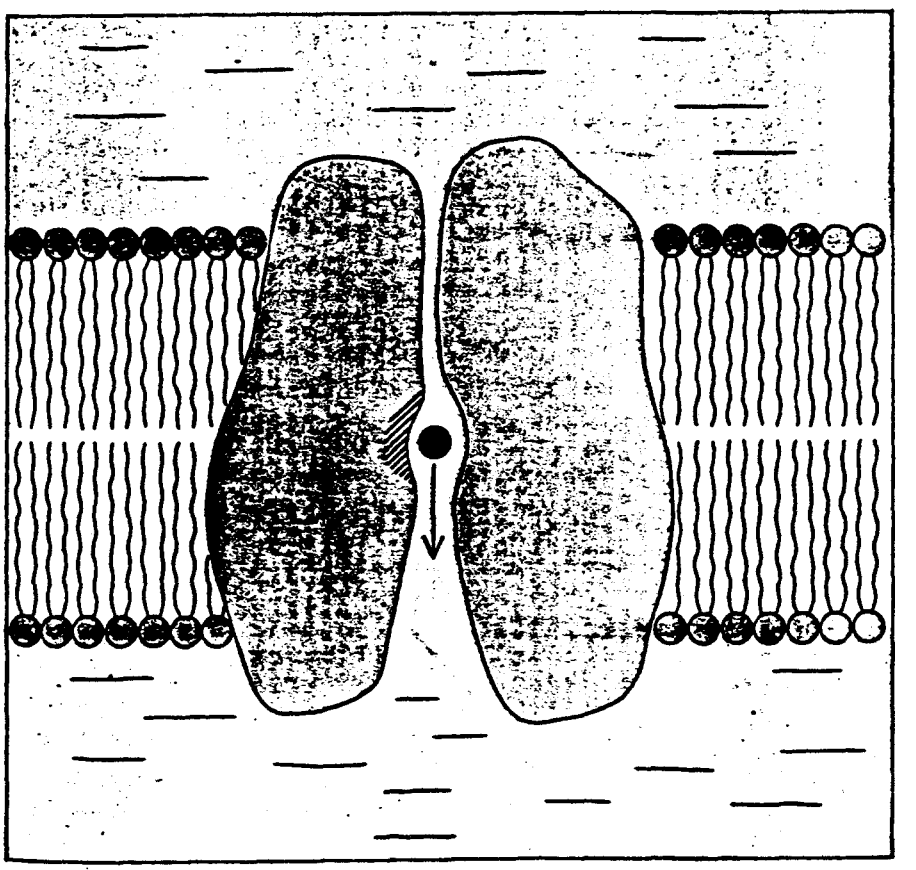




Whatever the precise mechanism of membrane carrier facilitation of sugar transport may be, the fact that the process is inhibited noncompetitively by parachloromercuribenzoate, gold and mercury indicates that one or more sulfhydryl groups is critically involved (272). An enhancement of cellular sugar transport consequent to inhibition of generation of high-energy phosphate compounds by anoxia, 2,4-dinitrophenol and salicylate also suggests the conversion of an inactive phosphorylated carrier to an active, dephosphorylated form (282).

Much evidence exists that insulin simply enhances the cell's normal sugar transport mechanism. The hormone accelerates sugar transport both into and out of adipocyte ghosts (268) and heart muscle cells (269), increases the rate of transport of nonmetabolizable sugars such as D-galactose and L-arabinose (29,271), but does not induce L-glucose uptake by either muscle (270) cells or adipocyte ghosts (268). Crofford and Renold (32), however, reported a significant insulin stimulation of L-glucose uptake by rat epididymal adipose tissue. Furthermore, countertransport also occurs in the presence of insulin (32). A nearly maximal (fourfold) stimulation of L-arabinose countertransport in rat heart muscle has been observed in the presence of 100 μU insulin per ml (269).

The Michaelis-Menten kinetics of glucose transport are preserved during insulin stimulation of the process (269). In heart muscle, the hormone causes a great increase in both the K_m (from 9 to 28 mM) and V_{max} (from 100 to 500 μ moles/g·hr.) of glucose transport (269). On the other hand, insulin stimulation of glucose uptake by adipose tissue involves a 3- to 13-fold decrease in K_m while V_{max} remains unaffected (32). Consequently, in insulin-stimulated muscle tissue, glucose phosphorylation (which is unaffected by the hormone) becomes rate-limiting for cellular uptake of the sugar through the physiologic

range of plasma glucose concentrations (>50 mg/100 ml, or 2.8 mM), where insulin exerts its maximum effect (269,275). Under these conditions, the intracellular glucose concentration can increase to as much as 30% of that outside the cell, resulting in a significant efflux of the sugar from the cell (270). Since glucose phosphorylation in muscle is already rate-limiting at plasma glucose concentrations greater than 200 mg/100 ml (11.1 mM, attained in diabetes), insulin effects on cellular glucose uptake are minimal under these circumstances (269,275).

Insulin enhancement of glucose uptake by muscle is independent of monovalent cation concentrations, but prior chelation of divalent cations with ethylenediaminetetraacetate renders the phenomenon insensitive to the hormone. Addition of Ca^{++} or Mg^{++} to the medium restores transport sensitivity to insulin, indicating that the presence of these cations is necessary for insulin potentiation of glucose transport in muscle (283,284). The role of sulfhydryl functions is indicated again, this time in insulin potentiation of transport, by the fact that N-ethylmaleimide (NEM) blocks insulin stimulation of 3OMG transport into brown fat cells without affecting basal transport rates or binding of the hormone to cell receptors (285). A 10-minute preincubation of the cells with 50 μU insulin/ml or agents such as hydrogen peroxide that stimulate glucose transport without binding to insulin receptors prevent NEM inhibition of insulin stimulation of transport. The mechanism of insulin stimulation of glucose transport appears to be unique, however, since the hormone can exert a striking effect on transport in muscle under anaerobic conditions (286), under which the K_m is decreased and the V_{max} is increased (269), in contrast to the insulin-induced increase in both K_m and V_{max} in this tissue.

Both the potential role of insulin as an effector of protein carrier dephosphorylation and the independence of insulin stimulation of glucose transport from the hormone's other effects, as facilitated glucose transport is independent of glucose metabolism, is underscored by the observation that a 5-minute preincubation of rat epididymal fat cells with 10^{-4} M ATP at 24°C leads to a 2.5-fold inhibition of augmented glucose transport in the presence of 100 μU insulin/ml without affecting insulin binding to receptors, basal glucose oxidation, insulin inhibition of lipolysis or basal glucose transport (222). The effect of ATP, which consists of a blocking of the insulin-induced decrease of transport K_m, accompanied by phosphorylation of a specific membrane protein, cannot be overcome by the addition of large amounts of insulin. The β, γ-methylene derivative of ATP, which cannot donate the terminal phosphate, not only can't suppress insulin-stimulated transport, but also inhibits both the ATP effect and membrane phosphorylation by ATP. Further, demonstration that insulin stimulation of glucose transport is dissociated from its effects on glycogen synthesis (287) and that high extracellular glucose concentrations do not reproduce insulin effects on cells (288) indicate that while an increase of glucose transport into cells is the primary effect of insulin on muscle and adipose tissue, it alone by no means leads to other effects of the hormone.

Another clue to the nature of the insulin-sensitive transport mechanism may be supplied by the work of Guidotti (208), who postulated the existence of an additional, insulin-sensitive membrane based on his studies of chick embryo hearts. He and his co-workers found that after the 7th day of development, when β-granules first appear in the endocrine pancreas, the heart exhibits normal, transport-limited kinetics and are sensitive to insulin. Prior to that time, however, glucose uptake by heart cells is insulin insensitive

and proceeds at a maximal (insulin-stimulated) glucose phosphorylation-limited rate, suggesting the formation of an insulin-sensitive structure of facilitated sugar transport coincident with the development of the insulin secretory apparatus.

2. Cyclic AMP. In 1957, Sutherland and Rall began studying the means by which glucagon and epinephrine exert their effects on liver, specifically the activation of glycogen phosphorylase, the enzyme catalyzing degradation of glycogen to its constituent glucose molecules. They found that exposure of liver slices to the hormones resulted in the rapid appearance of a water-soluble heat-stable factor identified as adenosine 3',5'-monophosphate (cyclic AMP; cAMP) via stimulation at the cell surface of a membrane-bound enzyme, adenylate cyclase (289-291). During the ensuing years, Sutherland and his associates and others succeeded in elucidating what is now considered the classic mechanism of cell response triggered by binding of catabolic hormones to cell-surface receptors, illustrated in figure 5.

Briefly, hormone binding to a cell-surface receptor activates a proximal adenylate cyclase and/or inhibits the phosphodiesterase that degrades cyclic AMP, resulting in a transient increase in intracellular cyclic AMP concentrations (10^{-8} to 10^{-6} M). Cyclic AMP binds to the regulatory subunit of a cyclic AMP-dependent protein kinase (also termed phosphorylase b kinase kinase, PbKK), causing its dissociation from the now active kinase. This kinase causes the phosphorylation of phosphorylase b kinase (PbK), activating it. The active PbK then causes phosphorylation of glycogen phosphorylase b, converting it to

FIGURE 5. SUMMARY OF GLYCOGEN SYNTHESIS AND DEGRADATION

Abbreviations

AC adenylate cyclase

cAMP cyclic AMP (3',5'-AMP)

GS glycogen synthetase

K kinase

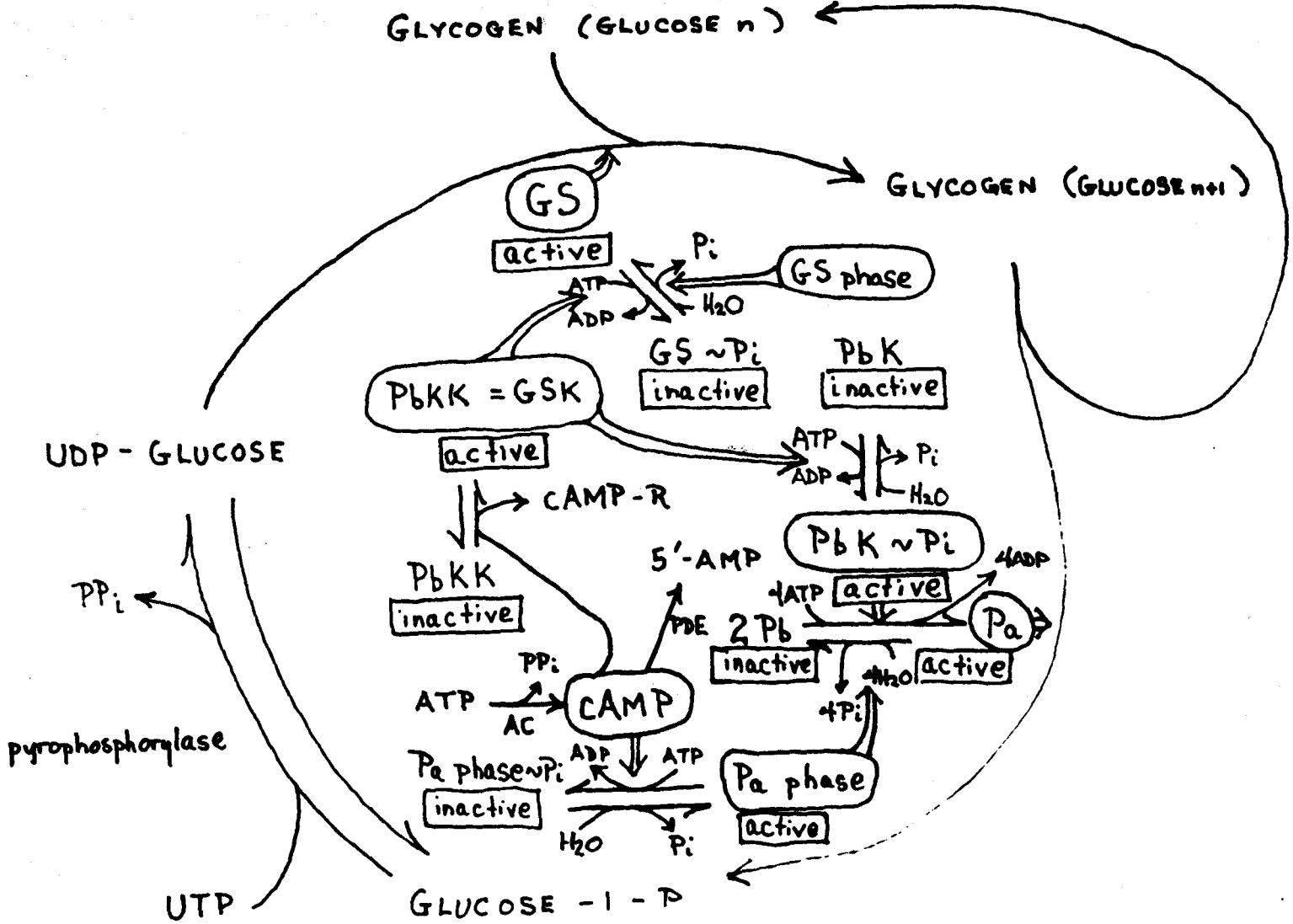
Pa glycogen phosphorylase a

Pb glycogen phosphorylase b

PDE phosphodiesterase

phase phosphatase

R regulatory subunit


GLYCOGEN (GLUCOSE n)
GLYCOGEN (GLUCOSE n+1)
GS
active
Pi
GS phase
ATP
ADP
H2O
GS ~Pi
inactive
PbK
inactive
PbKK = GSK
active
UDP - GLUCOSE
ATP
ADP
Pi
H2O
cAMP-R
PbK ~Pi
5'-AMP
4ADP
PbKK
inactive
4ATP
active
PDE
2 Pb
Pa
inactive
4H2O
active
PPi
4Pi
ATP
AC
cAMP
PPi
Pa phase~Pi
inactive
ADP
ATP
Pa phase
active
H2O
Pi
PPi
pyrophosphorylase
UTP
GLUCOSE - 1 - P

the active a-form, which causes removal of terminal glycogen residues as glucose-1-phosphate molecules. Meanwhile, the cAMP-dependent protein kinase also catalyzes phosphorylation of glycogen synthetase and glycogen phosphorylase a phosphatase, inactivating both enzymes and generally promoting glycogen breakdown (292-296). The same kind of cAMP-mediated activation of adipocyte lipase has been demonstrated to explain the stimulatory effect of epinephrine on lipolysis (295,296).

One of the most striking features of this mechanism is its amplification potential. The action of this hormone initiates a "cascade" in which the effect of the original hormonal stimulus is amplified at least 100 times at each of the four enzymatic steps in the process, for a total amplification of 10^8 or more; thus a single molecule of glucagon or epinephrine can theoretically trigger the release of about 100 million glucose molecules (296). In addition to glucagon and catecholamines, increases in intracellular cyclic AMP concentrations have been found to accompany the mediation of adrenocorticotropin, luteinizing hormone, vasopressin, thyrotropin, parathyroid hormone, melanocyte-stimulating hormone, histamine, thyrotropin-releasing hormone and certain prostaglandins effects (293).

From this work evolved four criteria for establishing a cyclic nucleotide's role as a mediator of hormone action: 1) elevation of intracellular cyclic nucleotide levels by the hormone; 2) activation of the nucleotidyl cyclase by the hormone in broken cell preparations; 3) recapitulation of the hormone's effects by addition of the cyclic nucleotide or its derivatives to a target cell preparation; and 4) potentiation of the hormone's effects in the presence of an inhibitor of the phosphodiesterase catalyzing that cyclic nucleotide's degradation (296). The fact that insulin antagonizes many cyclic AMP-mediated

hormone effects on its target tissues has led several investigators to postulate that the effects of this hormone are mediated by a decrease in the intracellular concentration of the cyclic nucleotide (297).

A number of reports, in fact, seem to support this supposition. Insulin antagonizes the increase in cyclic AMP levels and output of glucose brought about by low concentrations of glucagon or catecholamines and the action of added cyclic AMP itself in perfused livers (297-300). Butcher et al. (301) showed that insulin lowers intracellular cyclic AMP levels in adipocytes. This effect was especially pronounced when cAMP levels had first been elevated by pretreatment of the tissue with epinephrine and caffeine. Also, cAMP-induced K^+ release from perfused rat liver was found to be significantly reduced in the presence of 25 mU insulin/ml to a cAMP concentration of about 5 x 10^{-5} M (297). In vivo administration of anti-insulin serum to rats causes a progressive increase in hepatic cAMP levels, accompanied by an activation of glycogenolysis (302), and a diabetes-induced, insulin-reversible increase in rat liver cAMP concentrations has been observed (297).

Insulin has been reported to alter adenylate cyclase activity in adipose tissue (303). Numerous investigators have described insulin inhibition of basal and hormone-stimulated adenylate cyclase in adipocytes (304), hepatocytes (304), cultured fibroblasts (305), and cell membrane preparations (195,222,304,306). In addition, insulin has been found to stimulate membrane-bound cAMP phosphodiesterase, either directly (307) or after treatment of cells with the hormone (308,309).

The results of experiments designed to demonstrate an effect of insulin on intracellular cyclic AMP levels are contradictory, however. Goldberg and Larner in 1967 found that in both muscle and liver cells one could obtain full

expression of insulin action either without any lowering of cAMP levels or before any lowering was detectable (296). Numerous reports demonstrating an inability of insulin to affect basal cAMP levels in liver, muscle and fat cells have appeared (219,297). Insulin does not inhibit the activation of glucose output in perfused liver by dibutyryl cAMP (normally a potent cAMP analog) (298-300) and the hormone can activate glycogen synthetase without causing any change in the activity of glycogen phosphorylase or a decrease in the concentration of cAMP (310,311), contrary to expectations for a process mediated by a decrease in cAMP levels. Insulin has also been found to inhibit cellular K^+ release without affecting glycogenolysis (298,300).

Evidently, cAMP-mediated mechanisms are not involved in other insulin effects, namely stimulation of glucose transport (312) and protein synthesis (313), and inhibition of urea production (298,300) and lipolysis (313,314). The inability to satisfy the important third and fourth criteria of cyclic nucleotide mediation of insulin action is exemplified by the fact that such agents as prostaglandins E_1 and adenosine, which cause decreases in intracellular cAMP concentrations, do not mimic insulin's effects on glucose transport and lipolysis (219,315). Finally, in a number of studies using cultured cells (including human fibroblasts), no correlation was found between insulin binding to cells and the activation or inhibition of of any component of the adenylate cyclase system, although binding does correlate with stimulating effects on thymidine incorporation into DNA and amino acid transport (316).

3. Cyclic GMP. Although guanosine 3',5'-monophosphate (cyclic GMP; cGMP) had been found in biological fluids previously, it wasn't until 1969 that the compound was detected in animal tissues, almost simultaneously with the

discovery of guanylate cyclase (317-319), because the concentration of cGMP in cells is as much as 100-fold less than that of cAMP (296). Development of highly sensitive radioimmunoassays for the compound, however, enabled a spate of cGMP measurements using a wide variety of species, tissues, and effectors. From these studies emerged guanylate cyclase, cGMP phosphodiesterase and a cGMP-dependent protein kinase as predominant components of the cGMP system. The cyclase has been found in both soluble and particulate cell fractions (320), while the phosphodiesterase is predominantly soluble (321). Substrates for the kinase include histones (322), nuclear acidic proteins (323) and membrane proteins (324,325).

What has also emerged from cGMP research is a tendency for cellular processes in opposition to those mediated by cAMP to be accompanied by increased levels of cGMP. These include uterine contraction, venoconstriction, platelet aggregation, polymorphonuclear leukocyte motility, lymphocytotoxicity, intestinal contraction, gallbladder contraction, release of lysosomal enzymes, ductus deferens contraction, and inhibition of lipolysis and heart contraction (296). One of the most important ideas resulting from these observations is the possibility that insulin is a cGMP agonist.

Insulin has been found to increase intracellular cGMP concentrations in adipose tissue (326-328), liver (326) and intestinal mucosa (329). In the first case, physiologic insulin concentrations cause a marked but transient rise in the cellular level of cGMP and a stimulation of membrane-bound guanylate cyclase (222). Much information has also been gained from studies of cultured fibroblasts. In such cells of mouse and hamster origin insulin causes a transient increase in cGMP with a concomitant decrease in cAMP concentrations (330-335), and mitogenesis induced in mouse fibroblasts by high concentrations of

the hormone is accompanied by a ten- to fortyfold increase in intracellular cGMP concentrations (332). In these cells, cAMP-induced decreases in leucine and hexose uptake are antagonized by addition to the medium of cGMP (336).

Unfortunately, cGMP mediation of hormone effects is not so clear-cut as has been found for cAMP. Direct stimulation of guanylate cyclase by any cGMP agonist has not yet been observed (321,337) and dissociation of cGMP-dependent protein kinase into catalytic and regulatory subunits by cGMP has proven difficult to demonstrate (321). Studies designed to establish cGMP mediation of insulin action are no less prone to inconsistencies than those involving cAMP. For instance, atropine, a potent cGMP antagonist, does not affect the insulin-induced increase in intracellular cGMP concentrations in fat cells (326). Furthermore, the presence of Ca^{++} is required, not only for cellular processes associated with increased cGMP levels, but also for the rise in intracellular concentrations of the cyclic nucleotide itself (296). Indeed, this fact may provide a key to the elucidation of a plausible mechanism for insulin action.

4. Calcium. The critical role of calcium in nerve and muscle function has long been known. During the past ten years, however, evidence has emerged implicating the ion of this metal, Ca^{++}, as a ubiquitous and pivotal mediator of many metabolic processes in a great variety of cells.

While certain investigators have found it necessary to postulate an unsubstantiated intracellular compartmentalization of cyclic nucleotides to explain observed inconsistencies in the behavior of these compounds during manipulation of cellular processes putatively mediated by them (297), such compartmentalization is well documented for Ca^{++}. The cytoplasmic

concentration of Ca^{++} in the typical mammalian cell is very low (apprx. 10^{-7} M) compared to the extracellular (10^{-3} M) and mitochondrial concentrations of the ion. The mitochondrion is the cell's primary repository of Ca^{++} (except in muscle cells, which employ the sarcoplasmic reticulum for this purpose), in which the total Ca^{++} concentration is 10^{-2} M. Because of the high intramitochondrial phosphate concentration, 99% of the Ca^{++} in this organelle is stored as insoluble calcium phosphate, leaving 10^{-4} M soluble Ca^{++}, which is still 1,000-fold greater than that of the cytoplasm. These large differences in Ca^{++} concentrations are maintained by ion pumps that require cellular energy expenditure—membrane-localized Ca^{++}-dependent ATPases, enabling precisely regulated, rapid changes in cytoplasmic Ca^{++} concentrations (337). This situation is particularly significant in view of the interrelation of intracellular Ca^{++} and cyclic nucleotide metabolism, exemplified by the Ca^{++} dependence of cGMP metabolism already mentioned.

The effects of Ca^{++} on cellular metabolism—especially where related to cyclic nucleotides—is tissue specific, another condition necessarily imposed on cyclic nucleotide mediation of extracellular effectors. While Ca^{++} antagonizes cAMP-mediated processes in adipose tissue, where the ion facilitates the conversion of the glucose-6-phosphate-dependent form of glycogen synthetase to the independent form (338), it stimulates the activation of glycogenolysis by phosphorylase b kinase in muscle (339), where an increase in free calcium concentrations from 10^{-8} M to 10^{-4} M produces a four- to eightfold rise in pyruvate dehydrogenase phosphatase activity (340), leading to activation of the dehydrogenase and promoting glucose oxidation.

A now-classic example of cyclic nucleotide-Ca^{++} co-mediation of hormone action is provided by the salivary gland of the blowfly, the cells of

which secrete isotonic potassium chloride in response to hormonal stimulation by serotonin at the cell surface. At first it was thought that this system represented a typical case of cAMP-mediated hormone effects, since it satisfied the experimental criteria previously iterated. The effect of serotonin on intracellular cAMP and on KCl secretion could be uncoupled, however, if the extracellular medium were Ca^{++}-depleted. It has been found subsequently that binding of serotonin to cell membrane receptors not only activates adenylate cyclase, but also increases plasma membrane permeability to Ca^{++}. In turn, cAMP increases mitochondrial membrane permeability to Ca^{++}. Consequently, intracellular Ca^{++} concentrations rise significantly. In the blowfly salivary gland, increased intracellular cAMP activates a K^+ pump that transports the cation out of the cell, against a concentration gradient, into the lumen. Meanwhile, Ca^{++} mediates increased membrane permeability to Cl^-. Furthermore, Ca^{++} inactivates adenylate cyclase, providing a feedback loop which is conspicuously lacking in classic cyclic nucleotide mediation mechanisms (337).

A different kind of interrelationship between Ca^{++} and cAMP exists in mammalian longitudinal intestinal smooth muscle. In this tissue, acetylcholine causes muscle contraction by increasing intracellular Ca^{++} concentrations while epinephrine causes muscle relaxation by decreasing intracellular Ca^{++} levels. Both effectors cause increases in intracellular cAMP levels, however. This apparent discrepancy was resolved when the actual mechanisms of effector action were elucidated. Acetylcholine directly causes contraction by promoting entrance into the cell of extracellular Ca^{++} and release of the ion from sarcoplasmic reticulum. Increased Ca^{++} concentrations inhibit cAMP phosphodiesterase, leading to an increase of the cyclic nucleotide, which promotes uptake

of Ca^{++} by sarcoplasmic reticulum. As cytoplasmic Ca^{++} levels fall, the phosphodiesterase is again activated and cAMP returns to basal levels. Thus, termination of both contraction and the termination signal is provided for by a dual messenger mechanism. Epinephrine, on the other hand, causes relaxation of smooth muscle by decreasing cytoplasmic Ca^{++} concentrations through an immediate increase in cAMP levels due to stimulation of adenylate cyclase by the hormone. The signal is terminated by degradation of cAMP by an increasingly more active phosphodiesterase (337). The blowfly salivary gland and the mammalian longitudinal intestinal smooth muscle are only examples of many well-documented cases of the diverse regulatory systems provided by a Ca^{++}-cyclic nucleotide synergism that enables the exquisite tailoring of biologic response to external stimuli underlying the integration and subordination of the functions of specific cell types to the requirements of the whole organism.

Particularly significant is the apparent role of Ca^{++} in cellular responses to insulin, especially those effects that have not been associated with other postulated mediators. Insulin antagonizes epinephrine-induced extrusion of Ca^{++} from adipocyte ghosts by Ca^{++}-dependent ATPase (341). An insulin-dependent material that stimulates Ca^{++} uptake by isolated mitochondria has been extracted from liver cytoplasm (342). However, McDonald et al. have found that insulin treatment of adipocytes does not alter total mitochondrial calcium, but causes an increase in the labile form of the cation and a decrease in the stable form (343). Procaine, lanthanum and the Ca^{++} ionophore A23187, which all increase intracellular Ca^{++} concentrations, mimic insulin's metabolic effects in liver and adipose tissue (344-346), satisfying an important criterion of hormone mediation lacking in cyclic nucleotide models of insulin action. It has been found that plasma membrane permeability to glucose and amino acids

is a function of membrane-bound Ca^{++} (347), and that the presence of Ca^{++} is required for insulin-induced protein synthesis (348). Furthermore, the activity of several enzymes involved in mediating the intracellular action of insulin is calcium-dependent (340,347,349). Seals, McDonald and Jarett (350-352), have demonstrated that insulin causes the activation of what may be a plasma membrane-associated Ca^{++}-dependent phosphatase (M.W. 120,000) that dephosphorylates the alpha subunit (M.W. 42,000) of pyruvate dehydrogenase, leading to activation of the latter enzyme in cell-free preparations of rat adipose tissue. Recently, Jarett and Seals (342) reported that insulin-treated muscle extracts contain a factor that directly stimulates pyruvate dehydrogenase activity in isolated adipocyte mitochondria in the presence of Ca^{++}.

A prime contender for the role of an insulin-mediating factor is one of a family of enzymically active Ca^{++}-binding proteins exemplified by calmodulin, found in a great variety of tissues, and troponin C in skeletal muscle (353). Although these proteins tend to serve as kinases after binding Ca^{++}, the great variability of their specific functions in different cell types makes it possible that they may also act as phosphatases, an enzymic role frequently associated with insulin responses.

Based on evidence available at this time, it is probable that insulin effects are mediated by a complex network of messengers, primarily Ca^{++}, Ca^{++}-binding proteins, cAMP and cGMP. Ca^{++}-mediated effects may include direct activation of Ca^{++}-dependent enzymes (including nucleotidyl cyclases and phosphodiesterases), dephosphorylation of membrane proteins affecting sugar, ion and amino acid transport, and phosphorylation and dephosphorylation of chromosomal proteins, ribosomal proteins and cyclic nucleotide-dependent protein kinases affecting enzyme induction and suppression, DNA, RNA and

protein synthesis, and phosphorylation-dependent enzyme activity; cyclic nucleotide-mediated effects may include cell proliferation, antagonism of catabolic hormone effects, and termination of the calcium signal. Thus, by increasing the intracellular Ca^{++} concentration (perhaps through a Ca^{++}-binding protein cascade commencing with one in direct proximity to the insulin receptor), insulin may trigger an incredibly complex train of events appropriate to a hormone of unequaled profundity in regard to mammalian metabolism.

CHAPTER IV

INSULIN ASSAYS

An assay for insulin is herein defined as a procedure carried out for the specific purpose of measuring the amount of insulin present in a given sample. Although a great deal of research has been conducted to determine the physiologic and metabolic effects and physico- and immunochemical properties of insulin and the molecular mechanisms of insulin action, only the use of such work in the development of reproducible, reliable, quantitative assays for the hormone will be reviewed in this chapter.

Ideally, any assay should be 1) convenient, enabling a maximum number of determinations with a minimum of manipulation; 2) reproducible, exhibiting an interassay variation of less than ten percent; 3) sensitive, being able to detect basal levels of the measured substance in the fluids being studied; 4) accurate, reflecting the true quantities of the substance and its actual fluctuations in experimental or physiologic processes; 5) precise, with an intraassay variation of less than five percent; and 6) specific, measuring only what it is supposed to measure, relative to an appropriate standard. In the case of insulin, this standard is usually pure crystalline monomeric pancreatic insulin.

Of course, few assays fully satisfy the above iterated criteria and assays for insulin are not among them. However, the history of insulin assays is one of steady progression toward this goal, hampered by certain complications, the

most conspicuous of which are the ignorance concerning the form of insulin existing in the peripheral circulation and the multiplicity of hormone properties that may be measured. One may assay insulin's physicochemical properties, immunoreactivity, or biological activity, which in most cases includes the integrity of the molecule's receptor-binding site. In the following account, the properties recognized by a given assay and the appropriate applications for that assay will be considered. Each assay will be evaluated in terms of the above criteria, with special attention devoted to the assay's sensitivity, precision and range of human serum insulin concentrations in normal individuals.

IN VIVO BIOASSAYS

The First Insulin Assays

Even before the isolation of insulin was accomplished, Paulesco and Banting and Best utilized the decrease in blood sugar of dogs as an assay for the potency of hormone extracts. Of course, an appropriate assay is necessary to the isolation and characterization of any biological effector. However, the use of dogs proved too costly to be used on a routine basis once insulin was being isolated on an industrial scale.

Rabbit Hypoglycemia Assay

Once Banting et al. (354) found that insulin-induced convulsions in rabbits were usually associated with a blood glucose concentration of 45 mg/dl, this method was adopted for routine assay of preparative hormone potency. The amount of insulin that produces this concentration in 2-4 hours was first used as a unit of activity, but soon the unit was formally defined as one-third the amount required to produce this effect in a rabbit starved for 24 hours (355).

The First International Standard for Insulin, adopted in 1925 and based on the "rabbit unit," was said to have a specific activity of 8 units per mg, and subsequently all preparations of insulin have been assayed directly or indirectly in terms of it.

By 1935, the unit was defined as 1/22 mg (356) and presently the bovine insulin standard of the International Union of Pure and Applied Chemistry is stated to have an activity of 25.0 international units (IU) per mg dry weight and to contain 0.439% of zinc and 14.9% nitrogen (357). With the expectation that the maximum specific activity of insulin has been attained, the unit is now based on the absolute weight of a highly purified composite preparation (358).

Because considerable variation in the sensitivity of rabbits to insulin made these first rabbit assays unsatisfactory, Marks recommended the comparison of one preparation in terms of another in two similar groups of rabbits (359). One group received the preparation of known activity, the second the preparation of unknown activity. On another day, the procedure was reversed to complete a "cross-over" test. By averaging the hypoglycemic responses to each preparation over both days, the influence of the variation in sensitivity between rabbits was considerably reduced and an estimation using the variable "rabbit unit" of activity was avoided. In a later modification, two such cross-over tests were performed, using two dose levels of both the standard and unknown preparations to provide an additional check on potency (360). This method is presently recommended by the U.S. Pharmacopeia (361), which requires a maximum assay deviation from the stated U.S.P. standard value of 7.5 percent.

All subsequent investigations of the rabbit hypoglycemia assay have employed cross-over designs. The reduction in blood sugar, the final concentration, the reduction or final concentration as percentages of the initial value, either at a given time after insulin or averaged over a period of time, have been used to determine insulin activity. Lacey (362) demonstrated a linear relationship between the log of the insulin dose and the percent reduction in blood sugar down to 0.4 U insulin, which brought about a 22% reduction in blood sugar, while Young and Romans (363) succeeded in reducing the time between insulin injection and determination of blood sugar to 50 minutes with results as precise as those obtained using longer times (364). Using three dose levels each of standard and test preparation and twelve rabbits in a protocol requiring three weeks to carry out, Bliss and Marks (365) obtained data (percent fall in blood sugar over 5 hours) for which coefficients of variation (C.V., the ratio of the standard deviation to the mean, expressed as a percent) may be calculated as follows: intraassay variation (between rabbits), 33.7%; interassay variation (between days), 19.9%.

The rabbit hypoglycemia assay has proven satisfactory as a means of assessing the biologic potency of preparative quantities of insulin, but obviously is far too insensitive to measure concentrations of the hormone in blood. In addition, the assay is costly, cumbersome, extremely time-consuming, and relatively imprecise.

Mouse Convulsion Assay

Milliunit quantities of insulin injected into mice produce convulsions (366), and the percentage of mice responding or a mathematical function of the percent response, known as the normal equivalent deviation, was found to

increase linearly with the log of the insulin dose (355,367). Weighting coefficients were applied, the highest being given to 50% response; responses at the extreme ends of the dose-response curve were given the lowest values because of the greater range in doses producing them (368). A sensitivity of 2 mU insulin was attained when mice fed on bread and milk and starved overnight were each injected subcutaneously with 50 μg promethazine hydrochloride 30 minutes before insulin administration (369). Hemmingsen obtained an interassay variation of 7-10% in a series of cross-over tests using 160 mice (370).

Typically, the numbers of mice that die, exhibit frank convulsions, or lie still for more than 2-3 seconds when placed on their backs are recorded (369). In an effort to overcome possible observer error in determining positive responses, mice, after injection with insulin, were placed on a wire-mesh screen inclined at an angle of 60° (371,372) or placed within a rotating cylinder similarly inclined (373). Mice exhibiting convulsive symptoms are unable to retain their foothold and fall from the screen or cylinder and are counted as positive responders. This "mouse-drop" assay, however, offered no increase in precision compared to the more conventional method employing continuous observation (369).

In a comparison of the rabbit hypoglycemia and mouse convulsion assays, Smith (374) found no significant difference between values for insulin standards obtained by the two methods. In addition, the mouse assay offers greater sensitivity (although still not sufficient to measure insulin in blood), while being more economical (four mice have an efficiency equivalent to one rabbit) and requiring far less time to obtain a result. Nevertheless, the rabbit assay is the one presently employed to determine the biologic potency of insulin prepared for therapeutic use (375).

Insulin-Sensitive Animal Assays

Removal of the hypophysis and adrenal glands renders an animal hypersensitive to insulin. Using rats prepared in this way, Gellhorn et al. (376) obtained a linear relationship between the blood sugar concentration and insulin dose from 0.3 to 1.0 mU insulin/100 g body weight. When diabetes is induced in these animals with alloxan, variations in response to injected insulin, caused by the presence of endogenous insulin, may be reduced. Employing rats from which the adrenal medullae had been removed, injected with alloxan and then hypophysectomized, Anderson et al. (377) increased the sensitivity of the assay to 125 μU insulin/rat (approximately 200 g).

The first direct measurements of human plasma insulin concentrations in normal individuals were made by Bornstein, who carried out total adrenalectomy in the hypophysectomized rat with diabetes induced by alloxan (ADHA rat). He obtained a linear regression of blood sugar depression vs. insulin dose from 50 μU to 500 μU insulin. Since 0.5 ml was injected, insulin concentrations as low as 100 μU/ml could be measured (378). Using this method (8 rats), Bornstein determined the normal fasting human plasma insulin level to be about 100 μU/ml (<100-130 μU/ml) in three individuals (379). After ingestion of 50 g glucose, plasma insulin levels were found to have risen to about 200 μU/ml (<100-400 μU/ml) at 1 hour and 340 μU/ml (180-700 μU/ml) at 2.5 hours, and then to have fallen to 220 μU/ml (100-500 μU/ml) at 3.5 hours, describing a pattern subsequently seen consistently in normal oral glucose tolerance tests. Intraassay variation ranged from about 18% (C.V.) at 340 μU/ml to 52% at 100 μU/ml. The sensitivity of the ADHA rat to insulin is not affected by exogenous cortisone, somatotropin or corticotropin (380).

Although mice are usually more sensitive than rats to insulin, no less than 250 μU insulin per mouse could be measured with the hypophysectomized alloxan-diabetic mouse (381), mainly because of an extreme lack of precision (C.V. = 300-400%). Since saline alone produces a fall in blood sugar, use of control animals is especially important in this assay.

Insulin assays using hormone-sensitive animals proved not to be of lasting significance because they were most useful where they were needed least. They were far less precise than the traditional rabbit and mouse assays that are suitable for ascertaining the biological activity of preparative quantities of insulin and they were not sufficiently sensitive to measure accurately plasma insulin concentrations in normal individuals. These assays have been superseded by the more sensitive and precise tissue and cell bioassays, immunoassays and receptor assays.

IN VITRO BIOASSAYS

Isolated Tissue Assays

1. Diaphragm assays. In 1952, Groen et al. (382) described an assay for insulin based on the net uptake of glucose from the medium by hemidiaphragms removed from starved rats. A dose-response relationship of glucose decrement (from a 200 mg/dl initial concentration) above control preparations with increasing insulin concentration after a 90 minute incubation at 37°C was obtained between 100 μU and 1.0 U insulin per ml. Vallance-Owen and Hurlock (383) managed to improve the sensitivity of the assay to 10 U/ml. Plasma from normal subjects mimics the effect of insulin in increasing glucose uptake by the rat diaphragm, while plasma from depancreatized dogs has no such effect. Sulfhydryl agents known to inhibit the activity of crystalline

insulin also abolish plasma insulin-like activity in this assay (382) and insulin antisera block the effect of plasma insulin-like activity on the diaphragm (384). However, the rat hemidiaphragm assay for insulin employing the metameter of glucose uptake has been plagued by imprecision. For an initial glucose concentration of 300 mg/dl, a tenfold increase in insulin concentration from 10 to 100 μU/ml produces a decrement of only 2% or 4% in glucose concentration superimposed on a decrement of 6.7% in the absence of insulin (385). Thus, in this physiologic insulin range, the insulin concentration must be increased about 225% to produce a 1% change in glucose concentration and in the range 100-1,000 μU/ml the insulin concentration must increase by about 115% to produce a 1% change in glucose concentration. Consequently, any experimental error exercises an inordinate influence on the results of the assay.

The precision of the hemidiaphragm assay was greatly improved by making glycogen synthesis the criterion of insulin activity. Incorporation of C-14 activity into muscle glycogen from a tracer quantity of glucose 1-[^{14}C] added to the medium is determined after extraction of glycogen from hemidiaphragms with 30% KOH in boiling water and precipitation with 66% ethanol. Using this method, Moody and Felber attained a sensitivity of 10 μ U insulin/ml for mouse diaphragm (386); Guenther (387) obtained a linear relationship between CPM C-14 activity incorporated per gram rat diaphragm glycogen and insulin dose from 50 to 250 μU insulin/ml, with a correlation coefficient (r) of 0.961, highly significantly different from zero ($P<0.001$), and a coefficient of variation for scatter about the line (C.V. ($S_{y.x}$)) of less than 6%.

An <u>in vivo</u> variation of this method has also been developed (388,389). Rats are injected intraperitoneally with a mixture consisting of a glucose solution, a tracer quantity of ^{14}C-glucose, and an insulin standard or unknown.

Two hours later, the rats are sacrificed, diaphragms removed, glycogen extracted and incorporation of C-14 activity determined as in the in vitro assay. Using this technique, Guenther (387) obtained a linear relationship between CPM/g and insulin dose up to 1 mU insulin/ml ($r = 0.993$, $P<<0.001$; C.V. ($S_{y.x}$) = 13%) with a sensitivity of about 75 μ U insulin/ml. Obviously, this method represents a considerable improvement, both in sensitivity and precision, over previous in vivo insulin bioassays.

A range of 0 to 120 μU insulin per ml human serum has been found for normal fasting subjects (390,391), with a threefold increase seen in normal postprandial subjects (391), using the in vitro rat diaphragm assay.

2. Fat pad assays. Insulin enhances glucose uptake, oxidation of glucose to CO_2 and incorporation of glucose carbon into ether-soluble lipid in the rat epididymal fat pad maintained in Krebs-Henseleit buffer (392). In 1958, Martin et al. reported development of the first rat epididymal fat pad assay for insulin based on the second of these effects, insulin induction of glucose oxidation to CO_2 in adipose tissue preferentially via the pentose phosphate pathway with concomitant production of NADPH and ribulose-5-phosphate (393).

The fat pad of each fed male rat is cut into three or six equal parts and each piece is placed in a separate metabolism flask in buffer containing 1-^{14}C-glucose and insulin standard or unknown. After two hours incubation at 37°C in an atmosphere of 95% O_2/5% CO_2, with shaking, the medium is acidified to liberate $^{14}CO_2$ remaining in solution; upon contact with the $Ba(OH)_2$ or NaOH in the cup suspended over the reaction mixture, $^{14}CO_2$ is converted to $^{14}CO_3^{=}$. The solution in the cup is assayed for C-14 activity and the square root or log of CPM per gram adipose tissue is a linear function of insulin concentration.

Using this method, Martin et al. (393) attained a sensitivity of 10 μ U insulin/ml and Guenther (387) obtained a linear relationship between log CPM/gram tissue and insulin dose up to 500 μU/ml ($r = 0.999$, $P<<0.001$; C.V. ($S_{y.x}$) = 3.1%) with an interassay variation of 14.3%. Other metameters used in the fat pad assay are glucose uptake (394) and total gas exchange (395).

A source of puzzlement for more than two decades has been the degree of discrepancy between the insulin values found in the sera of normal fasting individuals using the diaphragm and fat pad assays, the latter yielding values ranging from 33 to 940 μU/ml with levels 2-5.5 times as high after the administration of glucose (396). Although there can be little question that the fat pad is very sensitive to the action of plasma insulin, the assay utilizing this tissue is notoriously lacking in specificity. Human serum insulin-like activity (ILA), as determined using the fat pad assay, is not extractable by acid ethanol (397), is not neutralized by anti-insulin serum (397), and to some extent persists after treatment of plasma with reduced glutathione (396). Fasting levels of ILA persist unchanged after pancreatectomy (396-398) and plasma ILA of pancreatectomized dogs actually increases after glucose administration (398). Although introduction of isolated tissue bioassays enabled routine measurement of plasma insulin concentrations for the first time, they also opened the bewildering and not yet resolved issue of insulin-like activity, or "atypical insulin," the nature of which is discussed in Chapter V.

Another disadvantage of isolated tissue bioassays is their particular sensitivity to the effects of insulin antagonists, especially catecholamines and free fatty acids. The addition of 0.01 μ g epinephrine/ml significantly reduces the effect of 0.5 mU insulin/ml on glucose uptake by rat diaphragm (399). Appreciable degradation of insulin may also occur due to the general action

of proteases and the specific action of the insulinase system when enzymes are liberated from cut diaphragm preparations (400). Furthermore, the potent insulin antagonism of diabetic serum has proven to be due to anti-insulin antibodies, the study of which led to the development of the radioimmunoassay (385).

The dilution of such insulin antagonists has been considered the cause of a disproportionate increase in the amount of ILA (as much as 24-fold) measured by the diaphragm assay with dilution of human sera (up to 8-fold) (391). However, quantitative recovery of insulin added to serum implies that the effect of antagonists is negligible (383,401,402).

Cell Cultures

The use of cultured cells for the assay of insulin is best exemplified by the free fat cell bioassay of Moody et al. (145). Short-term cultures of cells isolated from rat epididymal fat pads are incubated with 0.1-0.5 μCi 2- or 3-^{3}H-glucose and insulin standard or unknown for 2 hours at 37°C. Total lipids are then simply extracted with a toluene-based scintillation fluid and beta-radiation assayed in a liquid scintillation spectrophotometer. Thus, one determines the conversion of glucose to lipid, which is greatly accentuated in the presence of insulin (125-405 times the basal value, which amounts to no more than 0.05% total counts).

Sensitivity of the assay for insulin is 2.5 μU/ml with a linear relationship between CPM/weight lipid and insulin dose from 4 μ U/ml to 15 μ U/ml and an intraassay variation of less than 10%. Both the well-recognized insulin dependence of lipid biosynthesis and the favorable comparison of biologic activities exhibited by insulin analogs as determined by this method and by in

vivo bioassays (see Table I) would lead one to expect a high degree of insulin specificity for this technique. Therefore, cultured cell assays for insulin can afford a degree of sensitivity, convenience and specificity decidedly superior to and a measure of precision comparable to the best of the bioassays. The compelling advantage of delineation of insulin and other effects provided by cultured cells will be examined in Chapter V.

CHEMICAL ASSAYS

Before development of immunoassays, attempts were made to devise specific tests for insulin based on the physicochemical properties of the hormone that would possess the precision characteristic of chemical assays. Two of the most promising were based on fibril formation and paper chromatography.

Fibril Assay

Waugh et al. (403) developed an insulin assay based on the fact that the precipitate formed when a solution of insulin in dilute acid is heated is composed of radially oriented fibrils (404). In this method, an acid solution of insulin is repeatedly heated and frozen until intense flow birefringence occurs. The fibrils so formed are broken by expulsion through a hypodermic needle and used to seed the standard and test solutions of insulin. These solutions are stirred continuously overnight at 48°C and the precipitate formed is collected, washed, dried and weighed. The weight of fibrils obtained was found to be proportional to the amount of insulin present in the seeded solution. The method could not be applied to samples containing less than 11 units insulin/mg and the ability of the assay to reflect losses of biologic activity is questionable (369).

Chromatographic Assay

In 1952, Robinson and Fehr (405) assayed the insulin content of protamine zinc insulin by paper chromatography. A solution of insulin was streaked across a paper and the chromatogram was developed with n-butanol/glacial acetic acid/water (3:1:4). The insulin spots were stained with bromocresol green, the color was intensified in ammonia vapor and the stained material was eluted with borate buffer. A linear relation was found between light absorption by the resultant solution and insulin concentration, from 0.25 to 1.5%.

Fenton (406) separated insulin from associated impurities by ascending paper chromatography with 2-butanol/1% acetic acid (1:1). Insulin was stained with bromocresol green, the color intensified in ammonia and eluted with 0.1 N NaOH/95% ethanol (1:1). The absorbance of eluate was shown to be linearly related to weight of insulin applied up to 110 μ g, with a sensitivity of about 0.5 unit. Good agreement of this method with the mouse convulsion assay was found.

These two chemical assays are capable only of measuring preparative quantities of insulin and are less specific and less indicative of the hormone's biologic activity than *in vivo* bioassays. The fibril method using gravimetric procedures requires a large volume of solution containing at least 1 unit insulin/ml. Grodsky (407) found that the insulin B-chain, esterified insulin and carboxypeptidase-inactivated insulin also form fibrils. The chromatographic methods estimate between 0.5 unit and 150 units insulin, and impurities associated with low-grade insulin may interfere.

Moreover, these methods are more precise and less costly than bioassays. Fenton (406) calculated that a single determination of insulin potency by paper chromatography was equal in precision to that obtained with 960 mice.

IMMUNOASSAYS

Radioimmunoassay (RIA)

After obtaining her Ph.D. in physics at the University of Illinois, Rosalyn Yalow accepted a position as physicist in the radioisotope service of the Bronx Veterans Administration Hospital, where in the early 1950s she began what was to be a profoundly fruitful association with the physician Solomon Berson. In order to test I.A. Mirsky's hypothesis that maturity-onset diabetes might not be due to a deficiency of insulin secretion, but rather to abnormally rapid degradation of insulin by hepatic insulinase (408), they studied the metabolism of ^{131}I-labeled insulin following intravenous administration to nondiabetic and diabetic subjects (409). Berson and Yalow observed that radioactive insulin disappeared more slowly from the plasma of patients who had received insulin, either for the treatment of diabetics or as shock therapy for schizophrenia, than from the plasma of subjects never treated with insulin. Suspecting that the retarded rate of insulin disappearance was due to binding of labeled insulin to antibodies that had developed in response to administration of exogenous insulin and presuming that these antibodies were likely to be present in such low concentrations that classic immunologic techniques would be inadequate to measure them, the two investigators introduced radioisotopic methods of high sensitivity for detection of soluble antigen-antibody complexes. Studying electrophoresis patterns of plasma samples after addition of ^{131}I-labeled insulin, they found that in plasma from insulin-treated patients the labeled insulin is

bound to and migrates with an inter β-γ globulin, while in control plasmas the radioactivity remains near the origin, as does free insulin (409,410).

When Berson and Yalow first attempted to publish this observation as evidence that humans produce insulin-specific antibodies in response to insulin treatment, their manuscript was rejected with a request for more rigorous proof that the insulin-binding plasma component is an antibody (411), probably because of widespread scepticism among immunologists at that time regarding the ability of any substance below 10,000 daltons molecular weight to serve as an immunogen (412). Previously, many of the immunological reactions observed in diabetic patients were attributed to impurities in the insulin preparations then in use (413), but much evidence suggesting the existence of insulin-specific antibodies in humans and other animals had been reported prior to the work of Berson and Yalow (414). This evidence includes development of insulin resistance by insulin-treated humans; the ability of sera from these individuals to prevent or reduce insulin-induced convulsions and other hypoglycemic effects in mice and to abolish the effect of the hormone on glycogen deposition in isolated rat diaphragm; demonstration that the insulin-neutralizing component of these sera is associated with the globulins or γ-globulins by fractional precipitation and starch-block electrophoresis, is non-dialyzable and is thermostable; induction of insulin-resistance in rabbits, guinea pigs and other animals by means of repeated subcutaneous injections of insulin in Freund's adjuvant; and demonstration that the sera of these animals display the same properties as sera from insulin-resistant humans outlined above. Recently it has been shown that injections into rabbits of USP insulin and high molecular weight contaminants (including insulin aggregates and proinsulin) found in it produces insulin-binding antibodies, while highly purified (monocomponent)

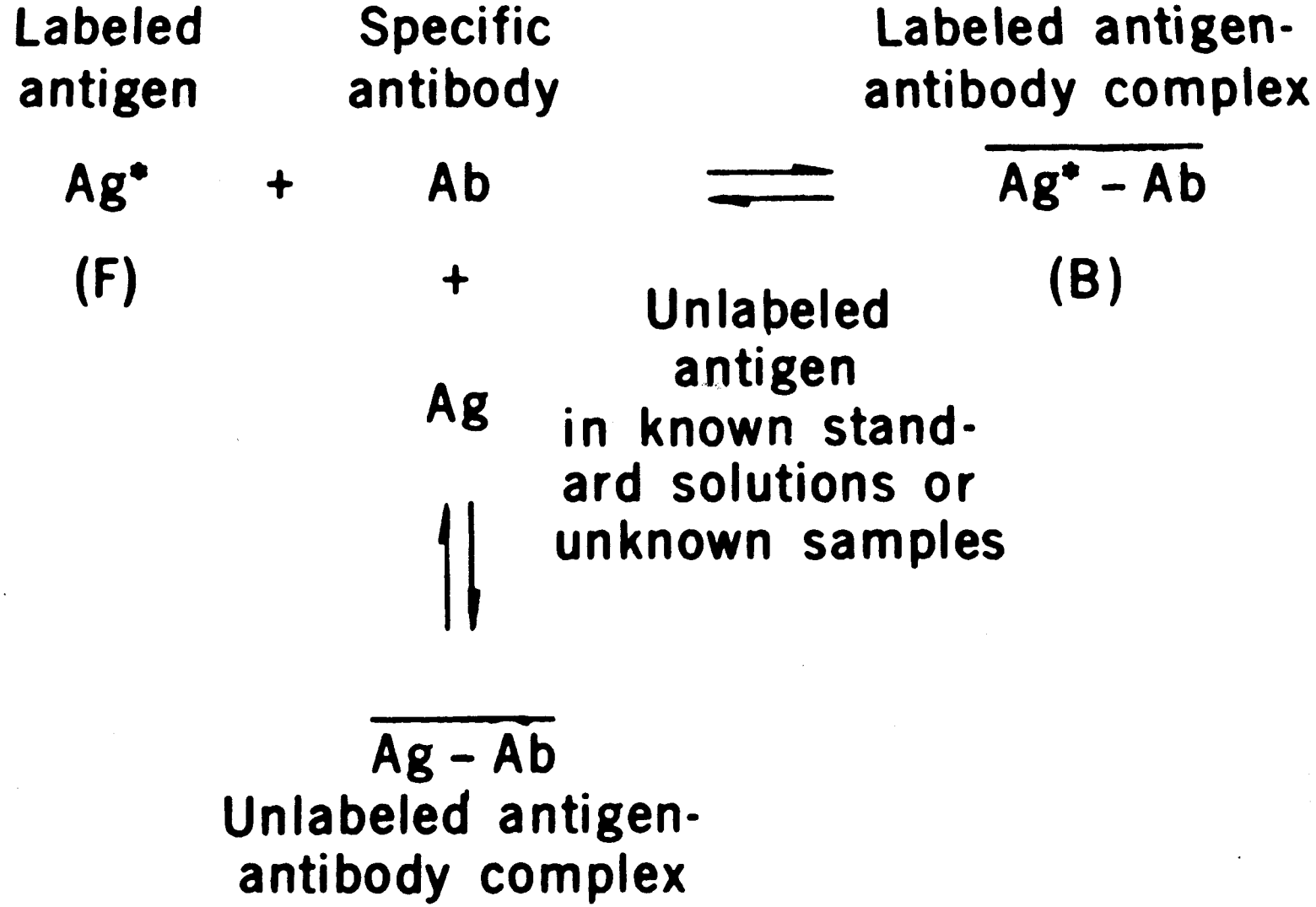
insulin does not, but that even monocomponent insulin is significantly immunogenic in humans (415,416).

Subsequent to Berson and Yalow's work, it was shown that erythrocytes coupled to insulin are agglutinated by sera from insulin-treated rabbits and humans (417-419) and that γ-globulin-bound labeled insulin can be precipitated with rabbit anti-human γ-globulin (420,421). In addition, it has been shown that generally the serum of insulin-treated animals fails to precipitate the hormone (417,422), confirming Berson and Yalow's suspicions. In response to the criticism of the reviewers, Berson and Yalow demonstrated that ultracentrifugation of serum to which labeled insulin has been added also shows that the hormone moves with the γ-globulins in the case of insulin-treated patients, but with the albumin fraction of serum from untreated patients, and that the addition of increasing amounts of unlabeled insulin to a mixture of insulin antibody and labeled insulin results in displacement of the labeled insulin (409). This latter observation led directly to development of the first radioimmunoassay (RIA).

The principle of RIA, as pictured in Figure 6, is based on competitive protein binding rather than simple isotope dilution. A tracer quantity of a radiolabeled form of the antigen to be measured (usually I-125 or I-131 bound to the tyrosyl phenolic ring in the case of proteins and H-3 or C-14 substituents in the case of other antigens), an aliquot of highly diluted xenogeneic antiserum specific for the antigen (usually prepared by hyperimmunization of rabbits or guinea pigs with the antigen in the presence of Freund's adjuvant), and a standard or sample containing unlabeled antigen are mixed in quantities appropriate to produce approximately 50% net binding (bound/free antigen, B/F ≈1) of labeled antigen by antibody at equilibrium in the absence of unlabeled

FIGURE 6. COMPETING REACTIONS THAT FORM THE BASIS OF RADIOIMMUNOASSAY.

(Reproduced, with permission, from Ref. 411.)


Labeled antigen
Specific antibody
Labeled antigen-antibody complex
Ag*
+
Ab
Ag* – Ab
(F)
+
(B)
Unlabeled antigen in known standard solutions or unknown samples
Ag
Ag – Ab
Unlabeled antigen-antibody complex

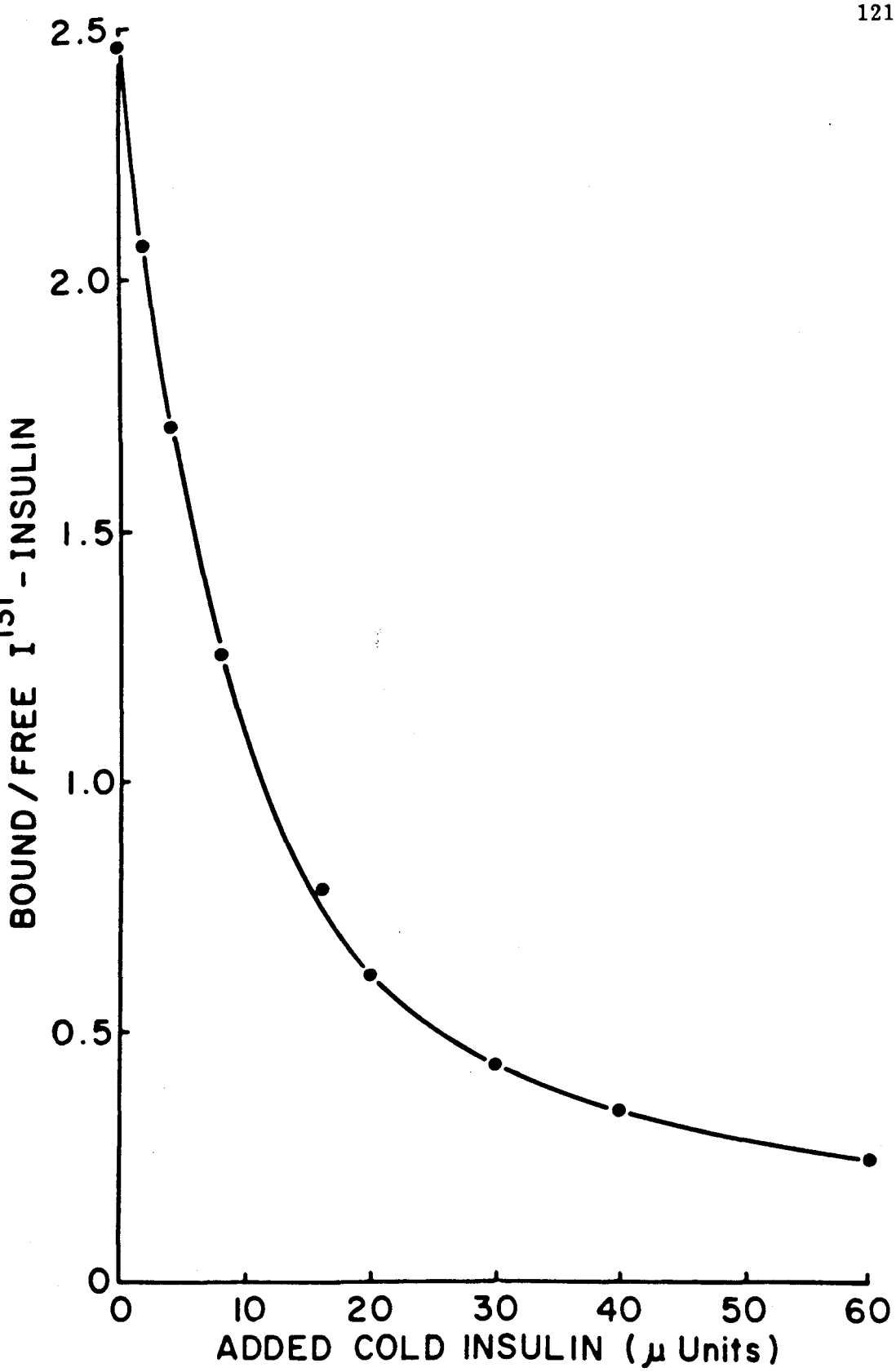
antigen. To attain this result, exceedingly small quantities, in the picogram to nanogram range, of antibody and antigen are required (411). Net fraction antigen bound, B/T, is determined as [total CPM (counts per minute) associated with antibody - nonspecifically bound (NSB) CPM]/[total CPM - NSB CPM] and net percent antigen bound, %B=B/T x 100. Nonspecifically bound antigen is the radioactivity found in the antibody fraction in the absence of specific antibody or in the presence of a large excess of unlabeled antigen.

When unlabeled antigen is added to the mixture, this species competes with labeled antigen for antibody binding sites and a significant reduction in the amount of radioactivity specifically associated with antibody protein is seen. As the concentration of added unlabeled antigen is increased, antibody-bound radioactivity decreases proportionately, resulting in a dose-response relation between fraction radiolabeled antigen bound to antibody (B/T) and concentration of unlabeled antigen, exemplified by the standard curve for insulin reproduced in Figure 7. The antigen concentration of an unknown sample may then be determined from the standard curve simply as that antigen dose corresponding to the proportion of net antibody-bound radioactivity obtained for the sample.

Unlike isotope dilution techniques, RIA does not require that labeled and unlabeled antigen exhibit identical behavior in the assay system, but only that antigen in unknown samples be immunochemically identical to the antigen standard (411). Even the competitive aspects of antigen-antibody interactions and attainment of equilibrium need not be strictly observed since successful RIAs have been developed based on principles of antigen displacement from antibody and approach to equilibrium, utilizing preincubation of unlabeled

FIGURE 7. DOSE-RESPONSE CURVE FOR INSULIN RIA, IN WHICH THE BOUND TO FREE RATIO OF RADIOLABELED INSULIN IS PLOTTED AGAINST THE ADDED COLD INSULIN.

(Reproduced, with permission, from Ref. 423.)


2.5
2.0
1.5
1.0
0.5
0
BOUND/FREE I^{131}-INSULIN
0
10
20
30
40
50
60
ADDED COLD INSULIN (μ Units)

antigen and antibody before addition of labeled antigen (424) and shortened incubation times (423), respectively.

Obviously, the "trick" of RIA is the complete and efficient separation of antibody-bound from free antigen. The first RIA, that developed by Berson and Yalow for assay of insulin in human plasma (425), utilized paper electrophoresis for the separation. Another separation method based on a difference in the physical properties of immunoglobulins and measured antigens, used in early RIAs, is gel chromatography (426). These methods are laborious and time-consuming, however, and proved unsuitable for routine clinical use. More rapid and convenient separation methods based on physicochemical properties of antibody and antigen include nonspecific precipitation of immunoglobulins with ammonium sulfate (427), alcohol (428) and polyethylene glycol (429), and nonspecific adsorption of antigen with talc (430) and charcoal coated with plasma proteins (431), albumin (432) or dextran (423). Although these techniques have found widespread use in clinical RIAs, one may question their use for more precise applications since anomalous values are likely to be found for fluids differing significantly in protein composition from normal serum (433) and precipitation methods tend to disturb the equilibrium of the antigen-antibody interaction (433,434). Furthermore, relatively high values for nonspecific binding of labeled antigen to antibody are likely to be found, including spurious contributions from nonspecific trapping of free antigen in precipitates and inability of charcoal to adsorb "damaged" tracer which may nonetheless retain immunoreactivity (433).

Immunoprecipitation of antibody with a second antibody specific for serum components, gamma globulins or IgG of the species in which the first antibody was prepared (double antibody method; 435) is well-suited to research

applications. Although more time-consuming, the double antibody method exhibits far less interference due to nonspecifically bound radioactivity and variation in fluid composition than nonspecific precipitation methods (433). Because antigen- and second antibody-binding sites are distinct (433), equilibrium distribution of bound and free antigen is relatively unaffected. A solid-phase variation of the double antibody method involves coprecipitation of first and second antibodies prior to addition of other components, which then require constant mixing with the insoluble antibody (433).

Solid-phase antibody systems offer a potentially popular means of RIA separation because they are extremely convenient and easily adapted to automated protocols, especially the coated tube method (436). Antibody is adsorbed onto polystyrene or polyethylene tubes, which may then be stored in dry form. After appropriate incubation with labeled and unlabeled antigen, bound antigen is separated from the free form simply by decantation or aspiration. Because the composition of the antibody phase is difficult to control, however, the sensitivity and precision of this method tend to be poorer than that of soluble antibody RIAs (433). With the development of more efficient means of immobilizing immunoglobulins via covalent coupling to insoluble matrixes, a wide variety of solid-phase RIAs have been attempted, among the most successful of which employs antibodies coupled to Sephadex (437,438). Although this last method exhibits a high degree of precision and accuracy (433,439), solid-phase RIAs tend to be costly and wasteful of antiserum (433).

Competitive protein binding assays, including RIA, provide dose response curves that exhibit severe nonlinearity. Thus, numerous attempts have been made to linearize the relation between labeled antigen bound to antibody and unlabeled antigen added in order to render data reduction compatible with

automated, relatively simple, low-cost computer systems. The most successful early attempt was that of Hales and Randle (424), in which C_o/C_i, where C_o is the radioactivity of the antigen-antibody complex in the absence of unlabeled antigen and C_i is the radioactivity of this complex at a given concentration of unlabeled antigen, is plotted against concentration of unlabeled antigen. An example of a Hales-Randle plot, obtained by Guenther (387), is shown in Figure 8. Using a coprecipitated double antibody complex and a microfiltration technique for separation of bound from free insulin, Guenther obtained a linear relation between C_o/C_i and insulin dose up to 200 μ U/ml with a correlation coefficient of 0.979 ($P<<0.001$, C.V. ($S_{y \cdot x}$)=13%). The sensitivity of this assay, however, was no better than 10 μ U insulin/ml.

A partial linearization of the dose response has been achieved using a semi-log plot of bound/total radioactivity (B/T) or percent total radioactivity bound (%B) vs. log unlabeled antigen added, resulting in a sigmoidal curve (Figure 9). Several attempts have been made to linearize this plot utilizing sigmoidal transformations, the most successful of which is the logit transform,

$$\text{logit } Y = \ln \frac{Y}{100 - Y} = 2.303 \log \frac{Y}{100 - Y},$$

where Y=(%B/%B in the absence of unlabeled antigen) x 100=$\%B_o$ (440). When logit ($\%B_o$) is plotted against log antigen dose a linear relationship between 10% and 90% (logit=-2.2 - +2.2) is obtained (Figure 10). It should be emphasized that the linear relation obtained results from mathematical manipulation of a non-linear function and does not necessarily reflect any intrinsic property of the RIA system (440).

FIGURE 8. DOSE-RESPONSE CURVE FOR INSULIN RIA, PLOTTED ACCORDING TO THE METHOD OF HALES AND RANDLE.

Each point is the mean of 10 determinations.

(From Ref. 387)

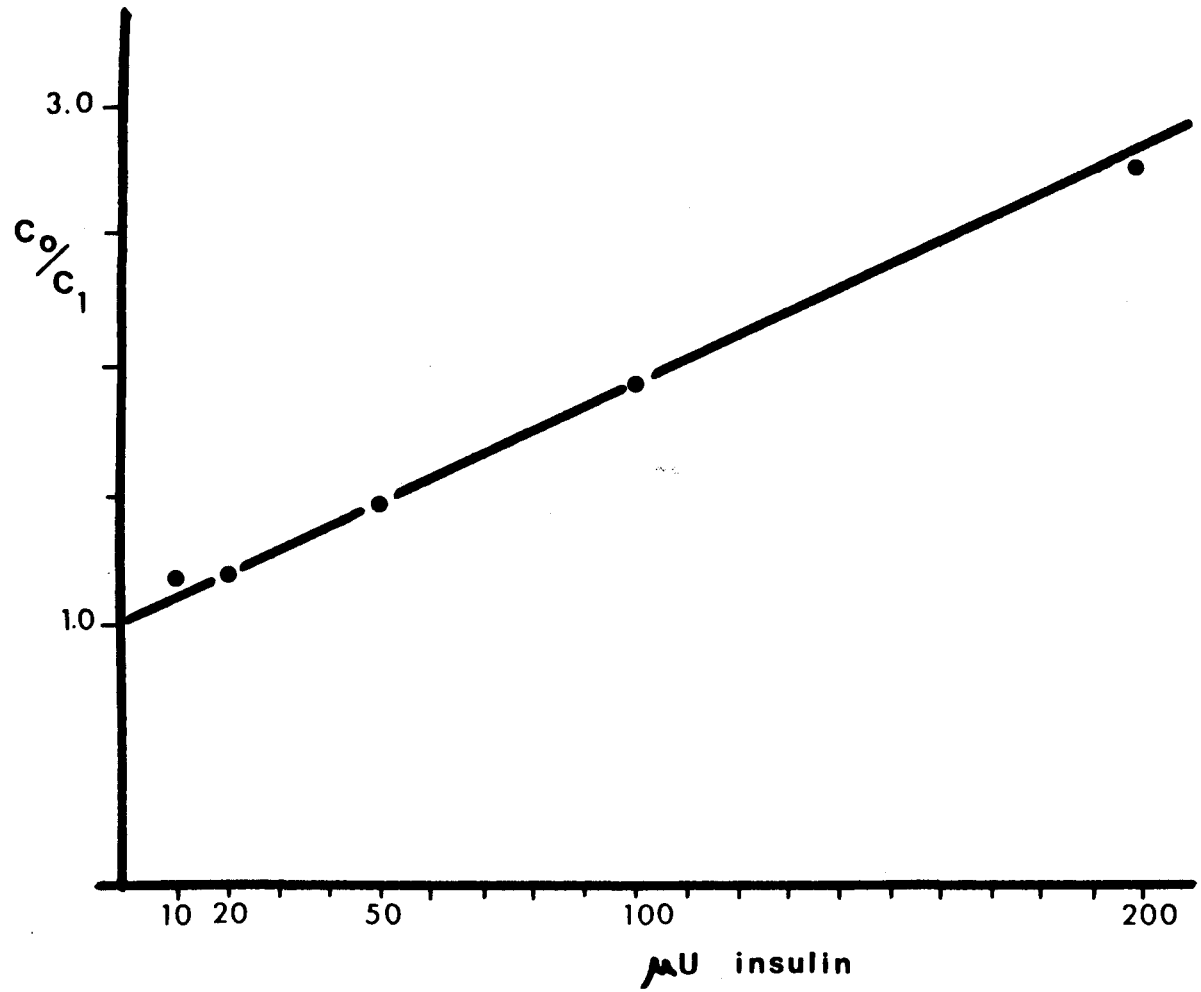

3.0
C_o/C_1
1.0
10 20 50 100 200
μU insulin

FIGURE 9. DOSE-RESPONSE CURVE FOR HUMAN LUTEINIZING HORMONE RIA: SEMI-LOGARITHMIC PLOT.

Bars indicate the range of 10 replicates.

(Reproduced, with permission, from Ref. 440. Copyright 1968 by the Endocrine Society.)

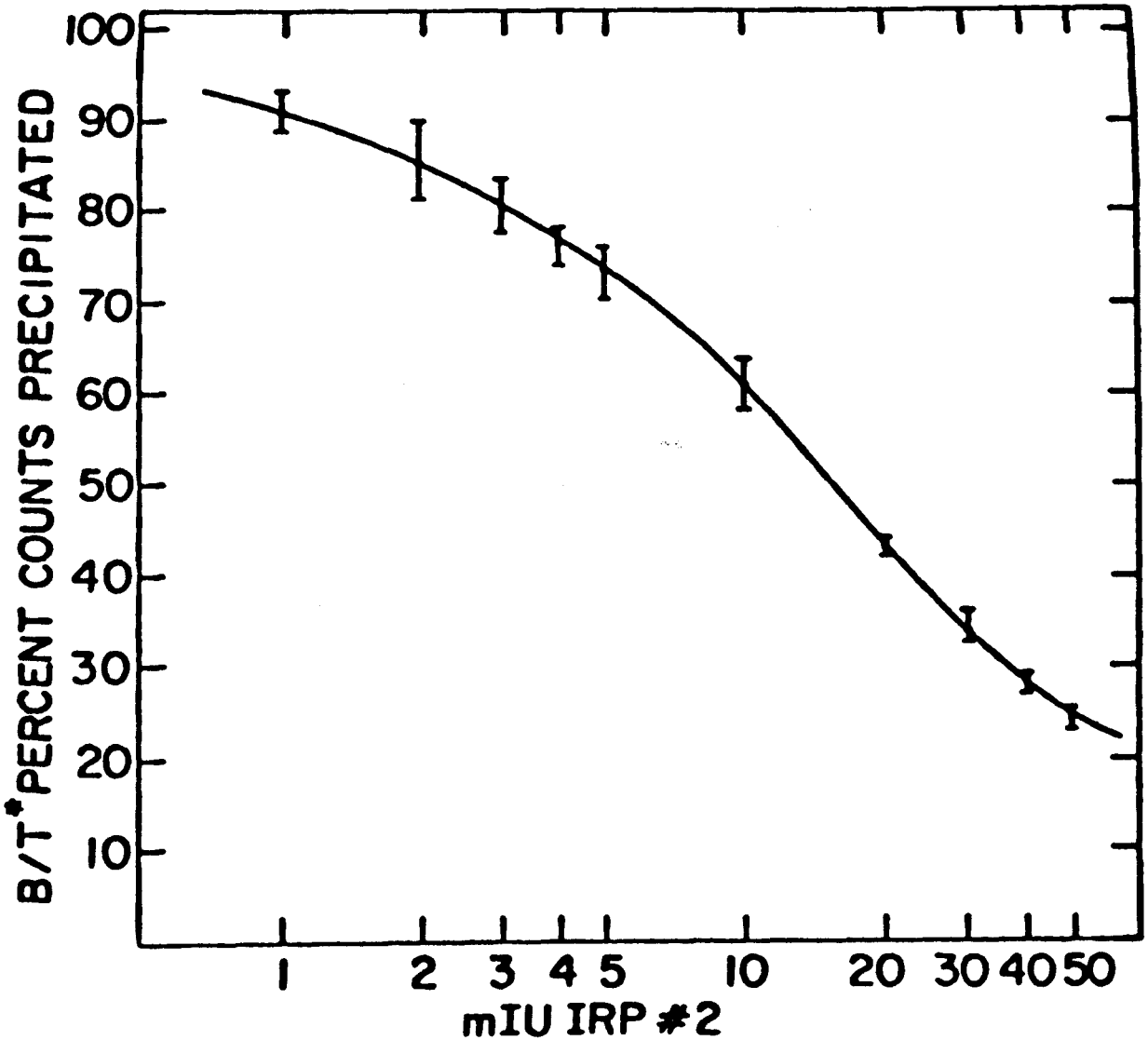
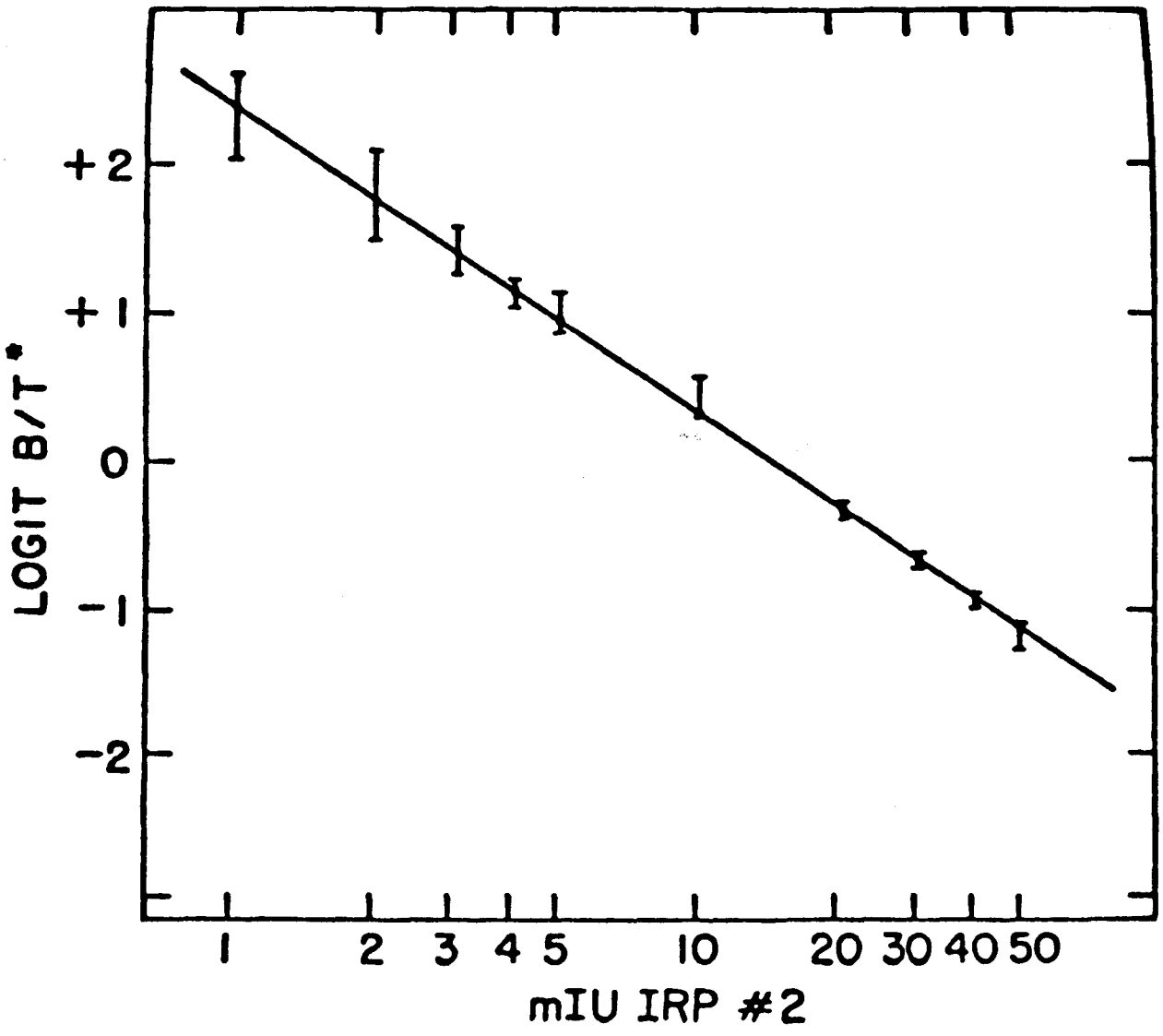

B/T* PERCENT COUNTS PRECIPITATED
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
10
20
30
40
50
mIU IRP #2

FIGURE 10. DOSE-RESPONSE CURVE FOR HUMAN LUTEINIZING HORMONE RIA: LOGIT-LOG PLOT.

Data of Figure 9.

(Reproduced, with permission, from Ref. 440. Copyright 1968 by the Endocrine Society.)


+2
+1
0
-1
-2
LOGIT B/T*
1
2
3
4
5
10
20
30
40
50
mIU IRP #2

Although it is technically feasible to apply linear regression analysis to logit-log plots and thereby determine the residual variance (scatter) about the regression line, the results of such analysis would be invalid and severely biased due to intensification of heteroscedasticity, or non-uniformity of the residual variance, by the logit transformation (440). Thus, a quality control approach has been found to be the most practical, expedient means of judging the suitability of established RIAs (440). The four most informative quality control parameters are B_O, the percent antigen bound in the absence of unlabeled antigen; the antigen dose at which $\%B_O=50\%$ (logit Y=0); the intraassay variation, the coefficient of variation for sample replicates assayed at the same time; and the interassay variation, the C.V. for samples assayed repetitively at different times.

Intraassay variation, a measure of assay precision, and interassay variation, a measure of assay stability and reproducibility, found for RIAs are typically less than 10% and 15%, respectively (440-443). Although the 90% B_O x-intercept in a logit-log plot is frequently considered the sensitivity of an RIA, the 50% intercept is a more suitable reflection of sensitivity for use as a quality control parameter, since it is situated at the center of the curve and thus is relatively unaffected by minor fluctuations of slope (440). As little as 1-3 μU insulin/ml is commonly detected by RIA (438,443,444), with a sensitivity of 0.3 μU/ml having been reported (445).

Before a given RIA can be considered a useful technique to which quality control tests may be applied, the validity of the assay must first be proven by certain established procedures. Assay specificity is generally verified by assaying dilutions of samples containing high concentrations of antigen; if the relation between sample dilution and %B parallels that of the standard so that

assignment of an antigen concentration to the sample based on the standard results in the superimposibility of the sample and standard dose-response curves, then one may conclude that antigen in the sample is identical to antigen in the standard and that the assay measures only that particular antigen in sample fluid (433,441-443). When an RIA is meant to measure one of a group of closely related antigens, such as the gonadotropins (luteinizing hormone, follicle-stimulating hormone and chorionic gonadotropin), somatotropin and prolactin, insulin and proinsulin, and the steroid hormones, assay specificity is often determined by assaying dilutions of the possibly cross-reacting substances in the RIA (433,441,443). The extent of cross-reactivity may be quantitated by comparing the doses at which 50% B_0 is attained.

Accuracy of an RIA may be ascertained by demonstrating "recovery" of exogenous unlabeled antigen from sample fluid. Antigen standard is diluted in sample fluid which is then assayed with the RIA. If the difference between results obtained for this preparation and that of untreated fluid closely approximates the antigen concentration of the added standard, the assay is considered capable of measuring accurately the quantity of immunoreactive antigen in that fluid (441-443). If RIAs for a particular antigen have already been developed, one may also compare the results of a newly-developed RIA for antigen concentrations in fluids from normal individuals and those suffering from well-characterized pathologic conditions with corresponding values reported using previously developed RIAs. Insulin levels in human sera from normal fasting individuals, determined by RIA, range from 1.0 to 20 μU/ml (443), with a mean of 8.44 0.35 μU/ml (S.E.M., n=75) having been reported (444). Serum insulin concentrations rise four- to tenfold after ingestion of 100 grams oral glucose (oral glucose tolerance test, OGTT), attain their

maximum values at 30 to 60 minutes, and thereafter gradually fall to the fasting level or below at two to four hours (443).

So precise and reproducible are RIAs for insulin that it has been possible to discriminate among normal, pre-diabetic, maturity onset diabetic and juvenile onset diabetic conditions from the pattern of serum immunoreactive insulin (IRI) levels during OGTTs (446,447), which have also been used to assess the probability of diabetic angiopathy, neuropathy and renal disease in maturity onset diabetics (448). Furthermore, the use of insulin RIAs for determination of serum IRI levels after administration of glucose, tolbutamide, leucine or glucagon has made possible the diagnosis of insulin-secreting tumors (insulinomas) with a high degree of reliability (446).

Radioimmunoassay is unsurpassed in its sensitivity, reproducibility, precision and specificity for a given antigenic species. Thus, the major drawback regarding RIA is the broader issue of accuracy. An antigen may lose its biologic activity, but retain its immunoreactivity, while a closely related or altered antigen may be capable of exerting a potent biologic effect, but have a much reduced affinity for antibody utilized in the RIA. Nowhere does this question have more important implications than in the fields of insulin and diabetes research, since there remains the possibility that insulin aggregates or protein-bound insulin constitute a physiologically important hormone reservoir that is largely undetected by RIA (see Chapter V).

Radioimmunoassay is also extremely convenient, provided one possesses the appropriate equipment for carrying it out. Not every laboratory can afford an automated gamma ray or liquid scintillation spectrophotometer, large centrifuges, and even a desk computer necessary for large-scale RIAs. For those possessing such equipment, antigen determinations can be made on several

hundred samples in a single assay (411). Of special importance to clinical and research applications alike is the ability of RIA to measure antigen levels in very small sample volumes (0.1 ml or less). Furthermore, the potential applications of RIA to biomedical research and diagnosis appear virtually unlimited because of recent advances in techniques for production of haptens (non-immunogenic compounds rendered capable of eliciting specific antibody production in animals after conjugation of the compounds to immunogenic proteins such as albumin) and radioisotopic labeling of organic compounds.

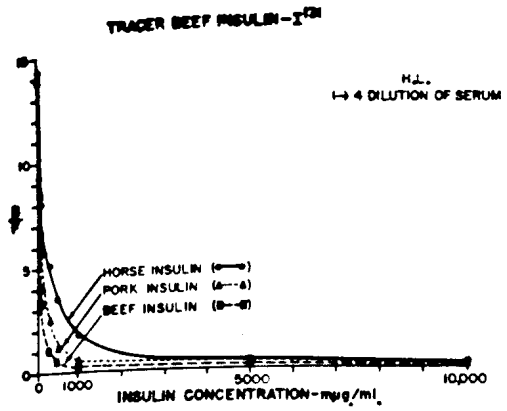
Thus, it is not surprising that during the past twenty years RIAs have been developed to measure a myriad of substances, including peptide and protein hormones, thyroid hormones, steroids, prostaglandins, neurotransmitters, non-hormonal serum proteins, drugs, vitamins, cyclic nucleotides, enzymes, viruses and tumor antigens (411). Plainly, RIA has had a major impact on those areas of study that could benefit from a striking increase in the ability to measure exceedingly small quantities of a given substance, and has even enabled the monitoring of fluctuations in intracellular concentrations of compounds reliably. This impact has been especially profound on the fields of endocrinology, cell physiology, oncology, and immunochemistry. Regarding this last application, RIA has been particularly useful in the immunochemical characterization of insulin.

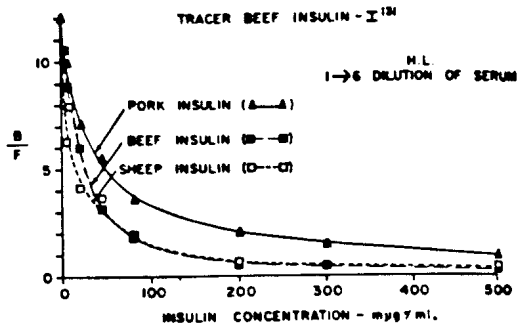
In their ability to inhibit binding of radiolabeled insulin to anti-insulin antibodies, insulins derived from various vertebrate species have the dual advantage of being extensively, but not completely, cross-reacting. Although the degree of inhibition per unit concentration of unlabeled insulin and thus the antigen-antibody affinity may differ among various insulins with a given antiserum, Berson and Yalow (449) found that bovine, porcine, ovine, equine

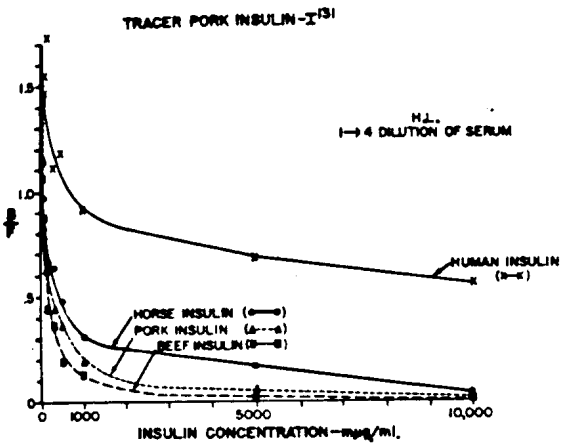
and human insulins were all capable of saturating antibody binding sites in human anti-insulin sera (inhibition curves shown in Figure 11). These investigators found that the kinetics of the association indicate that insulin is probably univalent and that there are at least two distinct orders of antibody-combining sites. Association constants for high affinity sites ranged from 3 x 10^8 M^{-1} (horse insulin) to 1.4 x 10^9 M^{-1} (beef insulin) and for low affinity sites from 3.4 x 10^6 M^{-1} to 3.2 x 10^7 M^{-1} utilizing serum obtained from an individual treated with a combination of bovine and porcine insulins. Lower affinities of iodinated, nitrated, desalanine-desasparagine (DAA), and desoctapeptide (DOP) insulins and proinsulin for guinea pig anti-insulin antibodies compared to native insulin in the RIA, represented by lesser slopes in Hales-Randle plots, have also been observed (136).

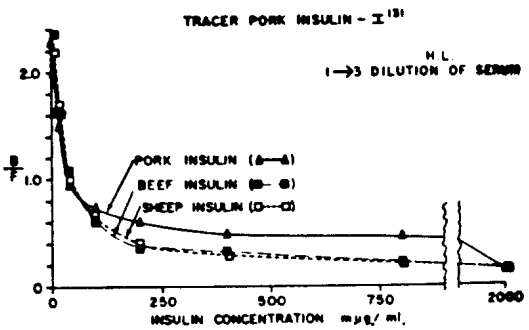
Apparently, the ability to discriminate among insulin from different species is dependent mainly upon the source of the insulin used for immunization and for radiolabeling. Serum from humans treated with porcine insulin cannot discriminate among bovine, porcine, human and DOP insulins, whether radiolabeled porcine or bovine insulin is used (450). When these subjects are further treated with bovine insulin, however, their antibodies exhibit a lower affinity for DOP insulin compared to the animal insulins studied if an ^{131}I-porcine insulin tracer is used and can discriminate between bovine and porcine vs. human and DOP insulins if an ^{131}I-bovine insulin tracer is used (450). At appropriate dilutions, sera from two of three individuals treated with a combination of bovine and porcine insulins can discriminate between bovine and porcine insulins, with bovine insulin exhibiting the greater affinity, provided a radiolabeled bovine or ovine insulin tracer is used (449).

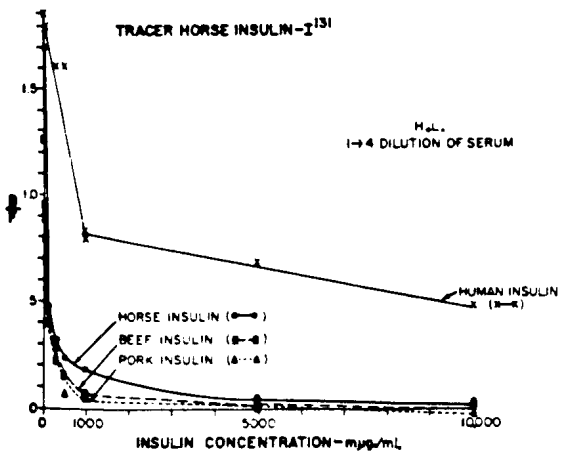
FIGURE 11. DOSE-RESPONSE CURVES FOR INSULIN RIA USING HUMAN ANTI-INSULIN SERUM H.L.

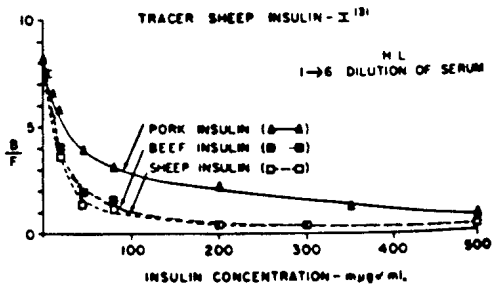

TRACER BEEF INSULIN-I¹³¹
H.L.
1→4 DILUTION OF SERUM
HORSE INSULIN
PORK INSULIN
BEEF INSULIN
INSULIN CONCENTRATION-mμg/ml.


TRACER BEEF INSULIN-I¹³¹
H.L.
1→6 DILUTION OF SERUM
PORK INSULIN
BEEF INSULIN
SHEEP INSULIN
INSULIN CONCENTRATION - mμg/ml.


TRACER PORK INSULIN-I¹³¹
H.L.
1→4 DILUTION OF SERUM
HUMAN INSULIN
HORSE INSULIN
PORK INSULIN
BEEF INSULIN
INSULIN CONCENTRATION-mμg/ml.


TRACER PORK INSULIN-I¹³¹
H.L.
1→3 DILUTION OF SERUM
PORK INSULIN
BEEF INSULIN
SHEEP INSULIN
INSULIN CONCENTRATION mμg/ml.


TRACER HORSE INSULIN-I¹³¹
H.L.
1→4 DILUTION OF SERUM
HUMAN INSULIN
HORSE INSULIN
BEEF INSULIN
PORK INSULIN
INSULIN CONCENTRATION-mμg/mL


TRACER SHEEP INSULIN-I¹³¹
H.L.
1→6 DILUTION OF SERUM
PORK INSULIN
BEEF INSULIN
SHEEP INSULIN
INSULIN CONCENTRATION-mμg/ml.

The results of these experiments suggest that the A8-10 apolar amino acids, which are the only ones that differ among bovine, porcine, ovine and equine insulins, are a site of antigenic determinacy (449). The antigenicity of the B30 residue, however, may be dependent upon the conformation of the insulin molecule, since sera from individuals treated with porcine insulin cannot discriminate between human and porcine insulins, which differ from each other only in the B30 residue (alanine in the pig, threonine in man), while anti-bovine insulin sera do discriminate between the two insulins, suggesting that the B30 alanine is presented in a more favorable way immunogenically by bovine insulin than by porcine insulin. This supposition is supported by the fact that antibodies to porcine insulin react well with desalanine porcine insulin and cannot discriminate between DOP bovine and intact insulins, while anti-bovine insulin sera readily distinguish DOP beef insulin from intact bovine and porcine insulins, but not necessarily from human insulin (450). Antisera from rabbits (whose insulin differs from human and porcine insulins only in containing B30 serine) immunized with porcine insulin also react with DOP bovine insulin (450).

That the conformation of the insulin molecule differs among species and that conformational differences affect the antigenicity of the hormone are supported by the finding that certain human anti-porcine, bovine insulin sera discriminate sharply between porcine and sperm whale insulins, which possess identical primary structures (450,451). Several years before the discovery of proinsulin, Berson and Yalow interpreted this observation to mean that the overall protein structure may not be determined by amino acid sequence alone (451). The concept that protein conformation is ultimately determined by primary structure was vindicated, however, when the insulin portion of the proinsulin molecule was shown to be in the same conformation as the free

insulin molecule (452). Thus, insulin conformation is dependent upon the tertiary structure of proinsulin, to which the primary structure of the connecting peptide (C-peptide, see Chapter V, "Insulin Precursors") would be expected to make a major contribution and, indeed, comparison of the amino acid sequences of human, porcine and bovine proinsulins reveal a high degree of variance within the respective C-peptide segments (453). This postulate is supported by the fact that oxidation of reduced A and B chains of sheep insulin yielded only 12-16% biologically active insulin (157,158), indicating that insulin is not in its thermodynamically most stable conformation, while reoxidation of fully reduced beef and rat proinsulins results in a high proportion of the native configuration, which could be converted to biologically active insulin by trypsinization (454), suggesting that the conformation of insulin is dictated by the thermodynamically most stable conformation of proinsulin.

It may be seen from Table I that the A14 and A19 tyrosines are important to insulin immunoreactivity. Unfortunately, RIA is a relatively insensitive means of investigating the effects of changes at these positions, since they are prime sites of radioiodination. Monoiodoinsulin is labeled equally at A14 tyr and A19 tyr (136), leaving half the labeled insulin intact at each site and able to bind antibodies specific for these epitopes. The partial competence of labeled insulin at these sites is indicated by findings of 37.7 and 58.4% immunoreactivity for mononitro- and monoiodoinsulin, respectively, by RIA and corresponding values of 6.7 and 3.9% by immune hemolysis inhibition (136). Table I also indicates that A1 glycine and A21 asparagine may be important immunodeterminants, while B1 phenylalanine and B29 lysine are relatively unimportant. Alteration of both B1 and B29 residues, however, leads to significant reductions of immunoreactivity. Arquilla has suggested that a

significant proportion of the antibodies involved with the insulin RIA may combine with determinants that include B10 histidine (136).

Previously iterated evidence for the immunogenicity of insulin, namely insulin resistance in animals treated with the hormone and the ability of these animals' sera to block insulin's biologic effects in both in vivo and in vitro systems, also provides a basis for the assertion that insulin immunodeterminants lie in or near the hormone's active site. This contention is also supported by the induction of experimental diabetes in animals via injection of hyperimmune anti-insulin sera (414). Thus, it is especially significant that the A1 glycine, A19 tyrosine and A21 asparagine—all associated with the insulin active site (135)—have also been implicated as insulin immunodeterminants. Although insulin aggregates are important to the immunogenicity of the hormone, immunodeterminant residues have been implicated in the hydrophobic aggregation regions of the monomer: B30 alanine in the dimer face and A14 tyrosine in the hexamer face. This contention is supported by the observation that compromised immunoreactivity of FITC-insulin derivatives parallels the progressive inability of these derivatives to form aggregates (136), leading one to expect that large insulin aggregates should exhibit decreased immunoreactivity. The immunogenicity of insulin aggregates may be related to an increased molecular size in excess of that required for processing by macrophages (455), which may then "read" dissociated monomer determinants.

Enzyme Immunoassays

Because RIA may involve exposure to potentially dangerous radioactivity, requiring special handling of materials and the use of expensive equipment, immunoassays utilizing enzyme-labeled antigen have been developed. Among

those procedures collectively known as enzyme-linked immunosorbent assays (ELISA), this technique is based upon the same principles as RIA, but determines quantity of labeled antigen bound to antibody by measuring enzyme activity in the presence of a large excess of substrate. Typically, the product of the enzyme reaction exhibits a characteristic color and may be quantitated in a simple colorimeter, giving ELISA a potentially great advantage over RIA in its applicability to small laboratories and fieldwork. The frequent use of specific antibody adsorbed to plastic test tubes or multiwell microtiter plates in ELISA increases the potential convenience of this assay (456,457).

Miedema et al. developed a solid phase ELISA for insulin utilizing anti-insulin serum adsorbed to polystyrene test tubes, insulin coupled to alkaline phosphatase with glutaraldehyde, and p-nitrophenylphosphate as substrate, the reaction product of which was monitored by measuring absorbance of 405 nanometer wavelength light (A_{405}) (458). The sensitivity of this assay was found to be no better than 75 μU insulin/ml, however, with a mean serum insulin concentration of 63 μU/ml (below the sensitivity) for normal fasting individuals and a normal range of 20 to 106 μU/ml, indicating that the sensitivity and precision of ELISA is far inferior to that of RIA for assay of insulin.

Enzyme Immunoelectrode

Mattiasson and Nilsson (459) described covering an oxygen electrode with a nylon net to which are coupled guinea pig anti-porcine insulin antibodies for the immunoassay of insulin. Insulin in a sample competes with glucose oxidase-labeled insulin for antibody sites on the nylon net. After a three-minute incubation, substrate (glucose) is added and the signal from the electrode,

reflecting the change in oxygen concentration because of the enzyme-catalyzed reaction, is recorded on a strip chart. After the assay, antigen is dissociated from antibody by washing with a pH 2.2 glycine buffer, and in about three minutes the electrode is ready for use again. Analysis of each sample requires about ten minutes. The sensitivity of the assay (about 10 μ g/ml) is many times poorer than that of RIA techniques and it is not yet applicable to routine testing (456).

RADIORECEPTOR ASSAYS

It became apparent soon after systematic studies of insulin-receptor interactions were begun that a competitive protein-binding assay for insulin could be developed based on the same principles as RIA, but utilizing specific cell receptors for the hormone instead of antibodies. Such as assay may possess the sensitivity and precision of RIA while measuring the biologic activity of the hormone. This promise has been realized through the development of insulin radioreceptor assays (RRA) utilizing both particulate (membrane) and solubilized receptor preparations.

Using a crude guinea pig kidney membrane fraction and ^{125}I-porcine insulin with separation of membrane-bound from free insulin effected by Millipore membrane filtration, Suzuki *et al.* obtained a sensitivity of 10 μ U insulin/ml with an intraassay variation of 8% (CV) and an interassay variation of 13% (460). Displacement of tracer from receptors by porcine proinsulin was 28% that of porcine insulin, while guinea pig insulin exhibited less than 1% of the potency of the porcine hormone in the RRA. Human serum insulin levels during OGTTs were found to increase from a mean of 19 μ U/ml (n=3) in fasting subjects to a maximum of 90 μU/ml (n=6) at 60 min., after which

they declined to 38 μU/ml at 180 min. Excellent correlation between RIA and RRA for serum insulin was found (r=0.952) with a correspondence of y=0.905x+1.24, where y=serum insulin measured by RIA (μU/ml) and x=serum insulin measured by RRA (μU/ml).

Ozaki and Kalant developed RRAs for insulin utilizing a membrane fraction derived from fresh normal human placentas and membrane receptors solubilized with Triton X-100 detergent (461). Receptor-bound insulin was separated from free hormone by centrifugation in the case of the membrane assay and by adsorption of free insulin to dextran-coated charcoal in the case of the soluble receptor assay. With the latter assay, these investigators were able to attain a sensitivity of 0.5 μU insulin/ml, which is understandable since the affinity of the receptor for insulin ($K=1.8 \times 10^{10}\ M^{-1}$) is similar to that of the antibody-hormone interaction using hyperimmune xenogeneic sera. The intraassay variation was 1.3% and the interassay variation was 10.9%, with an RIA correspondence of y=0.85x+2.7 (r=0.944), where the parameters are the same as iterated above. In this assay, proinsulin exhibits 4-5% cross-reactivity with the soluble receptor; thus, the reactivity of the prohormone in the RRA approximates its biologic activity.

The RRA may well prove to be the ultimate assay for insulin, combining the best features of RIA and bioassay, but it is unlikely that it will be adopted for routine clinical use for the following reasons: 1) The lifetime of a receptor preparation is several months, compared to several years for an antiserum; thus, it would be necessary to prepare new receptor fairly often. Even if such a preparation were made available commercially, each lot may exhibit significantly different binding properties and require recharacterization of the assay. 2) Characteristic levels and fluctuations of immunoreactive insulin in

health and disease have already been determined and have proven more than adequate for clinical purposes. 3) Serum insulin concentrations determined by RRA are nearly identical to those found by RIA.

Neither RIA nor RRA can be expected to exert much impact on the state of ignorance concerning the nature of circulating insulin, however, since there remains the possibility that the hormone may be bound to a carrier protein or itself (aggregates), masking both antibody- and receptor-binding structures. Investigation of serum insulin-like activity, including this possibility, requires a bioassay with a sensitivity within the physiologic range of immunoreactive insulin and the ability to discriminate between "typical" insulin and other serum factors that mimic the effects of the hormone in the traditional bioassays. The use of cells in culture may provide such an assay.

CHAPTER V

INSULIN-LIKE ACTIVITY (ILA)

Serum insulin-like activity or "atypical" insulin is distinguished from true monomeric or "typical" insulin by the fact that, while the former mimics the latter in one or more assay systems, the two differ in their physicochemical, immunochemical, and/or particular biological properties. The properties of typical and atypical insulin species discussed in the following sections are summarized in Table V, at the end of this chapter, to facilitate comparisons that may enable one to formulate conclusions regarding the nature of serum ILA.

TYPICAL INSULIN

Obviously, before the subject of atypical insulin may be broached, it is first necessary to define what is meant by typical insulin, which is the hormone possessing the physical, chemical and biologic properties described in previous chapters. Reiterating some of the more important of these properties, typical insulin is the monomeric species of the hormone, exhibiting a molecular weight between 5,500 and 6,000 daltons and consisting of two polypeptide chains covalently joined by two disulfide bonds. It exhibits a sedimentation coefficient ($S_{20,w}$) of 1.2 S, a diffusion coefficient ($D_{20,w}$) of 1.6 x 10^8 cm^2/sec., and a frictional ratio (f/f_0) of 1.12, indicative of a nearly spherical molecule. This species also has an electrophoretic mobility between that of albumin and α_1-

globulins, is soluble in acid ethanol, forms radially oriented fibrils when heated in dilute acid solution and rhombohedral crystals in the presence of zinc.

Theoretically, one mole of anti-insulin IgG antibodies will bind two moles of typical insulin. Thus, typical insulin will bind 13 times its weight in anti-insulin IgG. Binding capacity of sera from insulin-resistant humans has been found to range from about 2 to 200 μg typical insulin/ml (366), corresponding to 25 μg-2.5 mg anti-insulin IgG/ml, and association constants found for anti-insulin antibodies and typical insulin range from 10^6 to 10^{11} M^{-1} (428,440). Association of antibodies specific for determinants present on a given antigen and that antigen will be inhibited most efficiently by compounds structurally most similar to the antigen. Therefore, IRI concentrations determined by RIA (using an antiserum raised against pure typical insulin) relative to a typical insulin standard will be maximal for typical insulin.

Binding capacity of cellular insulin receptors is 40 to 250,000 molecules typical insulin (TI) per cell, 0.2 to 18 pmole TI per mg membrane protein, and 0.3 to 7 pmole TI per mg soluble receptor protein. Association constants found for receptor-TI interactions range from 10^7 to 10^{10} M^{-1}. Because this interaction is dependent upon the integrity of the hormone's receptor-binding site, insulin concentrations determined by radioreceptor assays are directly proportional to specific biologic activity relative to typical insulin.

Intravenous injection of 125 μg typical insulin into a 2 kg rabbit starved for 24 hours will cause an approximately 50% reduction in blood glucose over a period of 2-4 hours. Intravenous injection of 40 ng TI into a 25 g alloxan diabetic, hypophysectomized mouse will cause a reduction in blood glucose concentration of about 40 mg/dl after 20 minutes (362). Addition of 100 μU (4

ng) TI/ml to the medium results in a 50% increase in glycogen synthesis by isolated rat diaphragm, while a concentration of 5 μg TI/ml causes a 2-fold increase (relative to insulin-free medium) in CO_2 production by rat epididymal fat pads (368). Addition of 10 μU (0.4 ng) TI/ml medium causes a 4-fold increase in conversion of glucose to total cell lipids by isolated rat fat cells (145).

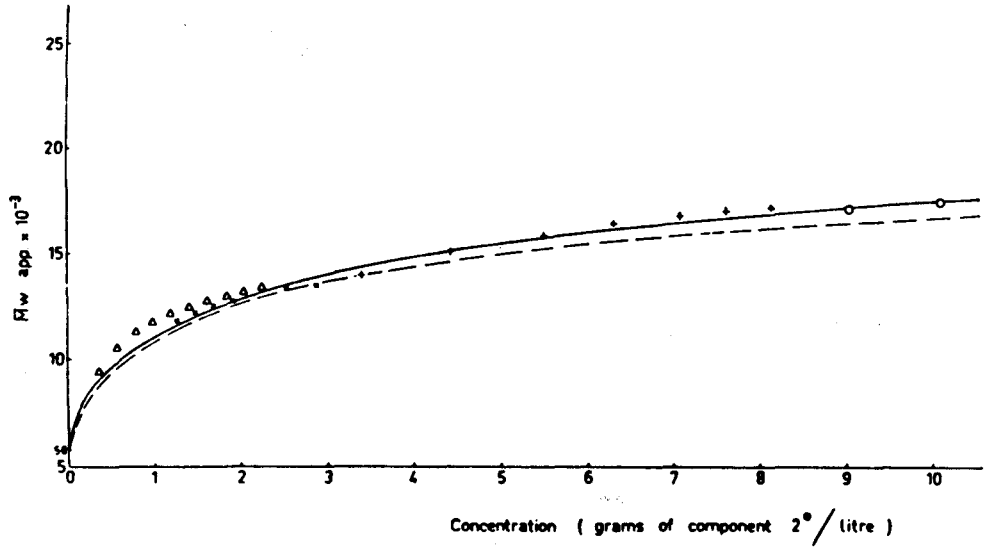
INSULIN AGGREGATES

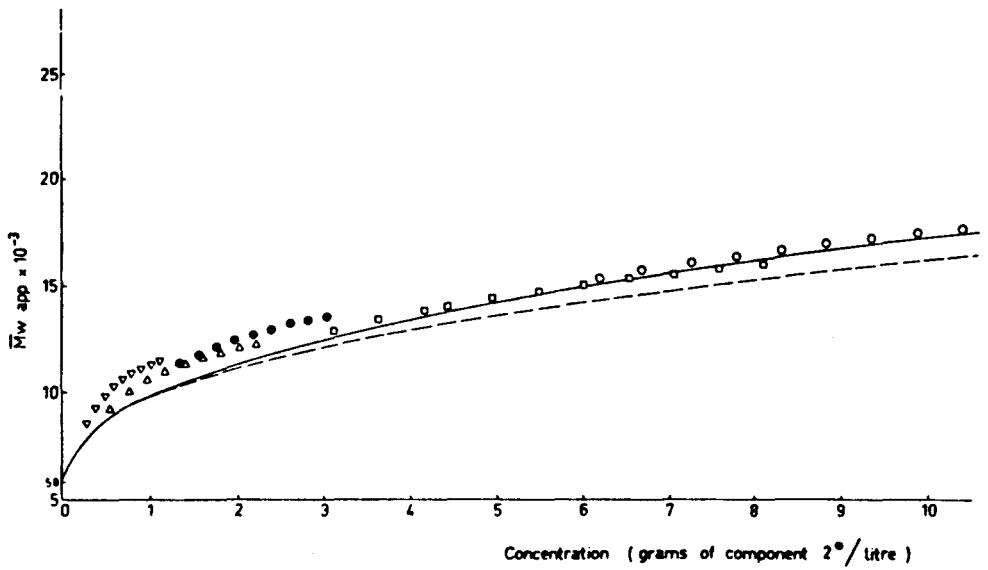
Insulin monomers associate non-covalently in solution to form stable dimers (2 monomers, M_2), which associate to form tetramers (M_4), which may combine with monomers to produce hexamers (M_6), which may themselves associate to form larger aggregates (M_{6n}) (462-465). As previously stated in Chapter III, insulin crystals consist of hexamers.

Insulin aggregation is dependent upon insulin concentration, pH, ionic strength and (for $M \geq 6$) divalent cation concentration. The change in weight fraction of various aggregates with increasing insulin concentration at pH 2.0, 25° C and ionic strength 0.1 is depicted in Figure 12. Under these conditions, disaggregation occurs below 0.1% insulin (<1 mg/ml) and the fraction of dimer attains a maximum between 0.1% and 0.25% insulin, declining thereafter concomitantly with a steady increase in the fractions of tetramer and hexamer. At neutral pH the average weight of zinc-free insulin ranges from the dimer to well above that of the hexamer in the concentration range 0.1-1.0% (466-468). Holladay et al. (469) found that, for zinc-free insulin at pH 7.4, 20° C and ionic strength 0.368, the proportion of insulin molecules that exist as dimers follows the pattern of Fig. 12, attaining a maximum of 32% between 0.05 and 0.8 mg

FIGURE 12. APPARENT WEIGHT-AVERAGE MOLECULAR WEIGHT OF INSULIN AS A FUNCTION OF CONCENTRATION; pH 2, IONIC STRENGTH 0.1. EACH SET OF SYMBOLS REFERS TO A PARTICULAR EXPERIMENT. THE SOLID LINE IS CALCULATED FROM THE EQUILIBRIUM CONSTANTS ASSUMING NO CHARGE EFFECTS AND IDEALITY; THE DASHED LINE IS CALCULATED ACCOUNTING FOR CHARGE EFFECTS. TOP: T = 15°C; BOTTOM: T = 25°C.

(Reproduced, with permission, from Ref. 462.)


25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
$\bar{M}w$ app x 10^{-3}
Concentration (grams of component 2° / litre)


25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
$\bar{M}w$ app x 10^{-3}
Concentration (grams of component 2° / litre)

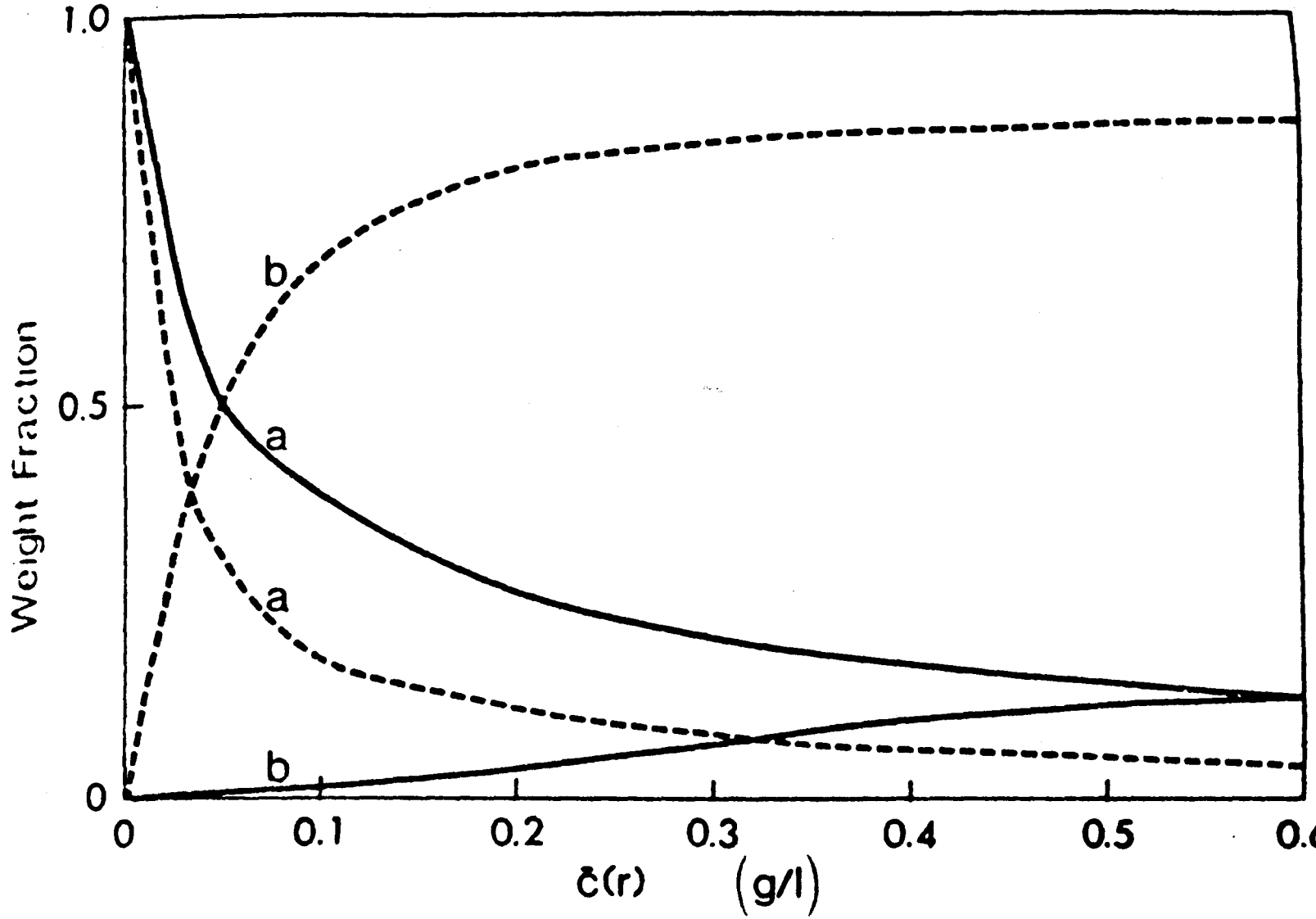
insulin/ml.

Insulin exists in the dimeric form below pH 3 at a concentration of 1% in 0.1M sodium chloride, while the hormone is dissociated to monomers below pH 3 at a concentration of 0.25% in 0.1 N sodium monophosphate (NaH_2PO_4); dissociation of the dimer also occurs in sodium chloride at pH 2.0-2.7 below 0.1% insulin concentration (464). Moderately dilute solutions (0.1-0.25%) of zinc-free insulin dissociate to the monomers upon increasing the pH to 9.1-9.5 (466,467,470). The role of increasing ionic strength promoting aggregation appears to lie in its effect on the net charge per monomer (number of protons bound less the number of counterions bound)(470). For a given degree of association of insulin molecules, the net proton charge (Z) has been found to remain the same (e.g. 2.5/monomer at 20,000 daltons) regardless of pH between 2.7 and 4.0, suggesting that the molecular weight of insulin in acidic media is a simple function of net charge and, indeed, the average molecular weight has been found to be a linear function of net charge over the range 7,000 daltons (Z = 4.1/monomer) to 25,000 daltons (Z = 1.8/monomer)(470). The anion-dependence of insulin association in acidic media ($CNS^- > I^- > NO_3^- > Br^- > Cl^- > H_2PO_4^-$) therefore reflects the ability of these species to serve as counterions in acidic media; e.g. the dissociation of the dimer in the presence of $H_2PO_4^-$ at pH 2 can be attributed to the poor ability of $H_2PO_4^-$ to act as a counterion at this pH, where the charge on the monomer approaches 5 proton units (471). A similar inverse relation between net monomer charge and insulin aggregation has also been found in alkaline media (470), implying repulsion of similarly charged molecules. Thus, the proportion of dimers increases with decreasing pH, insulin concentration and ionic strength.

The effect of divalent cations in promoting aggregation of insulin beyond the tetramer stage is striking, as illustrated in Figure 13. Zinc is the only metal found in crystalline insulin prepared in the absence of external metal sources, but rhombohedral crystals also form in the presence of Cd^{++}, Co^{++}, or Ni^{++}, with 3 atoms of metal found per hexamer (464). Cu^{++}, Mn^{++} and Fe^{++} can also facilitate formation of rhombohedral insulin crystals, which requires a minimum of 2 metal atoms per hexamer (153, 473), while metal-free insulin crystallizes in orthorhombic form (474). The first 2 zinc atoms taken up in the formation of the hexamer appear tightly bound, but insulin—both in solution and in crystalline form—is capable of binding more loosely at least an additional 7 zinc atoms per hexamer (464).

Insulin does not bind zinc (or other metals) below pH 3.5 (464) and the low incidence of hexamer in acid solution is about the same as for metal-free insulin in neutral solution (compare Figs. 12 and 13). Insulin binds zinc continuously with increasing pH above 4.5 to at least an additional 7 atoms per hexamer at pH 8.0 (466,475). At neutral and moderately alkaline pH, the proportion of hexamers increases with increasing zinc content from a few hundredths of a percent of zinc to about 0.35%, or 2 atoms per hexamer, where the population of hexamers is predominant when the insulin concentration is greater than 0.1 mg/ml (466, 470, 472, 473). Above 2 atoms zinc per hexamer, a 72,000 dalton unit occurs and with further increases above 6 atoms per hexamer, a polydisperse distribution in the range of 200,000-300,000 daltons predominates. With increases of pH above 8.0 dissociation again becomes predominant even in the presence of high zinc content (3-6 atoms per hexamer); under these conditions a minimum molecular weight of 36,000 is again obtained in the pH range 9-10

FIGURE 13. A COMPARISON OF THE WEIGHT-FRACTIONS OF MONOMER (a) AND HEXAMER (b) AS A FUNCTION OF TOTAL WEIGHT CONCENTRATION FOR THE ZINC-FREE INSULIN (————) AND ZINC-INSULIN (- - - - - -) SYSTEMS IN TRIS-HCl/NaCl BUFFER AT pH 7.0, IONIC STRENGTH = 0.2 AND 25°C.


1.0
0.5
0
Weight Fraction
b
a
a
b
0
0.1
0.2
0.3
0.4
0.5
0.6
C(r) (g/l)

(470).

Although the degree of insulin aggregation in blood is unknown, study of the hormone's self-association behavior under various conditions strongly suggests a marked predominance of the monomer at physiologic insulin concentrations (0.1-3 ng/ml)(476). Calculations of insulin aggregate concentrations at pH 8.0, 25°C, ionic strength 0.1 and an insulin concentration of 10^{-8}M, in the presence of mammalian serum concentrations of Zn++ (1.5-3.7 x 10^{-5}M), based on known insulin association constants, yield values no greater than 10^{-26}M for the 2-Zn++ hexamer, 10^{-13}M for the 2-Zn++ tetramer and 2.1 x 10^{-11}M for the dimer (464).

The physicochemical properties of insulin aggregates, including association constants and molecular weight, are summarized in Tables IV and V. The considerable enhancement of tetramer and hexamer formation in the presence of zinc is due to the affinity of sites on the insulin molecule for divalent metal ions. Two orders of affinities for the first 2 zinc ions bound and additional zinc binding are confirmed by equilibrium dialysis experiments at pH 8.0, at which maximum zinc binding occurs. This method yields an association constant of 2 x 10^5M^{-1}-5 x 10^6M^{-1} for each of the first 2 Zn^{++} bound and K = 7 x 10^3M^{-1}-3.5 x 10^4M^{-1} for each subsequent Zn^{++} bound, with 10-12 insulin binding sites per hexamer available for the weaker associations (464, 477).

The fact that agents known to disrupt hydrophobic interactions, such as guanidine hydrochloride, urea, detergents, dimethylformamide, trifluoroacetic acid/ether, pyridine, dioxane, and butanol/dichloroacetic acid, induce dissociation of insulin aggregates (464) indicates that such interactions play an

TABLE IV. SELF-ASSOCIATION OF INSULIN

Species Formed	Process	pH	$K_{assoc.}$	- ΔG (kcal/ mole)	Comments	Ref.
Dimer	$2M \rightleftarrows D$	2.0	$8 \times 10^3 M^{-1}$			462
			1.02×10^4 M^{-1}	5.2	$\mu = 0.1$	463
		7.0	$1.1 \times 10^5 M^{-1}$			476
		7.4	$2 \times 10^5 M^{-1}$			478
			$2.2 \times 10^4 M^{-1}$	5.8		469
		8.0	$2 \times 10^5 M^{-1}$			479
Zinc-Free Tetramer	$2D \rightleftarrows T$	2.0	$7.8 \times 10^2 M^{-1}$		$\mu = 0.1$	463
		7.0	$1.7 \times 10^4 M^{-1}$			476
Zinc Tetramer	$4M+2\ Zn^{++} \rightleftarrows TZn_2$	7.4	$10^{29} M^{-5}$			464
Zinc-Free Hexamer	$D+T \rightleftarrows H$	2.0	$6.7 \times 10^2 M^{-1}$		$\mu = 0.1$	463
	$3D \rightleftarrows H$	2.0	$5.3 \times 10^9 M^{-2}$		Z = +5.0	464
		7.4	$8.6 \times 10^9 M^{-2}$	13.3		469
		8.0	$2.0 \times 10^9 M^{-2}$		Z = -3.2	464
Zinc Hexamer	$6M+2Zn^{++} \rightleftarrows HZn_2$	7.4	$10^{32} M^{-7}$			464
Polyhexamer	$2H \rightleftarrows H_2$	7.0	$5 \times 10^3 M^{-1}$			476

Abbreviations:

M	monomer
D	dimer
T	tetramer
H	hexamer
μ	ionic strength
Z	net charge

important role in the hormone's self-association. This supposition is verified by study of the molecule's tertiary structure based on x-ray diffraction analysis of the insulin crystal, which several lines of evidence indicate is essentially the same as the insulin dimer and zinc hexamer in solution (464). This x-ray diffraction analysis reveals that regions of contacts among insulin molecules are largely composed of nonpolar residues. As mentioned in Chapter III, the insulin monomer exhibits two faces—one involved in dimer formation and the other in dimer-dimer associations—each containing a number of surface apolar residues with their side-chains contacting solvent in the dissociated state.

In the dimer, about 36 of 111 short atomic separations between the monomeric association faces involve the side chains of 10 nonpolar residues, the B12 valine, B16 and B26 tyrosines, and B24 and B25 phenylalanines of each molecule. In addition, B11 leucine, although not involved in short intermolecular separations, also forms part of the nonpolar core of the dimer (464). A twofold relationship between the molecules of the dimer means that the C-terminal residues of the B-chains run anti-parallel to each other, making possible the formation of an anti-parallel β-pleated sheet structure involving the B21-B30 residues and containing 4 hydrogen bonds between the monomers. These hydrogen bonds, between the backbone amido and carbonyl functions of the B24 and B25 phenylalanines and B26 tyrosine of each molecule, serve to stabilize the dimer in aqueous solution after formation due to hydrophobic interactions. In fact, all monomer-monomer contacts involve B-chain residues that contribute to α-helical and β-pleated sheet structures (143). The dimer constructed from x-ray diffraction analysis has an oval appearance and is about 40 Å from end to end and 25 Å across (143).

Of the 99 short intermolecular separations between dimers, about 39 involve the nonpolar side chains of B1 phenylalanine, B14 valine, A 14 tyrosine and the A13, B6 and B17 leucines (464); B18 valine may also be involved in hydrophobic interactions as well as the other α-helical residues, B14 and B17 (143). Dimer-dimer interactions are also stabilized by hydrogen bonds, between the B1 α-amino and A17 glutamate γ-carboxy functions and between the A14 tyrosyl phenolic hydroxyl groups across the surface of the molecules (143,464). The 2 tightly bound zinc ions in the hexamer lie on the threefold axis, about 18 Å apart, each coordinated to the N_3 imidazole nitrogens of 3 B10 histidines. The coordination of each Zn++ is completed by water molecules and is apparently intermediate between octahedral and trigonal prismatic (143). The 7 remaining, weaker metal-binding sites probably involve the B13 and A17 glutamyl γ-carboxy and the B1 α-amino functions (464). Recently, Sudmeier et al. (480) offered evidence from Cd-113 nuclear magnetic resonance studies that the 6 B13 glutamyl γ-carboxy functions normally form a specific Ca++ -binding site in the center of the 2-Zn++ hexamer. The threefold axis, consisting of B9 serine, B10 histidine and B13 glutamate residues connected by an elaborate water structure, constitutes a hydrophilic core in the center of the hexamer, which has the shape of a flattened disc about 50 Å across and 35 Å high with prominent channels lined with solvent molecules running between the dimers from the central zinc ions to the edge of the molecule (143).

Conclusions regarding roles played by specific residues in dimer-dimer interactions based on x-ray diffraction analysis tend to be supported by study of the aggregative properties of rat insulins. Rat insulins I and II both differ from porcine insulin in substitutions at A4 (glu → asp) and B3 (asn → lys), while rat

insulin I contains a further substitution at B9 (ser→pro) and rat insulin II at B29 (lys →met). Yet rat insulin I forms hexamers less easily than rat insulin II, reflecting the importance to hexamer formation of the B9-B19 α-helix and B10 histidine conformation, which would be affected by a nonconservative substitution at B9 (481).

The equatorial surface of the hexamer is smooth, but the top and bottom surfaces are broken by projections from residues of the A6-A11 loop regions on each surface. Between these projections large pockets about 10 Å across result. In the crystal these are partially filled by the A-chain loop regions from molecules above and below the hexamer in the direction of the 3-fold axis. The segregation of polar and nonpolar residues is more clearly defined in the hexamer than in any of the subunits: the surface is largely covered with polar side chains; even the surface pockets and channels are lined with groups such as the B5 histidine, B9 serine, B13 glutamate, and the hydroxyl functions of both B-chain tyrosine residues. The apolar residues exposed at the surface of the hexamer—the A14 tyrosines, B25 phenylalanines and A10 isoleucines—are utilized in interhexamer contacts in the crystal and probably in solution, where hexamer associations occur at relatively high hexamer concentration. Interhexamer contacts are formed between the A14 tyrosine and A15 glutamine residues and between the A18 asparagine and B25 phenylalanine residues. Besides filling of the large hexamer pockets by A6-A11 loops, two B5 histidine residues from adjacent hexamers come into close contact (464). The importance of B29 lysine to the association of hexamers is illustrated by rat insulin II, which possesses a methionine at this position and which forms cubic instead of rhombo-hedral crystals in the 2-Zn^{++} form (481).

Assessment of insulin aggregate behavior in established assay systems is precluded by the inability 1) to deliver solutions of known aggregate composition to tissues (due to probable dissociation in serum) in in vivo systems and 2) to obtain meaningful results in in vitro systems using the high concentrations of insulin at which significant proportions of aggregates occur. Thus, it is necessary to infer the insulin-like activity of aggregates in bioassays and immunoassays from knowledge of the hormone's tertiary structure and from the effects of substituent alterations.

Of the residues implicated in the dimerization face, B25 phenylalanine is known to be involved in the receptor binding site and the B30 residue may affect insulin immunoreactivity, while the A14 tyrosines, which make contact in dimer-dimer interactions, are important immunodeterminants. Since chemical alteration of the A14 tyrosine leads to a 96% reduction in insulin immunoreactivity (see Table I), it is expected that the insulin-like activity of the hexamer in immunoassays would be attenuated accordingly. A more precise estimate of aggregate activity in assay systems relative to monomeric insulin is provided by studies involving FITC-insulin conjugates. Mono-FITC insulin, which forms hexamers as well as native insulin, retains about 50% biologic activity (mouse convulsion assay) and 24-56% immunoreactivity (immune hemolysis inhibition); di-FITC insulin, which forms only monomers and dimers, possesses 4% biologic activity and 3.3-31% immunoreactivity; tri-FITC insulin, which exists only as monomers, exhibits no detectable biologic activity and 0.001-3.8% immunoreactivity (136). Thus, net values are bioactivity = 8%, IRI = 14-55% for di-FITC insulin and bioactivity = 0%, IRI = 0.2-6.8% for tri-FITC insulin.

Furthermore, desoctapeptide (DOP; lacking B23-B30 residues, involved in

the β-pleated sheet structure of the dimer) and guinea pig insulins do not form aggregates; DOP insulin possesses 0-15% biologic activity and 0.08-11% immunoreactivity relative to native monomeric bovine and porcine insulins, while the corresponding values found for guinea pig insulin are 10% biologic activity and 0.001% immunoreactivity (136,138, 18, 217). Also, insulin dimers do not exhibit negative cooperativity, the site for which is situated in the receptor-binding region of the monomer (217). Thus, insulin aggregates should be virtually undetectable by existing bioassays and immunoassays for insulin. Certainly, superhexameric aggregates are undetectable by these assays, since the A5 glutamines, A19 tyrosines, A21 asparagines, and B25 phenylalanines, residues important to insulin biologic and immunologic activity that are exposed at the hexamer surface, are covered by interhexameric contacts.

INSULIN PRECURSORS

Proinsulin

In 1965, Givol et al. (482) proposed the use of disulfide interchange enzyme as a probe for the thermodynamically most stable form of proteins. They based this proposition on their observation that treatment of oxidized native bovine pancreatic ribonuclease and chymotrypsinogen A with the enzyme and a thiol, such as β-mercaptoethanol (β-ME), did not significantly alter the activity of the proteins (the latter measured after conversion to α-chymotrypsin with trypsin), while their tryptic digestion products, the three-chain "C-protein" derivative of RNase and α-chymotrypsin, rapidly lost activity under the same circumstances. The authors concluded that the primary structure of "stable"

single-chain protein precursors dictates a tertiary structure in which cysteine residues are properly apposed to form the disulfide bonds of their products. Observing that treatment of insulin with disulfide interchange enzyme and β-ME results in chain separation and loss of immunoreactivity, they postulated that the hormone exists in a metastable state, which is the product of a "zymogen-like" conversion of a stable single-chain precursor.

Within two years, Steiner and coworkers had reported the isolation of such a precursor from tumors of human islets of Langerhans and normal isolated rat islets (483,484). When slices of human islet cell adenoma were incubated with ^{3}H-L-leucine or ^{3}H-L-phenylalanine, the acid-ethanol extractable material precipitated with ethanol-ether (see specific isolation procedures, below) yielded 3 radioactive fractions upon Sephadex G-50 chromatography with 1 M acetic acid. The first, designated peak a, eluted with a large protein peak at the column exclusion volume. The third, peak c, eluted with a smaller protein peak and was identified as insulin. The second, "peak b", eluted at a position corresponding to a protein of molecular weight 10,800 daltons and possessed insulin immunoreactivity (determined as insulin-displacable anti-insulin globulin binding). Tryptic digestion of peak b material yielded a radioactive fraction eluting with porcine insulin and another, lower molecular weight, radioactive peak that was not seen following tryptic digestion of radiolabeled insulin. Column chromatography of peak b after sulfitolysis (which dissociates insulin A and B chains) revealed no generation of insulin chains or apparent change in molecular weight. Neither was insulin released from peak b material by extremes of pH, high ionic strength, 50% acetic acid, nor 8M urea (483).

Incubation of tumor slices with ^{3}H-L-leucine resulted in the appearance

on G-50 chromatography only of radioactive peaks a and b after 40 minutes, with the appearance and progressive increase in radioactive peak c after 80 minutes. When isolated rat islets were pulsed with ^{3}H-L-leucine for 40 minutes, about 70% of peak b radioactivity had shifted to peak c 140 minutes after a non-radioactive leucine chase was begun, even in the presence of cyclohexamide, an inhibitor of protein synthesis. The investigators concluded that peak b represents an insulin precursor that is converted to the hormone by a proteolytic process in the β-cell with a half-life of about 1 hour, and termed the precursor protein proinsulin (484,485).

The following year, Roth et al. (486) reported the discovery of a serum component that possesses insulin immunoreactivity, but elutes ahead of normal or "little" insulin on Sephadex G-50 columns. They found that this "big insulin" accounted for as much as 50% of total insulin immunoreactivity in normal human serum, in which the two species apparently are not easily interconvertible, since rechromatography of each fraction did not yield significant amounts of the other and addition of large quantities of pancreatic insulin to serum did not result in the recovery of increased amounts of "big insulin". These investigators demonstrated that the pancreas was the source of "big" as well as "little" insulin by infusing a dog with glucose and collecting blood from the pancreatic vein and the femoral artery. At 10 minutes and 25 minutes after completion of the glucose infusion, pancreatic venous plasma had a more than 50-fold greater IRI concentration than did arterial plasma. At both times the concentrations of "little" and of "big" insulin were distinctly higher in the pancreatic effluent than in the peripheral arterial plasma.

At about the same time, Steiner and coworkers demonstrated the

presence of proinsulin in human serum and urine by G-50 chromatography and RIAs for proinsulin and insulin (487). They observed that serum proinsulin, when immunoassayed in serial dilutions, showed immunologic identity with a standard of pancreatic human proinsulin and they provided evidence that the lower molecular weight product of proteolytic proinsulin conversion, known as connecting peptide (C-peptide), is secreted with insulin from the β-cell in equimolar amounts (485). They found that, in a patient with an islet cell adenoma, the molar fraction of proinsulin in acid ethanol-extractable material reacting with anti-insulin serum was 52% for the tumor and 77% for fasting serum; the proinsulin fraction in serum did not exceed 10% after removal of the tumor (485). Berson and Yalow's group verified that high proportions of serum "big insulin" are largely limited to persons with insulinomas when they reported that, while "big insulin" comprised 24-55% serum IRI in such individuals, this fraction accounted for less than 20% serum IRI in 32 subjects without tumors, including obese normals, diabetics and myotonic dystrophy patients, and did not exceed 10% in 32 of 59 serum samples collected from these persons under diverse circumstances (488).

Finally, Roth and coworkers (489) demonstrated that "big insulin" is indeed circulating proinsulin as follows: 1) when human "big insulin", isolated from plasma by G-50 chromatography, was mixed with ^{125}I-porcine proinsulin and rechromatographed, the peaks of immunoreactivity and radioactivity were coincident; 2) when plasma was extracted by a method used for the isolation of insulin and proinsulin from pancreas, the location and relative proportions of "big" and "little" insulins upon G-50 chromatography were the same as those obtained by chromatography of whole plasma; 3) 90% of "big insulin" was

converted to "little insulin" by treatment with 20-200 μg trypsin; 4) trypsinization of human "big insulin" mixed with ^{125}I-porcine proinsulin resulted in >90% conversion of both to "little insulin"; and 5) guinea pig anti-porcine proinsulin serum absorbed with porcine insulin bound both porcine proinsulin and human "big insulin", but not porcine insulin and human "little insulin".

Steiner's recommended procedure for isolation of proinsulin from insulinoma tumor tissue is based on Davoren's insulin extraction method (108). About 170 mg tissue is homogenized with 2.2 ml acid ethanol (375 ml 95% ethanol + 7.5 ml conc. HCl) + 0.8 ml water. After 6 hours at 4°C, the supernate is decanted and 0.7 ml acid ethanol + 0.3 ml water is added to the precipitate. After 6 hours at 4°C, the second supernate is combined with the first, which are then clarified by centrifugation, adjusted to pH 8.3 with 6N NH_4OH, allowed to stand 15 minutes at 4°C, again clarified by centrifugation and adjusted to pH 5.3 with 4N HCl. After addition of 0.025 ml 2M ammonium acetate per ml acid ethanol extract, insulin and proinsulin are precipitated with 3 volumes absolute ethanol + 5 volumes ethyl ether for 12 hours at 4°C. The suspension is then centrifuged at 4°C; the precipitate is dried and dissolved in 1-2 ml 3M acetic acid, followed by chromatography on a 1 x 50 cm Bio-Gel P-30 column in the same solvent (485, 490).

Islets of Langerhans are isolated from rat pancreas by digesting the minced tissue with collagenase and are extracted by homogenization in acid ethanol. The supernate is adjusted to pH 8 with 6M NH_4OH, clarified by centrifugation and adjusted to pH 5.3 with 6M HCl; 2 drops 2M ammonium acetate, pH 5.3, per ml extract are added, followed by 2 volumes ethanol + 4 volumes ethyl ether. The remainder of the isolation procedure is the same as

that employed for tumor tissue (490).

Proinsulin and various two-chain intermediate forms are most readily purified from commercial preparations of crystalline insulin with which they cocrystallize during preparation of the insulin; precursors represent 2-6% of this material, whereas they represent only about 0.0005% by weight of whole pancreas (453, 490). A crude precursor fraction is obtained by chromatography of the first crystals of bovine insulin on G-50 with 1M acetic acid or on P-30 with 3M acetic acid. This fraction is then chromatographed on carboxymethylcellulose (CM-cellulose) with 0.01M sodium citrate, 7M urea, pH 5.5, yielding the two proinsulin intermediates des arg_{31},arg_{32} proinsulin and des lys_{59},arg_{60} proinsulin. Further elution of the column with 0.2M NaCl in starting buffer yields proinsulin and the insulin dimer, which are separated by DEAE-cellulose chromatography utilizing a 400 ml linear gradient of 0-0.2M NaCl in 0.02M Tris-HCl, 7M urea, pH 7.6 (490).

Chance (141) has isolated proinsulin and its intermediates from crystals of porcine insulin by a single chromatographic procedure on a 5 x 140 cm column of DEAE-cellulose utilizing a 12-liter gradient of 0-0.09M NaCl in 0.01M Tris-HCl, 0.001M EDTA, 7M urea, pH 8.1. This procedure resulted in the isolation of proinsulin, des lys_{62},arg_{63} proinsulin, proinsulin split between residues leu_{54} and ala_{55}, desnonapeptide proinsulin (lacking residues 55-63), arginyl insulin (+ arg_{31}), and diarginyl insulin (+ arg_{31},arg_{32}) (See discussion of proinsulin structure, below). Further purification of some of these fractions could be achieved by a subsequent step of gel filtration. The two rat proinsulins have been isolated from crystals of rat insulin using gel filtration, ion-exchange chromatography and preparative gel electrophoresis at pH 4.4 (111, 491). Similar

techniques have also been applied to the isolation of small amounts of proinsulin from the cod (492) and the angler fish (493). Aliquots of human serum (2-10 ml) are diluted with 2 volumes water and extracted with 7.5 ml acid ethanol per ml serum before purification and separation of IRI components as outlined for insulinoma tissue (490).

The molecular weight of proinsulin has been found by gel filtration to be 8,000-9,000 daltons (486-487). Based on the amino acid composition of human proinsulin (461), the molecular weight of this species is 9,430 daltons. Aggregation with increasing concentration at pH 7, equilibrium constants of self-association at acid and neutral pH, and Zn^{++} -binding properties of proinsulin are the same as insulin under corresponding circumstances (452). The physicochemical properties of proinsulin are summarized in Table V.

An important early indication that proinsulin is a single-chain precursor of insulin was Steiner's demonstration of N-terminal phenylalanine in peak b material (483). It may be seen from the primary structure of porcine proinsulin, pictured in Figure 14, that the entire insulin B-chain makes up the N-terminal portion of proinsulin, followed by a 29-amino acid C-peptide segment, and that the entire insulin A-chain comprises the carboxy end of the molecule. The C-peptide is connected to the insulin segments by two basic dipeptides that are readily removed by proteases with tryptic specificity. C-peptide lacks aromatic residues and cysteine, is largely composed of amino acids that can confer additional solubility on the molecule, and exhibits a high degree of variability in amino acid composition among species (Fig. 15). More than half the C-peptide residues are variant among human, bovine and porcine proinsulins and only 5 amino acids—which may be important to proper protein folding—are invariant

FIGURE 14. AMINO ACID SEQUENCE OF PORCINE PROINSULIN.

(Reproduced, with permission from the American Diabetes Association, Inc., from Ref. 453. Copyright 1972 by the American Diabetes Association, Inc.)

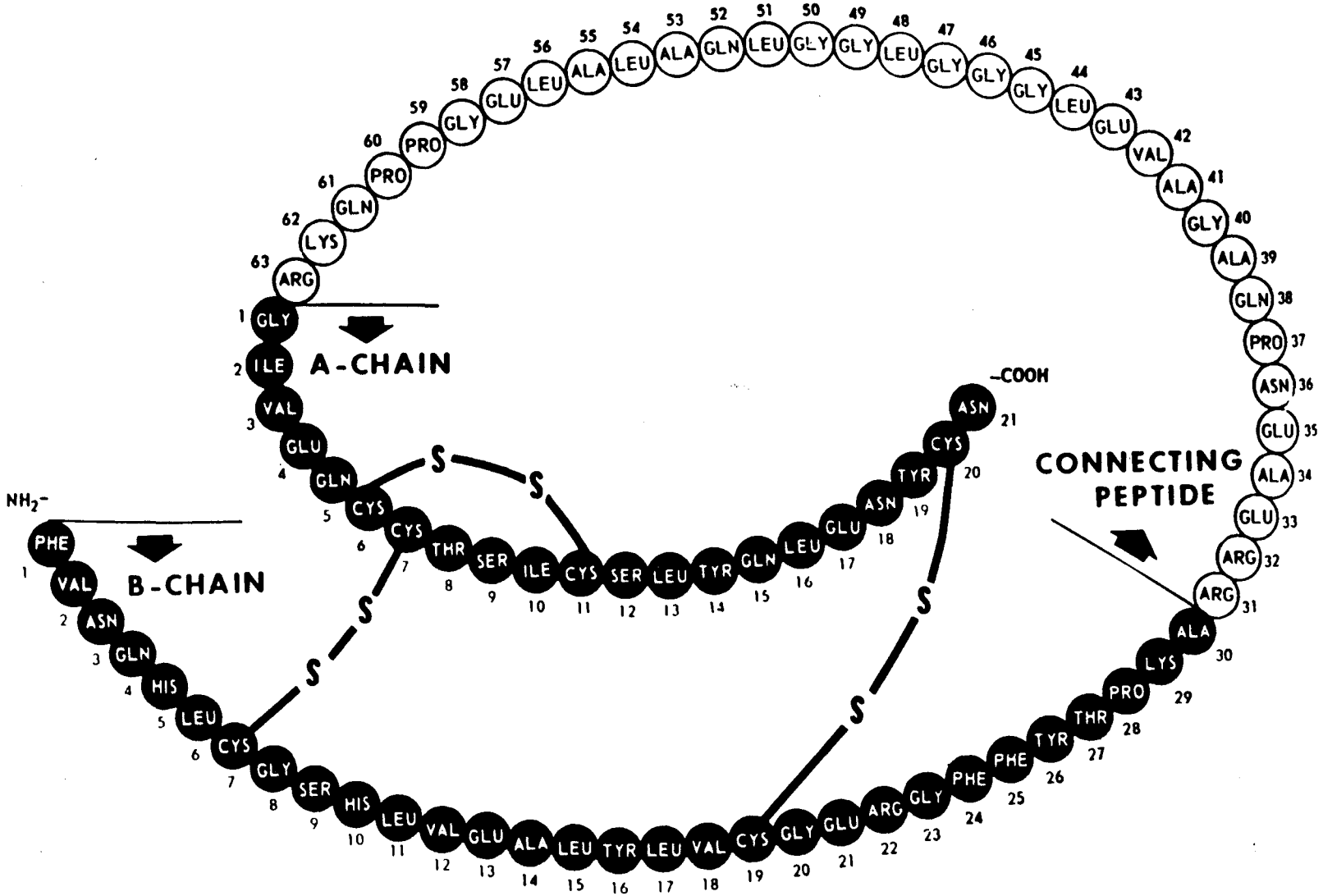

NH2-
PHE VAL ASN GLN HIS LEU CYS GLY SER HIS LEU VAL GLU ALA LEU TYR LEU VAL CYS GLY GLU ARG GLY PHE PHE TYR THR PRO LYS ALA ARG ARG GLU ALA GLU GLN ALA LEU GLY GLY LEU GLU VAL ALA GLY ALA GLN PRO ASN GLU ALA GLN LEU GLY GLY LEU ALA LEU GLU GLY PRO PRO GLN LYS ARG
B-CHAIN
A-CHAIN
GLY ILE VAL GLU GLN CYS CYS THR SER ILE CYS SER LEU TYR GLN LEU GLU ASN TYR CYS ASN
-COOH
CONNECTING PEPTIDE
S
S
S
S

FIGURE 15. THE C-PEPTIDE IN INSULINS OF VARIOUS SPECIES. INVARIANT RESIDUES ARE INDICATED BY BOLD TYPE, DELETIONS BY DASHES.

	B Chain . . . . Arg	Arg	1 Glu	2 Ala	3 Glu	4 Asp	5 Leu	6 Gln	7 Val	8 Gly	9 Gln	10 Val	11 Glu	12 Leu	13 Gly	14 Gly	15 Gly	16 Pro	17 Gly	18 Ala	19 Gly	20 Ser	21 Leu	22 Gln	23 Pro	24 Leu	25 Ala	26 Leu	27 Glu	28 Gly	29 Ser	30 Leu	31 Gln	Lys	Arg . . . . A Chain
Human																																			
Monkey							Pro																												
Horse							Pro													Leu		Gly							Ala		Pro	Gln		
Pig						Asn	Pro		Ala		Ala							Leu		–		–			Ala						Pro	Pro		
Ox, Sheep				Val		Gly	Pro				Ala	Leu			Ala							Gly		–	–	–	–	–			Pro	Pro		
Guinea Pig				Leu			Pro					Thr				Met	Gln	Leu				Gly					–	–	Gln		Ala			
Rat I				Val			Pro			Pro		Leu							Glu			Asp			Thr					Val	Ala	Arg		
Rat II				Val			Pro			Ala												Asp								Val	Ala	Arg		
Dog			Asp	Val		–	–	–	–	–	–	–	–		Ala		Ala			Glu		Gly									Ala			

among the mammalian species studied thus far. The two basic dipeptides are also invariant, reflecting the importance of enzymic cleavage by proteases with tryptic specificity.

Evidence for the existence in the β-cell of additional enzymes with carboxypeptidase B (CPB) and perhaps chymotrypsin specificities comes from identification of bovine and porcine proinsulin intermediates isolated from crystalline insulins (453). Proinsulin intermediates lacking either dipeptide (arg_{31}-arg_{32} or lys_{62}-arg_{63} in porcine proinsulin) and monoarginine insulin (arg_{31} attached to ala_{30} of the B chain), as well as insulin itself, suggest the action of enzymes with trypsin and CPB specificities. Diarginine insulin (+ arg_{31}-arg_{32}), porcine split proinsulin (leu_{54}-ala_{55} bond cleaved), and porcine desnonapeptide proinsulin (lacks sequence 55-63) suggest the action of enzymes with trypsin, chymotrypsin, and both trypsin and chymotrypsin specificities, respectively.

As mentioned previously, the fact that native insulin was recovered in poor yield after reformation of reduced disulfide bonds served as evidence that insulin is formed from a single-chain precursor protein in which the native hormone exists as the thermodynamically most stable conformation. Soon after its discovery, it was noted, in fact, that proinsulin reoxidizes in high yield after complete reduction by mercaptoethanol in urea (454). Furthermore, CD studies indicate that the conformation of insulin in proinsulin, as well as in split proinsulin and desnonapeptide proinsulin, is the same as in free insulin (452). Thus, it has been concluded that the main function of the C-peptide is to direct, interact and hold the A and B chains in a conformation favorable for correct and efficient pairing of cysteinyl residues (461).

The fact that both proinsulin and split proinsulin possess no more than

one-third the biologic activity of desdipeptide proinsulin, desnonapeptide proinsulin and mono- and diarginine insulins (in which the A1 glycine is exposed), which exhibit nearly as great an activity as free insulin (Table V), serves as additional evidence that the α-amino group of the A1 glycine is an important part of the insulin active site (452). Cross-reaction of anti-insulin antibodies with proinsulin is highly variable, with 25-75% immunoreactivity compared to insulin being observed in early work (485, 488). When antibodies are generated against the proinsulin or C-peptide of a given species, they exhibit little or no cross-reactivity with proinsulins and C-peptides from other species. Antigenic determinants have been implicated within the 33-54 segment, a requirement for leu_{54} has been demonstrated, and the probable major antigenic determinant has been identified as the 41-54 sequence, which is an unusually hydrophobic region and also one of considerable variability among species, explaining the lack of cross-reactivity (461). The increase in IRI with anti-insulin serum following cleavage of the arg-A1 gly bond (Table V) also supports the contention that the C-peptide covers important insulin immunodeterminants, especially the A19 tyrosine.

Proinsulin Precursors

In 1973, Yalow and Berson (494) reported the discovery of a high molecular weight insulin precursor in plasma samples from an insulinoma suspect. When the plasma was chromatographed on G-50 with 0.02M veronal buffer, pH 8.6, 45-100% of the IRI eluted in the exclusion volume. This material, designated "big, big" insulin", behaved similarly to γ-globulin in gel chromatography and exhibited a sedimentation velocity between γ-globulin and

albumin in ultracentrifugation, indicating a molecular weight of 100,000-150,000 daltons. "Big, big" insulin has about one-half the mobility toward the anode of porcine or crystalline human insulin on starch gel electrophoresis and exhibits about one-third the mobility of crystalline human insulin on starch block electrophoresis, indicating that it is more basic than insulin. This protein exhibited complete cross-reactivity with insulin for guinea pig anti-insulin antibody binding sites and was more than 80% convertible to insulin by trypsin treatment. These investigators found that "big, big insulin" comprised 0.7-1.0% of the IRI extracted from human insulinoma and normal pancreatic tissue with acid ethanol and subjected to G-50 chromatography under the same conditions as the plasma samples.

Identification, isolation and study of the human insulin gene and its messenger RNA (mRNA) product have revealed the true proinsulin precursor, termed preproinsulin, to be proinsulin with an additional 24-amino acid N-terminal extension, which serves as a signal peptide to direct the molecule to the secretory machinery of the β-cell (495). The molecular weight of human preproinsulin, based on its 110-amino acid sequence, is 11,981 daltons. Therefore, "big, big insulin" cannot be a proinsulin precursor and most likely represents an aggregate form of proinsulin, since Yalow and Berson carried out their G-50 chromatography at pH 8.6 and they did not detect IRI where proinsulin would be expected to elute (494).

The 330-nucleotide sequence for preproinsulin is completely contained within the human insulin gene, but in a discontinuous fashion. The mRNA sequence consists of 465 nucleotides, the first 59 and last 76 of which are untranslated, completely contained within a 1,431-nucleotide precursor (nuclear)

RNA sequence, which represents the immediate transcription product of the insulin gene. In the insulin gene, the mRNA sequence is interrupted after the 42nd nucleotide by the first of two intervening sequences, which consists of 179 nucleotides. The second intervening sequence, comprising 787 nucleotides, begins after the 246th mRNA nucleotide, which is the first nucleotide coding for the val_{63} of preproinsulin. The complete insulin gene consists of 1,726 nucleotides, the first 260 of which comprise a "cap site" and the last 35 of which comprise a termination site, both untranscribed (495).

Insulin Biosynthesis

The present understanding of insulin synthesis in the pancreatic β-cell is as follows:

1. The insulin gene is transcribed into a precursor RNA that is clipped and spliced in the nucleus to yield a messenger RNA (495, 496).

2. The messenger RNA (mRNA) is translocated to the rough endoplasmic reticulum (RER), which is contiguous with the nuclear membrane. There, the central portion of the mRNA is translated by polyribosomes into preproinsulin (495-497).

3. The amino-terminal "signal peptide" of preproinsulin enables the molecule to be secreted into the cisternae of the RER and directed in an energy-requiring step to the Golgi apparatus. The signal peptide is removed from preproinsulin very soon after its synthesis; in the rat, conversion of preproinsulin to proinsulin proceeds with a half time of about 1 minute (495-497).

4. By the time proinsulin is packaged into vescicles in the Golgi apparatus, the molecule has folded into its proper conformation and disulfide bonds have been

formed. The vescicles, which contain immobilized on their inner membrane surfaces proteases with the specificities of trypsin, carboxypeptidase B and chymotrypsin, bud off from the Golgi apparatus (496, 497).

5. As the vescicles traverse the cytoplasm, the proteases convert proinsulin to insulin through split proinsulin and arginyl insulin intermediates. The newly formed insulin condenses into hexameric crystals, receding from the edge of the vescicles and terminating the enzymic reactions before the hormone can be degraded to its desalanine and desoctapeptide forms (104, 496, 497).

6. At the β-cell periphery, mature vescicles, termed secretory granules, fuse with the plasma membrane and release their contents—equimolar quantities of insulin and C-peptide and some unreacted proinsulin—into the lumen (emiocytosis), from whence they are transported into the systemic circulation (104, 485, 498).

Insulin synthesis—from preproinsulin to mature secretory granules—requires about one hour (497). An increase in the extracellular concentration of glucose—the most important signal for insulin secretion from β-cells—stimulates this process at both translational and emiocytotic levels, independent of cAMP (497,499,500). Glucose stimulation of insulin release, but not biosynthesis, is Ca^{++}-dependent (497). Insulin secretion and biosynthesis can be stimulated further in the presence of glucose through activation of the β-cell adenylate cyclase system by glucagon and β-adrenergic agonists (497).

"BOUND INSULIN"

The phenomenon of "atypical" insulin-like activity (ILA) was recognized with the development of bioassays sufficiently sensitive to measure insulin in serum or plasma. Using both *in vivo* and *in vitro* assays, Beigelman and coworkers detected ILA in β-globulin fractions prepared from human peripheral venous serum by fractional precipitation and paper electrophoresis (501-504). The ILA was measured by blood glucose decrement in alloxan-diabetic hypophysectomized (ADH) mice and stimulation of glucose uptake in rat epididymal fat pads. Randle and Taylor demonstrated anti-insulin serum (AIS)-neutralizable ILA in these serum fractions by stimulation of glucose uptake and glycogen synthesis in isolated rat diaphragm (505-507). That the β-globulin form of ILA may be a transport form of insulin was suggested by the observation that in two samples of plasma from portal venous blood most of the ILA was in the albumin- α_1 globulin fraction (116), an observation indicating that insulin may be secreted in the free form by the pancreas and become incorporated elsewhere into the β-globulin fraction.

Beigelman *et al.* found that Cohn Fractions II + III and III-0, and the Plasma Globulin Precipitate fraction (PGP) obtained by zinc precipitation from blood plasma collected in acid-citrate-dextrose solution (ACD), possessed ILA measured *in vivo* (ADH rats), but that identical plasma fractions obtained from blood collected not in ACD, but through cationic exchange resin were devoid of ILA (501,502). Antoniades and coworkers found that when human whole blood or serum was passed through Dowex 50 x 8 or 50 x 2 or IRC 50 cation exchange resins, while typical insulin was not retained by the resin, a considerable quantity

of ILA was retained and eluted from the resin by acid (0.1N H_2SO_4, 0.1N HCl or 0.2M citrate, pH 2-3) or alkali (0.1 or 1.0N NH_4OH, pH 10.5-11) (247,508-510). This ILA, which Antoniades designated "bound insulin" (BI), was measured by induction of hypoglycemia in ADH rats (508, 509) and stimulation of $^{14}CO_2$ production from glucose-1-^{14}C by rat epididymal adipose tissue (509, 511); BI ILA in the fat pad was abolished by 20-40 mM reduced glutathione (511). Furthermore, this ILA was found to stimulate incorporation of uniformly labeled glucose carbon into muscle and adipose tissue glycogen and into fat of adipose tissue in intact rats (512) and into fat and glycogen of isolated rat epididymal adipose tissue (511). BI was observed to migrate in electrophoresis with a mobility similar to β-and γ-globulins, to possess a molecular weight between 60,000 and 100,000 daltons by Sephadex gel chromatography, and to precipitate with Cohn Fractions II + III and III-0 (compared with endogenous typical insulin activity, which is found in Cohn Fractions II + III, IV-1 and V) (511-513). Unlike the β-globulin ILA described by Beigelman and Randle and Taylor, however, BI is virtually undetectable by glucose uptake in the isolated rat diaphragm and is unreactive with anti-insulin antibodies (247,509,512,514).

Antoniades, however, observed an increase of BI ILA in the rat diaphragm assay after various treatments of the fraction. Treatment with acid (0.2M citrate, pH 2-3) or alkali (pH 9.8), an aqueous extract of rat adipose tissue, or acid-ethanol produced a many-fold increase in BI ILA measured by this assay (509,511). The diaphragm ILA increment induced by pH extremes (with subsequent neutralization) was especially pronounced when followed by centrifugation, leading Antoniades to conclude that such treatment liberated free insulin from an insulin-protein complex by rendering the cationic carrier

protein insoluble (509). A maximum adipose tissue extract (ATE)-induced increment in diaphragm assayable BI ILA was obtained after 30 minutes preincubation of the BI fraction with ATE, which exerts no effect on the ILA exhibited by crystalline insulin in the diaphragm assay (514). The BI ILA increment increased progressively with increasing ATE protein concentration, attaining a maximum of about 1,000 μU/ml eluate at an ATE nitrogen content of 18 μg/ml incubating medium (514). Further purification of ATE revealed that the factor responsible for the ILA increment migrates in electrophoresis with albumin and the α_1-globulins (514) and is heat stable, suggesting a small molecule (512).

Examination of the soluble and insoluble products of resin eluates subjected to pH extremes provided further evidence for an insulin-protein complex. ILA remaining in the supernate of BI fractions treated with citrate, pH 2.0, migrated on paper electrophoresis at pH 7.6 as an anion, in the albumin/α_1-globulin zone (as does typical insulin), and exhibited a molecular weight below 40,000 daltons by gel chromatography (509,512). the redissolved (0.1M acetate buffer, pH 4.0) precipitate obtained from BI fractions adjusted to pH 9.8 migrated on paper electrophoresis, pH 7.4, anodally to γ-globulins and stained with ninhydrin, suggesting a very cationic protein with an isoelectric point near 10 (509). The supernatant ILA was neutralized by anti-insulin serum (AIS), indicating that this, indeed, represented free insulin (509). No change in the diaphragm-assayable ILA of BI incubated with rat adipose tissue and AIS less than 2 hours was seen, but a significant decrease of ILA, which became greater with incubation time, occurred after 2 hours (511). A significant decrease in ILA of acid ethanol extracts of BI fractions was observed in the presence of AIS

within 90 minutes (511). This finding should be contrasted with the potentiation of BI diaphragm-assayable ILA after treatment with acid ethanol.

Antoniades postulated conversion of "free" to "bound" insulin in the liver (512), but was unable to demonstrate binding of exogenous radiolabeled insulin to a cationic protein in vitro or in vivo. Human serum incubated directly with ^{131}I-insulin or obtained from nondiabetic subjects injected with ^{131}I-insulin showed no significant loss of radioactivity after passage through cation exchange resin (509). He determined the mean concentration of BI (400 μU/ml) in fasting nondiabetic humans to be about 10 times that of free insulin (512). It is noteworthy that insulin-antibody complexes were not absorbed by the cation exchange resin (509).

Confirmation of Antoniades' observations came with the work of Shaw and Shuey, who demonstrated an AIS-neutralizable ATE-induced 12-fold increase in normal fasting human serum ILA, from 50 μU/ml to 600 μU/ml, measured by both glucose uptake and glycogen synthesis in the rat diaphragm (390). They obtained essentially the same results with a serum BI fraction eluted from Dowex 50w-x8 with 0.02M NH_4OH. Gundersen and Lin extended the concept of bound insulin by demonstrating that heparin also causes increase of BI ILA in the rat diaphragm to about that found with the fat pad assay (391). They found that addition of AIS to BI-heparin mixtures decreased diaphragm-assayable ILA 24-95% and fat pad-assayable ILA 25-80%. These investigators also showed a striking increase of ILA with dilution (to 1:8) of normal and diabetic human serum in both the diaphragm and fat pad. Heparin increased the ILA of dilute serum 8-fold in the diaphragm, but not in the fat pad.

Several investigators pointed out apparent contradictions in the data of

proponents of bound insulin, however. Berson and Yalow (515) noted that the results of Antoniades and coworkers in three separate reports show that total plasma insulin ("bound" plus "free") decreases strikingly after intravenous glucose administration and that earlier observations by Vallance-Owen et al. (516) demonstrated high insulin concentrations in plasma of diabetic subjects by the rat diaphragm without ATE. Using rat diaphragm assays based on glucose uptake and glycogen production, the latter 25 times more sensitive than Antoniades' assay, Meade et al. (517) obtained ILA values for BI fractions intermediate between what Antoniades observed with and without ATE and were unable to increase the ILA by treatment with ATE, acid alcohol, alkali or heparin. These investigators also found no ILA in vivo (mouse hypoglycemia) for BI fractions, whether prepared by themselves or obtained from Antoniades, they reported that different batches of Dowex 50-x8 or 50w-x8 resin did not always yield BI, and they pointed out that dialysis tubing—if not properly prepared—could contribute to ILA. Anderson et al. (518) have extracted insulin from plasma under conditions that presumably would dissociate BI and found with an in vivo assay that the concentration of plasma insulin in the fasting dog is only 30 μU/ml. Christopher et al. (519) demonstrated that the significant quantities of diaphragm-assayable ILA that could not be increased by ATE, acid alcohol, alkali or heparin found by others in BI fractions could be due to prolonged alkalinity of eluates, which would lead to irreversible dissociation of BI. These investigators first demonstrated a 4-fold increase in undialyzed human plasma ILA (measured by glucose uptake in the rat diaphragm) by ATE (which exerted no effect on crystalline insulin ILA) to a level about equal to dialyzed plasma ILA, which was further increased 2.5-fold by ATE. Plasma BI fractions eluted from Dowex 50 x

8 resin with 0.1N NH_4OH, however, exhibited high ILA that could not be increased significantly by ATE. When the plasma was incubated 15 minutes at pH 10, followed by neutralization, passage through the cation exchange resin and alkaline elution, about 90% of the BI ILA shifted to the plasma-saline effluent (not adsorbed), providing additional evidence that BI represents ILA bound to a cationic substance.

By 1965, proof of BI awaited only two developments: 1) demonstration of IRI by RIA following treatment of BI fractions in a way known to release "free" insulin, the ILA of which had been reported to be AIS-suppressible; and 2) demonstration of reversible binding of exogenous radiolabeled insulin to a serum protein. The latter development appeared to be achieved when several investigators reported migration of radioactivity with α- and β-globulins following incubation of exogenous radiolabeled insulin with human serum. Using immunoelectrophoresis and radioautography, Clausen et al. (520) demonstrated binding of ^{125}I-insulin to α_2- and β_2-macroglobulins. Mitchell (521) showed that, while ^{131}I-insulin alone migrated just behind albumin on cation exchange resin paper electrophoresis and with the β-/γ-globulins after incubation with AIS, the radioactivity was associated with the α-/β-globulins after incubation of the radiolabeled hormone with normal human serum. Prout et al. (522) found that ^{131}I-insulin alone migrated ahead of albumin on agar electrophoresis, but in both agar- and immunoelectrophoresis followed by radioautography the radioactivity migrated with γ-globulins after incubation of ^{131}I-insulin with AIS and with α_1-globulins after incubation with normal human serum.

In several reports and reviews, however, Berson and Yalow dealt a serious blow to the concept of bound insulin from three directions:

1. Demonstration that association of radioactivity with serum protein fractions following incubation of exogenous radiolabeled insulin with human serum represents irreversible binding of "damaged" insulin to proteins and thus constitutes an artifactual observation. Berson and Yalow reported that the labeled insulin-protein species observed in the studies mentioned above fail three primary criteria of physiologically significant hormone-protein association: a) reversibility of binding, indicated by release of free labeled insulin upon addition of a large excess of unlabeled (cold) insulin; b) competitive inhibition of binding with cold insulin; and c) demonstration of labeled insulin binding to protein in vivo, following injection of the labeled hormone into an animal (515). Observing that insulin may be damaged during the radiolabeling procedure and during incubation with plasma, Berson and Yalow found that incubation-damaged radiolabeled insulin binds to α-globulins and albumin in a non-saturable and irreversible manner and that nearly all ^{131}I-insulin may be damaged if incubation with plasma is sufficiently prolonged (515). They showed that, when care is taken to avoid artifactual results due to insulin damage, labeled insulin in buffer or normal human plasma migrates just behind albumin on starch block electrophoresis and is adsorbed at the site of application on paper electrophoresis (409). In addition, ^{131}I-insulin sediments in the ultracentrifuge with the same velocity in the presence or absence of plasma (410).

2. Inability to enhance IRI by methods employed to release "free" insulin from BI. Berson and Yalow (523) incubated fasting sera from 5 diabetic and 5 non-diabetic subjects with ATE according to the method of Antoniades and were unable to find any increase in plasma insulin as measured by immunoassay. Meade et al. (524) found that serum or pancreatic extract IRI is not changed by

passage through a cation exchange column. Also, the cation resin eluate did not yield any IRI, either before or after attempts to convert "bound" to "free" insulin. Furthermore, Leonards (397) reported that AIS had little or no effect on normal human serum ILA.

3. Inconsistencies between the concept of bound insulin and established observations. Plasma levels of ILA do not change following total pancreatectomy (515) and are then increased after glucose administration, despite symptoms of severe diabetes and complete absence of plasma IRI (398), suggesting that BI ILA is of extrapancreatic origin. Total serum insulin activity (TSIA, which is the diaphragm-assayable ILA found after treatment of plasma with acid alcohol and dialysis, yielding an insoluble suspension in which the diaphragm is incubated) exceeds 3 mU/ml plasma (525, 526). Yet, Froesch et al. (527) reported human serum concentrations of AIS-suppressible ILA (measured by the rat fat pad assay) in the fasting state and after glucose almost identical to those found by RIA. Calculations of normal daily insulin secretion rates on the basis of these values and the kinetics of insulin metabolism yield estimates of about 50 U/day, about equal to the insulin requirements of totally depancreatized subjects (528, 529). Berson and Yalow (528) also pointed out that the physiologic properties on insulin, namely a biologic half-life of less than 40 minutes, which is consistent with the short-lived effects of exogenous insulin in vivo and the rapid onset of diabetic symptoms after pancreatectomy, are inconsistent with two protein-hormone models: a) thyroxine, which is transported by thyroxine-binding globulin in the blood. The biologic half-life of thyroxine is about one week and the onset of the symptoms of hypothyroidism occur weeks after thyroidectomy; and b) diabetics with severe insulin resistance

due to large concentrations of serum anti-insulin antibodies may require very large doses of exogenous insulin for control of an acute episode of ketosis, following which, in the absence of any further insulin therapy, repeated insulin reactions may occur for days as a result of slow dissociation of insulin-antibody complexes. Finally, Sheps et al. (396) reported that some plasma ILA persists after treatment of plasma with reduced glutathione.

Recently, however, Guenther (387) reported an increase in the IRI of BI fractions following treatment with heparin. He also demonstrated an increase of the diaphragm-assayable ILA of these fractions by biotin, implicating the vitamin as the active factor in ATE. Since Guenther utilized gentler cation-exchange methods for the isolation of BI than those employed previously by Antoniades and others and since heparin had not been used in previous studies of BI IRI, the entire question of the identity of BI has again been opened to dispute.

THE SYNALBUMIN INSULIN ANTAGONIST

An alternate explanation for the observations leading to the postulation of bound insulin was advanced by Vallance-Owen and coworkers based on the existence in blood of a substance that antagonizes the effect of insulin on diaphragm muscle. These investigators found significant insulin antagonism in this system by albumin precipitated with trichloroacetic acid from the plasma of nondiabetic and diabetic humans and normal cats (530-532). This antagonist, which they termed the "synalbumin insulin antagonist" (SIA) (533), was noted at a concentration of 1.25% albumin derived from prediabetic and diabetic humans and 3.5-5.5% albumin derived from normal humans and cats (530-532, 534), but

not in 1.25% albumin from normal humans or in whole plasma from obese, untreated diabetic and normal humans (383, 516, 530, 534). The SIA was noted, however, in the whole plasma of cats after pancreatectomy, leading Vallance-Owen to conclude that normally SIA in whole plasma is completely masked by the production of adequate amounts of insulin by the pancreas (535). Since 3.5-5.5% normal human plasma albumin completely inhibits the effect of 1,000 μU insulin/ml on the diaphragm (531), normal human plasma apparently contains more than 1 mU insulin/ml, a conclusion consistent with the observation of Gundersen and Lin (391) that 8-fold dilution of normal and untreated diabetic sera yielded diaphragm-assayable ILA up to 780 and 2,100 μU/ml, respectively.

Vallance-Owen and Lilley (532) reported that the albumin of two adrenalectomized patients was free of SIA, but in one case the antagonist appeared after treatment with cortisone. Vallance-Owen et al. also noted that the SIA disappears from the albumin of diabetic and nondiabetic humans following hypophysectomy (531), but Lowy et al. observed no change in SIA after hypophysectomy or pituitary destruction by other means and demonstrated no effect of albumin fractions on fat-pad assayable ILA (536). Thus, the SIA appears to be adrenal glucocorticoids, which are known to antagonize insulin-stimulated glucose utilization by muscle (537) and which are transported in blood by albumin and transcortin, an α-globulin (538).

The observation that SIA inhibits insulin action on muscle, but not on fat led to development of the concept that a protein-bound form of insulin in serum may be alternatively explained by the presence of SIA (539). When Vallance-Owen and coworkers repeated Antoniades' experiment with serum, they found diaphragm-assayable ILA in the 0.1M NH_4OH eluate from Dowex 50-x8 resin

without the use of ATE. Reasoning that this ILA may represent dissociated "free" insulin, these investigators reapplied the neutralized eluate to the resin, but the ILA was again retained by the resin and NH_4OH elution once more yielded an AIS-suppressible, ATE-independent diaphragm-assayable ILA. Since preincubation of serum with AIS yielded no detectable ILA upon Dowex cation exchange resin chromatography, even in the presence of ATE, there arose the possibility that insulin itself may behave like a cation under the conditions of the experiment. They found that insulin behaves like an anion, passing through the resin, above pH 7.0, but behaves like a cation and is retained by the resin below pH 6.6. This was verified by the observation that crystalline insulin, when submitted to electrophoresis, behaves like an anion above pH 6.8 and like a cation below pH 6.6. Furthermore, 16% of exogenous insulin added to serum was eluted from the resin with NH_4OH by the method of Antoniades (539).

The SIA was also retained by Dowex cation exchange resin and eluted with NH_4OH. The antagonist was inactivated by preincubation with diaphragm or adipose tissue and with alcoholic extracts of adipose tissue (ATE) or muscle (MTE). It was noted that subsequent to incubation with ATE or MTE SIA eluted from the resin with NH_4OH was inactivated. Following incubation of serum with ATE or MTE, a significant elevation in glucose uptake by diaphragm muscle above serum controls was observed (539). Interpretation of Antoniades' results in terms of inactivation of SIA is contradicted, however, by persistence of serum ILA following pancreatectomy (515).

ATYPICAL INSULIN

Approaching the problem from a different direction, Samaan, Fraser and coworkers concluded that a protein-bound form of insulin exists in the peripheral circulation after observing that only 30% of fat pad-assayable ILA (measured by conversion of ^{14}C-glucose to $^{14}CO_2$ or ^{14}C-fatty acids) in human peripheral venous blood is AIS-neutralizable while about 90% of portal venous serum ILA was neutralized (540, 541). These investigators pointed out that, although the majority of peripheral serum fat pad-assayable ILA cannot be neutralized by a quantity of AIS capable of neutralizing 300 mU typical insulin, this entity, which they termed "atypical insulin" (AI), increases the conversion of glucose to CO_2 and fatty acids by adipose tissue, is acid ethanol extractable (during which some AI is converted to typical insulin), is abolished by 0.04M cysteine, and is eliminated—along with typical insulin—from the circulation of the rat by injection of AIS, indicating that AI contains typical insulin (541, 542). Consistent with an insulin-binding protein being produced in the liver, AI was found to be low in liver disease, while typical insulin levels are normal or slightly elevated (542). AI levels were found to be one-third normal in juvenile ketotic diabetics, lowered in thin adult diabetics, and very high in obese adult diabetics (542).

Samaan et al. (543) concluded that BI and AI are not the same, however, after observing that serum AI increases after glucose administration whereas BI decreases. Other observations also indicated that AI may not contain typical insulin. Although 90% of portal venous serum ILA is AIS neutralizable, total ILA concentration at this site is 5 times that of peripheral circulation, meaning that AI concentration is about the same in both. Likewise, infusion of 40-60 U insulin

into dogs resulted in a 100-300% increase in AI levels, but the increase in AI was only 2-4% as great as the increase in typical insulin (385). Fraser and Samaan reported that pancreatectomy of dogs leaves some, but a reduced level of serum AI, along with diabetes mellitus of varying severity (542), while Leonards and coworkers (397, 544) found that plasma AI persisted at least 16 days after pancreatectomy of dogs. Also, there is little change in serum AI during glucose tolerance tests (542). On the other hand, hypophysectomy of dogs resulted in a 40% reduction in serum ILA from the average preoperation value of 463 μU/ml (545), indicating a pituitary involvement.

NONSUPPRESSIBLE INSULIN-LIKE ACTIVITY (NSILA)

By far the most extensive investigation of atypical serum ILA has been carried out by a group of Swiss scientists, most notably E.R. Froesch, who termed fat pad-assayable (net gas exchange and glucose uptake) serum ILA in the presence of an excess of AIS "nonsuppressible insulin-like activity" (NSILA) (527). Only 7% (mean = 13 μU/ml, range = 0-93 μU/ml) of normal fasting human serum ILA (mean = 180 μU/ml, range = 68-587 μU/ml, n = 22) is AIS suppressible (SILA) and presumably represents pancreatic IRI, since serum levels of SILA compare well with RIA-assayable serum insulin and fluctuate with varying physiologic and pathologic conditions as expected for typical insulin (527, 546). Serum levels of NSILA don't change under physiologic or pathologic conditions and the species doesn't disappear after pancreatectomy (although it decreases considerably after alloxan induction of diabetes in rats), indicating that NSILA does not mimic insulin's effects in vivo (527, 546). Human studies

indicate that exogenous insulin is not converted to NSILA in vivo (527).

When acetone powders prepared from lyophilized human plasma or Cohn fraction B, which lacks albumin and gamma globulins, were treated with acid ethanol, it was found that 5-10% of NSILA was soluble and this species was thus designated NSILA-S (546-548). The NSILA-S was eluted from Sephadex G-75 with 5M acetic acid/0.15M NaCl in a sharp protein peak corresponding to molecular weight 6,000-10,000 daltons. When NSILA-S was chromatographed on Sephadex G-100 in 0.1M ammonium acetate, pH 7.2, it eluted in a broad peak, corresponding to a protein of molecular weight 50,000-70,000 daltons. When rechromatographed on G-75 at neutral pH after treatment with 5M acetic acid/0.15M NaCl, this material eluted as a protein of molecular weight 6,000, indicating binding to a carrier protein rather than aggregation (547). NSILA-S was later determined to be a heat-stable (80°C, 3 hours) single-chain polypeptide of 7,500 daltons molecular weight that is 40-50% inactivated by glutathione, but is not inactivated by rat liver (546, 547).

Acid ethanol-precipitable NSILA (NSILA-P), which accounts for more than 90% serum NSILA, was found to be a heat-labile (inactivated at 80°C in 15 minutes) protein of 100,000-150,000 daltons molecular weight that is not convertible to NSILA-S and is not dissociable by acid, urea or EDTA, but is more than 90% inactivated by mercaptoethanol and urea (546, 547). While only 0-10% NSILA-P was retained by Dowex 50 cation exchange resin, 27-80% of NSILA-S was retained and two-thirds of the serum ILA eluted from Dowex 50 with 0.02M NH_4OH eluted from G-75 with 5M acetic acid-0.15M NaCl at the same column volume as NSILA-S (547), indicating that BI may be NSILA-S.

NSILA-S has been characterized more extensively than NSILA-P. In 1976,

Rinderknecht and Humbel (549) reported the results of a systematic purification and characterization of NSILA-S. Starting with a crude extract that contained 50-80 mU NSILA-S/g, they achieved about 150-fold purification with a yield of 85% by extraction with 0.5M acetic acid followed by G-75 chromatography in 0.5M acetic acid. Chromatography on G-50 with 1M acetic acid yielded at least 80% of starting NSILA-S with an average specific activity of 30 mU/mg. When this material was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 55% of total NSILA-S activity was recovered in a single fluorescamine-positive band. This product, with a specific activity of 300 mU/mg, exhibited a molecular weight of 5,500-5,800 daltons by Bio-Gel A-5m chromatography in 6M guanidine HCl, G-50 chromatography in 1M acetic acid, and SDS-PAGE. No change in molecular weight was noted after reduction with dithiothreitol. The isoelectric point of NSILA-S was found to be 8.2 ± 0.4.

Ion-exchange chromatography of this material on SE-Sephadex C-25 with a gradient of 0.05M pyridine-acetate, pH 2.5/2.0M pyridine-acetate, pH 5.0, at 55°C yielded two NSILA peaks. The less cationic protein was termed NSILA I and the more cationic fraction, after further purification by gel electrophoresis at pH 4.3, was termed NSILA II. Both purified NSILAs possessed a specific activity of about 400 mU/mg and together represented about 30% of starting activity. Both proteins lack histidine and tryptophane. NSILA II also lacks methionine. Each form of NSILA-S was found to contain 4 cysteines per molecule, but no free sulfhydryl groups, indicating 2 intrachain disulfide bonds. Amino acid analysis also indicated a molecular weight of 5,757 daltons for NSILA I and 5,904 daltons for NSILA II. Biological activity of the polypeptides was fully conserved after heating to 70°C at pH 7.6 for 15 minutes, exposure to 8M urea,

6M guanidine HCl or 0.1% SDS, storage in 50% acetic acid at 4°C for several weeks, or repeated freezing and thawing, but was abolished by oxidation with performic acid or reduction and alkylation with iodoacetate or ethyleneimine, indicating that the disulfide bonds are necessary for the molecules' biological activity.

Determination of the amino-terminal sequences (the first 31 residues of 52 for NISLA I and 53 for NSILA II) of the two polypeptides revealed 73% identity between them through residue 30 and 47% homology between NSILA I and the human insulin B chain. The only B-chain invariant residue substituted in NSILA-S is tyr 16. Changes in conservatively variant insulin residues include the B10 histidine and the B14 alanine, suggesting that NSILA-S does not form aggregates in the presence of zinc, and B5 histidine. The degree of similarity (50%) between insulin B chains of man and coypu is the same as that between coypu insulin B chain and NSILA II and the highest degree of sequence identity (57%) is found between NSILA I and tuna fish insulin, indicating that NSILA-S and insulin diverged after duplication of an ancestral gene early in vertebrate evolution (549, 550).

Complete sequencing of NSILA I, which was found to contain 70 amino acids, revealed two segments having nearly 50% (24/51) homology with the porcine insulin A and B chains (5-25 ≈B6-B26, 42-61 ≈A1-A20) with a 12-residue "connecting peptide" showing no homology with proinsulin and an extra 8 residues at the carboxy terminus (551). The arrangement of cystines in NSILA I is identical to that of insulin and 17 of 19 invariant insulin residues are retained. The homology of NSILA I to porcine insulin thus found is intermediate between hagfish insulin and relaxin. Insulin residues involved in dimerization are

conserved or are conservatively substituted in NSILA I, but B10 histidine is changed to glutamate 9 and 3 of 10 dimer-dimer contacts are significantly changed, suggesting that NSILA I probably forms dimers but not hexamers.

The lack of NSILA immunoreactivity with AIS is explained by the fact that the antibody-binding regions are much altered: B5 histidine corresponds to threonine 4, A14 tyrosine corresponds to arginine 55, and A19 tyrosine, corresponding to tyrosine 60, is probably covered by the C-peptide and the carboxy extension. Eight of eleven receptor residues are conserved or are conservatively varied, but the A1 and A21 residues are not terminal. Alteration of these residues has resulted in retention of no more than 2% insulin bioactivity (see Table I). Using computer graphic programs, Blundell et al. (551) were able to construct a three-dimensional model of NSILA I very similar to the tertiary structure of insulin and possessing the same hydrophobic core.

NSILA-S mimics most of insulin's effects on adipose tissue and muscle in vitro and in vivo (552, 553). It stimulates glucose transport, CO_2 production and lipogenesis while inhibiting lipolysis, glycogenolysis and epinephrine action, including cyclic AMP release, in adipose tissue (527, 548, 554), and stimulates glucose transport and glycogen synthesis in muscle (554). NSILA-S exhibits 20% of insulin activity in stimulating glucose transport in heart muscle (548), but the highest nonsuppressible fat pad ILA found for this species is 470 mU/mg (555), indicting that NSILA-S possesses about 2% insulin activity in adipose tissue (548, 549). This fact and the finding of normal levels of NSILA-S in the sera of diabetics suggest that this substance does not exert an important physiologic insulin-like effect (527, 556, 557), although intravenous injection of NSILA-S into normal and adrenalectomized rats elicited hypoglycemic responses (558, 559).

Other studies, however, suggest that the physiologic role of NSILA-S is that of a growth factor. This substance has been found to be a potent sulfation factor in both chick and rat cartilage (560, 561), being 50-100 times more effective than insulin in stimulating [^{35}S] sulfate, [^{3}H] uridine and [^{3}H] or [^{14}C] leucine incorporation into chick cartilage and activity of ornithine decarboxylase (549, 561, 562). NSILA-S is also 20 times more potent than insulin in promoting DNA synthesis, cell proliferation, glucose uptake and lactate production in cultured chick embryo fibroblasts (527). Thus, NSILA-S is effective as a growth factor at concentrations of 0.1-10 μU/ml, while exerting insulin-like effects at concentrations greater than 10 μU/ml (557).

Poffenbarger (563) systematically characterized what he termed serum nonsuppressible insulin-like protein (NSILP), which apparently corresponds to NSILA-P. He isolated a NSILA protein of molecular weight 90,000 daltons, which under reducing conditions exhibited 2 SDS-PAGE bands corresponding to molecular weights of 42,000 and 56,000 daltons. NSILP has a pI of 6.2, Stokes radius of 42.7 A, $S_{20,w}=4.27 \times 10^{-13}$ sec., carbohydrate content of 16%, and partial specific volume of 0.724 ml/g. Amino acid analysis revealed a relatively high proportion of glutamic acid (or glutamine), glycine, arginine and tryptophan. A NSILP immunoassay indicated a normal human serum concentration of 1.2 μg/ml and anti-NSILP serum suppresses 70-80% total serum ILA.

Reber and Liske (564) developed an RIA for NSILA-S that exhibited a sensitivity of 150 pg/ml (0.07 μU/ml), an intraassay variation of 8.3% and an interassay variation of 16.8%. They found a range of human plasma NSILA-S concentrations of <0.15-25 ng/ml (11.8 μU/ml; mean = 4 ng/ml, or 1.9 μU/ml)

for adults and <0.15-20 ng/ml (9.4 μU/ml) for children. These values are about 10-20% of those determined by bioassay (527, 546). RIA of acid-ethanol extracts of human plasma, however, yielded a tenfold higher concentration of NSILA-S (564), again suggesting the existence of a plasma carrier protein for this substance. In contrast to humans, plasma NSILA-S in rats declines from about 20 ng/ml at 2 weeks of age to about 8 ng/ml at 4 weeks and then rises steadily to about 60 ng/ml by 6 months (564). Displacement of ^{125}I-NSILA-S from antibody by goat, dog, rat and human plasma parallels the human NSILA-S standard dose-response curve, indicating that this compound is immunochemically very similar in all these species and thus is highly conserved through mammalian evolution.

When Zapf et al. (556) attempted to measure human serum levels of NSILA-S with a solubilized fibroblast receptor assay, instead of displacement of radiolabeled NSILA-S, they found increased binding of label as the serum concentration was raised, further suggesting binding of NSILA-S by a serum component. These investigators then systematically demonstrated and characterized a specific NSILA-S carrier protein in human serum (557). Using inhibition of adsorption of ^{125}I-NSILA-S to albumin-coated charcoal as the criterion for this binding, they demonstrated that a maximum of about 0.8 μU exogenous NSILA-S is bound by 1 ml serum at a concentration of 10-20 μU ^{125}I-NSILA-S/ml. These observations and the RIA data indicate that about 90% of human NSILA-S is bound at physiologic concentrations of the substance, lending further support to the contention that sufficient concentrations of NSILA-S to exert insulin-like effects do not exist in vivo. More than 90% of ^{125}I-NSILA-S bound by 1:4 diluted human serum is displaced by 250 μU unlabeled NSILA-S/ml and efficiency of displacement is directly proportional to the biologic activity of

the unlabeled compound. Insulin, HGH and ACTH do not compete for the NSILA-S binding site in serum. Chromatography of ^{125}I-NSILA-S-serum on Sephadex G-200 at pH 7.5 revealed three peaks of radioactivity in the large molecular weight region and a fourth one corresponding to low molecular weight unbound labeled NSILA-S. An excess of unlabeled NSILA-S during preincubation led to the disappearance of the two major large molecular weight peaks and to a concomitant increase of the peak eluting in the low molecular weight range.

Binding of NSILA-S is saturable and concentration dependent, with half-maximal binding attained at 4-5 μU exogenous NSILA-S/ml, and is time dependent, with maximal binding attained after 24 hours, but is temperature independent between 0 and 30°C. Scatchard plots indicate a single affinity of association with a pH optimum of 7-8. The binding protein is not albumin, since 2% HSA binds less than 20% of the ^{125}I-NSILA-S bound by 1:4 human serum, is heat labile (>98% inactivated at 100°C for 5 minutes), and exhibits a molecular weight of about 70,000 daltons in Sephadex gel chromatography (548, 557).

NSILA-S activity in the insulin RRA using cultured human lymphocytes and purified rat liver plasma membranes is the same as in the insulin bioassay, i.e. NSILA-S exhibits 2% cross-reactivity with the insulin receptor (548-565). Zapf et al. (556) demonstrated specific binding of ^{125}I-NSILA-S to cultured chick embryo fibroblasts and developed an RRA for NSILA-S using solubilized receptors prepared from the fibroblasts by Triton X-100 extraction. Binding of NSILA-S to its receptor is time-, temperature-, and pH-dependent, and exhibits saturation kinetics. Linear Scatchard plots indicate a single binding affinity, with a K_D of 8×10^{-10} M (≃2 μU/ml; $K_{assoc.} \simeq 10^9$ M^{-1}), which is about the same NSILA-S concentration (1-2 μU/ml) at which half-maximal stimulation of

^{3}H-thymidine incorporation into fibroblast DNA is achieved. A similarly good correlation between binding and the biologic activity of NSILA-S was obtained with adipocytes (556) and the perfused rat heart (566). There are 18,000-24,000 NSILA-S binding sites per fibroblast cell and the pH optimum of binding is between 7.5 and 8.0. Cross-reactivity of insulin with the NSILA-S receptor is 0.001-1% and no displacement of ^{125}I-NSILA-S from fibroblasts by glucagon, ACTH or HGH was noted.

SOMATOMEDINS

In 1957, Salmon and Daughaday (567) demonstrated that growth hormone stimulates incorporation of labeled sulfate into the costal cargilage of hypophysectomized rats via a secondary factor, measurable in serum, which they termed "sulfation factor." Sulfation factor was shown to stimulate incorporation of ^{14}C-leucine into glucosaminoglycans (568), conversion of ^{14}C-uridine-proline into collagen-hydroxyproline (569), incorporation of ^{3}H-uridine into RNA (568) and incorporation of ^{3}H-thymidine into DNA (570) in rat cartilage and uptake of α-aminoisobutyric acid, cycloleucine and metabolizable amino acids into chick cartilage (571). The term somatomedin, indicative of serum factors that mediate the effects of somatotropin, has been applied to substances exhibiting the properties of sulfation factor (572) and is more generally defined as all growth hormone-dependent factors that exert an anabolic insulin-like action on their target tissues (573). Most bioassays of somatomedin measure incorporation of radiolabeled sulfate into chondroitin sulfate of cartilage glucosaminoglycans (574). Daughaday et al. (575) found a linear relation between the uptake of

labeled sulfate into costal cartilage of hypophysectomized rats and log of serum concentration. A more convenient assay, utilizing incorporation of labeled sulfate into cartilaginous pelvic leaflets from 11- or 12-day-old chick embryos, was developed by Hall (576, 577). Numerous bioassays for somatomedins have also been developed based on incorporation of radiolabeled thymidine into DNA (578-580).

Somatomedins have been found to be effective in the cartilage of many vertebrates, including monkey, horse, calf, dog, pig, sheep, rat and frog (573, 581), but no such substances were found in the blood of the carp or atlantic bluefish (582, 583). somatomedins have also been found to stimulate incorporation of $^{35}SO_4^=$ into polysaccharides of human fetal lung fibroblasts (584) and of ^{3}H-thymidine into DNA of human glial cells in culture (585). Rat plasma somatomedins stimulate incorporation of radiolabeled leucine into hypophysectomized rat diaphragm (586), indicating that these substances are also effective on muscle. The somatotropin dependence of somatomedins is reflected in findings of high serum levels of somatomedins in human acromegalics (575-577, 587). Total hypophysectomy in man was followed by falling levels of somatomedin (575, 587) and the half-life of somatomedin in three such patients was 9-18 hours (587). Low levels of somatomedin were found in pituitary dwarfs (575, 588). In retarded growth induced by other hormonal imbalances such as thyroid deficiency or cortisone administration normal somatomedin levels were obtained (577). Administration of HGH always caused a prompt rise of somatomedin in pituitary dwarfs (588-591). During long-term treatment of pituitary dwarfs with HGH a correlation was seen between serum levels of somatomedin and growth rate (592). Furthermore, an increase of somatomedin

was found in normal humans at the time of puberty by Almqvist and Rune (593). In newborns and children less than 4 years of age, the levels were lower than in adults (575, 589, 593). Somatomedin-like activity in frog serum is decreased following hypophysectomy and is restored to normal by administration of somatotropin or triiodothyronine (581). Perfusion of rat liver and kidney slices with growth hormone was accompanied by production of a somatomedin-like factor in the medium (594-596), indicating that these organs are sources of the substance.

Acid-ethanol extraction of human serum yielded a 20-40% recovery of somatomedin activity purified 50- to 200-fold (577, 578). Further purification by gel chromatography, ion-exchange chromatography, isoelectric focusing and paper electrophoresis revealed that somatomedins migrate on electrophoresis with β-globulins (573, 577, 578). Thus, 10-20% plasma somatomedins were recovered from acid-ethanol extraction of Cohn Fraction IV. Sephadex G-75 chromatography with 1% formic acid containing 0.01M 2-mercaptoethanol yielded a single peak of activity corresponding to molecular weight 6,000-8,000 daltons and representing an overall purification of 20,000-50,000-fold (577). Further purification of this material by charge, including paper electrophoresis, Dowex 50 x 2 ion-exchange chromatography and isoelectric focusing yielded three polypeptides of about the same molecular weight (7,000 daltons), but differing in their physical and biologic properties. A neutral (pI = 7.1-7.5) polypeptide that stimulates sulfate incorporation into chick cartilage was designated somatomedin A (SMA; 577, 585). This material was recovered from human plasma in a yield of 2% and a maximum specific activity of 7,000 U/mg (1 unit SMA activity is equivalent to 200 μU porcine insulin; 597), representing an

overall purification of 1-2 x 10^6-fold (577). An acidic (pI = 5.9-6.4) polypeptide that promotes incorporation of thymidine into DNA by human glial cells was termed somatomedin B (SMB; 585). A basic (pI = 9-10) polypeptide that stimulates sulfate and thymidine incorporation by rat cartilage was designated somatomedin C (SMC; 598, 599).

In addition to its effect on chick cartilage, partly purified SMA was found to exert a totally insulin-like effect on rat adipose tissue (600). During optimal stimulation of rat adipocyte lipolysis by such lipolytic hormones as epinephrine, norepinephrine, glucagon, ACTH, TSH, LH and parathyroid hormone SMA potentiated glycerol release, but during unstimulated and slightly stimulated lipolysis, SMA caused a dose-dependent depression of glycerol release. Whereas the adenylate cyclase-stimulating lipolytic hormones increase the uptake of labeled calcium by these cells, both SMA and insulin cause an inhibition of calcium retention. Thus, SMA, insulin and calcitonin exert a similar effect on lipolysis as well as on uptake of glucose and calcium by rat adipocytes (600). Partially purified human plasma SMA also stimulates transport of α-aminoisobutyric acid into muscle (573). In a study of both healthy children and children with certain growth disturbances, a correlation was found between serum SMA levels and dental maturity as well as body height growth (601). The mean serum level of SMA in 28 normal children (7-11 yrs.) was found to be 0.92 ± 0.06 (SEM) U/ml, while those of 29 short and 9 tall children were 0.64 and 1.24 U/ml, respectively (601). A specific RIA for SMB indicated a mean plasma concentration of 9.8 ± 1.7 μg/ml for normal adult males, 6.6 ±0.5 μg/ml for 17 adult males with diagnoses of chromophobe adenoma and growth hormone deficiency, and 19.3 ± 2.3 μg/ml for 22 adult males with clinical acromegaly

and proven hypersecretion of growth hormone (602).

Whereas purified SMB has no effect on adipose tissue, SMC has been shown to exert an insulin-like effect. Partially purified SMC stimulates oxidation of ^{14}C-glucose to $^{14}CO_2$ in isolated fat cells, 1 unit of SMC being equivalent to 30-50 μU insulin (573). SMC also inhibits glycerol release in vitro in epinephrine-stimulated rat epididymal fat tissue. In this system 1 unit of SMC is equivalent to 120 μU porcine insulin (603). SMC, as well as somatotropin, stimulates hypothalmic somatostatin release, resulting in inhibition of pituitary somatotropin excretion, and also inhibits stimulation of somatotropin release by a delayed, direct mechanism that completes a negative feedback loop further indicating that SMC is a mediator of somatotropin action (604). A 37% inhibition of somatotropin release from cultured rat adenohypophyseal cells by a pharmacologic dose (625 μU/ml) of insulin was also observed (604). Amphibia is the most primitive class of vertebrates in which a SMC-like substance has been detected, but displacement of ^{125}I-SMC from specific antibodies by frog serum does not parallel displacement by normal human serum. DNA synthesis and mitosis in frog lens epithelium is abolished by hypophysectomy and is restored by administration to the animals of SMC, which causes resumption of the cell cycle in G_0 phase and continuation through S phase. Somatotropin, frog prolactin and thyrotropin, which enhance synthesis of SMC in mammalian liver (TSH via thyroid hormones), stimulate mitogenesis in hypophysectomized frog lens epithelium in vivo, but not in vitro. SMC also stimulates mitogenesis in vivo. Likewise, somatotropin and triiodothyronine restore lens cell proliferation in vivo, but not in vitro (581). Using a specific SMC RIA, Furlanetto et al. (582) found that serum SMC levels were lowest at birth (0.38 ± 0.05 (SEM) U/ml) and

rose dramatically during early childhood to reach 1.30 ± 0.16 U/ml by 4 years of age. The mean serum SMC concentration in 23 normal adults was 1.50± 0.10 U/ml (apprx. 150 ng/ml). The levels were 6.28± 0.37 U/ml in 14 active acromegalics and <0.2 U/ml in 19 hypopituitary children.

Experimental observations with SMA and SMC iterated above indicate that somatomedins exert their anabolic effects by a mechanism that antagonizes adenylate cyclase-activating hormones and, in fact, a partially purified somatomedin preparation has been found to suppress the adenylate cyclase activity of membrane fractions obtained from rat lymphocytes, adipocytes and hepatocytes, and from chick embryo cartilage (605). A similar mechanism has been postulated for insulin action (see Chapter III) and, indeed, extensive cross-reaction of somatomedins, proportional to their ILA, has been reported. SMC competes with ^{125}I-insulin for membrane binding sites on adipocytes and the displacement curves obtained with the two hormones are parallel. The apparent ratio of SMC to insulin of 50 μU insulin per unit of SMC is very similar to their relative biologic activities. Scatchard plots of ^{125}I-insulin binding in the presence of both insulin and SMC are compatible with simple competition for the same receptor sites. SMA also competes with ^{125}I-insulin for binding to adipocytes and particulate adipocyte membrane preparations (606,607). SMC has also been found to compete with insulin for binding to rat liver (606) and human placental (598,608) membranes. SMA in doses above 1 μg/ml displaces ^{125}I-insulin from placental membrane and 1 μg SMA is as potent as 1.25 ng insulin (4.5 μ U ILA/U SMA) in this respect (609), but SMB up to a concentration of 10 μ g/ml has no effect in this system.

Furthermore, SMA and SMC have been shown to have specific binding

sites on placental membrane (608-610). The specifically bound ^{125}I-SMA was readily displaced by unlabeled SMA. Insulin in high doses could also displace bound ^{125}I-SMA, but was 100-1,000 times less potent than SMA in this respect (610). The Scatchard plots for insulin and SMA in this system are biphasic, indicating at least two types of binding sites. The calculated apparent association constant of the high-affinity sites is 3.8×10^8 M^{-1} for insulin and 2.7×10^7 M^{-1} for SMA. The corresponding amounts of binding sites are 5.4 nmole and 19 nmole per milligram of membrane protein, respectively (609). A placental membrane radioreceptor assay for SMA yieldedmean serum values of 0.57 and 3.2 U/ml for pituitary dwarfs and acromegalics, respectively, while corresponding values of 0.41 and 1.61 U/ml were found using a bioassay (609). Despite their definition based on selectivity of biologic activity, specific binding of SMA to rat cartilage and of SMC to chick cartilage has been observed (573,608). Displacement of ^{125}I-SMC from human placental membranes by frog serum also did not parallel displacement by human serum (581).

The biologic, membrane-binding, and physicochemical properties of SMA and SMC are very similar to those of NSILA-S (577,597,603). Hall and Uthne (573,597) found that SMA and NSILA copurified from human plasma and, in fact, the two are inseparable. NSILA-S also stimulates incorporation of radiolabeled sulfate into rat and chick cartilage and is regulated by somatotropin (560,573). Serum NSILA levels are increased in acromegalics and decreased in pituitary dwarfs (572). In addition, evidence for somatomedin carrier proteins in serum has been obtained (573,585,598,602,611,612). The lack of similarity between SMB and other somatomedins is reflected by the fact that anti-SMB serum doesn't react with NSILA-S or SMA (613). Neither SMB nor SMC compete with

NSILA-S for binding to liver plasma membranes, but SMA is 10% as active as NSILA-S in competing for the NSILA-S receptor (614).

SERUM GROWTH FACTORS

Serum has been known to be a necessary component of cell and tissue cultures since their inception at the beginning of this century (615). Nearly all animal cells in culture require serum for proliferation, although transformed cells (those that form tumors when injected into syngeneic animals) generally have lower serum requirements in vitro than do their normal counterparts (616). Serum has been shown to stimulate the rates of growth and DNA synthesis in cultures of normal chicken, mouse and hamster cells (617-620). Griffiths (621) found that 10% fetal bovine serum (FBS) and amino acids present in minimal essential medium (MEM) cause protein and DNA synthesis and cell division in confluent cultures of MRC-5 human diploid fibroblasts, which normally exhibit decreased growth, protein, DNA and RNA synthesis, and amino acid uptake at confluency. Addition of amino acids at this point has no effect on DNA synthesis, but causes an increase in protein synthesis, while addition of serum stimulates DNA synthesis and amino acid uptake by the cells, but not cell division. Thus, the synergism of the two additives results in cell division. Stimulation of ^{3}H-thymidine incorporation into the DNA of human skin fibroblasts was observed in the presence of 1.25% FBS, was half-maximal with 2.5% FBS, and was maximal with 5% FBS (555).

Plasma has been found to have little growth-promoting activity for cultured cells compared to serum (622-625), although factors necessary for cell

survival in vitro are present in both plasma and serum (616). Since cells in vivo are exposed to plasma except in wounds, where they are exposed to serum, it may be concluded that plasma growth factors, such as NSILA-S, epidermal growth factor (EGF) and the somatomedins, are important to systemic growth requirements while serum growth factors, deriving from platelets and enzymic conversion of plasma precursors during clotting may be important for wound healing (616).

In introducing their 1959 report of the first defined medium supporting growth of mammalian cells in culture at rates comparable to those obtained with serum, Lieberman and Ove (626) outlined three major reasons for identifying the growth factors of serum: 1) the desirability of better defined media for biochemical and other studies, 2) the elucidation of growth requirements not detectable in experiments with whole animals, and 3) the accumulation of a baseline of knowledge for future investigation of comparative biochemistry and nutrition on a cellular level. Using a bicarbonate-buffered basal medium composed of a mixture of amino acids, inorganic salts, glucose, reducing agents, antibiotics and vitamins, these investigators were able to replace serum with serum protein (flattening) factor isolated from serum, catalase, insulin and versene (disodium ethylenediaminetetraacetate, Na_2EDTA) in supporting proliferation of the human epithelial cells appendix A_1 and HeLa, the latter derived from a carcinoma of the cervix. The medium did not support long-term survival or cloning (propagation of single cells) of these cells, however, and, with the exception of the basal medium and catalase at low inocula (20,000 cells per flask), growth was not prevented by the omission of any single component (including serum). The serum protein factor was later found to be the

globulin fetuin, a glycoprotein of molecular weight 45,000 daltons that probably promotes cell proliferation by acting as a cationic bridge between the cells and their glass or plastic suport (626-628).

It was not until the late 1960s, with the general availability of separation methods permiting isolation of small quantities of active polypeptides from the complex mixture of serum proteins and improvements in cell culture techniques enabling investigators other than tissue culture specialists to study the effects of serum factors on isolated cells in vitro, that serum growth factors such as multiplication stimulating activity (MSA) were identified and the growth-promoting properties of plasma factors such as NSILA-S and the somatomedins were fully elucidated. Difficulties due to mitotic inhibitors (chalones) and toxic factors in serum also had to be overcome (616). Prior to this time, only growth factors such as nerve growth factor (NGF) and EGF, which are produced in significant quantities by specific tissues and whose effects are demonstrable in vivo, were discovered. Furthermore, as indicated by the studies of Lieberman and Ove and others, survival factor and migration factor activities are not related to the stimulation of DNA synthesis and mitosis (629).

Since serum growth factors may appear to possess ILA in certain tissues due to elicitation of a positive pleiotypic response, i.e. stimulation of metabolic parameters secondary to a primary induction of cell proliferation, major serum growth factors affecting insulin target cells in culture will be reviewed in this section.

Nerve Growth Factor

In 1948, Bueker (630) reported the increased sensory innervation of a mouse sarcoma implanted in the body wall of a chick embryo and soon afterward, Levi-Montalcini and Hamburger (631) showed that the tumor also caused hypertrophy of the sympathetic ganglia of the embryo, suggesting that the sarcoma cells released a diffusible nerve growth factor into the circulation. In 1954, NGF was isolated from male mouse submaxillary glands, as a factor capable of stimulating the growth and differentiation of immature nerve cells (632). Significant quantities of this factor have also been found in snake venoms (633-635). NGF is secreted in vitro by mouse neuroblastoma cells (636), 3T3 mouse fibroblasts (637), L-cells (virus-transformed 3T3) (637,638), rat glioma cells (639), and chick embryo fibroblasts (640). Synthesis of this factor is stimulated by androgens (641).

In vivo, the mitogenic (i.e., processes preceeding and causing cell division, primarily DNA synthesis) effect of NGF is restricted to the early part of embryonic life (642). In vitro, the factor is vital to survival of embryonic sensory and sympathetic nerve cells. When it is absent, most nerve cells disintigrate; when it is present, nerve cells survive in excellent condition and axons grow vigorously (643). However, no mitogenic effect of NGF on nerve cells or fibroblasts has been reported (616). In the picogram range, NGF enhances survival of embryonic sensory and sympathetic nerve cells in culture (643). In the nanogram range, it stimulates neurite outgrowth (644), neurotubule polymerization (644), anabolic metabolism (644,645), electrical excitability (646), and acetylcholine sensitivity (646). In the microgram range, it induces the

regeneration of adrenergic fibers (647), stimulates an increase in cell-cell and cell-substratum adhesiveness (648), inhibits the synthesis of mucopolysaccharides in chondrocytes (649), and induces the enzyme systems involved in adrenergic neurotransmitter synthesis (650). NGF elicits a positive pleiotypic response in vitro that includes increased transport of glucose (651) and synthesis of RNA (645,652), lipids (653), proteins (652), and the specific enzymes of adrenergic nervous transmission, especially tyrosine hydroxylase and dopamine β -hydroxylase (650).

The high molecular weight NGF complex, the 7S species (M.W. 140,000), consists of 3 subunits termed α, β and γ (654,655). The β subunit, in which the biologic activity of NGF resides, consists of a dimer of two identical monomers of molecular weight 13,259 daltons, containing 118 amino acids and 3 disulfide bonds (655-658). The γ subunit of 7S NGF is an arginylesteropeptidase that activates the β zymogen (molecular weight 22,000 daltons) by cleavage at a specific arginine residue (659). A 16 percent homology between the amino acid sequences of mouse NGF and human proinsulin suggest that both polypeptides evolved from a common ancestral gene (616,660).

Epidermal Growth Factor (EGF)

In 1962, Cohen (661) reported the isolation from male mouse submaxillary glands of a substance that promotes premature opening of eyes (7 days instead of 12-14 days) and eruption of incisors (6-7 days instead of 8-10 days) in newborn mice. Also effective in newborn rats, this factor, which Cohen termed tooth-lid factor, caused stunting of mouse growth (1/3-2/3 normal weight at 10 days) and inhibition of hair growth (661). This epidermal growth factor (EGF) is present in

mouse serum at a level of 1 ng/ml and stimulation of adrenergic receptors by phenylephrine in normal adult mice leads to a marked increase in the serum level, reaching 150 ng/ml in 1 hour (662). Levels of organ EGF may be controlled by androgens (663).

Mouse EGF stimulates epithelial cell proliferation in a variety of organ culture systems, from the cornea of chick embryo or human fetus (664,665) to epithelial cells of mouse mammary glands and mammary carcinomas in organ culture (666,667). It also stimulates keratinization and proliferation of mouse epidermal tissue in vivo (668). Nanogram amounts of EGF are mitogenic in vitro for epithelial cells of mouse mammary glands and mouse mammary carcinomas (666), epidermal cells from chick embryo and human fetal cornea (664,665), bovine corneal endothelial cells (669), human foreskin fibroblasts (670,671), 3T3 cells (672) and human glial cells (673), stimulating DNA, RNA and protein synthesis (670,674), polyribosome formation and small molecule transport (including amino acids, nucleotides and sugars) and inducing ornithine decarboxylase (616,675). Human EGF not only induces DNA synthesis in human foreskin fibroblasts, but also increases the final cell density attained in the presence of 1% or 10% calf serum (676). Thus EGF, by itself,cannot completely satisfy the serum requirement of mammalian cells in culture. EGF has also been shown to stimulate cell migration in aggregates of chick embryo epidermal cells (668) and the synthesis of prostaglandins in cultures of canine kidney cells (677), but is not mitogenic for WI38 human lung fibroblasts (678), nor does it exhibit ILA in adipocytes (652).

Cohen (661) originally purified EGF from homogenates of male mouse submaxillary glands by centrifugation, streptomycin and 56% ammonium sulfate

precipitations, dialysis, carboxymethyl (CM)-cellulose and DEAE-cellulose ion-exchange chromatography, Sephadex G-75 gel chromatography and Sephadex G-25 gel filtration, achieving a 20% yield and 150-fold purification. The product was homogeneous in ultracentrifugation, paper chromatography and paper electrophoresis, was heat-stable (100°C for 30 mimutes), but was destroyed by 0.1 N sodium hydroxide at 100°C for 1 hour, 0.2 N hydrochloric acid at 100°C for 2 hours, chymotrypsin and bacterial proteinase. It exhibited a sedimentation coefficient of 1.25 S and a pI of 4.2. Amino acid analysis revealed the absence of lysine and phenylalanine and a miminum molecular weight of about 15,000 daltons. Taylor et al. (679,680) have isolated mouse EGF by the method of Cohen (661) with additional gel filtration steps and have shown it to be a single-chain polypeptide of molecular weight 6,045 daltons, consisting of 53 amino acids and 3 disulfide bonds, all of which are necessary for biologic activity. It exhibits a pI of 4.6 and possesses no carbohydrate, lysine, phenylalanine or alanine. The amino acid sequence of EGF reveals no homology with insulin (681,682). Human urine EGF differs from mouse EGF in amino acid composition, a more neutral pI and lower molecular weight (5,300-5,500 daltons) (676).

The mitogenic effect of EGF on human fibroblasts is inhibited by dibutyryl cyclic AMP, theophylline and cholera toxin (674), suggesting that this factor acts by reducing intracellular cyclic AMP levels. Specific EGF receptors have been found on human, mouse, rat and chick cells (674). Human foreskin fibroblasts can bind 10^5 EGF molecules per cell with a K_D of 2-4 x 10^{-10}M (678). EGF receptors are lost after transformation of cells with murine or feline sarcoma viruses, but not after transformation with the DNA tumor viruses polyoma and SV40 (683). No other peptide hormones complete for EGF receptors

(674); likewise, EGF does not compete for insulin or NSILA-S receptors (614,670). Mouse EGF has been shown to be associated with a high molecular weight carrier protein in crude homogenates of submaxillary gland. This carrier protein possesses arginyl esterase activity and may be responsible for the release of active EGF from a higher molecular weight precursor since active EGF has a C-terminal arginine (679,681).

Multiplication Stimulating Activity (MSA)

About 10 years ago, Temin suggested that, since growth factors in serum must be produced by some cell in the body, and since all cells in an organism possess the same genetic information, it might occur that cells could adapt to serum-free culture by producing their own growth factors. This consideration led Temin to investigate the components of a medium conditioned by the growth of Coon rat liver cells, a cell line that does not require serum. He and his co-workers purified the growth-promoting activity in this conditioned medium, which stimulated DNA synthesis in chick fibroblasts, and termed it multiplication stimulating activity (MSA) (629). Previously, Pierson and Temin (684) had purified MSA from calf serum. These investigators found that MSA stimulates chick embryo fibroblast (CEF) DNA synthesis and mitosis, as well as cell proliferation. The factor also stimulates uridine, glucose and α - aminoisobutyrate (AIB) uptake (629). The stimulation of glucose uptake is primary, i.e. it is not inhibited by actinomycin D or cycloheximide, but stimulation of AIB uptake was inhibited by actinomycin D, indicating that MSA-enhancement of amino acid transport is dependent on protein synthesis. They found a definite correlation between stimulation of glucose uptake and DNA

synthesis by MSA, but not by serum, suggesting that increased glucose uptake does not necessarily cause DNA synthesis. MSA had no effect on leucine or phosphate uptake or cell migration or survival. This factor possesses sulfation factor activity and NSILA, leading Smith and Temin (629) to conclude that MSA belongs to a family of polypeptides, including insulin, NSILA-S and the somatomedins, that exhibits ILA. MSA was also shown to support growth of and exhibit mitogenic activity including stimulation of DNA synthesis (although to a lesser extent than serum) in rat embryo, 3T3 and human fibroblasts (685-688).

Pierson and Temin (629) utilized Antoniades' method of BI isolation for initial purification of MSA from calf serum. Dowex 50w cation exchange chromatography of the whole serum resulted in retention of 20-30% total CEF mitogenic activity by the resin. This material was further purified by gel filtration and polyacrylamide gel electrophoresis, yielding a fraction that was 6,000-8,000 times more active than whole calf serum in stimulating CEF proliferation, but possessing only 0.5% insulin ILA. A molecular weight of 4,000-5,000 daltons was determined for this preparation, which was stable to heat and acid and sensitive to periodate, chymotrypsin and dithiothreitol. When Dulak and Temin (685,689) purified MSA from Coon rat liver cell conditioned medium by salt precipitation, ion-exchange chromatography, and gel filtration, they obtained 4 differently charged polypeptides (pI = 5.7-7.1), all exhibiting a molecular weight of about 10,000 daltons.

Specific MSA receptors have been demonstrated in CEF and rat liver membranes (687,690). Cell binding of MSA was inhibited by NSILA-S and SMA. Proinsulin and insulin inhibited MSA receptor binding in CEF and human fibroblasts, while SMB, EGF, fibroblast growth factor (FGF) and NGF had no

effect on MSA binding; SMC exhibited 1% cross-reactivity with MSA receptors (690). Cells established in culture from human fibrosarcomas were unable to bind MSA, but human renal cell carcinomas, chondrosarcoma and glioblastomas in culture retain the ability to bind MSA (691). MSA competes with insulin for binding at rat liver cell membranes (629) and is as active as NSILA-S in competing for the NSILA-S receptor (614).

Fibroblast Growth Factor (FGF)

While isolating well-characterized hormones such as thyrotropin and luteinizing hormone from pituitary glands, several investigators noted that partly purified preparations exhibited growth-promoting and mitogenic effects in 3T3 cells, but highly purified hormones did not, suggesting that the pituitary gland produces an active mitogen besides the familiar mélange of hormones (618,692,693). The first isolation and purification of FGF, from bovine pituitary glands, was reported by Gospodarowicz in 1975 (694). A growth factor has also been purified from bovine brain which is physically and biologically similar to pituitary FGF (695).

FGF is a potent mitogen for cells derived from the embryonic mesoderm, including mouse and human fibroblasts, chondrocytes, adrenal cells, vascular endothelial cells, bovine myoblasts, primate smooth muscle, glial cells, cornea endothelial cells and amniotic cells (616,669,695-704), but not for ectodermal (e.g., epidermal and adenohypophyseal) or endodermal (e.g., liver and pancreas) cells (669), nor for WI38 fibroblasts (698), avian fibroblasts (including CEF) and myoblasts (701), and SV40 and polyoma-transformed cells (705).

Initiation of DNA synthesis in resting sparse cultures of 3T3 cells can be

obtained at concentrations of FGF as low as 10 pg/ml, and a plateau has been observed at 1 ng/ml in 3T3 cells maintained quiescent in the presence of glucocorticoids (695). However, the effect of FGF alone on the initiation of DNA synthesis is 30% that of serum, and a combination of FGF and glucocorticoids is required to replace serum. FGF and glucocorticoids not only initiate DNA synthesis in 3T3 cells, but also induce cell division in both sparse and confluent cultures (697). In density-inhibited cultures, FGF alone or FGF plus glucocorticoids provokes a morphological transformation that makes the cells appear virus-transformed (706). Upon removal of FGF, the cells revert to their normal morphology.

Human foreskin fibroblasts respond to nanogram concentrations of FGF (698). However, the effect of FGF on these cells differs from that observed with 3T3 cells in two respects: 1) glucocorticoids have no effect or are inhibitory, and 2) the mitogenic effect of FGF is dependent on the serum concentrations in which the cells are maintained. In low serum (<0.6%), FGF has little or no effect. In high serum (>2.5%), it has a strong mitogenic effect, additive over the effect of serum, and it reduces the doubling time of the human foreskin fibroblasts from 48 to 24 hours, indicating that the division of early passage fibroblasts is not controlled by the same system of steroids and macromolecules as is the division of 3T3 cells.

In susceptible cells, FGF stimulates transport of sugars, nucleotides and amino acids, polyribosome formation, and synthesis of messenger RNA, transfer RNA and proteins as well as DNA (707), but the factor does not possess ILA (616), nor does it compete for NSILA-S receptors on liver plasma membranes (614).

FGF purified from bovine pituitary glands by acid and ammonium sulfate precipitation, dialysis, and ion-exchange and gel chromatography exhibits a pI of 9.5 and a molecular weight of 13,400 daltons (694,700). The factor does not inhibit adenylate cyclase, indicating that decreased intracellular levels of cAMP do not mediate its effects, but it may stimulate guanylate cyclase activity in 3T3 cell plasma membranes (708).

Other Growth Factors

Ross et al. (624) demonstrated that addition of medium in which platelets had been incubated with thrombin renders plasma as effective as serum in supporting growth of pigtail macaque arterial smooth muscle cells. Kohler and Lipton (623) found that addition of platelet extract to 3T3 cells maintained in low concentrations of plasma induces a resumption of growth similar to that observed with serum. Gospodarowicz et al. (625) confirmed these results and showed that FGF and glucocorticoids could substitute for the platelet extract, suggesting that FGF could be similar to the platelet factor. Paul (709) showed that 3T3 DNA synthesis and cell division in response to FGF, insulin and dexamethasone requires the presence of two serum factors, a 158,000-dalton survival factor and a 500,000-dalton serum growth factor. SV-40-transformed 3T3, the growth of which is also serum dependent, require the serum factors but not the hormones for mitogenesis.

Katsuda et al. (710) found that most serum mitogenic activity for Yoshida ascites tumor cells was associated with an α-globulin and the albumin fraction. Holmes and Wolfe (711) isolated α-globulin- and albumin-containing serum protein fractions with growth-promoting activities for HeLa and Chang

endothelial cells. Healy and Parker (712) found that serum growth-promoting activity for mouse embryo fibroblasts was associated with an α_2-macroglobulin and an α_1-acid glycoprotein. The role of α_2-macroglobulin appears to be protective, since it could be replaced by nonprotein polymers. In later studies, these investigators isolated a serum fraction composed of three components that could reduce (at 250 μg/ml) the doubling time of mouse embryo fibroblasts from 4 days to 1 day (628). Holmes has prepared an α-globulin from human serum that can stimulate (at concentrations as low as 0.4 μg/ml) the proliferation of HeLa, conjunctiva and human heart cells, an effect that cannot be duplicated by insulin (713).

Holley and Kiernan (714) purified a serum growth-promoting activity for 3T3 cells from β-globulins by gel chromatography at low pH. This fraction, which stimulated 3T3 division at 1 μg/ml, exhibited a molecular weight of about 20,000 daltons and required one or more other serum factors to elicit an effect comparable to that of serum. Hoffmann et al. (715) isolated from FBS two proteins, termed S_1 and S_2,that together optimally stimulated the growth of embryonic rat fibroblasts. S_1 was found to be a complex of insulin and an α_2-macroglobulin, both of which are necessary for growth stimulation. S_2 exhibits a molecular weight of 26,000 daltons and a pI of 4.9, and does not cross-react immunologically with S_1.

Houck and Cheng (716) purfied a serum sialoglycoprotein mitogenic for WI38 fibroblasts. It has a molecular weight of 120,000 daltons and a pI of 5.2-5.4; besides sialic acid, it contains hexose and its activity is destroyed by both trypsin and neuraminidase. The factor comprises 0.5% of total serum protein and 50 μg/ml is as effective as 10% FBS in supporting WI38 proliferation. Since

other serum growth factors such as MSA, EGF and FGF are roughly one-tenth the molecular weight and active at one thousandth the concentration, this species may represent a complex between a smaller active molecule and its carrier protein, a possibility supported by the fact that activity is partially lost during ion-exchange chromatography and high-current electrophoresis.

ILA, Revisited

When Antoniades et al. (717) re-examined serum substances retained by Dowex 50w x 8 cation exchange resin (BI) for growth-promoting activity in vitro, they found that the ability of human serum to cause 3T3 division almost completely resides in BI. This fraction induces DNA synthesis and mitosis in confluent 3T3 cells, but does not promote cell survival and does not possess insulin immunoreactivity. The mitogenic activity is heat stable (100°C, 20 min.), but was 75% abolished by trypsin or chymotrypsin and completely abolished by mercaptoethanol. The BI ILA eluted from Sephadex G-100 in a molecular weight range of >100,000 to about 30,000 daltons, while mitogenic activity was found at 10,000-35,000 daltons. Isoelectric focusing revealed at least two mitogens, one co-eluting with ILA (pI = 7.3-9.4) that exhibited a pI of 8.6-8.8 and a molecular weight of about 30,000 daltons and the other free of ILA, with a pI of 9.6-9.9 and a molecular weight of 13,000 daltons. About 8 ng protein (equivalent to 8 ml serum) from the latter fraction was sufficient to induce DNA synthesis in 87% of 3T3 cells in a confluent monolayer and cell division, and to cause the cells to assume a random orientation characteristic of transformation. Thus, the latter mitogen may be FGF while the ILA fractions probably contained NSILA-S and somatomedins.

As previously mentioned, sulfation factor activity of NSILA-S at concentrations found in blood and the somatotropin dependence of these concentrations indicate that the physiologic role of NSILA-S is that of a growth factor. This substance also displays significant growth-promoting activity *in vitro*. Morell and Froesch (554) found a 1.5- to 4-fold increase in DNA synthesis by CEF 8 hours after addition of 10-100 μU (21-213 ng) NSILA-S/ml. Furthermore, 10-100 μU NSILA-S/ml caused a twofold increase in CEF glucose uptake and lactate production 21 hours after addition, and a twofold increase in cell number after 4 days in the presence of 300 μU NSILA-S/ml was noted, but NSILA-S was far less potent than FBS in this respect. Rinderknecht and Humbel (549) observed a linear response of thymidine incorporation into CEF DNA in the presence of 0.75-25 ng NSILA I or II/ml with a maximal 4-fold stimulation attained at 250 ng/ml. Half-maximal stimulation occurred at 1.6 nM (9.3 ng/ml). Stimulation of CEF ornithine decarboxylase activity by NSILA-S has also been demonstrated (718).

Rechler *et al.* (555) showed that for human fibroblasts NSILA-S increases the fraction of cell nuclei incorporating thymidine and stimulates thymidine uptake as well as incorporation into DNA. Stimulation of DNA synthesis was detected at 50 ng NSILA-S/ml, half-maximal stimulation occurred at 150 ng/ml, and a maximal 5.4-fold stimulation of DNA synthesis was attained at 500 ng/ml whereas 5% FBS induced a maximal 50-fold increase in DNA synthesis. Destruction of disulfide bonds by reduction and aminoethylation abolished the ability of NSILA-S to stimulate DNA synthesis. Combined additon to human fibroblast cultures of maximally effective concentrations of insulin and NSILA-S did not result in additive stimulation, suggesting that insulin and NSILA-S act at

a common point while serum induces DNA synthesis by a different mechanism.

In view of its growth-promoting properties and its striking homology with insulin, Rinderknecht and Humbel (550) proposed a new designation for NSILA-S: insulin-like growth factor (IGF) and termed NSILA I and II IGF I and II. During purification and characterization of MSA, it became apparent that this factor possesses physicochemical properties very similar to NSILA (684), and Poffenbarger (563) found that an antiserum raised against NSILP also reacts with MSA.

Salmon and Hosse (719) found that partially purified somatomedins prepared from calf serum stimulate the growth of HeLa cells in a defined medium deprived of serum. When this preparation was present in a concentration of 23 μg protein/ml, cell proliferation after 3 days was twice that of control cultures. SMB stimulates initiation of DNA synthesis in cultured glial cells at a concentration of 15 μg/ml, whereas 45 ng/ml is sufficient to induce a 30% increase in lung fibroblast proliferation (720). SMB also stimulates DNA synthesis in fibroblasts (573). However, Heldin et al. (721) have found that the mitogenic effect of SMB on cultured human glial cells is neutralized by rabbit anti-EGF IgG and that their preparation of SMB competes with ^{125}I-EGF for binding to these cells, 10 μg SMB containing the equivalent of about 2 ng EGF, indicating that SMB mitogenesis may be due to contaminating EGF. SMC is mitogenic for cultured human fibroblasts and fetal rat hepatocytes, with a 3- to 4-fold stimulation of DNA synthesis by the latter cells in the presence of 0.01-1 μgSMC/ml being observed (598,722). Thus, NSILA-S, SMA, SMC, MSA and perhaps NGF may belong to a family of closely-related IGFs.

Curiously, the oldest known growth-potentiating factor in vitro is

insulin. Within three years after Banting and Best isolated the hormone, Gey and Thalhimer reported an insulin-induced enhancement of CEF growth and glucose uptake in tissue culture (723). Subsequent studies demonstrated that these are consistent effects of the hormone on cultured fibroblasts, but pharmacologic doses (>0.8 mU/ml) of insulin were always required to elicit an increment in cell proliferation. However, several investigators reported an insulin-induced potentiation of fibroblast growth, but not glucose uptake, in the presence of 8-20% serum (724-726).

Lieberman and Ove (626) found a fourfold stimulation of Appendix A1 and HeLa proliferation in culture by 40 mU insulin/ml. The same effect was obtained with 5-times recrystallized insulin and was abolished by treatment of insulin with cysteine, indicating that it was due to the hormone rather than a contaminant. No increment in the rate of glucose uptake by A1 cells during log-phase growth in the presence of 130 μU-130 mU insulin/ml was seen, but 40 mU/ml caused a 67% increase of this rate in confluent cultures. Temin showed that insulin (>8 ng or 200 μU/ml) can stimulate thymidine incorporation into CEF DNA to one-sixth the maximum achieved with serum, but the hormone is 250,000 times less potent than serum in this regard (727). He also demonstrated that both serum and insulin elicit their mitogenic effects by inducing cells to move out of G1 phase.

Griffiths (621) found that 1 mU insulin/ml causes protein synthesis and cell division as well as DNA synthesis in confluent MRC-5 human diploid fibroblasts and also potentiates the effects of serum. He postulated that the hormone's mitogenic effect may be due to its stimulation of amino acid uptake and protein synthesis. De Asua et al. (331) showed that 8 μg insulin/ml with serum could induce baby hamster kidney (BHK) cells to reach higher final

densities than with serum alone, while Clarke et al. (619) showed that 10 μg insulin/ml potentiates the effect of serum on BHK DNA synthesis. In 1973, Morell and Froesch (554) reported a 3- to 4-fold stimulation of CEF DNA synthesis by 10-100 mU insulin/ml as well as a 33-167% stimulation of glucose uptake and a 30-150% stimulation of lactate production in these cells by 1-100 mU insulin/ml. These investigators observed a 1.5-fold increase in CEF proliferation after 4 days with 300 mU insulin/ml, half that obtained with 5% FBS, but could demonstrate no effect of physiologic insulin concentrations (<1 mU/ml) on CEF and WI38. Nor could they find any insulin effect on fibroblast growth and glucose consumption in the presence of as little as 5% FBS, contrary to previous studies.

Leffert (722) showed a 3- to 6-fold stimulation of DNA synthesis in cultured fetal rat hepatocytes by 10 ng (250 μU)-10 μg insulin/ml, but 10% FBS caused a 4.5-fold greater stimulation of DNA synthesis than 10 μg insulin/ml. Rechler et al. (555) reported that stimulation of DNA synthesis in human fibroblasts could be detected with 20 ng insulin/ml, half-maximal stimulation occurred at 0.4 μg/ml and a maximal 2.5-fold stimulation was obtained with 20 g insulin/ml. Insulin also increased the fraction of cells incorporating thymidine and thymidine uptake. A 40% stimulation of DNA synthesis in human foreskin fibroblasts was observed by Hollenberg and Cuatrecasas at insulin concentrations as low as 40 ng/ml (222).

The finding that pharmacologic doses of insulin exhibit sulfation factor activity in rat cartilage (560) supports the contention that pharmacologic doses of insulin exert growth-potentiating effects on cells and pharmacologic concentrations of IGFs exhibit ILA due to cross-reaction with each other's

receptors (728) and that insulin and the IGFs derive from a single ancestral gene via duplication and divergence early in vertebrate evolution.

In summary, serum ILA, or "atypical insulin," apparently is due to IGFs whose role in vivo is that of growth-promoting agents. However, the results of Guenther (387), who found heparin-induced IRI in BI fractions and heparin and biotin enhancement of BI ILA in the rat diaphragm assay, revive the possibility of a serum insulin-binding protein important to systemic, insulin-dependent metabolic homeostasis. Futhermore, demonstration of serum binding proteins for IGFs dispels the notion that polypeptide hormone binding proteins cannot exist and makes a serum insulin-binding protein a plausible expectation. The methods employed by Zapf et al. (557) to elucidate the NSILA-S binding protein of serum should be applied to this question using heparin and biotin as possible potentiators of complex dissociation. These agents should also be utilized in a systematic study of serum fractions with an in vitro bioassay capable of discriminating the effects of physiologic concentrations of insulin from those of growth factors, the kind of assay that proved vital to the elucidation of serum growth factors: mammalian cells in culture.

TABLE V. PROPERTIES OF CERTAIN COMPOUNDS EXHIBITING ILA AND OF GROWTH FACTORS

Compound	Mol. Wt. (kd)	Electr. Mobility or (pI)	Sol. in acid-ethanol	Biol. Act. (% TI)	RRA (% TI)	IRI (% TI)	Reference
Typical Insulin	5-7.5	albumin-α_1	S	100	100	100	102,104,115 116
Insulin Aggreg.							
Dimer	10-12.3			0-15	1.1-1.9	0-11	136,138,182, 464
Hexamer	35.1-36			<15	<1.9	<11	143,464
>Hexamer	72 200-300			0	0	0	143,464
Insulin Precurs.							
Proinsulin	8-9		S	2.5-18	20-28	45	182,452,460 461,486,487 489,490
Split Proins. (leu_{54}-ala_{55} bond split)				3-20		45	452,461
Desdipeptide Proinsulin (lys_{62}-arg_{63} absent)				58-100		61	461

TABLE V. (CONTINUED)

Compound	Mol. Wt. (kd)	Electr. Mobility or (pI)	Sol. in acid-ethanol	Biol. Act. (% TI)	RRA (% TI)	IRI (% TI)	Reference
Desnonapeptide Proinsulin (sequence 55-63 absent)				60-90		61	452,461
Diarginine Insulin ($+arg_{31}$-arg_{32})				60-90		80	452,461
Monoarginine Insulin ($+arg_{31}$)				60-90		67	461
"Bound Insulin"	60-100	β-γ	I	8-13[a] 100[b]		0	390,391,501-504,511-514
NSILA						0[c]	
NSILA-S	7.5	(8.2)	S	2	2		548,549,555,565
NSILA-S/carrier	50-70		S				547
NSILA I	5.8		S	1.6			549
NSILA II	5.9		S	1.6			549

TABLE V. (CONTINUED)

Compound	Mol. Wt. (kd)	Electr. Mobility or (pI)	Sol. in acid-ethanol	Biol. Act. (% TI)	RRA (% TI)	IRI (% TI)	Reference
NSILA-P	100-150		I				546,547
NSILP	90	(6.2)					563
Somatomedins	6-8	β	S				573,577,578
SMA	7	(7.1-7.5)	S	5.6	0.1		573,577,597, 599,609,612
SMB	5	α (5.9-6.4)	S		<0.01		585,602,609 729
SMC	7	(9-10)	S	0.8-1.4	1.4	0	573,598,599, 606-608,612
				3.4			603
Growth Factors							
Nerve Growth Factor (NGF)	13.3	(9.3)					656
Epidermal Growth Factor (EGF)	6.0	(4.6)		0	0		614,670,679, 680

TABLE V. (CONTINUED)

Compound	Mol. Wt. (kd)	Electr. Mobility or (pI)	Sol. in acid-ethanol	Biol. Act. (% TI)	RRA (% TI)	IRI (% TI)	Reference
Multiplication Stimulating Activity (MSA)	10	(5.7-7.1)		0.5		0	629,684,685, 689
Fibroblast Growth Factor (FGF)	13.4	(9.5)		0			616,694,700
WI38 Growth Factor	120	(5.2-5.4)					716

a. diaphragm assay

b. fat pad assay; diaphragm assay + ATE or heparin

c. by definition

CHAPTER VI

MATERIALS AND METHODS

DEXTRAN-CHARCOAL INSULIN RIA

The method is a modification of that described by Herbert et al. (423), as follows:

Barbital-acetate buffer, pH 7.4, consists of 1.471 grams sodium barbital, 0.971 grams sodium acetate, 7.65 grams sodium chloride, and 50 ml 0.1 N hydrochloric acid per liter aqueous solution.

Albumin buffer is prepared by adding 7 ml 5% human serum albumin ("Albumisol", Merck, Sharp and Dohme, West Point, Pa.) to 93 ml barbital-acetate buffer to a final albumin concentration of 3.5 mg/ml (0.35%).

Dextran-coated charcoal suspension consists of 2.5 grams Norit A charcoal (Matheson, Coleman and Bell, Norwood, Ohio) and 0.25 grams Dextran T-70 (Pharmacia Fine Chemicals, Piscataway, N.J.) per 100 ml barbital-acetate buffer.

Antiserum

Guinea pig anti-porcine insulin serum was a gift from Dr. Peter Wright, was diluted 1:1,000 with albumin buffer, divided into aliquots, and stored at -65°C. One milliliter of this solution is mixed with 199 ml albumin buffer to make a working antiserum dilution of 1:200,000. One-tenth ml of working antiserum is added to each assay tube for a final antiserum dilution of 1:1,600,000.

Insulin standard is prepared by dissolving 0.58 units human insulin (lot 258-1064B-27, obtained from Dr. Mary K. Root, Eli Lilly and Co., Indianapolis, Ind.) in 5.8 ml 0.01 N HCl. This 100 mU/ml solution is diluted 1,000-fold with albumin buffer to provide a 100 μU/ml stock solution that is stored at -20°C. This standard is diluted with albumin buffer to produce 5, 10, 15, 20, 30, and 40 μU/ml standards.

^{125}I-insulin

^{125}I-bovine insulin was purchased from Amersham/Searle Corp., Arlington Heights, Ill., in quantities of 5 microCuries (μCi)/0.1 μg in 5 ml albumin buffer. The contents of one vial are mixed with 195 ml albumin buffer to make a stock label of 0.5 ng ^{125}I-insulin/ml with an activity of approximately 8,800 dpm/0.1 ml.

Assay

The final incubation volume in each tube is 0.8 ml; 0.1 ml standard or sample (undiluted, 1:5 or 1:20 dilution of serum) is mixed with 0.1 ml 1:200,000

antiserum solution. Neither component is added to nonspecific binding (NSB) tubes for standards and 0.1 ml of an appropriate pooled serum dilution alone is used for NSB tubes for samples; 0.1 ml antiserum alone is used for the zero-standard. The volume of each tube is brought to 0.7 ml with albumin buffer. After a 2-day incubation at $4^{o}C$, 0.1 ml ^{125}I-insulin solution is added to each tube and incubation is continued at $4^{o}C$ for another 2 days. Four-tenths ml dextran-charcoal suspension is then added to each assay tube, which is vortexed and centrifuged at 500 x g for 10 minutes. The supernate is decanted into another 12 x 75 mm culture tube and both are counted in an LKB 80000 gamma ray spectrophotometer for one minute each.

Data Reduction

Percent ^{125}I-insulin bound to antibody (not adsorbed to charcoal) in each sample is calculated as

$$\%B = \frac{\text{CPM sample spnt. - CPM NSB}}{\text{(CPM sample spnt. + ppt.) - CPM NSB}} \times 100;$$

$\%B_o$=%B standard or sample /%B zero standard.

The insulin concentration of samples was determined from a linearized dose-response curve prepared by plotting $\%B_o$ of standards relative to their insulin concentrations on logit-log graph paper. Sample $\%B_o$ and insulin concentration in μU/ml were calculated from supernate and precipitate CPM using a Wang desk computer program into which was entered the parameters of 50% intercept, y-intercept, and slope from the manual plot.

The insulin RIA described above was employed routinely during 1974-1975 for clinical determination of serum insulin levels for the Foster G. McGaw Hospital, Maywood, Illinois.

Addition of Heparin to Insulin Standards

Twenty or 200 mg sodium heparin (Nutritional Biochemicals, Cleveland, Ohio) was dissolved in 10 ml barbital-acetate buffer to produce 2 mg/ml and 20 mg/ml solutions, respectively. Equal volumes of these solutions and insulin standards were mixed and incubated 3 hours at 25°C; 0.2 ml solution was added to assay tubes instead of 0.1 ml standard for a final heparin concentration of 0.25 or 2.5 mg/ml.

DOUBLE-ANTIBODY INSULIN RIA

The assay is a modification of the method of Morgan and Lazarow (435).

Reagents

1. Albumin buffer: 0.35% bovine serum albumin (BSA) (Sigma Chem. Co., St. Louis, Mo.) in barbital-acetate buffer, pH 7.4.
2. EDTA: 0.1 M Na_2EDTA (Mallincrodt, Inc., Paris, Ky.).
3. Standards: 5, 10, 15, 20, 30, and 40 μU/ml human insulin standard (Burroughs Wellcome, Research Triangle Park, N.C.) in albumin buffer.
4. Controls and unknowns: human plasma or serum samples undiluted or diluted appropriately with albumin buffer.

5. Anti-insulin serum (AIS): 1:50,000 AIS (Burroughs Wellcome) in albumin buffer.

6. Trace: 0.5 ng/ml ^{125}I-bovine insulin (New England Nuclear, Boston, Mass.) in albumin buffer, after purification through 25 ml Sephadex G-50.

7. Second antibody: 1:8 anti-guinea pig serum (Burroughs Wellcome) in albumin buffer.

8. Carrier protein: 1:200 guinea pig serum (Gibco, Grand Island, N.Y.) in albumin buffer.

Assay

The reagents are mixed in 12 x 75 ml glass culture tubes and treated according to the protocol outlined in Table VI. Precipitates (antibody-bound radioactivity) were counted in a Picker Pace-1 automatic gamma ray spectrophotometer (Picker Corp., Highland Heights, Ohio).

Data Reduction

$$\%B = \frac{\text{CPM sample} - \text{CPM NSB}}{\text{CPM total count} - \text{CPM NSB}} \times 100.$$

Logit-log standard curves were prepared as described for the dextran-charcoal assay. Insulin concentrations of samples were also calculated using a Wang desk computer program.

TABLE VI.

DOUBLE-ANTIBODY INSULIN RADIOIMMUNOASSAY PROTOCOL

Sample	Albumin	Standard buffer	Unknown or	EDTA control	AIS	^{125}I-insulin
	ml added					
Total count	0.9	0	0	0	0	0.1
NSB	0.8	0	0	0.1	0	0.1
B_o	0.7	0	0	0.1	0.1	0.1
Stds.	0.6	0.1	0	0.1	0.1	0.1
Unknowns & Control	0.6	0	0.1	0.1	0.1	0.1

Incubate 72 hr. at 4°C.

Add 0.1 ml 1:200 guinea pig serum + 0.1 ml 1:8 anti-GPS to each tube, except Total Counts.

Incubate 48 hr. at 4° C.

Centifuge at 2,000 x g 20 min., 4° C.; aspirate supernate; wash 1X with barbital-acetate buffer; count precipitates (except Total Counts).

CATION-EXCHANGE CHROMATOGRAPHY OF HUMAN PLASMA

The method is that described by Guenther (387). Ten grams Sephadex C-50 (carboxymethyl-Sephadex, Pharmacia) was allowed to swell in 900 ml 0.005 M ammonium carbonate buffer, pH 7.8, overnight at room temperature.

Two units of outdated citrate-phosphate-dextrose (CPD) plasma from separate donors, obtained from the Foster G. McGaw Hospital blood bank, were pooled and centrifuged at 1,500 x g for 20 minutes, 4°C. The 450 ml recovered was mixed with the gel after aspiration of the supernatant buffer and stirred for 3 hours at 4°C. The mixture was poured over a double layer of cheesecloth in a Buchner funnel to which was applied mild suction. The residue was kept constantly covered with a wash solution of cold deionized water until the filtrate was clear.

A 2.5 x 100 cm glass column (Bio Rad Laboratories, Richmond, Ca.) was packed with the gel (column and accessory equipment were kept in a cold room at 4°C) and 0.005 M $(NH_4)_2CO_3$, pH 7.8, was run through the column until a steady baseline (as monitored by an LKB Uvicord III, LKB Instruments, Inc., Rockville, Md., and recorded on an LKB Biocal recorder Type 6520-5) was obtained.

A one-liter salt gradient of 0.005 to 1.5 M potassium chloride was applied to the column using a two-chamber plexiglass gradient mixer and dispenser, specially constructed by the Experimental Medical Shop, University of Missouri, the flow was maintained at 30 ml per hour with an LKB 10200 Perpex peristaltic pump, and 5 ml fractions were collected with a Buchler

Fracto Mette 200 automatic fraction collector (Buchler Instruments, Inc., Fort Lee, N.J.). The column was further eluted with 1.5 M KCl for 24 hours.

The gradient was traced by determining the osmolality of selected fractions using a model 68-3L Advanced osmometer (Advanced Instruments, Inc., Needham Heights, Mass.). Fractions exhibiting ultraviolet absorption at 280 nm (A_{280}) were pooled. The protein content of each major fraction was estimated from A_{280} using $E^{0.1\%}_{280} = 1.0$ and 2 ml of each were reserved for determination of IRI with and without heparin using the dextran-charcoal RIA. Fractions were adjusted to pH 7.3 0.1 with 0.01 or 1.0 N acetic acid or 0.001 or 0.01 N potassium hydroxide, were then lyophilized, and were stored dessicated at -20°C. Fractions exceeding 140 ml volume were concentrated through UM 10 membranes (molecular weight cutoff = 10,000 daltons; Amicon Corp., Danvers, Mass.) in an ultrafiltration cell (Amicon) to 50 ml and those exceeding a salt concentration of 0.3 M were diluted to 0.005 M by repeated addition of water and concentration through UM 10 membranes before lyophilization.

For the RIA, lyophilized fractions were dissolved or suspended in water at a protein concentration of 2.5-10 mg/ml and a salt concentration of 0.05-0.31 M. Heparin was added to pooled fractions at a concentration of about 40 μg/mg protein and to reconstituted fractions at a concentration of 100 μg/ml (10-40 μg/mg protein), which were then incubated at 33°C for 30 minutes and assayed relative to standards containing a comparable amount of heparin.

IMMUNOREACTIVE ENDOGENOUS INSULIN DETERMINATION AND EXOGENOUS INSULIN RECOVERY FROM VARIOUS SERUM AND PLASMA SAMPLES

Serum: N - pooled normal serum

SS (stripped serum) - pooled serum treated with Amberlite IRA 400 (anion exchanger; J.T. Baker Chem. Co., Phillipsburg, N.J.) 24 hr. at 4°C.

Plasma: P - citrated plasma from a single donor (outdated)

SP (stripped plasma) - citrated plasma from two donors, treated with Amberlite IRA 400 24 hr. at 4°C.

Recoveries: 69 or 104 μU of human insulin standard was added to 1.0 ml of each of the above samples. Significance of comparisons was determined by Student's t test (730).

Heparin: 11 mg sodium heparin (Sigma) was dissolved in 1.1 ml deionized water. Ten μl was added to 1.0 ml each of N, SS, P, SP, and recoveries.

Biotin: 8 mg of D-biotin (Sigma) was dissolved in 8 ml hot saline. Ten μl (10 μg) was added to 1.0 ml each of N, SS, P, SP, and recoveries.

For replicate samples, 10 mg D-biotin was dissolved in 0.1 N NaOH dropwise. The alkaline solution was brought to 10 ml with barbital-acetate buffer, pH 7.4, for a 1 mg/ml solution. Ten μl (10 μg) was added to each sample immediately before incubation. Separate standard curves for control, heparin and biotin additions were included. All assay tubes were incubated 30 minutes at 37°C before assay with the dextran-charcoal RIA.

SEPHADEX CHROMATOGRAPHY OF UNTREATED AND TREATED HUMAN SERUM AND PLASMA SAMPLES CONTAINING EXOGENOUS RADIOLABELED AND UNLABELLED INSULIN

Initial Chromatography

^{125}I-insulin (10 μCi) was purchased from New England Nuclear Corp. and was twice purified on a 25 ml Sephadex G-50 disposable column. The purified product was divided into three equal parts, which were lyophilized and stored at -20°C for no more than one week before use. A serum pool of 30 ml was prepared from three normal donors and stored at -20°C in three aliquots of 9.9 ml each before use. Biotin solutions were prepared as in the recovery study immediately before use. A 2.6 x 90 cm (477 ml) Sephadex G-200 column was prepared and equilibrated with barbital - acetate buffer. The column was calibrated with a mixture of 25 mg blue dextran (Pharmacia), 40 mg catalase (2 ml Boehringer crystalline suspension, no. 15674, Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 53 mg hexokinase (Nutritional Biochemicals), 75 mg bovine serum albumin (BSA, Sigma Fraction V), and 23 mg chymotrypsinogen A (Worthington Biochemicals, Freehold, N.J.) in 8 ml barbital-acetate buffer. All elutions were with barbital-acetate buffer at 4°C using an LKB 12000 Varioperpex peristaltic pump set at 1.5 x 10 counterclockwise (flow = 17-21 ml/hr.), an LKB Uvicord II UV monitor set for 280 nm, and an LKB 6520-3 recorder, recording at 20 mm/hr.

For the test runs, a serum aliquot was thawed in a 37°C water bath while a lyophilized ^{125}I-insulin aliquot was reconstituted with 0.3 ml deionized, distilled water. The serum was added to the labeled insulin and mixed. The

following was added immediately: control, nothing; biotin, 0.1 ml (100 μg) biotin solution. After mixing, 0.1 ml was removed for counting and the remainder applied to the column. The eluate was monitored as before and two-ml fractions were collected with an LKB fraction collector. Fractions were counted for radioactivity in a Searle 1185 automatic gamma-ray spectrophotometer for 2 minutes each. Fractions of protein and/or radioactive peaks were pooled, sterilized by filtration, and stored at 4°C prior to lyophilization, after which they were stored dessicated at -20°C. Radioactive fractions were each reconstituted with 20 ml distilled water and dialyzed against 100 volumes of barbital-acetate buffer, pH 7.4 (3 changes in 24 hrs.). Fractions designated A (A_1) were dialyzed through Fisher cellulose (M.W. 12,000 cutoff) and B (B_1) fractions were dialyzed through Spectrapor membrane (M.W. 3,500 cutoff). Dialysis retentates were lyophilized and stored at -20°C.

Rechromatoghraphy of Pooled A + B Fractions

Another 475 ml Sephadex G-200 column was prepared and calibrated as before. All A_1 and B_1 fractions were reconstituted to their respective dialysis retentate volumes and pooled. The pool (108 ml) was concentrated to 15 ml through Amicon UM 2 membranes and was centrifuged at 8,000 RPM for 10 minutes. The supernate was applied to the column and fractions collected as before. A 105 ml (2.5 x 21 cm) G-200 column was prepared and calibrated.

Rechromatography of the A_2 Fraction

Pooled fraction A (A_2) from rechromatography was concentrated to 15 ml through UM 2 membranes and lyophilized. Two-thirds of the lyophilizate

was reconstituted and applied to the small column; two-ml fractions were collected. The pooled A_3 fraction was concentrated, lyophilized, reconstituted, and incubated with 1,000 μU bovine insulin before chromatography on the small column (A_3-I).

Chromatography of Stripped Plasma (SP)

CPD (citrate-phosphate-dextrose) plasma from a single normal donor was obtained from the Foster G. McGaw Hospital blood bank after preparation of packed red cells, maintained continuously at 4°C. Fifty ml plasma was stirred with 25 grams Amberlite IRA-400 at 4°C overnight, after which the resin-plasma suspension was centrifuged at 1,700 x g 20 minutes at 4°C. The supernate was dialyzed through cellulose (cutoff M.W. 12,000) vs. normal saline (3 changes of 200 volumes in 2 days) at 4°C.

Radioiodinated insulin (^{125}I-insulin) was obtained from New England Nuclear Corp. (NEX-104, lot 135-264). The contents of one vial were reconstituted with 0.31 ml distilled, deionized water (to 50 μCi/ml + 0.050 ml 5% bovine serum albumin (BSA, in 0.01 M phosphate-buffered saline, PBS) and applied to a column of Sephadex G-50 fine, bed volume 25 ml. One-ml fractions were collected and assayed for I-125 activity in a Picker Pace-1 auto gamma counter, 0.1 min. each. After addition of 0.070 ml 5% BSA to each, insulin peak tubes were pooled and lyophilized.

Approximately 0.5 μCi of this purified ^{125}I-insulin was reconstituted with 0.3 ml water, mixed with 9 ml ion-depleted plasma, incubated 30 minutes at 37°C, and chromatographed on 477 ml Sephadex G-200 as previously described. In addition, 0.2 ml 5% BSA was added to each peak B tube before

pooling and lyophilization. The procedure was repeated with the addition of 2.25 mU unlabeled bovine insulin to the incubation mixture (SP-I).

Chromatography of Long-Incubated Stripped Plasma (L)

^{125}I-insulin (Lot 135-278) was obtained from New England Nuclear Corp.; 14.5 μCi was reconstituted with 0.3 ml distilled, deionized water + 0.1 ml 3.5% BSA in 0.01 M phosphate-buffered saline (PBS). The resultant solution was applied to a 25 ml column of Sephadex G-50 fine and eluted with barbital-acetate buffer, pH 7.4. Each 1 ml fraction, collected in a 12 x 75 mm Falcon polystyrene tube, was counted 0.1 min. in a Picker Pace-1 auto gamma counter. Fractions from the second radioactive peak were pooled; 2.5 μCi was diluted for the double-antibody RIA and the remaining 3.1 μCi (in 6.1 ml) was dialyzed through Spectrapor 3 tubing vs. 3 changes of 1 liter 1/10 normal saline (0.085%) at 4°C. The retentate was lyophilized and reconstituted with 1 ml water.

Normal CPD plasma from a single donor (O-neg.; #29-00236), kept at 4°C continually after collection, was obtained from the Foster G. McGaw Hospital blood bank; 50 ml of this plasma was stirred with 25 grams Amberlite IRA-400 anion exchange resin at 4°C overnight and then was centrifuged at 2,000 x g at 4°C. Twenty ml of the supernate was dialyzed through Fisher cellulose tubing vs. 3 changes of 4 liters normal saline at 4°C. Ten ml of the retentate was mixed with the reconstituted purified ^{125}I-insulin, the mixture was sealed in a vial and kept at 4°C 5 days. Following this incubation the mixture (2.5 μCi) was applied to a 475 ml column of Sephadex G-200. Chromatography and collection of eluted fractions was carried out as for SP.

Preparation of Rabbit Anti-HSA (human serum albumin)

A New Zealand white rabbit (approx. 3 kg) was immunized with multiple subcutaneous injections of 1 mg HSA in complete Freund's adjuvant three times at weekly intervals. One week after the last challenge, the rabbit was exsanguinated by heart puncture.

The efficacy and specificity of the serum vs. HSA was tested with precipitin ring tests, immunodiffusion, and hemagglutination using HSA conjugated to sheep red blood cells with chromic chloride (731). The serum was found to have an excellent titer against HSA (0.5-1.0 mg antibody/ml) and no detectable reactivity with bovine serum albumin (BSA).

Immunodiffusion

Chromatography fractions were tested for HSA using the Ouchterlony double diffusion technique (732). A hexagonal array of six small wells surrounding a larger center well is cut out of a highly purified agar plate using a planchet. Antiserum is put into the center well and the samples to be tested into the peripheral wells. If a sample contains the antigen to which the antiserum reacts, a precipitin band forms between that well and the center well within 24 hours of incubation at room temperature. The plate is then washed in normal saline twice in 48 hours and distilled water twice in 24 hours, allowed to dry 24 hours, stained with Naphthalene Black 30 minutes, and washed several times with acetic acid/methanol/water 1:7:2.8. The bands stain dark blue. For serum chromatography and rechromatography, 10 mg of each lyophilized fraction was dissolved in 1 ml water for testing. Unlyophilized fractions were used directly.

Amounts of radioactive peak C and A and protein peak II lyophilizates from the three CPD plasma/^{125}I-insulin columns (control, cold insulin added, and 5-day incubation) containing 10 mg protein each were reconstituted with 1 ml water. The resultant solutions or suspensions were dialyzed through Spectrapor 3 tubing vs. 3 changes of 200 volumes normal saline at 4°C. The retentates (C-fractions were first centrifuged to remove insoluble material) were then tested without dilution (1% protein) and at 0.1% protein dilutions for BSA, HSA, and human IgG vs. the appropriate antisera by the Ouchterlony double diffusion technique. Rabbit anti-HSA, anti-BSA, and anti-human IgG sera were obtained from Miles Laboratories, Inc., Elkhart, Ind.

Protein Determinations

Protein in each fraction was assayed using the method of Lowry (733). Dissolved lyophilized fractions were assayed at a concentration of 0.5 mg/ml; partially soluble lyophilized fractions were incubated with 0.1 ml 1 N NaOH/ml at room temperature overnight and then diluted twenty-fold for assay; unlyophilized fractions were assayed directly or, if they represented a large absorbance peak at 280 nm, were first diluted tenfold. Total protein was calculated on the basis of total lyophilizate recovered or total fraction volume.

Dextran/Charcoal Analysis of Radioactive Fractions

Each group consisted of three 12 x 75 mm plastic culture tubes:

1. 0.1 ml trace + 0.9 ml albumin buffer.
2. 0.1 ml trace + 0.1 ml 1:10,000 guinea pig anti-insulin serum (AIS) + 0.8 ml albumin buffer.

3. 0.1 ml trace + 0.1 ml AIS + 0.1 ml human insulin standard (0.1 mU) + 0.7 ml albumin buffer.

4. 0.1 ml trace + 0.9 ml albumin buffer in 8 M urea.

5. 0.1 ml trace + 0.1 ml AIS + 0.8 ml albumin buffer in 8 M urea.

The above tubes were incubated 2 days at $4^{o}C$.

For the following three groups, 78 mg (25 mg protein) of 5-day incubation A-fraction (A_L) lyophilizate was reconstituted with 1-2 ml water for each group of three tubes and dialyzed through Spectrapor 3 tubing vs. 3 changes of 200 volumes barbital-acetate buffer at $4^{o}C$ 2 days. The retentate was diluted to 3 ml (group 6) or 2.7 ml (groups 7 and 8).

6. A_L alone (1.0 ml).

7. 0.9 ml A_L + 0.1 ml insulin standard (0.1 mU).

8. 0.9 ml A_L + 0.1 ml 1:10,000 AIS.

Groups 7 and 8 were further incubated 2 days at $4^{o}C$.

9. 78 mg A_L lyophilizate was reconstituted with 2 ml water and dialyzed through Spectrapor 3 tubing vs. 500 ml barbital-acetate buffer in 8 M urea at $4^{o}C$ (3 changes in 2 days). The retentate was diluted to 3.0 ml with barbital-acetate buffer in 8 M urea and 1.0 ml was put in each of three tubes.

To each of the 27 tubes was then added 0.5 ml of a cold dextran-charcoal suspension (0.25g Dextran T-70 + 2.5g Norit A/100 ml barbital-acetate buffer). The contents of each tube were agitated and then centrifuged at 2,000 RPM 10 minutes at $4^{o}C$. The supernates were decanted into 12 x 75 mm

culture tubes. Both supernatant ("bound") and precipitate ("free") tubes were assayed for I-125 activity in the Picker Pace-1 auto gamma counter.

SDS Polyacrylamide Disc Gel Electrophoresis

1. The calibration mixture consisted of 4 mg each bovine insulin, chymotrypsinogen A, BSA, catalase, and 8 mg glucose oxidase dissolved in 10 ml barbital-acetate buffer, pH 7.4.
2. Six mg (2.1 mg protein) long incubation A-peak (A_L) was reconstituted in 0.4 ml deionized water + 0.6 ml barbital-acetate buffer (B/A).
3. Solutions were dialyzed vs. 500 ml 2.5% sodium dodecyl sulfate (SDS) in 0.01 M sodium phosphate buffer, pH 7.2, 3 hours at 25°C.
4. Dialysis was continued in 500 ml 0.1% SDS in 0.01 M sodium phosphate buffer, pH 7.2, with 1.6 M urea and 0.05% dithiothreitol 17 hours at 25°C.
5. Gels consisted of the following:

A_1 1% SDS	40 ml
1 M sodium phosphate buffer, pH 7.2	40 ml
deionized water	to 180 ml
A_2 acrylamide	40g
bis-acrylamide	1.04g
water	to 180 ml
B_2 Ammonium persulfate	90 mg
TEMED	0.05 ml
water	10 ml

For 12 gels, 22.5 ml A_1 + 22.5 ml A_2 + 5 ml B_2 are mixed, deaerated 30 seconds to 1 minute, 2.5 ml poured into each glass tube, overlayed with water, and allowed to stand until solidified, about 30 minutes. Gels measured approximately 10 cm length x 0.7 cm diameter.

6. 0.1 ml of each solution was applied to duplicate gels.

7. Gels were electrophoresed (15-20 v, 2-3 ma/gel) in 0.1% SDS in 0.1 M phosphate buffer 16 hours, until a bromphenol blue dye marker was 0.5 cm from the end of the gel.

8. Gels were removed from tubes with a stream of ice water and transferred to a fixative solution consisting of methanol/glacial acetic acid/water 45:10:45.

9. The gels were agitated 5 days at 25°C with 12 changes of fixative, until all residue was removed.

10. Gels were stained with 1% fast green in fixative 2 hours at 25°C, washed once with tap water, and agitated in 3 changes of fixative 1-1.5 days.

11. The gels were destained 5-6 days in 2 changes of fixative and stored in fixative at 25°C.

Affinity Chromatography

1. Four grams CNBr-activated Sepharose 4B (Sigma Chem. Co.) were swollen in 20 ml 0.001 M HCl in a scintered glass funnel 15 minutes and then washed with 800 ml 0.001 M HCl.

2. The gel was then washed and resuspended to 28 ml with 0.1 M bicarbonate buffer, 0.5 M NaCl, pH 8.65.

3. The gel was divided into two equal portions; to one portion was added 50 mg BSA in 1.5 ml bicarbonate buffer, the same concentration of HSA to the other.

4. The suspensions were rocked at 4°C overnight and then washed with bicarbonate buffer.

5. Each gel was resuspended in 7 ml 1 M ethanolamine, pH 8.0, and rocked 2 hours at 25°C.

6. The gels each were washed alternately 5 times with 0.1 M acetate buffer, 0.5 M NaCl, pH 4.0, and 0.1 M borate buffer, 0.5 M NaCl, pH 8.45, followed by 0.01 M PBS, pH 7.4.

7. The gels were resuspended to 7 ml each and stored in 0.01 M PBS with 0.1% sodium azide before being poured into two glass columns.

8. Fourteen ml rabbit anti-BSA (Miles lot no. R148, Miles Laboratories, Inc., Elkhart, Ind.) was passed through the HSA-Sepharose column to remove cross-reacting antibodies; the retained antibodies were eluted with glycine-HCl buffer, pH 3.0, and the eluate was immediately neutralized with 0.1 N NaOH.

9. The first eluate (hereafter called the eluate) was then passed through the BSA-Sepharose column.

10. The neutralized eluted retentate (hereafter called the retentate) was pooled with a second retentate obtained by passing the eluate through the column again, the pool was concentrated to 10 ml through a PM 10 membrane, and dialyzed through cellulose tubing vs. 2 liters 1/10 normal saline at 4°C with 3 changes in 2 days.

11. Rabbit anti-HSA (9.5 ml; Miles lot no. RHA 2) was passed through the HSA column and two pooled retentates were processed as was the anti-BSA.

12. Pure anti-BSA and anti-HSA were lyophilized after dialysis and reconstituted in 1 ml bicarbonate buffer each.

13. The anti-BSA (5.8 mg) and anti-HSA (7.6 mg) solutions were added to respective gel suspensions and the proteins coupled to CNBr-activated Sephorose 4B as described above.

14. 312.5 mg (100 mg protein) A_L was dissolved in 20.7 ml water; 5 ml was assayed for I-125 activity in a Picker Pace-1 auto gamma ray spectrophotometer in a plastic centrifuge tube.

15. The A_L solution was passed through the anti-BSA column until no protein was eluted with glycine-HCl, six times.

16. The pooled anti-BSA retentates were concentrated to 8.0 ml through a PM 10 membrane; the resultant retentate, filtrate, and membrane were assayed for radioactivity (membrane activity was added to retentate activity).

17. The final anti-BSA eluate was passed through the anti-HSA column twice and the pooled retentates concentrated to 7.2 ml through a PM 10 membrane.

18. The final eluate was concentrated to 9.0 ml through a PM 10 membrane and the resultant filtrate concentrated to 8.4 ml through a UM 2 membrane. All fractions were assayed for radioactivity as for the anti-BSA retentate.

19. Immunochemical identification of fractions was made using the Ouchterlony double diffusion technique and protein was determined using the method of Lowry.

Dextran-Charcoal Binding Study

Immunoreactive insulin content of the UM 2 retentate was ascertained alone, with AIS, and with AIS + unlabeled insulin using dextran-coated charcoal as described above.

MAINTENANCE OF HUMAN CELLS IN CULTURE

Culture media, L-glutamine, trypsin and antibiotics (unless otherwise indicated) were purchased from Grand Island Biological Co. (Gibco), Grand Island, N.Y. Hy-Clone fetal bovine serum (FBS) was purchased from Sterile Systems, Inc., Logan, Utah (incubated at 56°C for 60 minutes to inactivate complement before use), and disposable polystyrene culture flasks were obtained from Corning Glass Works, Elmira, N.Y.

WI38 Fetal Lung Fibroblasts

WI38 cells in the 15th passage level were obtained in frozen ampuoles from the American Type Culture Collection, Rockville, Md. After thawing rapidly in a 37°C water bath and centrifugation in warmed Eagle's Basal Medium with Earle's salts (BME) containing 10% FBS and 2 mM L-glutamine (BME CM), the cells (in 5 ml BME CM) were placed in a T-250 culture flash and incubated at 37°C in an atmosphere of 5% CO_2/95% air in a Hotpack CO_2

incubator (Hotpack Corp., Philadelphia, Pa.). Confluent monolayers were subcultivated in BME CM using 0.25% trypsin or 0.125% trypsin/0.01% Na_2EDTA. Antibiotics (100 U penicillin-100 μg streptomycin/ml or 100 μg gentamicin/ml) were added after the 20th passage level. The cells were used up to the 30th passage level (54 generations).

SK-HEP-1 Hepatoma Cells

Samples of this cell line were generous gifts from Dr. Jørgen Fogh at the Sloan-Kettering Institute for Cancer Research, Rye, N.Y. Two samples of SK-HEP-1 growing in glass culture tubes were transferred to a 37°C incubator in an atmosphere of 5% CO_2/95% air. The tube mouths were covered with sterile gauze to enable gas equilibration. Within one week, the cells were removed from the tubes by treatment with 0.25% trypsin (Gibco) and transferred to T-250 culture flasks, which were kept in the CO_2 incubator. When cells attained confluency, they were subcultivated into other T flasks by trypsinization as before. SK-HEP-1 was cultivated in MEM alpha medium with 15% FBS and 100 U penicillin/ml-100 μg streptomycin/ml.

Other Cell Lines

LT2 squamous-cell vulvar carcinoma cells were established in continual culture and characterized as described (734). AU471 ovarian carcinoma cells were obtained in frozen ampuoles from W.A. Nelson-Rees, Naval Biosciences Laboratory, Oakland, Ca., and BeWo choriocarcinoma cells were obtained from R.A. Pattillo, Medical College of Wisconsin, Milwaukee. All three cell lines were maintained in RPMI-1640 medium with 15% FBS (CM).

REPLICATE CULTURE TECHNIQUES

Cells maintained in adherent culture in 75 cm^2 plastic culture flasks (Corning) were washed with 10 ml Dulbecco's phosphate-buffered saline (PBS) without Ca^{++} and Mg^{++} and detached with 2-10 ml trypsin/EDTA for 1-15 minutes at 37°C. The reaction was stopped by addition of at least an equal volume of complete maintenance medium (containing FBS) and the cell suspension was centrifuged at 400 x g for 10 minutes at room temperature. The cells were resuspended in 5 ml PBS; to 0.1 ml of this suspension was added an equal volume of 0.4% trypan blue in normal saline and number of viable cells (those excluding dye) was determined using a hemacytometer and a light microscope (American Optical Scientific Instruments, Buffalo, N.Y.)

The cells were centrifuged once more and resuspended in maintenance medium to a concentration of 1 x 10^6 cells/ml. This suspension was further diluted with maintenance medium with or without varying concentrations of human (Wellcome) or bovine (Sigma) insulin to a concentration of 5 x 10^3 (WI38, SK-HEP-1, AU471, and BeWo) or 1 x 10^4 (LT2) cells/ml. One-ml aliquots were distributed among the center 8 wells of 24-well (2 cm^2 area/well) polystyrene cluster plates (Costar, Cambridge, Mass.) using a Manostat (Manostat Corp., New York, N.Y.) dispenser. Proliferation of cells in the peripheral wells of these cluster plates had been found to be inconsistent and thus these wells were each filled with 2 ml sterile distilled water. Periodically, the medium from 2-8 replicate wells was removed, centrifuged and assayed for glucose in a Beckman glucose analyzer (Beckman Instruments, Inc., Fullerton, Ca.). Aliquots were frozen for double-antibody insulin RIA. The cells in these wells were retrieved

with 1 ml trypsin/EDTA. After centrifugation, cells were resuspended in 0.1 ml PBS + 0.1 ml trypan blue and counted as described above.

Log-phase growth rates (doubling time, t_D) were determined from the linear portion (established by least-squares regression analysis) of plots of log viable cell number (N) vs. time. Significance of difference in growth rates with and without insulin was determined from $t = (b_c - b_i)/\sqrt{S_{b_c}^2 + S_{b_i}^2}$, where b_c = slope of control, b_i = slope with insulin and S_b is the standard error of the slope (730). For cells in lag phase (before log phase) and stationary phase (in medium lacking serum or at confluence), rate of glucose uptake, r, was determined as u/Nt, where u is glucose uptake in mg, N is average number of viable cells in millions, and t is time in days. For cells in log phase, $r = u/N_g t$, where N_g is the geometric mean of viable cell number (in millions) = antilog {(log initial cell number + log final cell number)/2}.

In addition, insulin effects on WI38 cells were examined using three replicate monolayer systems: 1) initial lag phase cultures. Five thousand single cells in one ml BME CM with or without various amounts of insulin were seeded in each well. At various times thereafter the supernates were assayed for glucose, then frozen for insulin RIA, and the cells were harvested for counting with trysin/EDTA; 2) log phase cultures. Twenty thousand cells were seeded in each well. Two days later, medium was replaced with one ml BME CM with or without various amounts of insulin. Supernates and cells were harvested at various times thereafter; 3) confluent cultures. One hundred thousand cells were seeded in each well. Two days later, medium was replaced with or without various amounts of insulin. Supernates and cells were harvested at various times thereafter. All operations until the final harvest were carried

out with sterile equipment and reagents, using sterile techniques in a laminar flow hood (Germfree Laboratories, Miami, Fla.).

Efficiency of plating (EOP) was determined by seeding duplicate Costar cluster wells with 100, 200, 500 and 1,000 cells in 1 ml each. After 24 hours in the CO_2 incubator, the medium was removed, the cells were washed with PBS, and the cells were fixed in 95% ethanol and stained with Giemsa 5 minutes each. The cells in each well were counted on an inverted microscope (Olympus Corp. of America, New Hyde Park, N.Y.).

CULTURED FIBROBLAST INSULIN BIOASSAY

The assay is a modification of the method of Morell and Froesch (615). One-ml aliquots of 2×10^4 viable WI38 cells in BME CM were distributed among the center 8 wells of Costar cluster dishes. After 1 day, the medium was aspirated, the cells were washed with 1 ml PBS/well, and 1 ml stationary medium (0.35% BSA in BME) was added to each well. After 2 days, medium was aspirated, cells washed and test samples in 1 ml stationary medium (aliquots reserved for glucose assay) were added to each well. After 4 days, medium was removed for glucose assay and cells were harvested for counting as described above.

The parameters of the assay are:

$I =$ u/N.

$\nu =$ number viable cells in test well/number viable cells in control well (stationary medium alone).

$R = r_t/r_c$, whre r_c = control r and r_t = sample $r = u/N_g t$ if $N_t > N_c$ or u/Nt if $N_t \leq N_c$.

$U = u_t/u_c$.

ILA, determined from a plot of U vs. insulin concentration.

Specific ILA = ILA_s, determined from a plot of R vs. insulin concentration.

$$t_D = \frac{0.301\ (t - 38\ \text{hrs.})}{\log (N/N_o)}.$$

FBS equivalent = $(22\ \text{hrs.}/t_D) \times 0.1$ ml.

EFFECT OF AIS ON INSULIN-TREATED CULTURES

Wellcome human insulin standard was reconstituted with stationary medium to a concentration of 100 μU/ml. To 2 ml of this solution was added 0.2 ml 1:5,000 normal guinea pig serum (Gibco) or 0.2 ml 1:5,000 guinea pig anti-insulin serum (Wellcome). The solution were sterilized by filtration through 0.2 μm Swinny filters (Gelman Sciences, Inc., Ann Arbor, Mich.), incubated at 37°C for 30 minutes and then were added to WI38 cells in replicate wells prepared for bioassay as described above.

CHARACTERIZATION OF CELL-SURFACE INSULIN RECEPTORS

Tracer Determination of Cell-Surface Insulin Binding

Radioiodinated (^{125}I) insulin (New England Nuclear) was purified as described for Sephadex chromatography of plasma and serum.

One-ml aliquots of SK-HEP-1 cells in 0.5% BSA/MEM spinner culture medium (MEM-SC) brought to 300 mg glucose/dl with 20% dextrose/MEM-SC solution at concentrations of 5 x 10^5 - 10^6 viable cells/ml were distributed among 12 x 75 mm polystyrene culture tubes. To each tube was added 1.33 μU or 13.3 μU ^{125}I-insulin and the tubes were shaken in a 37°C water bath before removal at 5 min., 30 min., and 5 hrs. The tubes were centrifuged at 300 x g 10 minutes and the pellets washed once with PBS. Pellets were assayed for I-125 activity in a Picker Pace-1 auto gamma ray spectrophotometer.

The same quantities of ^{125}I-insulin were added in 1 ml aliquots of 0.5% BSA/MEM-alpha or CM to adherent SK-HEP-1 or LT2 cells (3.5-5.0 x 10^4 viable cells) in Costar cluster wells. After 30 minutes and 5 hours, supernates and a 1-ml PBS wash were pooled and assayed for activity as above.

Cells from adherent cultures of SK-HEP-1 and LT2 were also removed with trypsin, washed with PBS, and treated with ^{125}I-insulin as described for suspension cultures.

Binding of ^{125}I-insulin to WI38 cells in suspension was studied by using cells harvested with trypsin/EDTA and washed twice with PBS, pH 7.4. Four million cells were resuspended in 0.6 ml 5% BSA/PBS; an equal number of cells were resuspended in the same buffer containing 1 U bovine insulin/ml to ascertain non-specific binding of ^{125}I-insulin. Aliquots of 0.05 ml cell suspension were placed in polystyrene culture tubes; 0.05 ml of various concentrations of ^{125}I-insulin were then added to each tube, followed by incubation at 25°C for 2.5 hours with occasional shaking. Four ml PBS was then added to each tube, the contents were mixed, and the tubes were centrifuged at 300 x g 10 minutes. The supernates were decanted and the precipitates assayed

for I-125 activity 20 minutes each in a Searle 1185 auto gamma ray spectrophotometer. Net percent ^{125}I-insulin bound to cells was determined as (CPM without cold insulin - CPM with cold insulin)/(total CPM - CPM with cold insulin).

Immunocytochemical Determination of Cell-Surface Insulin Binding

Anti-insulin peroxidase/anti-peroxidase (PAP) staining of SK-HEP-1, LT2, WI38 and BeWo cells was performed by a modification of the method of Sternberger (735) as follows:

1. Cells dispersed from adherent culture with trypsin/EDTA were resuspended in 1 mU or 10 mU bovine insulin in 0.5% BSA/PBS or MEM-SC at a concentration of 10^6 cells/ml and incubated 5 minutes at 37°C.
2. The cells were washed once in PBS and resuspended in 0.1 ml FBS. Four smears were made on standard glass microscope slides. Subsequent treatments were carried out by placing 3 drops of solution on an area in the center of the slide, demarcated by an ink circle. Slides were washed in a Coplin jar.
3. 0.75% gelatin in 0.01 M PBS, pH 7.6, 30 min. 25°C. Blot.
4. 1:2,000 guinea pig anti-insulin serum (Wellcome) in 0.1% gelatin/PBS 46 hrs. 4°C, 2 hrs. 25°C. Wash 3 times in 0.1% gelatin 5 min. each. Blot.
5. 1:8 rabbit anti-guinea pig serum (Wellcome)/0.1% gelatin 30 min. 25°C. Wash 3 times (5 min.) in PBS. Blot.
6. Two slides 1:10 goat anti-rabbit serum (Polysciences, Inc., Warrington, Pa.)/0.1% gelatin 30 min. 25°C, two slides 1:10 normal goat serum (controls); 3 x 5 min. PBS wash. Blot.

7. 1:50 rabbit PAP (Polysciences)/0.1% gelatin 30 min. 25° C; 3 x 5 min. PBS wash. Blot.

8. 0.05% diaminobenzidine tetrahydrochloride (Sigma) in 0.01% hydrogen peroxide/PBS 8 min. 25°C; 3 x 2 min. water wash. Blot.

9. One control and one test slide were counterstained with 0.1% toluidine blue in 0.1 M acetate buffer, pH 4.2, 5 min.; 3 sec. rinse.

BULK FRACTIONATION OF HUMAN SERUM

Normal human serum in 500 ml bottles was purchased from Gibco, filtered through 0.2 μm Nalgene units (Nalge Co., Rochester, N.Y.) and stored at 4°C; 488 ml of this serum was concentrated to 142 ml through Amicon PM 10 (M.W. cutoff 10,000 daltons) membranes in a stirred cell under positive nitrogen pressure at 4°C. The filtrate was then concentrated to 52 ml through UM 2 (M.W. cutoff 2,000 daltons) membranes. One-half of each retentate (PM10r1, 3.44x conc., and UM2r1, 6.60x conc.) was lyophilized. The remaining PM 10 retentate was diluted to 502 ml with water and was reconcentrated through a PM 10 membrane to 84.5 ml (PM 10r2, 2.89x conc., 5.94x desalted); the remaining UM 2 retentate was diluted to 300 ml with water and was reconcentrated through a UM 2 membrane to 24.5 ml (UM2r2, 7.00x conc., 12.24x desalted). These retentates and both UM 2 filtrates (UM2f1 and UM2f2) were lyophilized.

For bioassay, various quantities of lyophilized fractions were reconstituted with stationary medium alone or containing 100 μg heparin/ml or 10 μg D-biotin/ml to produce a 10% increase in medium tonicity and were then

sterilized by filtration. In addition, the PM10r1 fraction was assayed by further 10-fold and 100-fold dilutions with corresponding stationary media. Various quantities of lyophilized fractions were also reconstituted with normal saline or albumin buffer for Lowry protein assay and double-antibody insulin RIA, respectively.

Treatment of the PM 10 Retentate

C (control). 64.8 mg PM10r2 was dissolved in 10 ml normal saline and incubated at 37°C for 30 minutes. The solution was then transferred to an ice bath and concentrated 10-fold through a PM 10 membrane. Both the retentate (CR) and filtrate (CF) were dialyzed in Spectrapor 3 tubing against 2 liters of 1/10 normal saline with 2 changes in 48 hours at 4°C. The dialysis retentates were each divided into 3 equal parts and lyophilized for bioassay as described above.

Equal amounts (64.8 mg) of PM10r2 were treated as above (C) with the following differences:

H. The fraction was dissolved in a solution of 100 μg heparin/ml normal saline.

B. The fraction was dissolved in a solution of 10 μg biotin/ml normal saline.

U. The fraction was dissolved in 2 ml normal saline, to which was then added 4.805 g urea in 8 ml normal saline (final urea concentration = 8 M).

AE. 259 mg PM10r2 was dissolved in 2.48 ml 0.1 N ammonium acetate, pH 7.2, and transferred to an ice bath; 7.44 ml 96% cold ethanol was added and mixed vigorously, followed by addition of 0.15 ml concentrated hydrochloric

acid and remixing. The suspension was then incubated at 4°C for 30 minutes and centrifuged at 12,000 x g for 30 minutes at 4°C. The supernate was then treated like the control, commencing with the concentration step, and the precipitate (AEP) was resuspended in 2 ml normal saline and treated like the control, commencing with the dialysis step.

Gel Chromatography of the PM 10 Retentate

A 2.6 x 70 cm (bed volume 371 ml) Sephacryl S-200 superfine (Pharmacia) column was prepared according to the manufacturer's instructions (736) and was equilibrated with 0.01 M sodium phosphate buffer, pH 7.4, at a flow rate of 26 ml/hour for 24 hours at 4°C. The column was calibrated with the following standards:

	Standard	Amount (mg)	Molecular Weight(kd)
1.	Blue Dextran	20	2,000
2.	glucose oxidase	100	186
3.	BSA	100	67
4.	chymotrypsinogen A	50	25
5.	myoglobin	50	16.9

The standards were dissolved in 7.5 ml phosphate buffer and the solution was clarified by centrifugation and filtration before application to the column, which was then eluted with phosphate buffer at a flow rate of 23 ml/hr. The effluent was monitored for ultraviolet absorbence at 280 nm.

Three grams PM10r1 were reconstituted with 10 ml deionized, distilled water, clarified by filtration, applied to the column and eluted as described for

the calibration; 2-ml fractions were collected. Twenty-four major fractions were prepared by pooling the contents of 5-49 collection tubes; 6 tubes were pooled for each of the 20 fractions (2-21) commencing with the first protein peak. One ml from each fraction was reserved for Lowry protein assay and RIA. Fractions were then lyophilized and one-third of each fraction was reconstituted for bioassay.

Dextran-Charcoal Analysis of Chromatography Fractions

One-tenth ml of each aliquot reserved from the Sephacryl fractions was mixed with 0.1 ml ^{125}I-insulin + 0.6 ml albumin buffer (NSB = 0.1 ml trace + 0.7 ml albumin buffer) and incubated for 3 days at 4°C; 0.4 ml dextran-charcoal suspension was then added to each tube, which was further treated as described for dextran-charcoal analysis of radioactive fractions.

Acid Treatment of Serum and Serum Fractions

One ml serum or reconstituted fraction was mixed with 9 ml 0.05 M glycine-HCl, pH 2.8, adjusted to pH 3.0 with concentrated HCl, and concentrated tenfold through PM 10 membranes. Filtrates were neutralized with an equal volume of 0.05 M PBS, pH 7.4, and adjusted to pH 7.4 with 1 N sodium hydroxide. Retentates were dialyzed against 200 volumes 0.001 N HCl in normal saline, followed by PBS. Both retentates and filtrates were assayed for IRI and dextran-charcoal adsorption. In the latter assay, 0.6 ml 8 M urea in albumin buffer or 1 mU bovine insulin in 0.6 ml albumin buffer was also added to 0.1 ml sample + 0.1 ml trace.

NSILA-S Screen

Fractions (46.3 mg fraction 9, 58.3 mg fraction 12) were each dissolved in 0.15 M acetic acid in 0.15 M sodium chloride. Each solution was concentrated tenfold through PM 10 membranes. All fractions were dialyzed against 4 liters 0.01 M phosphate buffer, pH 7.4, in Spectrapor 3 tubing. Each fraction was then divided into 3 equal parts and lyophilized for bioassay.

Comparative RIA

Double-antibody insulin RIA of selected fractions was carried out with ^{125}I-labeled and unlabeled porcine insulin (Pharmacia), as well as with ^{125}I-bovine insulin and human insulin standard.

GEL CHROMATOGRAPHY OF HUMAN SERUM ACETONE POWDER

The lyophilizate from 200 ml human serum (Gibco) was suspended in 500 ml acetone (-20°C) and stirred for 1 hour at 4°C. The suspension was filtered through 2 layers of Whatman no. 42 paper in a Buchner funnel and the residue was washed 4 times with 500 ml acetone (-20°C) and dried *per vacuo* for 25 minutes at room temperature and *in vacuo* for 45 minutes at room temperature (547, 737). Three grams of acetone powder were homogenized with 10 ml 0.01 M phosphate buffer, pH 7.4, in a 30-ml syringe. Undissolved residue was removed and the clarified solution was applied to a 2.6 x 71.5 cm (380 ml bed volume) Sephacryl S-200 column and eluted with 0.01 M phosphate buffer, pH 7.4, at a flow rate of 25 ml/hr. Two-ml fractions were collected and pooled to produce 24 major fractions as described above.

STATISTICAL METHODS

Means ($\bar{x}$), standard deviations (SD), coefficients of variation (CV(%) = [SD/$\bar{x}$] x 100) and standard errors of the means (SEM) for RIA quality control and standard curve parameters and recovery data, dextran-charcoal analysis of affinity chromatography fractions, and cell number, glucose uptake and rates of glucose uptake were determined by standard variance analysis (730). Significance of differences between means was determined by both paired and unpaired Student t-tests.

Semi-log growth curve and glucose analyzer response parameters were determined by least-squares linear regression analysis (730). Significance of differences between log-phase proliferation slopes was tested as described for "replicate culture techniques." Normal populations were established for the bioassay parameters U and ν by determining these parameters for each control well relative to the average glucose uptake and cell number, respectively, of two control wells per plate. The normal range was determined from the mean of $|$control parameters -1$|$ $\pm$ standard deviation x t-statistic of 95% confidence limit for control population degrees of freedom. Thus, any test-well parameter falling outside this normal range exhibited a minimum significance (high or low) of $P<0.05$. Specific ILA significance was determined relative to control r-values.

CHAPTER VII

RESULTS

DEXTRAN-CHARCOAL INSULIN RIA

A typical parabolic dose-response curve for this assay, obtained by plotting percent ^{125}I-insulin bound to antibody (%B) relative to concentration of unlabeled insulin added, is reproduced in Figure 16. Plots of logit (%B_o) relative to log [unlabeled insulin] (Fig. 17) were linear between 5 μU/ml and 15-20 μU/ml. Based on the 90% intercepts of logit-log plots for the 9 dextran-charcoal RIAs utilized in the research described herein, this assay exhibits a minimum sensitivity of 2.1 μU/ml (0.21 μU or 8.4 pg insulin in the assay tube). The interassay variance of curve parameters (slope and 50% intercept) was less than 10% (Table VII).

Nonspecific binding (NSB) of ^{125}I-insulin in albumin buffer ranged from 6-14% and in undiluted serum (diluted 8-fold in the assay tube) from 6-38%. Where NSB exceeded 20%, data were obtained from dilute sera, if possible. B_o ranged from 38% to 50% in these assays. Interassay variance of serum controls was approximately 10% (Table VII) and intraassay variance of a normal serum pool run 12 times in one assay was 9.5% (Table XI). Recovery of 69 μU exogenous insulin/ml from this pooled serum using this RIA was virtually 100% (Table X).

The mean insulin concentration of 17 normal fasting human sera determined using the dextran-charcoal RIA was 6.2 ± 4.9 (S.D.) μU/ml, yielding a

FIGURE 16. DEXTRAN-CHARCOAL INSULIN RIA: STANDARD CURVE.

Ordinate: Free insulin is adsorbed by dextran-coated charcoal, while antibody-bound insulin is not. Therefore, % ^{125}I-insulin bound to antibody,

$$\%B = \frac{\text{CPM in the supernate - CPM nonspec. bound*}}{\text{Total CPM - CPM nonspecifically bound}} \times 100.$$

Abscissa: Concentration of unlabeled insulin added to the assay tube. Since 0.1 ml standard is added to each tube, the range of absolute quantity of unlabeled insulin in the assay tubes is 0.5-3.0 μU.

* CPM in supernate in the absence of antibody or standard.

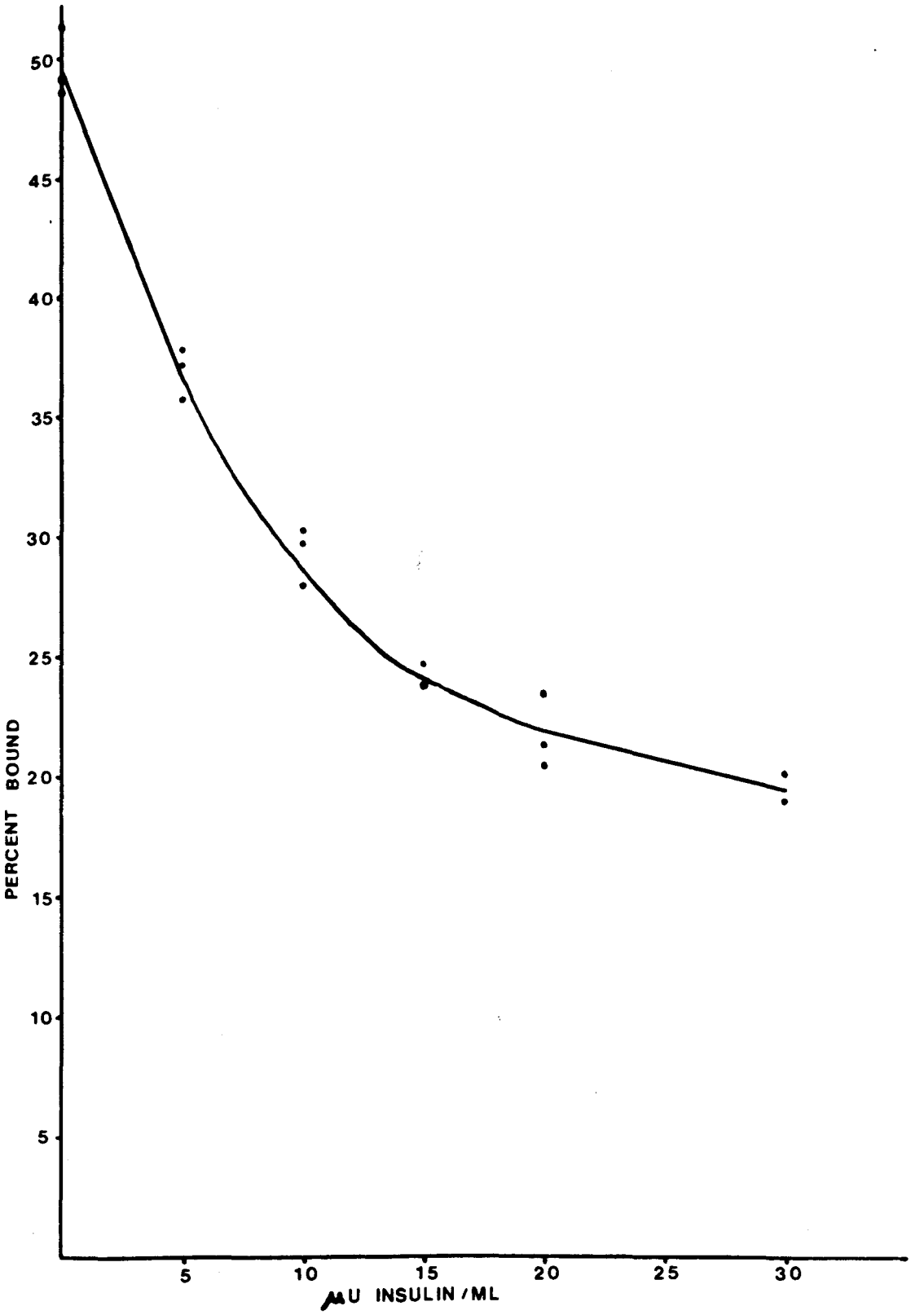

50
45
40
35
30
25
20
15
10
5
PERCENT BOUND
5
10
15
20
25
30
μU INSULIN / ML

FIGURE 17. DEXTRAN-CHARCOAL INSULIN RIA STANDARD CURVE: LOGIT-LOG TRANSFORM.

Ordinate: (left) $\%B_o$ = percent of CPM specifically bound to antibody in the absence of unlabeled insulin.

(right) logit $(\%B_o) = \ln \frac{\%B_o}{100 - \%B_o}$

Abscissa: $\log_{10}$ (insulin concentration)

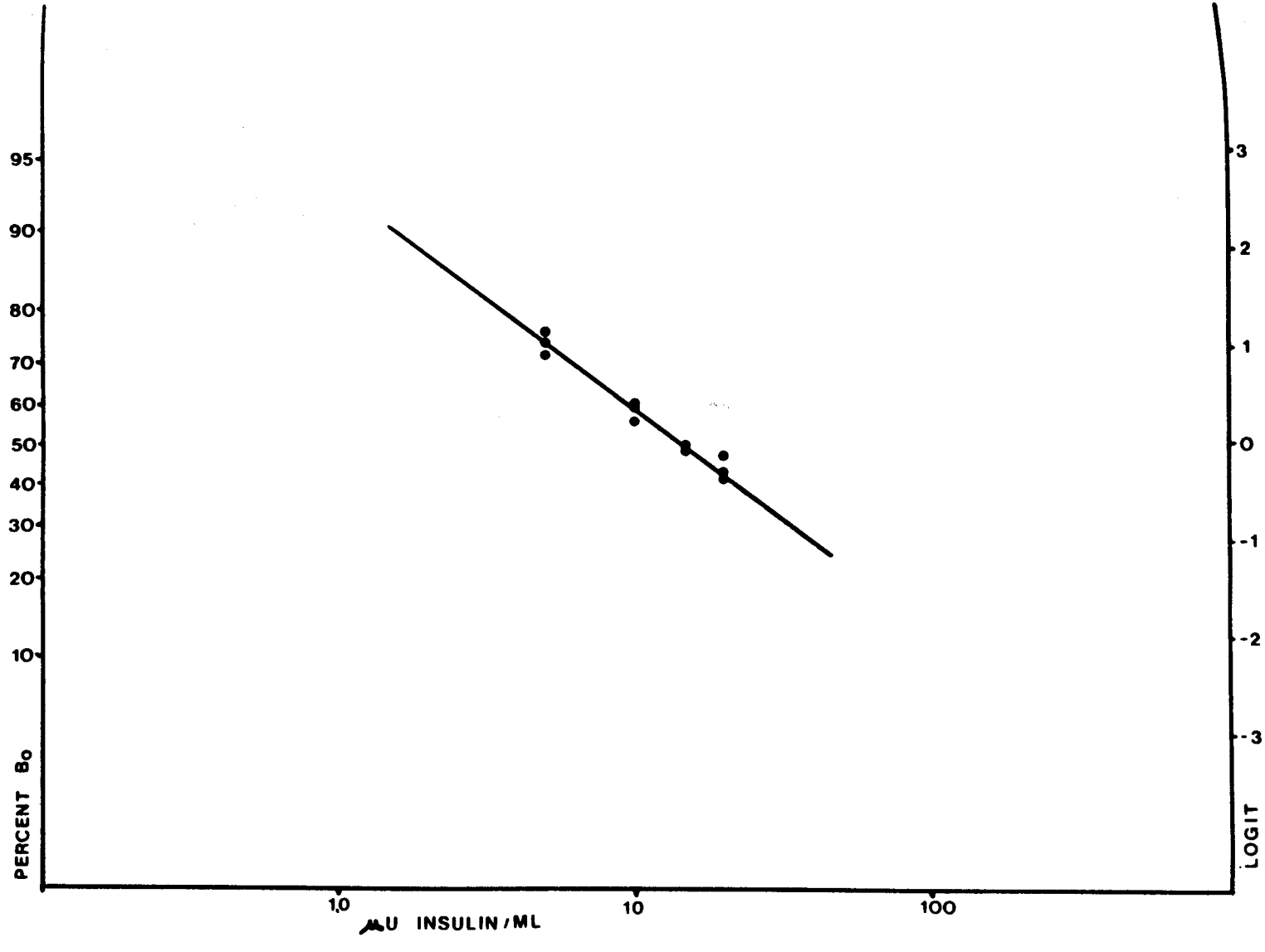

95
90
80
70
60
50
40
30
20
10
PERCENT B_0
3
2
1
0
-1
-2
-3
LOGIT
1.0
10
100
μU INSULIN / ML

TABLE VII. DEXTRAN-CHARCOAL INSULIN RIA: INTERASSAY VARIANCE

	Logit-Log Parameters	
	Slope	50% Intercept (μU/ml)
Number	9	9
Mean	-2.43	13.4
Standard Deviation	0.21	0.9
Coefficient of Variation (%)	8.8	7.0

	Serum Controls (μU/ml)	
	Normal Pool	High Serum
Number	8	6
Mean	42.2	116
Standard Deviation	4.3	10
Coefficient of Variation (%)	10.1	8.9

95% confidence range of 0-16.6 μU/ml, close to the observed range of 0-15.4 μU/ml. This RIA also yielded values for the Phadebus Reference Serum within the range found by the Phadebus insulin radioimmunoassay and for sera provided by the College of American Pathologists within the range found by other laboratories using their own radioimmunoassays, and showed expected fluctuations in human insulin levels in response to a glucose load or tolbutamide administration in both normal individuals and maturity-onset diabetics.

EFFECT OF HEPARIN ON THE DEXTRAN-CHARCOAL INSULIN RIA

Heparin was found to inhibit binding of ^{125}I-insulin to antibody at concentrations above 100 μg/ml in the assay tube. Although dose-response curves were parallel, 0.25 mg heparin/ml depressed %B 19% and 2.5 mg heparin/ml depressed %B 45%.

DOUBLE - ANTIBODY INSULIN RIA

Assay Characteristics

The range of variability of triplicate standards in the dose response curve (Fig. 18) indicates a useful range for this assay of 1-30 μU/ml with a resolution of 1 μU/ml at 5 μU/ml to 2 μU/ml at 30 μU/ml. The 90% intercept on the log-logit curve (Fig. 19), which is linear between 5 and 40 μ U/ml, indicates a sensitivity of 3.6 μU/ml. NSB for both buffer and sera was consistently less than 4%. Therefore, samples and standards were not corrected for NSB in this assay. B_0 varied between 50 and 72%.

FIGURE 18. DOUBLE-ANTIBODY INSULIN RIA: STANDARD CURVE.

- ● Insulin standards in albumin buffer
- O Serum D.N.-CPD plasma
- ▲ Serum D.N.-CPD plasma, diluted 10-fold with albumin buffer
- ■ Serum D.N.-CPD plasma, diluted 20-fold with albumin buffer

Insulin concentration of D.N. serum was determined by assay of serum diluted with albumin buffer.

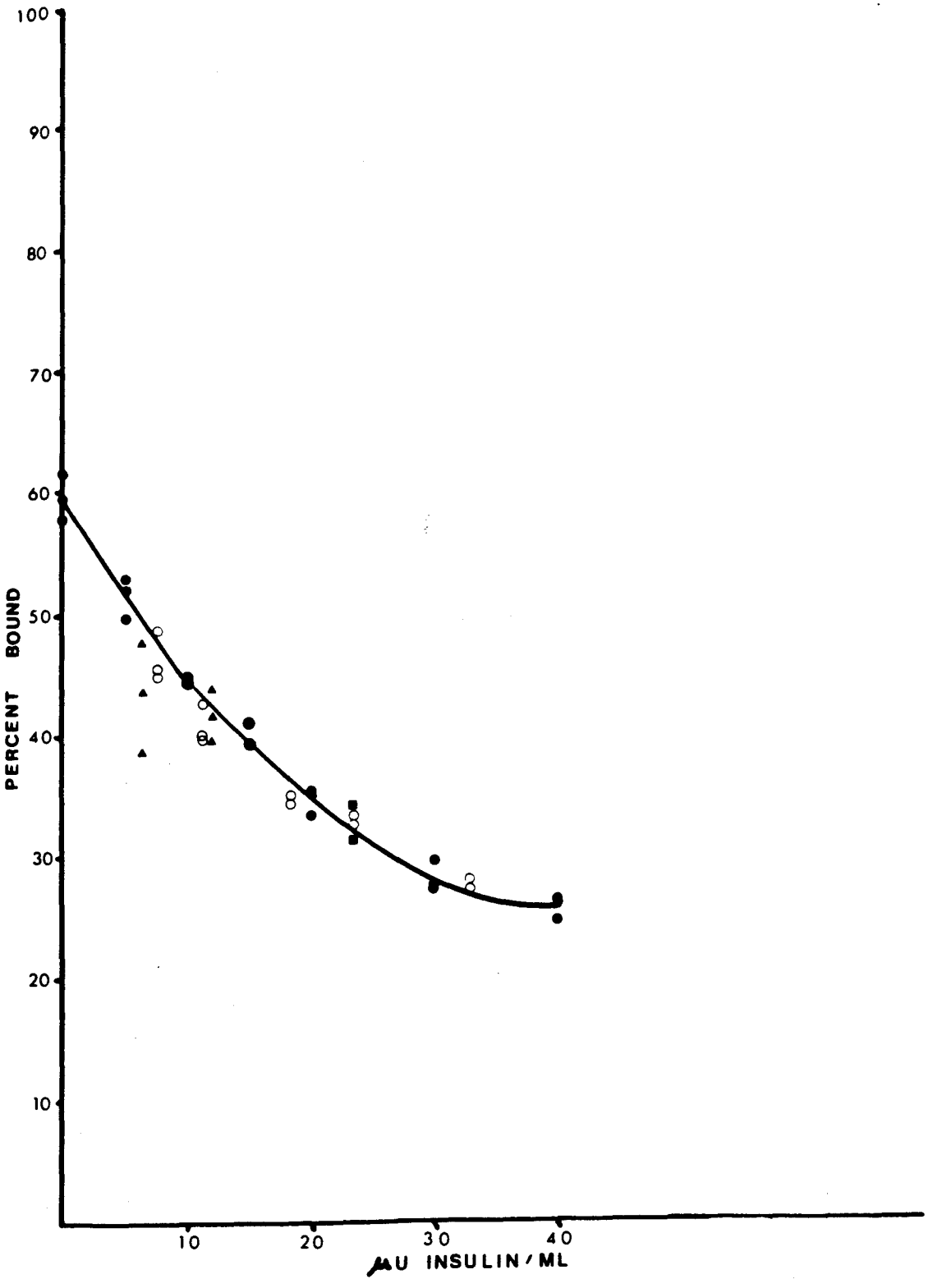

100
90
80
70
60
50
40
30
20
10
PERCENT BOUND
10
20
30
40
μU INSULIN / ML

FIGURE 19. DOUBLE-ANTIBODY INSULIN RIA STANDARD CURVE: LOGIT-LOG TRANSFORM.

Range of insulin standards = 5-40 μU/ml.

Insulin quantity in assay tube is one-tenth of insulin standard concentration.

LOGIT
3
2
1
0
-1
-2
-3
PERCENT B_0
95
90
80
70
60
50
40
30
20
10
5
1.0
10
100
μU INSULIN / ML

When a patient sample showing a high concentration of insulin (D.N., 496 μU/ml) was serially diluted with CPD plasma low in insulin, displacement of radiolabeled insulin was nearly superimposible on the standard curve (Fig. 18) over several dilutions. Recovery of exogenous human insulin standard added to CPD plasma was essentially 100% from about 10 μ U/ml to 300 μ U/ml (Table VIII). High assay values at low concentrations indicate an actual sensitivity and resolution of about 4 μU/ml.

Quality Control

Intraassay variation was determined by assaying 15 replicates of three human serum and plasma samples, ranging from about 7 to 500 U insulin/ml, in one assay. Coefficients of variation for samples exceeded 10% only at low insulin concentrations (Table IX). Interassay variation of curve parameters was less than 15%, but exceeded 15% for serum controls. The 23% CV for N_1 underscores the inaccuracy of the assay for insulin levels less than 10 μ U/ml.

Patient Samples

Fifty patient samples, including four oral glucose tolerance tests, showing normal glucose levels were obtained from Dr. Marion Brooks. The distribution of insulin concentrations determined for 27 fasting samples among these is skewed to the right with a median of 4.5 μU/ml. Although the arithmetic mean is 8.8 μU/ml, treatment of the population as a normal distribution yields a 95% confidence range of 0-19.4 μU/ml (or approximately 0-20 μU/ml), rather close to the observed range of 2.3-18.2 μU/ml.

Insulin concentrations assayed for the glucose tolerance tests show

TABLE VIII. RECOVERY OF HUMAN INSULIN STANDARD ADDED TO CPD PLASMA BY DOUBLE-ANTIBODY RIA

Insulin Added (μU/ml)	Percent Recovered	± Standard Deviation
303	107	10.6
129	130	19.7
80.8	106	2.5
35.8	105	16.8
20.8	100	20.7
6.3	163	11.2
2.8	236	94.8

CPD = citrate-phosphate-dextrose

Each recovery determination is based on triplicate samples.

$$\text{Percent recovered} = \frac{\text{Total } \mu U/ml - \text{plasma } \mu U/ml}{\text{Insulin added } (\mu U/ml)} \times 100.$$

TABLE IX. DOUBLE-ANTIBODY INSULIN RIA QUALITY CONTROL

	Number	Mean	Std. Dev.	Coeff. Var. (percent)
Intraassay Variation				
N_1 (μU/ml)	15	7.38	0.90	12.2
N_{10} (μU/ml)	15	124	10	8.1
D.N. (μU/ml)	15	496	29	5.8
Interassay Variation				
Slope	22	-2.59	0.31	12.0
50% intercept (μU/ml)	22	27.5	2.5	9.1
N_1 (μU/ml)	20	6.4	1.5	23.4
N_{10} (μU/ml)	19	121	22	18.2

TABLE X. SERUM AND PLASMA IRI AND RECOVERY OF EXOGENOUS INSULIN BY RIA: AVERAGE OF TRIPLICATE DETERMINATIONS

Sample	IMMUNOREACTIVE INSULIN (μU/ml) (Recoveries)		
	No Additions	+ 100 μg/ml heparin	+ 10 μg/ml biotin
N	51.1	44.6	38.6
P	0.3	0.3	1.5
SS	13.8	12.4	10.2
SP	4.4	4.4	6.7
+ 69 μU/ml			
N	120 (99.9%)	114 (100.6%)	96.6 (84.1%)
P	72.3 (104.3%)	65.1 (93.9%)	58.9 (83.2%)
SS	85.6 (104.1%)	70.0 (83.5%)	63.1 (76.7%)
SP	73.3 (99.9%)	68.1 (92.3%)	54.4 (69.1%)

TABLE XI. SERUM AND PLASMA IRI AND RECOVERY OF EXOGENOUS INSULIN BY RIA: STATISTICAL ANALYSIS

UNTREATED SAMPLES

Sample (no. replicates)	IRI μU/ml ±S.D. (S.E.M.)	% Recovery ±S.D. (S.E.M.) + 104 μU	Comparison with % Recovery of N
N (12)	42.2±4.0 (1.2)	---	---
P (12)	0.1±0.3 (0.1)	---	---
SP (12)	3.0±0.7 (0.2)	---	---
SS (12)	21.2±5.1 (1.5)	---	---
N 100 (12)	132 ±17 (4.7)	86.5±16.3 (4.5)	---
P 100 (12)	74.3±7.2 (2.1)	71.3 ±6.9 (2.0)	significantly lower ($P<0.01$)
SS 100 (12)	93.2±7.6 (2.2)	69.2 ±7.3 (2.1)	significantly lower ($P<0.005$)
SP 100 (12)	79.9±8.9 (2.6)	73.9 ±8.6 (2.5)	significantly lower ($P<0.05$)

+ 10 μg/ML BIOTIN

Sample	IRI	% Recovery +99.8 μU	Comparison with IRI of untreated samples	Comp. with % Recovery of N	Comp. with % Rec Untr. Smpls
N (12)	41.7 ±3.9 (1.1)	---	no sign. diff. ($P>0.5$)	---	---

TABLE XI. (CONTINUED)

Sample	IRI	% Recovery + 99.8 µU	Comparison with IRI of untreated samples	Comp. with % Recovery of N	Comp. with % Rec. Untr. Smpls.
P (12)	0	---	no sign. diff. (P>0.2)	---	---
SP (12)	2.2 ±0.3 (0.1)	---	sign. lower (P<0.005)	---	---
SS (12)	15.4 ±2.1 (0.6)	---	sign. lower (P<0.005)	---	---
N 100 (12)	113 ±8 (2.4)	71 ± 8 (2.4)	---	---	sign. lower (P<0.01)
P 100 (12)	66.5 ±10.0 (2.9)	66.6±10.0 (2.9)	---	no sign. diff. (P>0.20)	no sign. diff. (P>0.1)
SP 100 (12)	75.2 ±5.9 (1.7)	73.1±8.6 (2.5)	---	no sign. diff. (P>0.4)	no sign. diff. (P>0.5)
SS 100 (12)	82.1 ±11.6 (3.3)	62.9±11.6 (3.3)	---	no sign. diff. (P>0.05)	no sign. diff. (P>0.1)

expected fluctuations and a comparison of insulin values of the 50 samples from this assay and from Dr. Brooks' clinical endocrinology laboratory (dextran-charcoal RIA) by the t-test for paired data shows no significant difference ($t = 0.207$; $P > 0.5$).

CATION-EXCHANGE CHROMATOGRAPHY OF HUMAN PLASMA

Chromatography of 450 ml CPD plasma on Sephadex C-50 was undertaken in order to determine the reproducibility of Guenther's observed heparin-potentiation of IRI in cationic serum fractions. About 10 μU IRI/ml plasma was adsorbed by the cation exchanger and 43% of this IRI was eluted in the most cationic fraction (Fraction XII), but heparin did not enhance IRI in this fraction or overall (Fig. 20). Heparin increased IRI 0.2- to 3-fold in Fractions I, VII, X and XI, but heparin-induced IRI in no case exceeded 1 μU/ml plasma. No significant heparin-potentiation of IRI was found in any lyophilized, reconstituted fraction. The fact that 4.8 mg protein/ml plasma was recovered from the gel suggests that most of the IgG was not eluted.

ENDOGENOUS IRI AND EXOGENOUS INSULIN RECOVERY FROM SERUM AND PLASMA

Since it was possible that insulin could be dissociated from a putative binding protein in CPD plasma and thus not be adsorbed by the cation exchanger, IRI in various serum and plasma samples was determined. Furthermore, reversible binding of insulin to a protein should be capable of detection by RIA as anomalous recovery of exogenous insulin from serum and plasma.

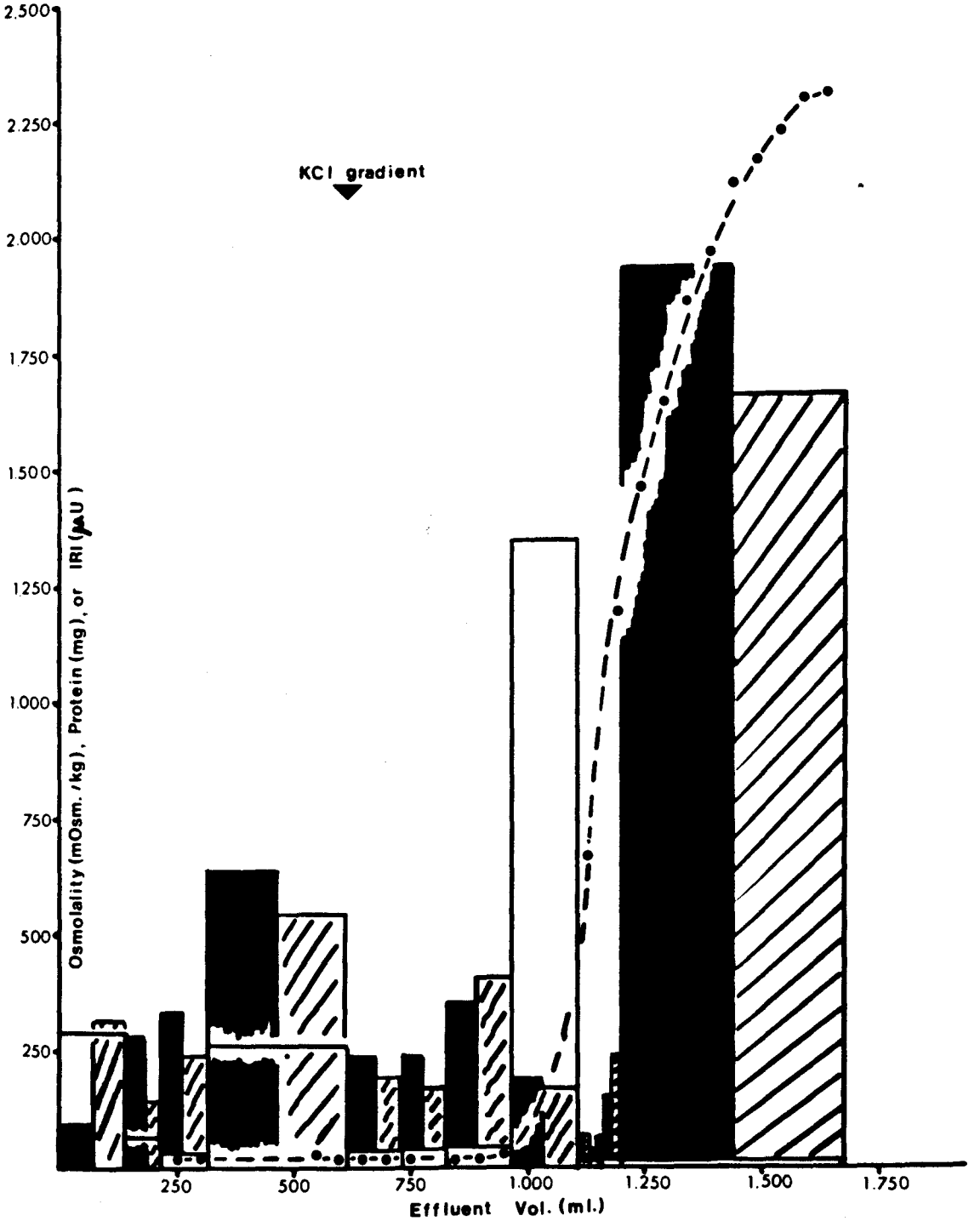
FIGURE 20. SEPHADEX C-50 CHROMATOGRAPHY OF HUMAN PLASMA.

●--● Effluent osmolality

Open bars: Protein

Black bars: IRI without heparin

Hatched bars: IRI with heparin


2.500
2.250
2.000
1.750
1.500
1.250
1.000
750
500
250
KCl gradient
Osmolality (mOsm./kg), Protein (mg), or IRI (μU)
250
500
750
1.000
1.250
1.500
1.750
Effluent Vol. (ml.)

Serum and plasma were also treated with an anion exchange resin (Amberlite IRA 400; "stripped" serum, SS, and "stripped" plasma, SP). If free insulin-binding protein exists in serum or plasma or anion stripping generates free binding protein, then recovery of IRI from such samples should be low. If heparin or biotin dissociates insulin from a binding protein, an enhancement of IRI should be observed.

It is evident from Table X that heparin caused no significant change in the IRI of any sample studied; 10 μg D-biotin/ml caused slight increases of IRI in plasma and stripped plasma and a decrease of IRI in normal serum. However, appreciably low recoveries of IRI were seen in heparin-treated stripped serum and all biotin-treated samples, but recoveries from untreated samples were complete. Statistical analysis of IRI in replicate untreated and biotin-treated samples (Table XI) revealed that recovery of exogenous insulin from CPD plasma, stripped serum and stripped plasma was significantly lower than from normal serum, representing a loss of approximately 30 μU added insulin/ml sample. Biotin treatment caused a small but significant decrement of IRI in stripped serum and plasma and reduced recovery of IRI from normal serum to that seen in other samples, but did not further reduce recoveries from plasma, stripped serum or stripped plasma.

GEL CHROMATOGRAPHY OF RADIOLABELED INSULIN IN HUMAN SERUM AND PLASMA

Since diminished recovery of exogenous IRI could also be caused by enhancement of serum or plasma proteolytic activity, a systematic examination of human serum and plasma for an insulin-binding protein using the methodology

employed by Zapf et al. (557) to elucidate the NSILA-S-binding protein was undertaken.

Purification of ^{125}I-insulin + 5% BSA using Sephadex G-50 resulted in elution of no more than 17% radioactivity in the albumin fraction and 5% in the iodide fraction. G-200 calibration curves, depicted in Figure 21, indicate that significant differences in the elution behavior of proteins can occur with different columns.

Despite differences in protein elution patterns of serum and plasma, the qualitative profile of ^{125}I-insulin elution is very consistent (Fig. 22). About 90% of the radioactivity (peak B) eluted at volumes consistent with globular proteins of molecular weight 26,500-39,000 daltons (Tables XII, XIV, XV), indicative of tetramer-hexamer aggregation. This possibility is supported by elution of the final serum protein peak (V) appreciably after peak B, which eluted at a position between BSA and chymotrypsinogen A, while plasma peak B eluted coincident with the final protein peak (IV), at the position of chymotrypsinogen A. Less than 5% of the radioactivity (peak A) eluted coincident with protein peak III, corresponding to molecular weight 117,000-158,000 daltons. Less than 1% of the radioactivity (peak C) in plasma eluted at the exclusion volume (M.W. >360,000 daltons). I-125 activity nonspecifically associated with eluted protein accounted for little more than 6% of recovered activity.

Approximately 90% of Fraction B radioactivity was lost between elution from the first column to dialysis and another 90% of that activity was lost by completion of dialysis. Losses are attributed to adherence of labeled insulin to glass. Essentially no Fraction A activity was lost, however, from elution to dialysis and approximately 75% was recovered after dialysis; thus, high

FIGURE 21. SEPHADEX G-200 CALIBRATION CURVES.

V_e/V_o = Elution volume (ml)/Exclusion volume (elution volume of blue dextran)

——— 2.6 x 90 cm columns:

● Initial chromatography

▲ Rechromatography of A_1 + B_1

■ Chromatography of stripped plasma and long incubated stripped plasma

2.5 x 21 cm column:

O--O Chromatography of A_2 and A_3-I

Standards: a. catalase

b. glucose oxidase

c. hexokinase

d. BSA

e. chymotrypsin A

f. ribonuclease A

g. insulin

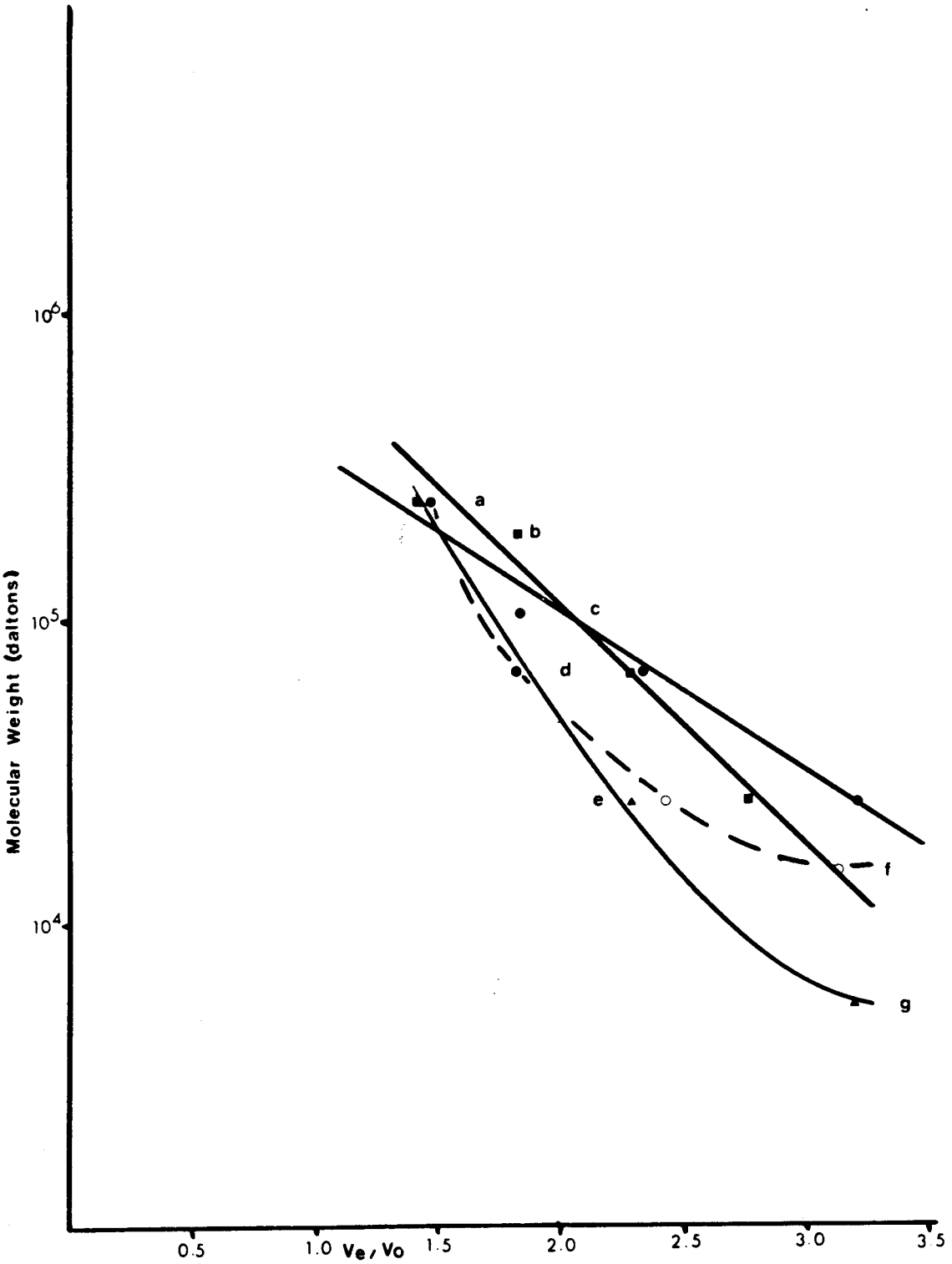

Molecular Weight (daltons)
10^6
10^5
10^4
a
b
c
d
e
f
g
0.5
1.0
Ve / Vo
1.5
2.0
2.5
3.0
3.5

FIGURE 22. SEPHADEX G-200 CHROMATOGRAPHY OF EXOGENOUS ^{125}I-INSULIN IN HUMAN SERUM AND PLASMA.

Open peaks: protein, designated by roman numerals.

Shaded peaks: radioactivity, designated by letters.

BD	blue dextran
cat	catalase
GO	glucose oxidase
hex	hexokinase
BSA	bovine serum albumin
chnA	chymotrypsinogen A
V_e	elution volume (ml)

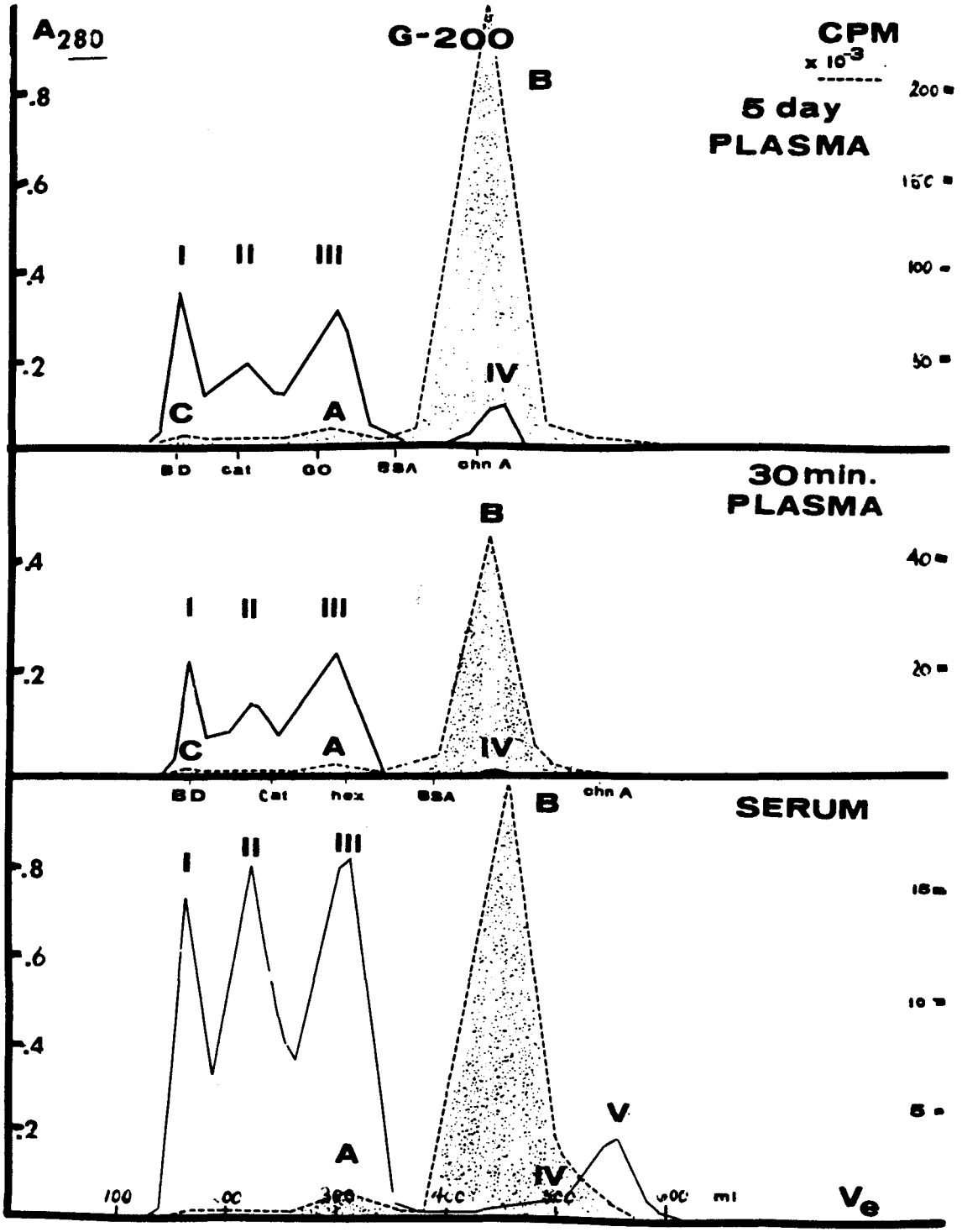

A280
G-200
CPM
x 10-3
5 day
PLASMA
B
I
II
III
IV
C
A
.8
.6
.4
.2
200
150
100
50
BD
cat
GO
BSA
ohn A
30 min.
PLASMA
40
20
hex
SERUM
V
16
10
5
100
200
300
400
500
ml
Ve

TABLE XII. SEPHADEX G-200 CHROMATOGRAPHY OF ^{125}I-INSULIN IN NORMAL HUMAN SERUM

Fraction	V_e (ml)	V_e/V_o	Molecular Weight (daltons)
CONTROL:			
I	162	1.0	>360,000
II	222	1.370	232,000
III	314	1.938	115,000
IV	500	3.086	28,000
V	555	3.426	18,200
A	312	1.926	117,000
B	456	2.814	39,000
BIOTIN:			
I	135	1.0	>360,000
II	190	1.407	220,000
III	260	1.925	117,000
IV	420	3.111	27,000
V	455	3.370	19,800
A	270	2.000	106,000
B	390	2.888	35,500

TABLE XIII. RECHROMATOGRAPHY OF G-200 ELUTION FRACTIONS

Fraction	V_e (ml)	V_e/V_o	Molecular Weight (daltons)
A + B Pool:			
I (IIIa)	142	0.861	>360,000
II (IIIb)	210	1.273	350,000
III (IIIc)	300	1.818	82,000
IV	432	2.618	<20,000
A	306	1.855	74,000
B	422	2.558	<20,000
A Control (A_2):			
I	63	1.848	62,000
II	106	3.109	<20,000
A	59	1.730	80,000
Insulin (A_3-I):			
I	68	1.994	47,500
II	109	3.196	20,000
A	67	1.965	49,500

TABLE XIV. SEPHADEX G-200 CHROMATOGRAPHY OF ^{125}I-INSULIN IN STRIPPED PLASMA (SP)

PROTEIN PEAKS

	Fraction	V_e (ml)	V_e/V_o	Molecular Weight	Protein (mg)
Control					
	I	162	1	>360,000	17.9
	II	222	1.370	365,000	100
	III	298	1.840	149,000	257
	IV	444	2.741	26,000	--
+ Cold Insulin					
	I	161	1	>360,000	34.7
	II	220	1.366	370,000	91.8
	III	294	1.826	151,000	235
	IV	440	2.733	26,500	--

RADIOACTIVE PEAKS

	Fraction	Ve (ml)	V_e/V_o	M.W.	Percent Recover. Act.	Insulin equiv. (μU)
Control						
	A	292	1.802	158,000	1.3	2.5
	B	440	2.716	27,500	89.9	177
	C	166	1.025	>360,000	0.3	0.5

TABLE XIV. (CONTINUED)

Fraction	V_e (ml)	V_e/V_o	M.W.	Percent Recover. Act.	Insulin equiv. (μU)
+ Cold Insulin					
A	294	1.826	151,000	1.5	3.0
B	432	2.683	29,000	91.1	188
C	166	1.031	>360,000	0.2	0.5

TABLE XV. SEPHADEX G-200 CHROMATOGRAPHY OF CPD PLASMA/ ^{125}I-INSULIN AFTER FIVE DAYS INCUBATION AT 4° C

PROTEIN PEAKS

Fraction	V_e (ml)	V_e/V_o	Molecular Weight (daltons)	Protein (mg)
I	159	1	>360,000	63.3
II	217	1.365	365,000	138
III	297	1.868	140,000	336
IV	446	2.805	22,800	--

RADIOACTIVE PEAKS

Fraction	V_e (ml)	V_e/V_o	M.W.	Percent recov. act.	Insulin equiv. (μU)
A	294	1.849	147,000	1.7	11.1
B	434	2.730	26,500	91.6	604
C	162	1.019	>360,000	0.3	2.2

molecular weight-associated I-125 activity is stable to dialysis. The elution profile (Fig. 22) resulting from G-200 chromatography of CPD plasma/^{125}I-insulin after a 5-day incubation at 4°C shows the effect of proteolysis during the extended incubation as an enlarged protein peak IV. Yet, the profile of radioactivity remains essentially the same as found for ^{125}I-insulin in stripped plasma after 30 minutes incubation.

The ^{125}I-insulin/stripped plasma (SP) G-200 B-fraction, which contained approximately 35% BSA (added after chromatography to prevent losses of radiolabeled insulin by adherence to glass or dialysis tubing) when applied to a 50-ml G-50 column, yielded elution of nearly half the activity around the exclusion volume, indicating association of ^{125}I-peptides with BSA. Decreased ability of this "damaged" labeled insulin to bind to AIS is demonstrated by incubation of elution fractions with AIS in the absence of unlabeled insulin (B_o) in the RIA (Fig. 23). Material eluted in the second peak showed a B_o characteristic of purified ^{125}I-insulin (trace). No downfield ^{125}I- peak was found.

Displacement of this second peak activity and trace by increasing concentrations of unlabeled insulin was parallel in linear RIA dose-response curves (Fig. 24). Dose-response behavior of second peak material was nearly identical to that of trace in logit-log plots, including values for 50% B_o x-intercept and slope. Thus, despite elution of B-peak activity from G-200 in a supramonomeric position, rechromatography of this fraction yielded material immunochemically identical to ^{125}I-insulin.

Rechromatography of pooled serum A + B peaks and the A peak (Table XIII) resulted in a shift of both peaks and their coeluting protein peaks to lower

FIGURE 23. SEPHADEX G-50 CHROMATOGRAPHY OF FRACTION B (L-B).

The final peak of radioactivity (B-peak) eluting from a Sephadex G-200 column after application of a plasma/^{125}I-insulin mixture incubated for 5 days at 4^{o} C was rechromatographed on a Sephadex G-50 column.

Double-antibody radioimmunoassay:

% NSB = % total radioactivity in "bound" fraction (guinea pig serum-antiguinea pig serum precipitate) in the absence of anti-insulin serum (AIS) and unlabeled insulin.

B_o = % radioactivity specifically bound to AIS in the absence of unlabeled insulin:

$$\% B = \frac{\text{CPM in bound fraction - NSB CPM}}{\text{Total CPM - NSB CPM}}$$

V_e = elution volume (ml)

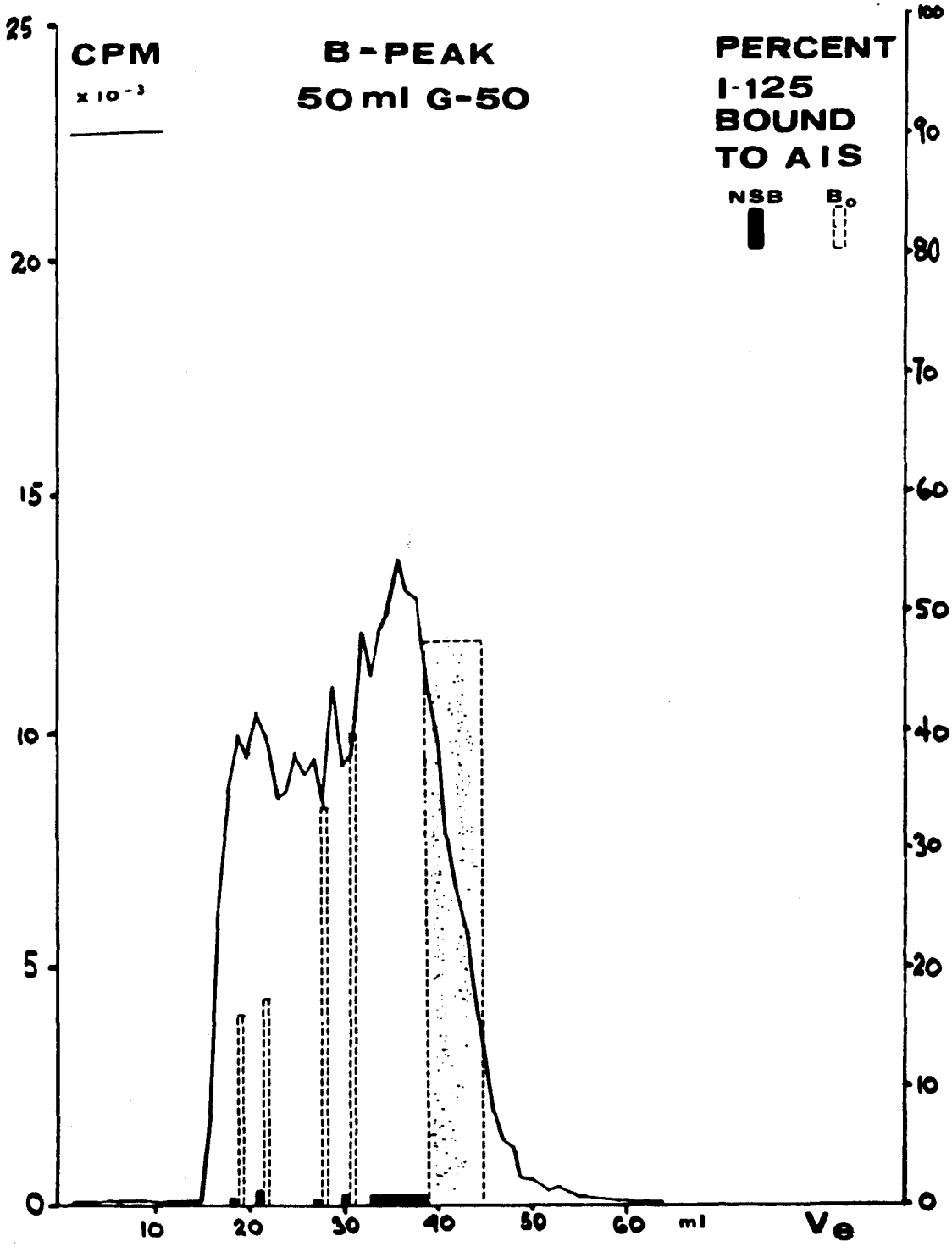

CPM
x 10⁻³
B-PEAK
50 ml G-50
PERCENT
I-125
BOUND
TO AIS
NSB
B₀
25
20
15
10
5
0
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
ml
Ve

FIGURE 24. DOUBLE-ANTIBODY RADIOIMMUNOASSAY OF FRACTION B.

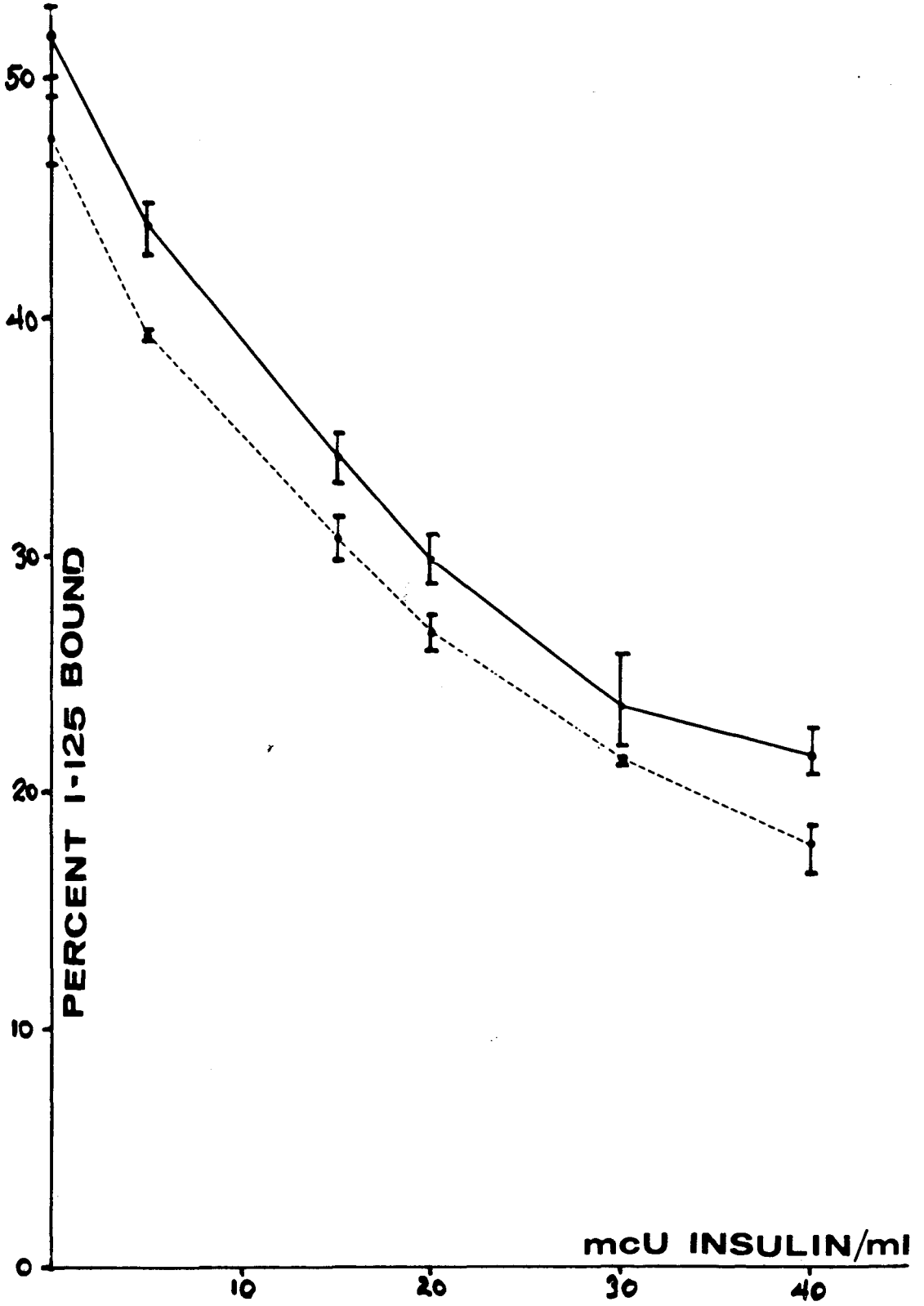
Solid line: ^{125}I-insulin

Broken line: Fraction B (L-B)

Bars: Range of triplicates

The second peak of radioactivity eluting from the Sephadex G-50 column was examined in the RIA for ability of unlabeled insulin to displace radioactivity from AIS.

Actual quantity of unlabeled insulin in the assay tube was one-tenth the concentration added (abscissa).


50
40
30
20
10
0
PERCENT I-125 BOUND
mcU INSULIN/ml
10
20
30
40

molecular weights (B <20,000, A= 74,000-80,000 daltons). Treatment of ^{125}I-insulin added to the serum A-peak (A_3-I), to serum or to plasma with biotin (Table XII) or excess unlabeled insulin (Tables XIII and XIV) resulted in no change in the elution pattern of radioactivity compared to controls and no shift of A-peak radioactivity to positions consistent with free insulin, although chromatography of A_3-I continued the trend of elution of both radioactivity and protein at higher elution volumes with successive rechromatography.

DEXTRAN-COATED CHARCOAL ANALYSIS OF FRACTION A

The data in Table XVI indicates that antibody-antigen reactions, represented by AIS-^{125}I-insulin, are a good model for study of reversible protein associations using dextran-coated charcoal. Only 8% of ^{125}I-insulin activity was not adsorbed to dextran-coated charcoal, while nearly 84% activity was transferred to this supernatant "bound" fraction in the presence of 1:10,000 AIS. Antibody binding of labeled insulin activity is nearly completely reversed by 0.1 mU unlabeled insulin, indicating competition for antibody binding sites by an immunochemically identical substance. Adsorption of more than 90% labeled insulin to dextran-coated charcoal is unaffected by 8 M urea, but half the antibody-bound insulin is dissociated under these conditions.

No dissociation of Fraction A activity is produced by these criteria by excess unlabeled insulin or 8 M urea, but a small fraction of "unbound" activity may have been transferred to the "bound" fraction by excess AIS.

TABLE XVI. ASSESSMENT OF PROTEIN-BOUND I-125 ACTIVITY USING DEXTRAN-COATED CHARCOAL

Preparation	"Bound" Fraction (spnt.)	"Free" Fraction (ppt.)	Percent "Bound" (± percent deviation*)
	CPM (± percent dev.*)		
^{125}I-insulin	393 (4.2)	4,517 (1.4)	8.0 (5.0)
^{125}I-insulin + AIS	4,064 (2.1)	787 (5.1)	83.8 (0.7)
^{125}I-insulin + AIS + cold ins.	797 (5.1)	4,073 (2.9)	16.4 (6.7)
^{125}I-insulin in 8 M urea	432 (7.2)	4,474 (1.4)	8.8 (8.0)
^{125}I-insulin + AIS in 8M urea	2,054 (4.1)	2,912 (1.9)	41.4 (2.4)
Fraction A (5 day inc.)	674 (21.8)	471 (9.1)	58.8 (6.1)
A + cold insulin	735 (1.2)	369 (2.9)	66.6 (0.8)
A + AIS	763 (7.0)	275 (9.0)	73.5 (1.4)
A in 8M urea	697 (19.8)	383 (18.5)	64.5 (2.0)

* percent deviation = $\frac{\text{determination of greatest diff. from mean} - \text{mean}}{\text{mean}} \times 100$

Triplicate determinations

cold insulin = 100 μU unlabeled human insulin

IDENTITY OF HIGH MOLECULAR WEIGHT RADIOACTIVE FRACTIONS

Immunochemical Analysis of Serum Proteins

Ouchterlony immunodiffusion of ^{125}I-insulin-serum G-200 fractions against specific antisera revealed that strong HSA reactivity resides in Fraction A. Protein found in Fraction B is due almost entirely to BSA added to stabilize insulin. Immunodiffusion of ^{125}I-insulin-plasma (SP and L) fractions (Table XVII) demonstrated that maximum concentrations of HSA and BSA (added to stabilize tracer) were both found to reside in A-fractions (protein peak III) and most of the human IgG was found in protein peak II, with lesser amounts in radioactive peaks A and C.

SDS Polyacrylamide Disc Gel Electrophoresis of A-Fraction

R_f values for SDS-PAGE bands were determined from band migration/dye migration from both the stained gels (Fig. 25) and densitometer scans of the gels. A calibration curve (Fig. 26) was constructed by plotting the R_f of the calibration bands vs. log M.W. An unexpected sixth band was assigned to the 60,000 dalton subunit of catalase.

The apparently homogeneous G-200 protein peak III (A-peak) was found to consist of more than seven distinct proteins, ranging in molecular weight from 70,000 (probably albumin) to 240,000 daltons (Table XVIII). A-peak albumin was estimated to be 9.5% A_L protein from the area under the densitometer scan peaks. Thus, albumin comprised slightly more than 7 mg of the 75 mg recovered protein and 4.4 mg HSA remained after subtraction of the 2.74 mg total BSA retentate recovered.

TABLE XVII. IMMUNOREACTIVE PROTEIN CONTENT OF CPD PLASMA/ ^{125}I-INSULIN SEPHADEX G-200 CHROMATOGRAPHY FRACTIONS

Fraction	vs. anti-BSA	vs. anti-HSA	vs. anti-human IgG
Control			
C	-	-	+
II	+++	+++	++++
A	++++	++++	-
Insulin			
C	±	-	++
II	+++	+++	++++
A	++++	++++	++
Five day incubation			
C	+	-	++
II	+++	+++	++++
A	++++	++++	+

Results of Ouchterlony immunodiffusion analysis. Scoring is based on intensity of band and proximity to antibody well, which reflects relative concentration of antigen.

FIGURE 25. SDS POLYACRYLAMIDE DISC GEL ELECTROPHORESIS OF A-FRACTION.

A-fraction was obtained by Sephadex G-200 chromatography of stripped plasma + ^{125}I-insulin incubated for 5 days at 4^{o} C.

Gels were stained with 1% fast green and fixed in methanol/acetic acid/water (45:10:45).

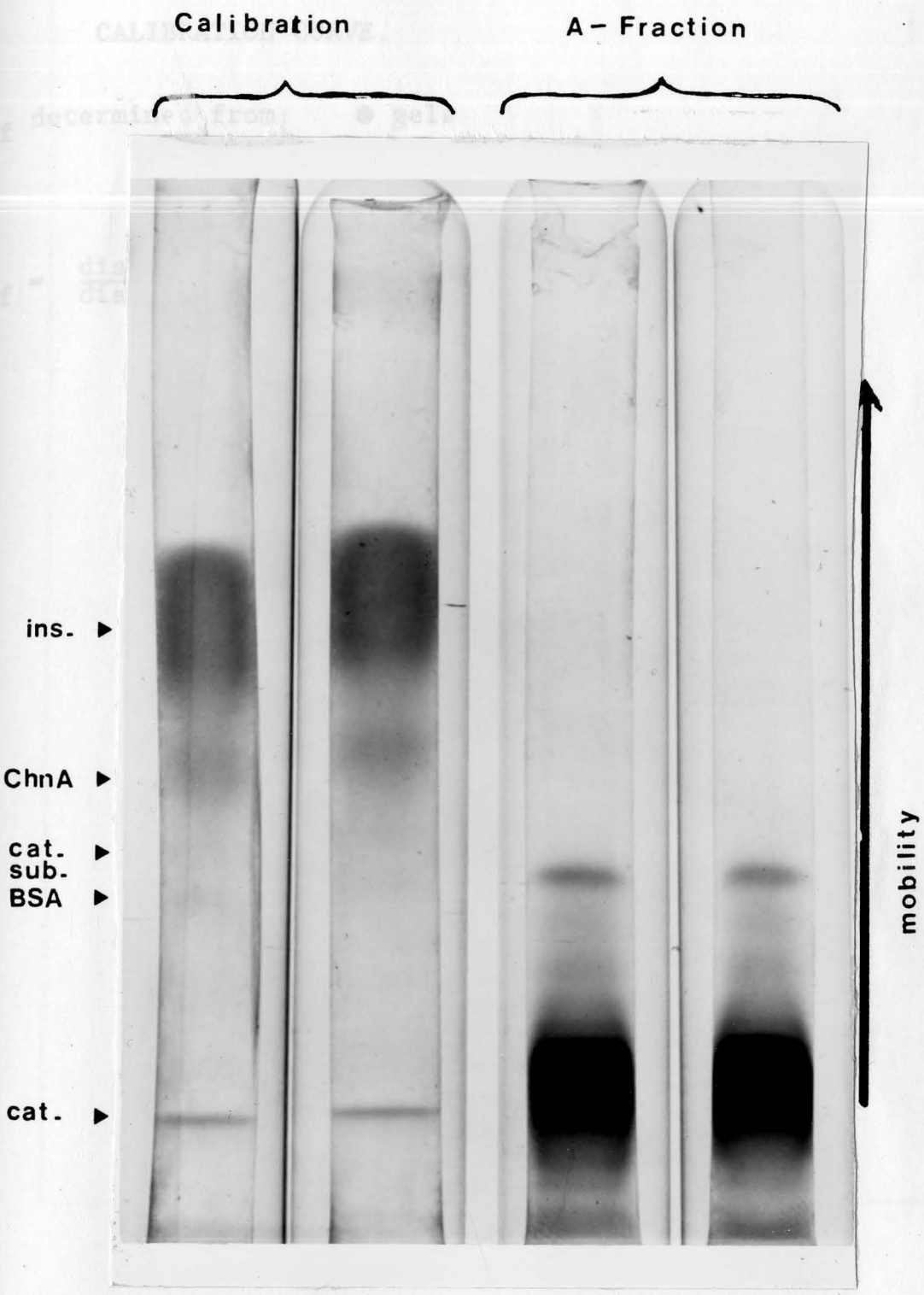

Calibration
A - Fraction
ins.
ChnA
cat.
sub.
BSA
cat.
mobility

FIGURE 26. SDS POLYACRYLAMIDE DISC GEL ELECTROPHORESIS CALIBRATION CURVE.

R_f determined from: ● gels

▲ densitometer scans

$$R_f = \frac{\text{distance of band from top of gel}}{\text{distance of bromphenol blue band from top of gel}}$$

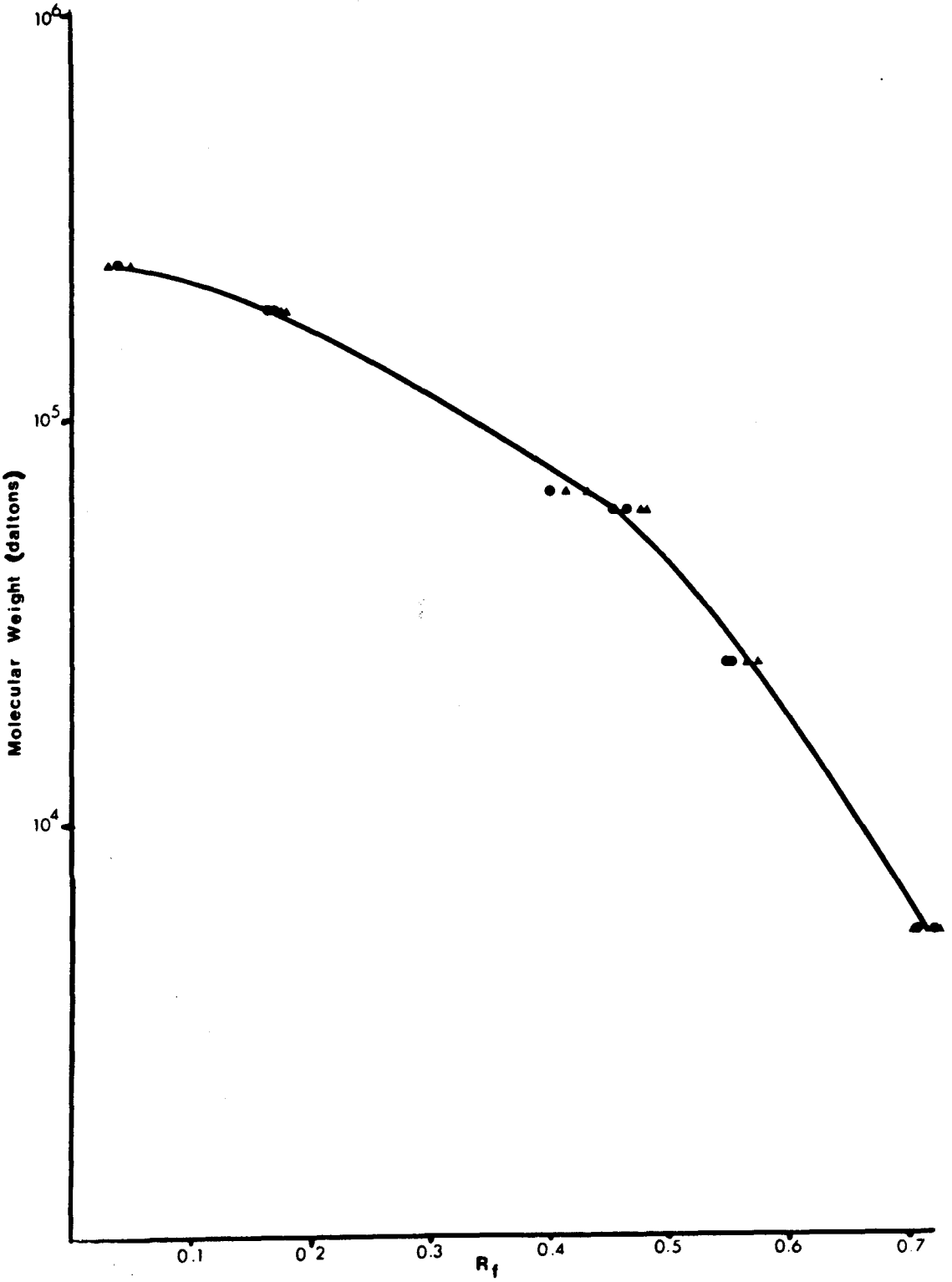

10^6
10^5
10^4
Molecular Weight (daltons)
0.1
0.2
0.3
0.4
0.5
0.6
0.7
R_f

TABLE XVIII. RESULTS OF SDS POLYACRYLAMIDE DISC GEL ELECTROPHORESIS

	Band	R_f	M.W. (daltons)
Calibration			
	1 (catalase)	0.039	240,000
	2 (gluc. ox.)	0.173	187,000
	3 (BSA)	0.418	67,000
	4 (catalase subunit)	0.471	60,000
	5 (chn A)	0.562	25,000
	6 (insulin)	0.717	5,500
Sample			
	1	0.030	243,000
	2	0.078	223,000
	3	0.191	172,000
	4	0.273	125,000
	5	0.315	106,000
	6	0.369	85,800
	7	0.414	71,700

Affinity Chromatography of the the A-Fraction

A fraction of 41.2% activity nonspecifically bound to protein was assigned from extrapolation of G-200 interpeak radioactivity. Thus, NSB activity for a given A_L protein fraction was calculated as A_L NSB activity (CPM) x percent total recovered A_L protein in the fraction. The results of affinity chromatography and ultrafiltration, summarized in Table XIX, show that about five-sixths of the activity in A_L was associated with BSA and HSA. The remaining activity exhibited a molecular weight less than 10,000 daltons and thus may correspond to the 20-27% radioactivity lost during dialysis of other A-Fraction preparations.

The 4% A-fraction radioactivity exhibiting a molecular weight between 2,000 and 10,000 daltons may correspond to the apparent 7% IRI found for A_L in previous dextran-charcoal binding studies, but this material did not behave like IRI in dextran-charcoal analysis (Table XX).

EFFECT OF INSULIN ON PROLIFERATION AND GLUCOSE UPTAKE BY CELLS IN CULTURE

WI38 fibroblasts exhibit strictly monolayer growth, elongated morphology and parallel orientation in confluent adherent cell culture (Fig. 27a). Cell number determined for WI38 in 4 replicate wells exhibited a variance less than 15% when in excess of 2,500 cells per well. WI38 fibroblasts in passage 15-20 plate with an efficiency of 45%. After an initial lag phase of about 18 hours, 5,000 cells in a 2 cm^2 cluster well enter log phase growth with a generation time of about 22 hours, until they attain confluency at about 10^5 cells (Figs. 28 and 29). The hepatoma line SK-HEP-1, with an efficiency of plating (EOP) of 36%,

TABLE XIX. PARTITION OF A-FRACTION ACTIVITY THROUGH AFFINITY CHROMATOGRAPHY

Species	mg protein	% recov. protein	CPM*	% recov. act.	Proj. NSB act. (CPM)	% A-peak act.
BSA	2.74	3.6	1,699	31.3	80.5	50.7
HSA	4.40	5.9	1,186	21.9	132	33.0
Other Protein	68.1	90.5	1,935	35.7	2,024	0
2,000-10,000 M.W.	1	--	126	2.3	--	3.9
<2,000 M.W.	0	0	359	6.6	0	11.3

* as of Feb. 7, 1977

TABLE XX. DEXTRAN-CHARCOAL ADSORPTION OF 2,000 - 10,000 DALTON MOLECULAR WEIGHT A - FRACTION

Preparation	Percent "Bound" ± S.D. (n = 4)
UM-2 Retentate	43.8 ± 4.5
UM-2 Retentate + 1:400,000 AIS	45.9 ± 4.1
UM-2 Retentate + 1:400,000 AIS + 40 μU insulin	40.4 ± 2.7

FIGURE 27. WI38 FIBROBLASTS.

a. Adherent cell culture; 300X magnification.

b. Detached cell, peroxidase-antiperoxidase stain for insulin; 350X magnification.

c. and d. Adherent cell culture in the presence of 6.9 mg human serum acetone powder Fraction 4/ml stationary medium; 300X magnification.

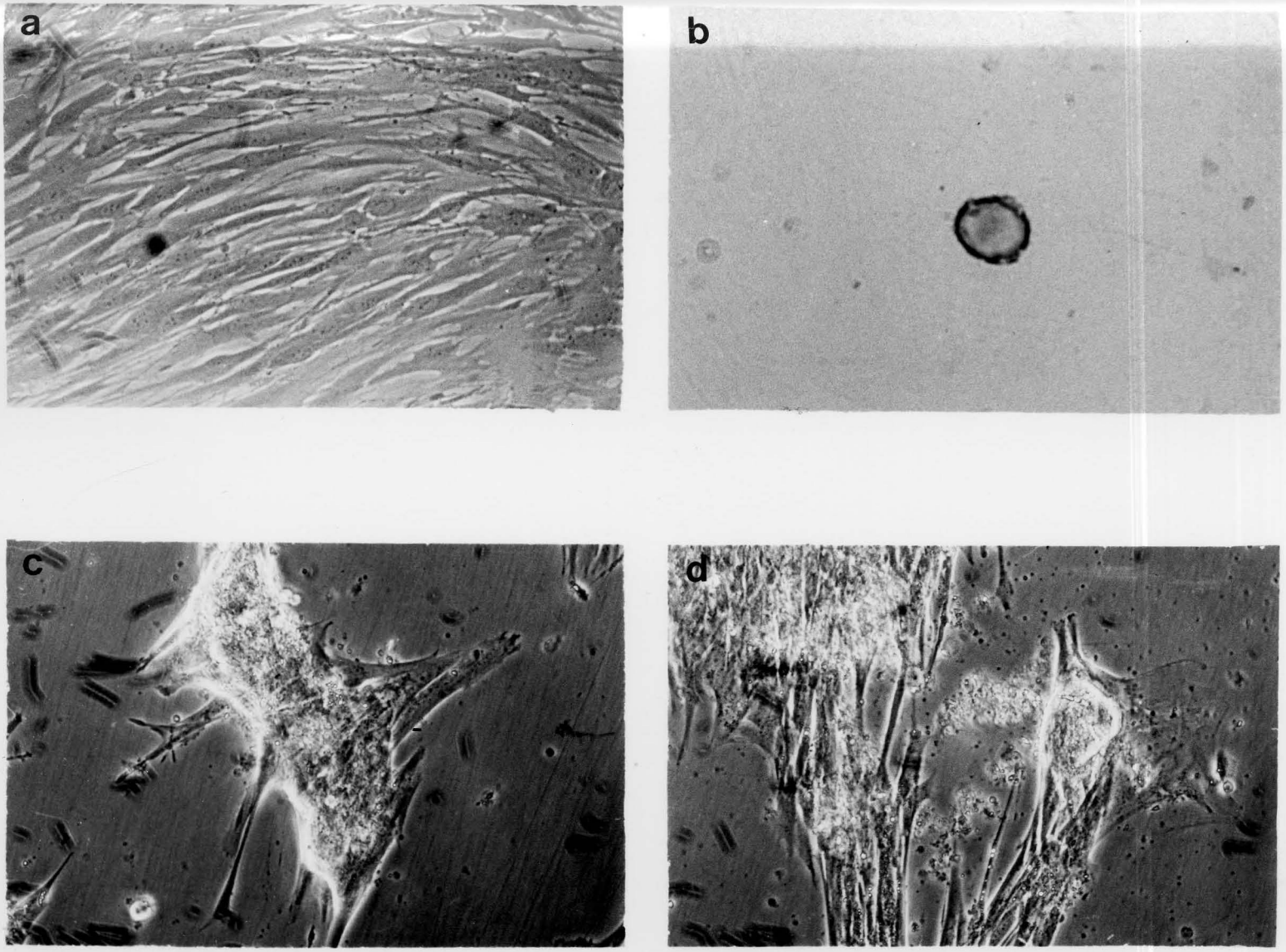

a
b
c
d

FIGURE 28. INSULIN EFFECTS ON WI38 CELL PROLIFERATION AND GLUCOSE UPTAKE IN BME WITH 10% FBS.

- ● BME CM, mean of 4 determinations
- ▲ 100 μU human insulin, average of duplicates
- ■ 10 mU bovine insulin, average of duplicates

Solid lines: cell number

Dashed lines: medium glucose

Bars: standard deviation

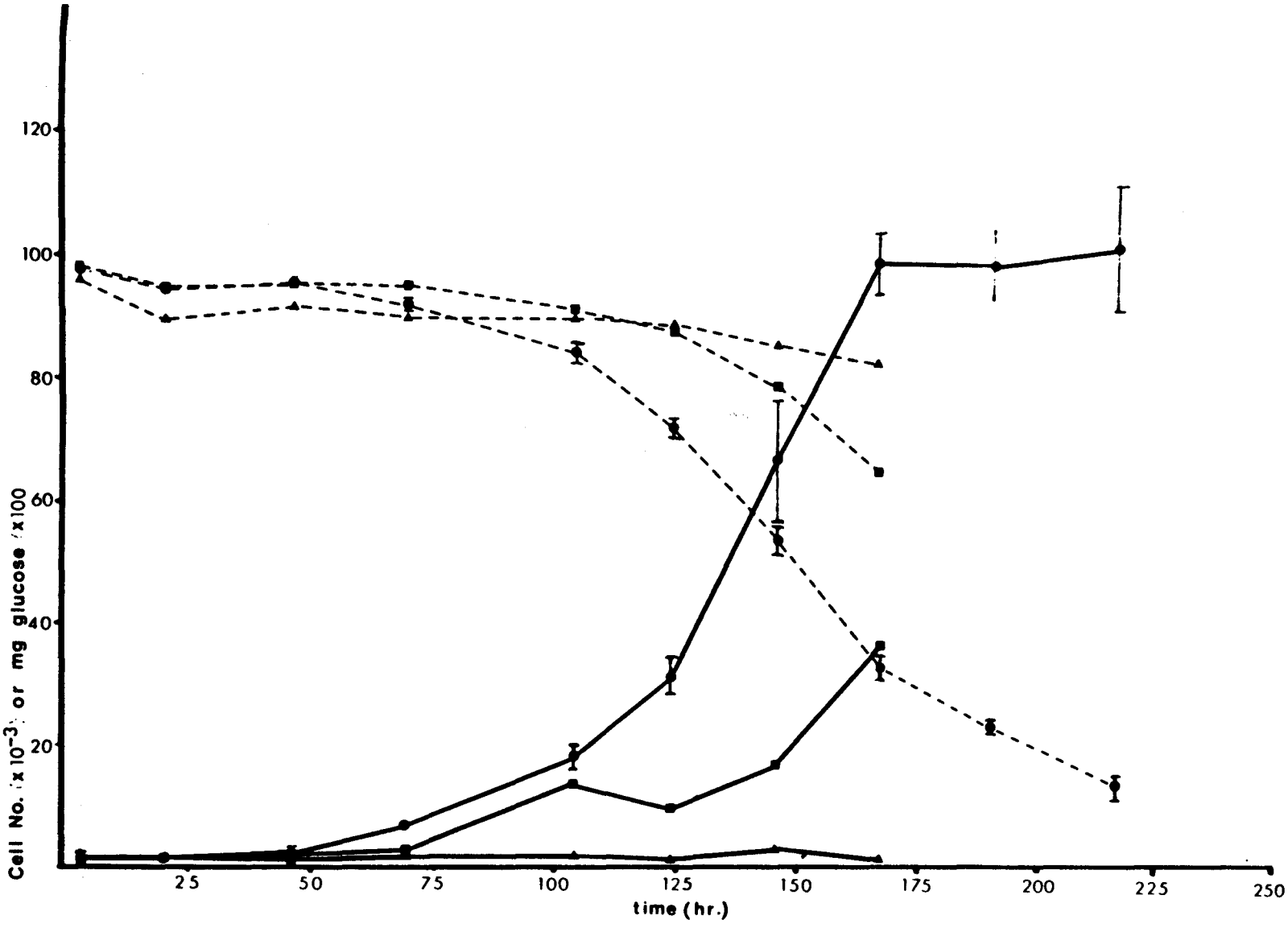

120
100
80
60
40
20
Cell No. (x 10⁻³) or mg glucose (x100
25
50
75
100
125
150
175
200
225
250
time (hr.)

FIGURE 29. INSULIN EFFECT ON WI38 CELL PROLIFERATION: SEMI-LOG PLOT.

Data of Figure 28.

- ● BME CM, time = 19.5-145.5 hours; n = 24, r = 0.990, t_D = 22.4 hours, t = 32.7, P<0.001.
- ▲ 100 μU human insulin, time = 3.5-167 hours; n = 16, r = 0.101, t_D = 665 hours, t = 0.381, P>0.1.
- ■ 10 mU bovine insulin, time = 3.5-167 hours; n = 16, r = 0.966, t_D = 31.9 hours, t = 13.9, P<0.001.

bars: standard deviation

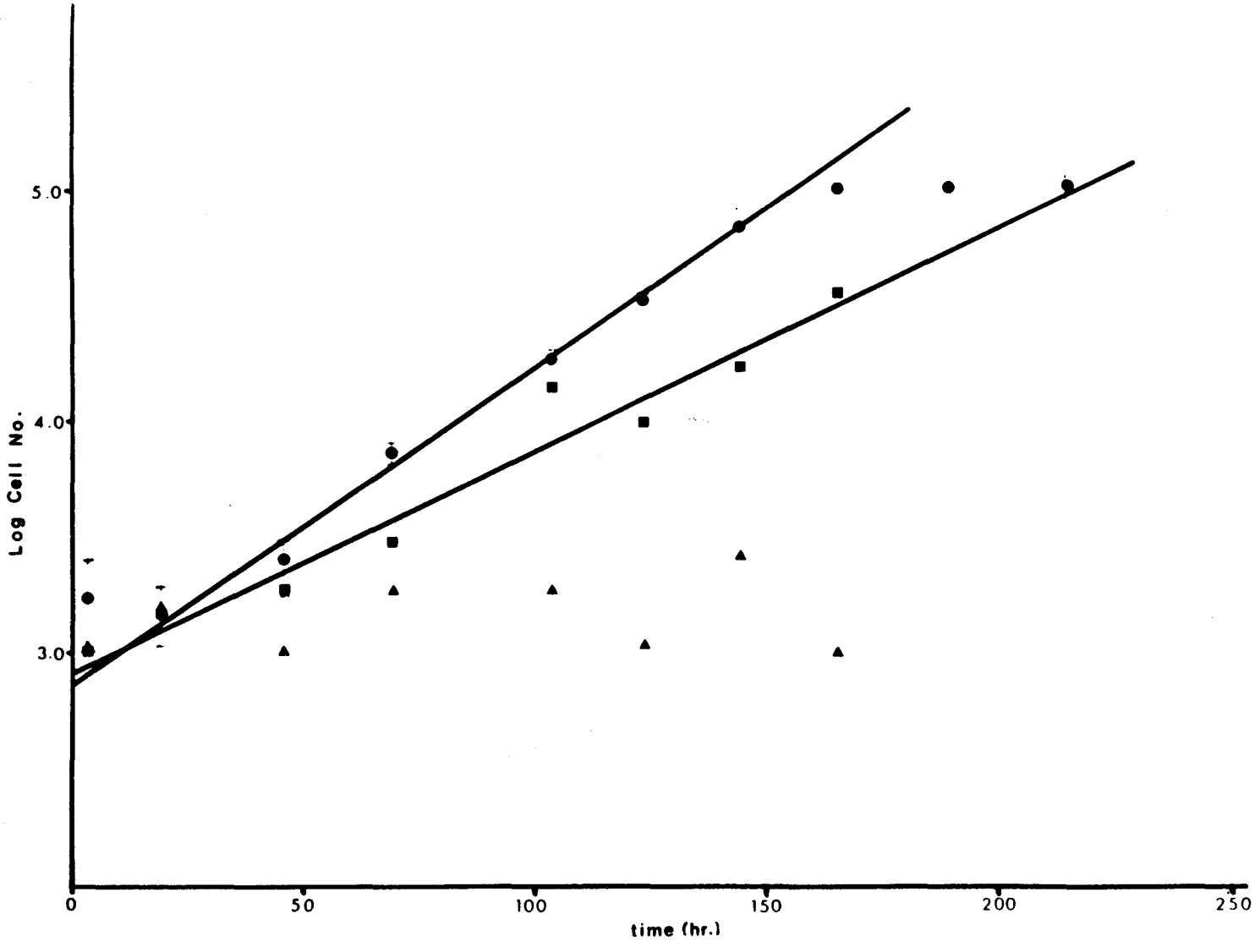
n = number of points

r = correlation coefficient

t_D = doubling time

t = Student's t-statistic (significance of slope difference from zero

P = probability that slope = 0


5.0
4.0
3.0
Log Cell No.
0
50
100
150
200
250
time (hr.)

exhibited a log-phase t_D of 26 hours, while the more differentiated squamous-cell carcinoma cell line, LT2, exhibited a 29% EOP and a t_D of about 61 hours (Table XXI). LT2 cells also attain confluence, while the less differentiated malignant cell lines SK-HEP-1, AU471 and BeWo did not attain confluence within 10 days.

Figure 30 indicates that the Beckman Glucose Analyzer exhibits a high degree of accuracy and reproducibility. Glucose consumption by WI38 fibroblasts increased proportionately with cell growth (Fig. 28) at an overall rate of -7.7 mg/10^6 cells/day (Table XXI) and slowed markedly to -0.9 mg/10^6·day at confluence. However, when 12,500 WI38 cells were plated in each well, log-phase r was -4.00 mg/10^6·day, although the t_D, 21.1 hours, was very similar to that found when 5,000 cells were plated per well. Furthermore, when glucose uptake was measured after 6.2 x 10^4 cells were allowed to attain confluence over a period of two days, the rate of glucose consumption was found to be only slightly below log phase uptake: -3.2 mg/10^6 cells/day. Log-phase r for other cell lines ranged from -3.5 to -8.6 mg/10^6·day (Table XXI).

In the presence of 10 mU bovine insulin, WI38 cells entered log phase with a significantly lower generation time of about 32 hours and, in the presence of 100 μU human insulin, these cells remained in lag phase, exhibiting no growth during a 7-day interval (Figs. 28 and 29). Glucose consumption by WI38 fibroblasts in the presence of 10 mU bovine insulin (r = -8.1 mg/10^6·day) is not significantly different from that of control cultures, but a 50% increment in r (-11.7 mg/10^6·day) was seen with 100 μU human insulin (Table XXI). Insulin added to these cells during log-phase growth had no apparent effect on growth rate, but glucose consumption rate followed the same pattern: no significant effect of 10

TABLE XXI. INSULIN EFFECTS ON CELL PROLIFERATION AND GLUCOSE UPTAKE BY SEVERAL CELL LINES

Cell Line	Medium/ Additive	t_D (hrs.)	Diff. from zero-slope		CM vs. insulin slope		-r (mg/10^6·day)
			t	P	t	P	
SK-HEP-1	CM	26.0	14.7	<0.001			3.52
	1 mU b.i.	30.0	10.3	<0.001	0.16	>0.1	3.88
	100 mU b.i.	28.7	5.67	<0.02	0.55	>0.1	4.28
WI38	CM	22.4	32.7	<0.001			7.69
	100 μU h.i.	665	0.38	>0.1	10.8	<0.001	11.7
	1 mU b.i.	31.9	13.9	<0.001	5.77	<0.001	8.14
AU471	CM	19.9	21.3	<0.001			4.93
	100 μU h.i.	34.5	9.8	<0.001	5.58	<0.001	8.91
BeWo	CM	27.8	14.9	<0.001			8.59
	100 μU h.i.	81.5	5.18	<0.001	6.98	<0.001	52.5
LT2	CM	60.8	10.9	<0.001			6.49
	100 μU b.i.	48.7	2.86	<0.05	0.56	>0.1	15.2
	10 mU b.i.	518	0.63	>0.1	4.26	<0.001	7.73

CM = complete medium (with FBS)

b.i. = bovine insulin

h.i. = human insulin

t_D = doubling time

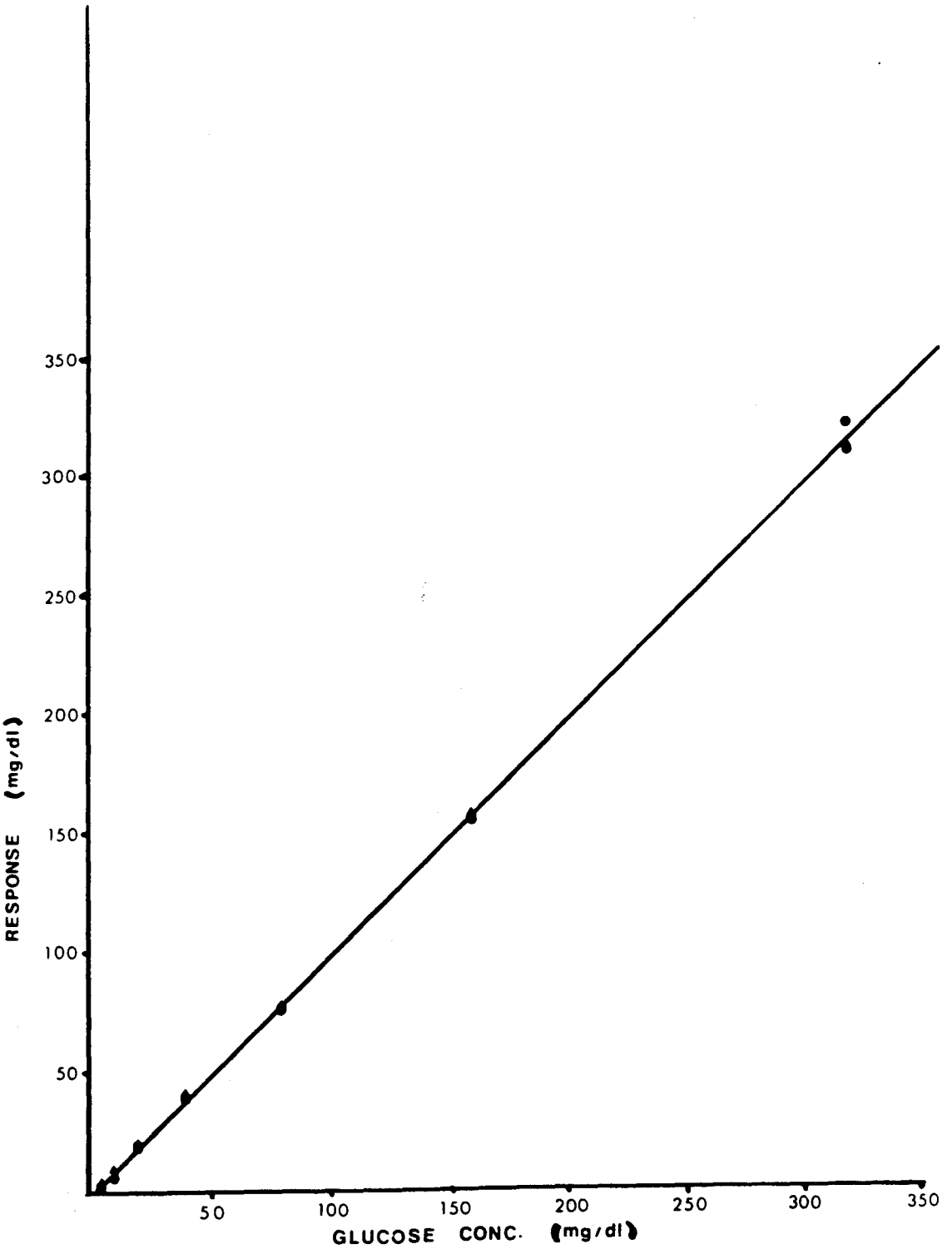
r = rate of glucose uptake (-mg glucose/10^6 cells/day)

FIGURE 30. BECKMAN GLUCOSE ANALYZER RESPONSE.

● and ▲ Separate assays

$r = 0.9998$

$y = 0.987x - 1.77$


350
300
250
200
150
100
50
RESPONSE (mg/dl)
50
100
150
200
250
300
350
GLUCOSE CONC. (mg/dl)

mU bovine insulin (-4.6 mg/10^6·day) and about 50% increment with 100 μU human insulin (-5.8 mg/10^6·day). Cell number was also unaffected by insulin added after cell confluence, while r was unaffected by 10 mU bovine insulin (-2.6 mg/10^6·day) and 100 μU human insulin caused a 25% increase in r (to -4.2 mg/10^6·day). An insulin-induced repression of cell proliferation and stimulation of glucose uptake rate was also observed for LT2, AU471 and BeWo, but not for SK-HEP-1 (Table XXI).

CELL INSULIN RECEPTORS AND DEGRADATION

After incubation with 1 mU bovine insulin/ml and washing, WI38 cells showed strong positive staining with anti-insulin/peroxidase-antiperoxidase (Fig. 27b). Although a mean specific ^{125}I-insulin binding of 0.90 ± 0.51% was found, no progressive change in this binding was seen in the range 0.95-19.0 μU ^{125}I-insulin. A specific association of about 0.2 μU (8 pg) insulin with 3.33 x 10^5 cells indicates a minimum of 2,400 insulin binding sites per cell for WI38 fibroblasts.

A consistent activity of 1-5% associated with suspended, adherent, and trypsinized SK-HEP-1 cells in quantities of 5 x 10^4-10^6 after 5 minutes-5 hours incubation with 1.33 μU and 13.3 μU ^{125}I-insulin indicates less than 200 insulin receptors per cell. Likewise, 0-2% activity was found associated with about 5 x 10^4 adherent or 10^6 trypsinized LT2 cells, indicating less than 120 insulin receptors per cell.

Immunocytochemical staining with PAP, able to detect less than 100 molecules antigen/cell, was plainly negative for SK-HEP-1 cells, despite heavy background staining, and definitely positive for LT2 cells, with a lighter

background, as well as for AU471 and BeWo.

RIA of replicate WI38 culture supernates revealed that insulin degradation occurred in cultures, resulting in 50% of the initial insulin concentration remaining after 22-48 hours in initial, log phase, and confluent cultures. The rate of insulin degradation decreased as medium insulin concentration decreased, with about 25% IRI remaining after 72 hours of log-phase growth. Bovine insulin RIA of supernates from SK-HEP-1 adherent cell cultures in the presence of 1 mU bovine insulin/ml showed no loss of immunoreactive insulin from the medium.

WI38 ASSAYS FOR INSULIN IN THE PRESENCE OF SERUM

Short-form assays utilizing confluent cultures of WI38 (Table XXII) yielded a glucose uptake rate of 3.21 mg/10^6·day, which was still about half that for log-phase cultures. Initial growth cultures reflect both glucose uptake and growth. Indices in all short-form assays were significantly increased in the presence of physiologic concentrations of human insulin.

The rate of glucose consumption in confluent cultures was seen to increase progressively in an apparent dose-response relationship in the presence of 10-100 μU human insulin, returning to control levels at higher insulin concentrations (≥1 mU). The same kind of dose-response increase in mg glucose consumed/10^6 viable cells (but less pronounced) and a decrease in cell number was seen for cells seeded in the presence of 10-100 μU insulin. No progressive changes were seen when 10 μU-100 mU insulin was added to log-phase cultures. The variance of these parameters relative to their mean changes in these assays was too great, however, to enable their use as bioassays for insulin.

TABLE XXII. EFFECTS OF INSULIN ON WI38 FIBROBLASTS IN THE PRESENCE OF SERUM

	t_D (hr.)	$-r$ ($mg/10^6 \cdot d$) ±SD (n)	I ($mg/10^6$)	t unpaired	t paired	P
Confluence				3.44		<0.005
BME CM		3.21±0.64 (27)				
100 μU hu. insulin		4.14±0.88 (9)				
Log Phase					9.29	<0.001
BME CM	21.1	6.28±1.86 (11)				
100 μU hu. insulin	21.1	7.17±1.86 (5)				
Initial Growth				2.32		<0.05
BME CM	22.4		2.00±6.81 (6)			
100 μU hu. insulin	∞		15.2±12.2 (6)			

CULTURED FIBROBLAST ASSAY FOR INSULIN

Figure 31 illustrates that the stationary medium (SM,0.35% BSA in BME) described by Morell and Froesch (615) does, indeed, cause WI38 fibroblasts to enter stationary phase 10 hours after complete medium (CM, BME with 10% FBS) is replaced with SM. These cells re-enter log phase 38 hours after SM is replaced with CM (based on a log-phase t_D of 21.1 hours). Therefore, 38 hours were subsequently subtracted from the time of SM culture when calculating the proliferation rates of cells in the presence of serum fractions. Cell number was unaffected by 100 μU human insulin after 3 days. This quantity of insulin, however, caused a significant 65% increase in glucose uptake by these cells relative to controls and this insulin-induced increment in r was completely reversed by 0.1 ml 1:5,000 AIS (Table XXIII). The mean r of -3.56 mg/10^6 cells·day for 182 determinations, with a standard deviation of 1.20 mg/10^6·day and a two-tailed t-statistic of 1.98 at P = 0.05 (730) yields a 95% confidence range for control culture r of -1.18 to - 5.94 mg/10^6·day. The normal ranges for U (n = 86, $\bar{x}$ = 0.044, SD = 0.051) and ν(n = 86, $\bar{x}$ = 0.11, SD = 0.084) were found to be 0.85-1.15 and 0.72-1.28, respectively. Thus, assay parameters falling outside these ranges are high or low at a minimum significance level of $P < 0.05$.

Both assay parameters, U and R, show a progressive, dose-response increase with increasing insulin concentration (Fig. 32). The U-response is curvilinear and is linearized by logit transformation, permitting extrapolation beyond 100 μU/ml. This parameter represents total ILA since it is independent of cell number and therefore includes the pleiotypic increment in glucose uptake due to the proliferative effects of growth factors. The R-response is directly

FIGURE 31. EFFECT OF STATIONARY MEDIUM, WITH AND WITHOUT INSULIN, ON WI38 CELL PROLIFERATION.

CM: Complete medium, BME with 10% FBS.

SM: Stationary medium, BME with 0.35% BSA.

- ● CM
- ▲ SM
- ■ 100 μU human insulin in SM

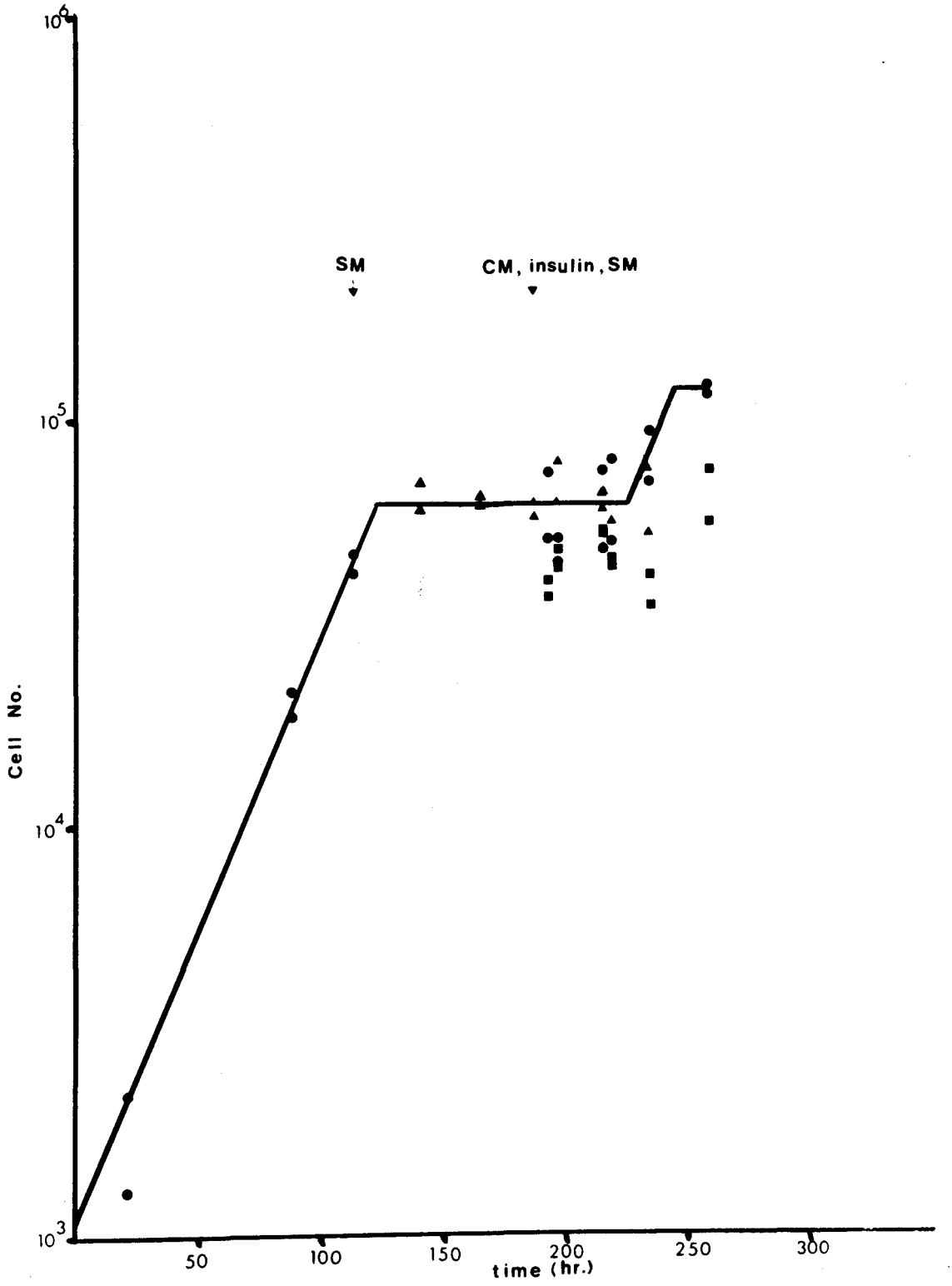

SM
CM, insulin, SM
10^6
10^5
10^4
10^3
Cell No.
50
100
150
200
250
300
time (hr.)

TABLE XXIII. EFFECT OF INSULIN ON GLUCOSE UPTAKE BY STATIONARY-PHASE WI38 CELLS

Medium	n	Mean r (-mg/10^6.d)	S.D.	CV (%)	S.E.M.	Comparison with Control	
						t	P
SM	182	3.56	1.20	33.7	0.09	---	---
SM + 100 µU human insulin	10	5.87	1.13	19.3	0.36	6.23	<0.001
SM + 100 µU human insulin + AIS	5	3.26	0.73	22.4	0.33	0.88	>0.10

FIGURE 32. CULTURED FIBROBLAST BIOASSAY FOR INSULIN: STANDARD CURVES.

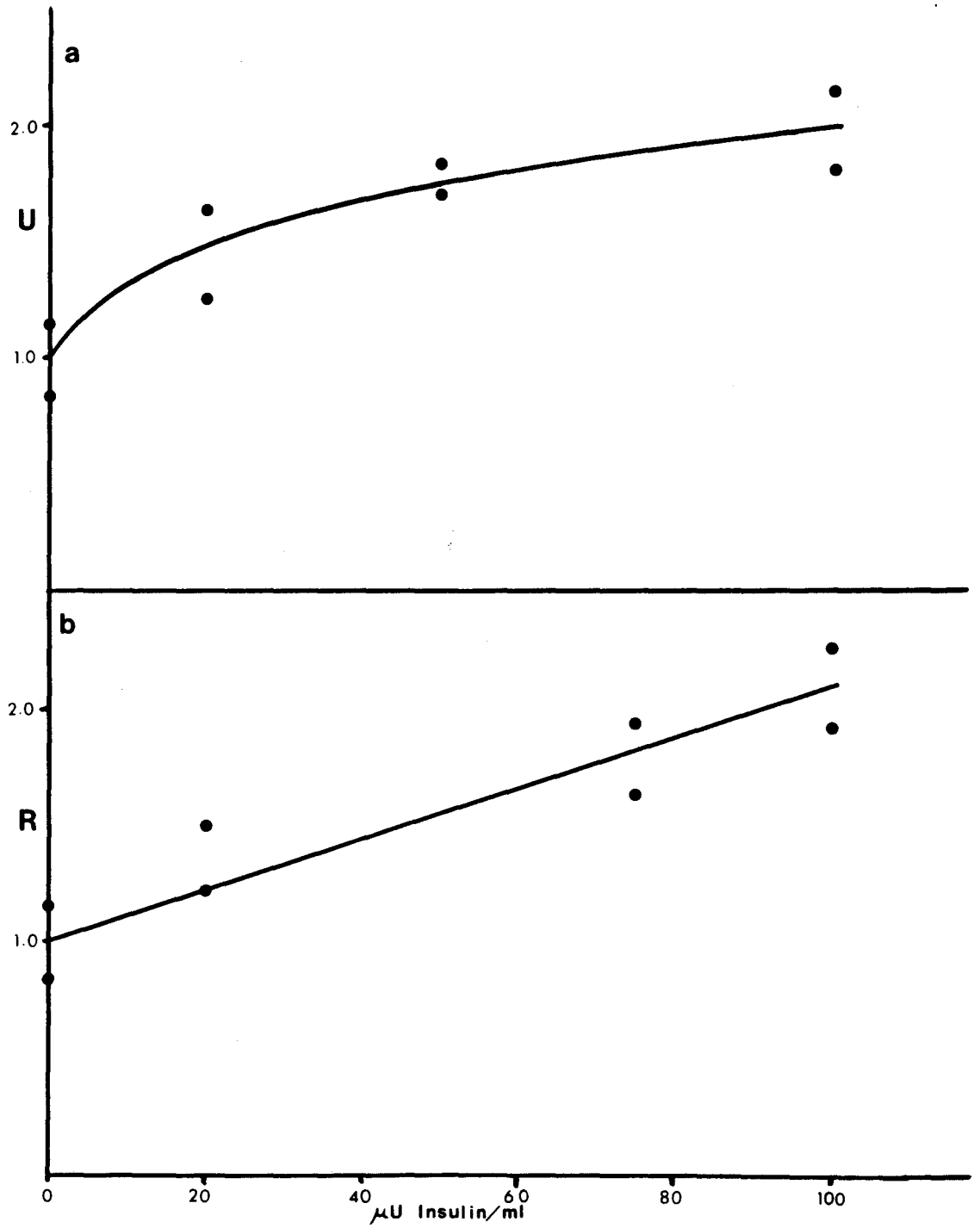
a. Total ILA

$$U = \frac{\text{glucose uptake (mg)}}{\text{control glucose uptake (mg)}}$$

b. Specific ILA

$$R = \frac{\text{rate of glucose uptake (mg/}10^6\text{ cells.day)}}{\text{control rate of glucose uptake (mg/}10^6\text{ cells.d)}}$$

Abscissa: Insulin concentration in assay well.


a
2.0
U
1.0
b
2.0
R
1.0
0
20
40
60
80
100
μU Insulin/ml

linear and represents specific ILA since it is corrected for increase in cell number, measuring insulin-like effects on r. Thus, this assay is capable of delineating the effects of growth factors and insulin, and was chosen as the in vitro bioassay for the systematic study of serum fractions.

BULK FRACTIONATION OF HUMAN SERUM

At least 99% of both serum proteins and IRI was retained by the 10,000-dalton M.W.-cutoff membrane (Table XXIVA). A nearly 60-fold enhancement of IRI was seen in the PM10r1 fraction compared to the PM10r2 fraction. Serum ILA was about 180 μU/ml (Table XXIVB) and ILA_S agreed well with this value. Human serum potentiation of WI38 proliferation was 13% of FBS. The enhanced IRI of PM10r1 was reflected in the ILA and ILA_S values for this fraction. A 10-fold dilution of PM10r1 yielded an ILA value that agreed well with the IRI. A large enhancement of growth potentiation was also seen in this fraction. The ILA_S of PM10r2 agreed well with IRI, while about two-thirds of serum ILA was recovered in this fraction, which possessed more growth-potentiating capacity than whole serum. Less than 2% of recovered serum ILA or ILA_S was found in UM2 retentates and filtrates. No heparin or biotin enhancement of ILA, ILA_S, or growth potentiation was seen in serum. Heparin enhancement of PM10r1 (100x) ILA, PM10r2, UM2f1 and UM2f2 ILAs and PM10r1 (100x) growth potentiation and biotin enhancement of UM2f1 growth potentiation was also noted.

Treatment of the PM10 Retentate

Re-concentration of PM10r2 through a PM 10 membrane resulted in nearly

TABLE XXIVA. BULK FRACTIONATION OF HUMAN SERUM: IRI

Fraction	Vol. (ml)	mg residue	Total Protein (mg)	Serum Equiv. (ml)	IRI			
					µU/ml	µU/mg Protein	Total µU	% Total
Human Serum	488	---	31,134	488	17.4	0.27	8,491	100
PM10r1	71.0	18,255	17,160	244	6,591	27.3	467,933	5,511
PM10r2	84.5	17,514	16,638	244	98.2	0.50	8,294	97.7
UM2r1	26.0	413	54	172	9.3	4.5	241	2.8
UM2r2	24.5	101	45	172	3.4	1.9	84	1.0
UM2f1	291	2,406	48	291	0	0	0	0
UM2f2	275	228	6.8	24	0	0	0	0

TABLE XXIVB. BULK FRACTIONATION OF HUMAN SERUM: BIOASSAY

Fraction	Treatment	ν	r ($-mg/10^6 \cdot d$)	ILA			ILA_s			t_D	FBS
				μU/ml	Total mU	μU/ml serum	μU/ml	Total mU	μU/ml serum	(hrs.)	equiv. (ml/ml serum)
Human Serum	Control	1.26	5.49	177*	86	177	208	101	208	173	0.13
	Heparin	1.31*	5.13	179*	87	179	88	43	88	148	0.15
	Biotin	1.33*	5.33	234*	114	234	140	68	140	140	0.16
PM10r1 (10x)	Control	2.82*	3.71	5,963*	423	1,735	0	0	0	36.0	1.78
	Heparin	2.35*	3.36	2,570*	182	748	0	0	0	43.7	1.46
	Biotin	3.28*	3.46	5,974*	424	1,738	0	0	0	31.5	2.03
(100x)	Control	1.38*	4.67	14,411*	1,023	4,195	3,500	249	1,018	125	5.13
	Heparin	1.85*	4.52	25,496*	1,810	7,419	2,000	142	582	65.4	9.80
	Biotin	1.58*	4.46	15,825*	1,124	4,606	0	0	0	87.9	7.28
PM10r2	Control	1.44*	4.69	322*	27	112	115	9.7	40	102	0.22
	Heparin	1.21	5.10	370*	31	128	245	21	85	194	0.11
	Biotin	1.60*	4.78	535*	45	185	58	4.9	20	78.8	0.28
UM2r1	Control	1.51*	3.20	81	2.1	12	0	0	0	90.7	0.04
	Heparin	1.38*	3.31	78	2.0	12	0	0	0	116	0.03
	Biotin	1.48*	2.85	0	0	0	0	0	0	95.3	0.03
UM2r2	Control	0.25*	10.3*	0	0	0	139	3.4	20	∞	0
	Heparin	0.40*	7.53*	0	0	0	78	1.9	11	∞	0
	Biotin	0.23*	11.4*	0	0	0	163	4.0	23	∞	0

TABLE XXIVB. (CONTINUED)

Fraction	Treatment	ν	r (-mg/10^6·d)	ILA			ILA_s			t_D (hrs.)	FBS equiv.
				μU/ml	Total mU	μU/ml serum	μU/ml	Total mU	μU/ml serum		(ml/ml serum)
UM2f1	Control	1.03	4.32	0	0	0	0	0	0	1,253	0.02
	Heparin	0.89	4.95	0	0	0	93	27	93	∞	0
	Biotin	1.33*	3.48	0	0	0	0	0	0	130	0.18
UM2f2	Control	0.27*	6.14*	0	0	0	3.4	0.9	39	∞	0
	Heparin	0.20*	8.74*	0	0	0	8.9	2.4	102	∞	0
	Biotin	0.27*	7.26*	0	0	0	5.8	1.6	66	∞	0

* Significantly different from control population. (See legend to Fig. 34.)

50% of IRI passing through the membrane, but less than 5% of IRI passed through the membrane after treatment of this fraction with heparin, biotin or urea (Table XXVA). Inability of these agents to enhance passage of insulin through PM10 membranes was reflected in ILA (Table XXVB), but ILA_S was found in heparin, biotin and urea filtrates. Growth potentiation followed the pattern of ILA. Heparin enhanced ILA in the heparin filtrate and the acid-ethanol precipitate, ILA_S in the control retentate, biotin retentate and filtrate, and urea filtrate and growth potentiation in the heparin filtrate. Biotin enhanced ILA in the control, heparin and biotin filtrates and the acid-ethanol precipitate, ILA_S in the urea filtrate and growth potentiation in the heparin filtrate and acid-ethanol precipitate.

Gel Chromatography of the PM10 Retentate

All of the PM10r1 IRI applied to the Sephacryl S-200 column was recovered in the eluate and more than 90% of the recovered IRI eluted as protein less than M.W. 12,000 daltons (calibration curve, Fig. 33) with a peak at about 6,500 daltons (Table XXVIA). A minor IRI peak also occurred in Fraction 4, corresponding to about 240,000 daltons, possibly coincident with a peak of growth potentiation since ν was increasing in Fraction 2. However, ILA data of Fractions 3-8 were obliterated due to toxicity of these fractions for WI38 cells, resulting in loss of all cells in Fractions 3-5 (Table XXVIB, Fig. 34).

ILA_S is not depicted in Fig. 34 wherever ν is significantly lower than the control population (resulting in spurious elevation of ILA_S, which is dependent upon cell number), but major ILA and ILA_S peaks occur at Fraction 10 (M.W. 37,000), coincident with a minor IRI peak. ILA peaks at Fractions 12 (M.W.

TABLE XXVA. TREATMENT OF PM10r2: IRI

Sample	Vol. (ml)	Serum equiv. (ml)	IRI		
			μU/ml	Total μU	% Recovered
CR	1.3	0.90	10.4	13.5	43.8
CF	10.5	0.80	0.9	9.9	32.1
HR	1.1	0.90	5.7	6.3	20.5
HF	10.9	0.82	0	0	0
BR	1.1	0.90	18.0	19.8	64.3
BF	10.9	0.82	0.1	0.9	2.9
UR	1.5	0.90	5.4	8.1	26.3
UF	10.5	0.79	0	0	0
AEP	5.1	3.6	0	0	0

C = control

H = heparin

B = biotin

U = 8 M urea

AEP = acid ethanol precipitate

R = PM 10 membrane (M.W. cutoff = 10,000) retentate

F = PM 10 filtrate

TABLE XXVB.TREATMENT OF PM10r2: BIOASSAY

Sample	ν	r (mg/10^6·d)	ILA µU/ml	ILA Total µU	ILA µU/ml serum	ILA_S µU/ml	ILA_S Total µU	ILA_S µU/ml serum	t_D (hrs.)	FBS equiv. (ml/ml serum)
CR	1.77*	3.55	301*	391	434	0	0	0	66.9	0.33
heparin	1.20	3.81	194*	252	280	59	77	86	210	0.11
biotin	2.29*	2.97	228	297	330	0	0	0	46.1	0.48
CF	0.99	4.30	0	0	0	0	0	0	∞	0
heparin	1.03	3.85	0	0	0	0	0	0	1,292	0.02
biotin	1.09	4.32	6.3	66	83	0	0	0	443	0.06
HR	2.24*	4.08	132*	145	161	0	0	0	47.4	0.46
heparin	2.17*	3.67	74	81	90	0	0	0	49.3	0.45
biotin	2.29*	3.77	97*	107	119	0	0	0	46.1	0.48
HF	0.95	5.37	0	0	0	1.7	18	22	∞	0
heparin	1.93*	4.70	7.2	78	95	0.4	4.5	5.5	61.6	0.39
biotin	1.25	4.77	2.6	28	34	0	0	0	182	0.13
BR	1.38*	4.72	124*	137	152	0	0	0	126	0.17
heparin	1.09	5.37	94*	103	115	20	23	25	470	0.05
biotin	1.63*	4.58	152*	167	186	0	0	0	82.9	0.27
BF	0.87	5.19	0	0	0	2.1	23	27	∞	0
heparin	0.80	5.65	0	0	0	8.7	95	115	∞	0
biotin	1.00	4.74	2.9	31	38	0	0	0	∞	0
UR	1.55*	4.79	84*	126	140	21	32	35	92.1	0.24
heparin	1.68*	4.28	64*	95	11	0	0	0	77.8	0.28
biotin	2.06*	4.02	75*	112	125	0	0	0	55.9	0.39

TABLE XXVB. (CONTINUED)

Sample	ν	r (mg/10^6·d)	ILA µU/ml	ILA Total µU	ILA µU/ml serum	ILA_S µU/ml	ILA_S Total µU	ILA_S µU/ml serum	t_D (hrs.)	FBS equiv. (ml/ml serum)
UF	0.88	5.03	5.2	55	69	7.7	81	103	∞	0
heparin	0.82	5.57	3.2	34	43	18	189	239	∞	0
biotin	0.77	5.82	0	0	0	23	243	308	∞	0
AEP	1.30*	4.63	21	109	30	0	0	0	177	0.12
heparin	1.32*	5.01	55	282	78	0	0	0	168	0.13
biotin	1.91*	3.19	56	287	80	0	0	0	71.9	0.31

* Significantly different from control population. (See legend to Fig. 34.)

FIGURE 33. SEPHACRYL S-200 CALIBRATION CURVE.

V_e/V_o = elution vol. (ml)/exclusion vol. (ml)

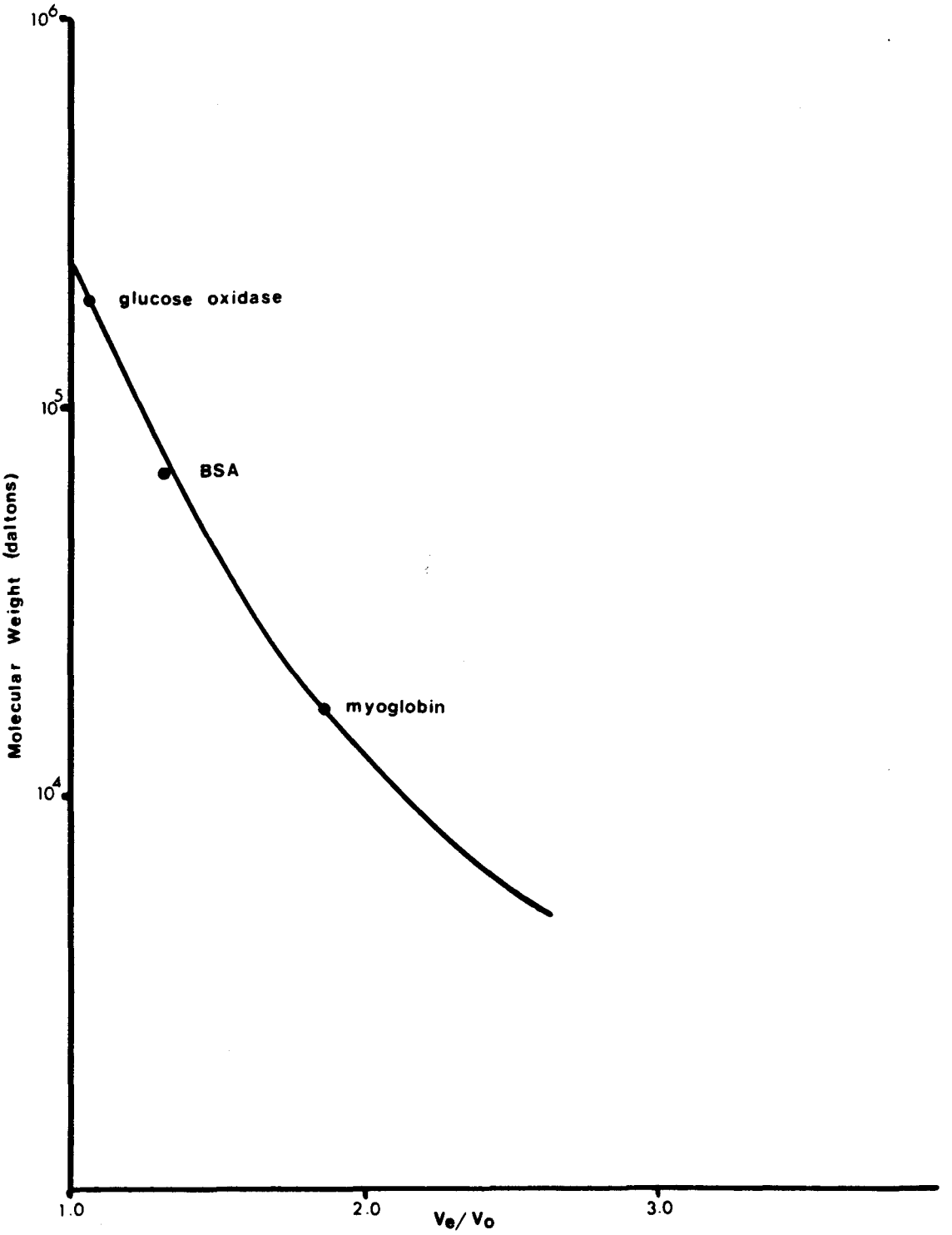

10^6
10^5
10^4
Molecular Weight (daltons)
glucose oxidase
BSA
myoglobin
1.0
2.0
3.0
V_e/V_o

TABLE XXVIA. GEL CHROMATOGRAPHY OF PM10r1: IRI

Fraction	M.W. (kd)	Total Protein (mg)	IRI			Dextran/ Charcoal
			Total μU	μU/ml serum	% total	% bound
1	>250	0	22	0.6	0.03	0
2	>250	12.2	120	3.0	0.2	1.0
3	>250	170	918	23	1.2	4.6
4	210->250	218	943	24	1.2	6.0
5	137-210	211	689	17	0.9	5.6
6	98-137	169	685	17	0.9	5.0
7	72-98	156	449	11	0.6	4.0
8	54-72	145	401	10	0.5	1.9
9	42-54	152	314	7.9	0.4	2.5
10	33-42	180	336	8.4	0.4	1.8
11	26-33	192	271	6.8	0.4	0.4
12	21-26	196	486	12	0.6	0.9
13	17.5-21	182	528	13	0.7	1.6
14	14.6-17.5	163	595	15	0.8	1.0
15	12.2-14.6	155	818	20	1.1	1.1
16	10.5-12.2	96.7	1,704	43	2.2	0.2
17	9.0-10.5	52.1	3,652	91	4.8	0
18	7.8-9.0	36.2	8,748	219	11.4	0
19	6.8-7.8	32.5	19,296	482	25.2	0
20	6.1-6.8	26.0	25,904	648	33.8	0.7
21	5.5-6.1	17.8	21,484	537	28.0	0

TABLE XXVIA. (CONTINUED)

Fraction	M.W. (kd)	Total Protein (mg)	IRI			Dextran/ Charcoal
			Total μU	μU/ml serum	% total	% bound
22	5.0-5.5	7.8	11,970	299	15.6	0
23	<5	2.5	5,480	137	7.1	0
24	<5	4.6	1,000	25	1.3	0.6
Total		2,577 (91.4%)	106,813	2,670	139	

TABLE XXVIB. GEL CHROMATOGRAPHY OF PM10r1: BIOASSAY

Fraction	Serum equiv. assayed (ml)	ν	r (mg/10^6·d)	ILA Total μU	ILA μU/ml serum	ILA % total	ILA_s Total μU	ILA_s μU/ml serum	t_D (hrs.)	FBS equiv. (ml/ml serum)	% total
1	1.4	1.71*	3.46	987*	25	1.4	636	16	63.8	0.02	1.4
heparin		1.36*	3.69	1,079*	27	3.6	795	20	111	0.01	1.0
biotin		1.38*	3.46	816*	20	1.2	564	14	106	0.01	0.7
2	1.3	1.77*	3.13	523*	13	0.8	0	0	59.9	0.03	1.6
heparin		1.59*	3.09	387*	10	1.3	0	0	73.8	0.02	1.6
biotin		2.19*	2.44	296	7	0.4	0	0	43.6	0.04	1.9
3	1.4					TOXIC					
4	1.3					TOXIC					
5	1.2					TOXIC					
6	1.2	0.27*	8.62*	0	0	0	5,712	143	∞	0	0
heparin		0.51*	4.79	0	0	0	1,572	39	∞	0	0
biotin		0.47*	5.82	0	0	0	2,687	67	∞	0	0
7	1.2	0.26*	7.36*	0	0	0	6,175	154	∞	0	0
heparin		0.26*	6.43*	0	0	0	4,973	124	∞	0	0
biotin		0.37*	5.26	0	0	0	3,429	86	∞	0	0
8	1.2	0.44*	5.75	185	5	0.3	4,670	117	∞	0	0
heparin		0.69*	4.34	501*	13	1.7	2,673	67	∞	0	0
biotin		0.54*	5.70	539*	13	0.8	4,620	116	∞	0	0

TABLE XXVIB. (CONTINUED)

Fraction	Serum equiv. assayed (ml)	ν	r (mg/10^6·d)	ILA Total μU	ILA μU/ml serum	ILA % total	ILA_s Total μU	ILA_s μU/ml serum	t_D (hrs.)	FBS equiv. (ml/ml serum)	% total
9	1.2	1.04	4.64	800*	20	1.2	875	22	1,089	0.002	0.1
heparin		1.24	4.29	801*	20	2.7	545	14	198	0.009	0.6
biotin		1.65*	3.86	937*	23	1.3	132	3	85.2	0.02	1.0
10	1.2	0.97	4.22	1,850*	46	2.7	2,311	58	∞	0	0
heparin		1.50*	3.02	1,254*	31	4.1	658	16	105	0.02	1.1
biotin		0.16*	19.5*	463*	12	0.7	23,032	576	∞	0	0
11	1.3	0.96	2.83	618*	15	0.9	1,144	29	∞	0	0
heparin		0.67*	4.35	683*	17	2.3	3,492	87	∞	0	0
biotin		1.09	2.40	604*	15	0.9	473	12	397	0.004	0.2
12	1.2	1.13	2.66	794*	20	1.1	883	22	280	0.006	0.4
heparin		0.96	3.04	766*	19	2.6	1,504	38	∞	0	0
biotin		1.01	2.98	1,004*	25	1.4	1,406	35	3,439	0.001	0.03
13	1.2	0.74	4.38	427*	11	0.6	1,950	49	∞	0	0
heparin		0.78	3.86	321*	8	1.0	1,300	33	∞	0	0
biotin		1.06	3.41	581*	15	0.8	748	19	681	0.003	0.1
14	1.3	1.05	3.83	532*	13	0.7	561	14	814	0.002	0.1
heparin		1.25	3.45	339*	8	1.1	240	6	178	0.01	0.7
biotin		1.13	3.89	505*	13	0.7	448	11	325	0.005	0.3

TABLE XXVIB. (CONTINUED)

Fraction	Serum equiv. assayed (ml)	ν	r (mg/10^6·d)	ILA Total μU	ILA μU/ml serum	ILA % total	ILA_s Total μU	ILA_s μU/ml serum	t_D (hrs.)	FBS equiv. (ml/ml serum)	% total
15	1.2	0.85	4.39	531*	13	0.8	1,618	40	∞	0	0
heparin		1.14	3.15	405*	10	1.4	219	5	303	0.006	0.4
biotin		1.00	3.43	461*	12	0.7	472	12	∞	0	0
16	1.2	0.85	3.79	444*	11	0.6	1,311	33	∞	0	0
heparin		0.91	3.32	296	7	1.0	776	19	∞	0	0
biotin		1.04	2.99	340*	9	0.5	242	6	940	0.002	0.1
17	1.3	0.93	3.60	389*	10	0.6	704	18	∞	0	0
heparin		0.59*	4.82	100	2	0.3	2,066	52	∞	0	0
biotin		1.09	2.79	271	7	0.4	0	0	428	0.004	0.2
18	1.4	1.32*	3.35	434*	11	0.6	237	6	154	0.01	0.6
heparin		0.59*	5.14	121	3	0.4	1,904	48	∞	0	0
biotin		1.04	3.54	260	7	0.4	487	12	1,088	0.001	0.07
19	1.5	1.26	3.86	507*	13	0.7	352	9	185	0.008	0.5
heparin		1.02	3.95	348*	9	1.2	420	11	2,156	0.001	0.05
biotin		1.35*	3.41	292*	7	0.4	190	5	142	0.01	0.5
20	1.5	0.03*	29.6	0	0	0	25,558	639	∞	0	0
heparin		0.63*	5.49	275*	7	0.9	2,470	62	∞	0	0
biotin		0.08*	35.1	0	0	0	30,745	769	∞	0	0

TABLE XXVIB. (CONTINUED)

Fraction	Serum equiv. assayed (ml)	ν	r (mg/10^6·d)	ILA Total μU	ILA μU/ml serum	ILA % total	ILA_s Total μU	ILA_s μU/ml serum	t_D (hrs.)	FBS equiv. (ml/ml serum	% total
21	1.8	1.06	4.39	358*	9	0.5	781	20	607	0.002	0.1
heparin		1.35*	2.96	205	5	0.7	0	0	118	0.01	0.7
biotin		0.95	3.88	279*	7	0.4	390	10	∞	0	0
22	2.1	0.92	3.09	183	5	0.3	166	4	∞	0	0
heparin		1.25	2.30	133	3	0.4	0	0	159	0.007	0.5
biotin		1.16	2.44	183	5	0.3	0	0	238	0.005	0.2
23	1.6	0.80	2.73	109	3	0.2	538	13	∞	0	0
heparin		0.90	2.35	79	2	0.3	225	6	∞	0	0
biotin		0.59*	3.72	182	5	0.3	1,700	43	∞	0	0
24	1.7					TOXIC					
Total				9,671	242	13.9				0.08	4.5
heparin				8,093	202	25.0				0.09	6.3
biotin				8,013	200	11.5				0.10	5.1

* Significantly different from control population. (See legend to Fig. 34.)

FIGURE 34. SEPHACRYL S-200 CHROMATOGRAPHY OF PM10r1.

Bioassay: Solid bars = no treatment

Open bars = heparin (100 μg/ml)

Hatched bars = biotin (10 μg/ml)

b.d. = blue dextran

g.o. = glucose oxidase

BSA = bovine serum albumin

Mb = myoglobin

IRI = immunoreactive insulin (measured by RIA)

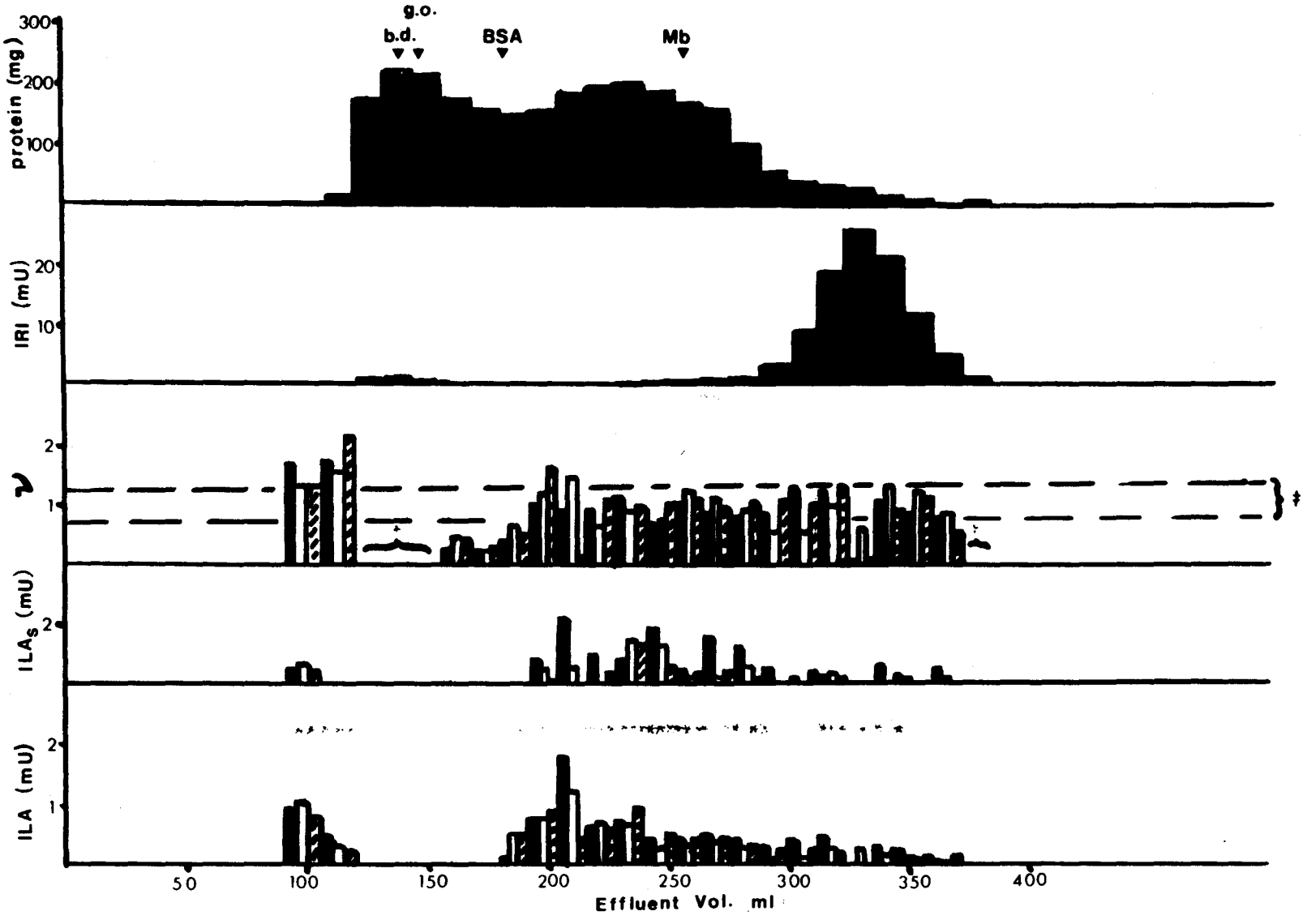
= cell number/control cell number

ILA_s = specific insulin-like activity

* Significantly higher than control population. A 95% confidence-limit control range was determined by assigning each control culture a U-value based on the average glucose uptake of two control cultures per plate. Range = $1 \pm$ (mean of $|U-1| + \{t_{0.05} \times S.D.\}$) = 0.85-1.15. Therefore, cultures of U>1.15 were judged to be higher than the control population at a minimum significance level of $P<0.05$.

† Toxic

‡ Control range (determined like ILA control range) = 0.72-1.28.


g.o.
b.d.
BSA
Mb
protein (mg)
300
200
100
IRI (mU)
20
10
2
1
ILA$_S$ (mU)
2
ILA (mU)
2
1
50
100
150
200
250
300
350
400
Effluent Vol. ml

24,000), 14 (M.W. 16,000) and 19 (M.W. 7,000), and ILA_S peaks at Fractions 13 (M.W. 19,000), 15 (M.W. 13,000), 21 (M.W. 6,000) and 23 (M.W.<5,000) were also observed. A peak of growth potentiation occurred at Fraction 18 (M.W. 8,000). Heparin-induced increase of ILA_S occurred in Fraction 12. Biotin-enhancement of both ILA and ILA_S was also seen in this fraction. Enhancement of cell proliferation by heparin occurred in Fraction 10 and by biotin in Fractions 2 (M.W. >250,000) and 9 (M.W. 48,000). Fractions 20 (M.W. 6,500) and 24 (M W< 5,000) were also toxic for WI38 fibroblasts. Heparin caused inhibition of ILA, ILA_S and ν in several fractions. Both heparin and biotin significantly decreased ILA and ILA_S in Fraction 10, and biotin also decreased ν in this fraction.

Because cytotoxicity of high molecular weight fractions could have been caused by lipidic material such as low-density lipoproteins and chylomicrons (738,739), bioassay was carried out on an acetone powder of Fraction 4. Loss of toxicity with a peak of ILA due to a growth-potentiating effect was noted in the treated fraction (Table XXVII).

Dextran-Charcoal Analysis of Fractions

Since appearance of large quantities of monomeric IRI in PM10r1 is consistent with dissociation of the hormone from a serum binding protein, dextran-charcoal analysis of S-200 chromatography fractions was undertaken to detect appreciable binding of ^{125}I-insulin by a liberated binding protein. A maximum net binding of 6% was found in Fraction 4 (Table XXVIA). Reversibility of this binding was tested by dextran-charcoal analysis of acid-treated human serum, PM10r2 and PM10r1 S-200 Fraction 5 PM-10 retentates. Although these fractions exhibited about 20% net binding of ^{125}I-insulin, no

TABLE XXVII. TREATMENT OF PM10r1 CHROMATOGRAPHY FRACTIONS

Fraction	Serum equiv. assayed (ml)	ν	r ($mg/10^6 \cdot d$)	ILA Total μU	ILA $\mu U/ml$ serum	ILA % total	ILA_s Total μU	ILA_s $\mu U/ml$ serum	t_D (hrs.)	FBS equiv. (ml/ml serum)	% total
4 AP	1.3	2.00*	2.59	766*	19	1.1	90	2	57.8	0.03	1.6
heparin		1.82*	2.64	740*	18	2.5	90	2	66.9	0.02	1.7
biotin		1.71*	2.66	654*	16	0.9	30	1	74.6	0.02	1.1
9 HOAcR	1.2	1.18	3.58	230	6	0.3	0	0	212	0.009	0.5
heparin		1.17	3.56	202	5	0.7	0	0	223	0.008	0.6
biotin		1.21	3.20	111	3	0.2	0	0	184	0.01	0.5
9HOAcF	1.1	1.43*	4.22	273	7	0.4	0	0	98.0	0.02	1.2
heparin		1.12	3.79	108	3	0.4	203	5	309	0.007	0.4
biotin		0.87	6.02*	307	8	0.4	756	20	∞	0	0
12HOAcR	1.2	0.67*	4.33	192	5	0.3	1,481	37	∞	0	0
heparin		0.91	3.44	248	6	0.8	461	12	∞	0	0
biotin		0.98	3.13	235	6	0.3	82	2	∞	0	0
12HOAcF	1.1	1.11	4.68	288	7	0.4	128	3	380	0.005	0.3
heparin		1.14	4.59	309	8	1.0	0	0	303	0.007	0.5
biotin		0.87	5.58	298	7	0.4	624	15	∞	0	0

* Significantly different from control population. (See legend to Fig. 34.)

AP acetone powder

HOAc acetic acid

R PM 10 retentate

F PM 10 filtrate

decrease in this binding was observed after treatment of fractions with 6M urea or 1 mU insulin despite apparent enhancement of monomeric IRI by acid treatment (Table XXVIII).

NSILA-S Screen

Acid treatment of PM10r1 S-200 Fraction 9 resulted in appearance of significant growth-potentiating activity in the PM 10 filtrate. Heparin and biotin did not enhance the activity of the filtrate. A biotin-enhanced ILA_S was also seen in acid-treated Fractions 9 and 12 (Table XXVII).

Comparative RIA

Appearance of large amounts of IRI in PM10r1 is also consistent with contamination by exogenous insulin, especially since porcine insulin was being purified by another investigator in the same laboratory. One possible mode of contamination could have been through common use of lyophilization flasks. Use of porcine trace and standards in the insulin RIA was undertaken in order to discriminate dose-response behavior of human and porcine insulins. Unfortunately, no significant difference in displacement of porcine trace by human and porcine standards was found, so that this question could not be answered.

GEL CHROMATOGRAPHY OF HUMAN SERUM ACETONE POWDER

In order to examine characteristics of the important exclusion region of chromatographed serum, S-200 chromatography was carried out on human serum

TABLE XXVIII. ACID TREATMENT OF SERUM AND SERUM FRACTIONS

Fraction	Vol. (ml)	IRI			Dextran-Charcoal	
		µU	% IRI recov.	% total recov.	Treatmt.	% bound
Human Serum						
Retentate	1.2	18.8	108	33.0	None	20.3
					6 M urea	26.0
					Insulin	30.2
Filtrate	20.1	38.2	220	67.0		
PM10r2						
Retentate	1.2	19.9	57.8	24.7	None	21.7
					6 M urea	25.8
					Insulin	29.6
Filtrate	20.2	60.6	176	75.3		
PM10r1 Fraction 5						
Retentate	1.0	131	69.0	64.5	None	20.3
					6 M urea	46.3
					Insulin	26.8
Filtrate	19.0	72.2	38.0	35.5		

acetone powder, the results of which are summarized in Tables XXIXA and B and Figure 35.

All of the IRI applied to the S-200 column was recovered in the eluate, but more than half of this material eluted at positions corresponding to molecular weights greater than 18,000 daltons. IRI peaks were seen at Fractions 3 (M.W. 240,000), 7 (M.W. 57,000), 11 (M.W. 21,000), 16 (M.W. 8,000) and 20 (M.W. 5,000). The expectation that acetone would extract toxic lipids from serum was realized since toxicity was completely eliminated from the exclusion fractions, verifying a pleiotypic ILA peak, probably at Fraction 3. Unfortunately, bioassay of untreated and biotin-treated Fraction 3 was unable to be completed because of microbial contamination.

A peak of growth potentiation was seen in Fraction 7 (coincident with an IRI peak), followed by an ILA peak at Fraction 8 (M.W. 43,000) and an ILA_S peak at Fraction 9 (M.W. 33,000). Another peak of growth potentiation occurred at Fraction 10 (M.W. 26,000), followed by an ILA peak at Fraction 11 and an ILA_S peak at Fraction 12 (M.W. 17,000). An ILA peak coincident with a ν peak and heparin- and biotin-inhibited ILA_S occurred at Fraction 15 (M.W. 10,000). Extensive cytotoxicity was seen below M.W. 9,000, from Fraction 16. ILA peaks were also observed at Fractions 5 (M.W. 110,000) and 13 (M.W. 14,000), and ILA_S peaks at Fractions 1 (M.W. >250,000) and 13.

Heparin stimulation of cell proliferation and ILA was noted in Fraction 1 and of ILA_S in Fractions 5, 7, and 10. The compound also appeared to exert a sparing effect on loss of cells in Fractions 12 and 14. Biotin stimulated cell proliferation in Fraction 1, ILA in Fractions 5-7 and 9, and ILA_S in Fractions 5, 7, and 10. Heparin inhibited ILA in the serum acetone powder and Fractions 2

TABLE XXIXA. GEL CHROMATOGRAPHY OF HUMAN SERUM ACETONE POWDER: IRI

Fraction	M.W. (kd)	Total Protein (mg)	IRI		
			Total μU	μU/ml serum	% Total
AP		2,032	631	17.0	100
1	>250	0.08	0	0	0
2	>250	32.8	20.4	0.5	3.2
3	190->250	125	78.0	2.1	12.4
4	130-190	126	44.4	1.2	7.0
5	92-130	122	39.6	1.1	6.3
6	65-92	132	24.0	0.6	3.8
7	49-65	152	61.2	1.6	9.7
8	37.5-49	157	46.8	1.3	7.4
9	28.5-37.5	146	2.4	0.1	0.4
10	23-28.5	140	0	0	0
11	18.7-23	154	16.8	0.5	2.7
12	14.9-18.7	158	0	0	0
13	12.5-14.9	150	0	0	0
14	10.7-12.5	143	0	0	0
15	8.9-10.7	146	0	0	0
16	7.6-8.9	125	10.8	0.3	1.7
17	6.8-7.6	109	9.6	0.3	1.5
18	5.9-6.8	73.2	48.0	1.3	7.6
19	5.2-5.9	32.8	63.6	1.7	10.1

TABLE XXIXA. (CONTINUED)

Fraction	M.W. (kd)	Total Protein (mg)	IRI		
			Total μU	μU/ml serum	% Total
20	<5-5.2	13.4	82.8	2.2	13.1
21	<5	3.7	75.6	2.0	12.0
22	<5	3.1	21.6	0.6	3.4
23	<5	2.1	14.4	0.4	2.3
24	<5	14.1	14.6	0.4	2.3
Total		2,261 (111% recov.)	675	18.2	107

TABLE XXIXB. GEL CHROMATOGRAPHY OF HUMAN SERUM ACETONE POWDER: BIOASSAY

Fraction	Serum equiv. assayed (ml)	ν	r (mg/10^6·d)	ILA Total μU	ILA μU/ml serum	ILA % total	ILA_s Total μU	ILA_s μU/ml serum	t_D (hrs.)	FBS equiv. (ml/ml serum)	% total
AP	0.1	1.96*	4.14	15,939*	430	100	3,875	104	58.7	0.37	100
heparin		1.98*	3.66	8,955*	241	100	0	0	57.9	0.38	100
biotin		2.09*	3.46	8,638	233	100	0	0	53.6	0.41	100
1	0.9	0.90	4.75	0	0	0	620	17	∞	0	0
heparin		1.41*	3.88	266	7	1.7	0	0	115	0.02	5.4
biotin		1.37*	3.63	0	0	0	0	0	126	0.02	4.6
2	1.7	1.45*	3.52	482*	13	3.0	197	5	106	0.01	3.3
heparin		1.30*	3.58	310*	8	1.9	77	2	151	0.009	2.3
biotin		0.31*	3.65	0	0	0	110	3	∞	0	0
3	1.5										
heparin		1.62*	4.24	661*	18	7.4	144	4	73.7	0.02	5.1
4	1.8	1.38*	4.54	222*	6	1.4	31	1	120	0.01	2.8
heparin		1.22	4.25	132	4	1.5	0	0	194	0.006	1.7
biotin		1.38*	4.13	156	4	1.8	0	0	120	0.01	2.5
5	1.7	1.13	3.82	380*	10	2.4	344	9	330	0.004	1.1
heparin		0.90	4.52	302*	8	3.4	833	22	∞	0	0
biotin		1.00	4.26	434*	12	5.0	644	17	∞	0	0
6	1.7	0.71*	5.42	229*	6	1.4	1,287	35	∞	0	0
heparin		0.90	4.91	374*	10	4.2	946	25	∞	0	0
biotin		0.82	5.63	463*	12	5.4	1,430	39	∞	0	0

TABLE XXIXB. (CONTINUED)

Fraction	Serum equiv. assayed (ml)	ν	r (mg/10^6·d)	ILA Total μU	ILA μU/ml serum	ILA % total	ILA_S Total μU	ILA_S μU/ml serum	t_D (hrs.)	FBS equiv. (ml/ml serum)	% total
7	1.6	1.72*	3.80	414*	11	2.6	0	0	74.4	0.02	5.1
heparin		1.33*	4.34	433*	12	4.8	214	6	142	0.01	2.6
biotin		1.25	4.70	514*	14	6.0	440	12	181	0.008	1.9
8	1.6	1.23	4.29	581*	16	3.6	531	14	172	0.008	2.2
heparin		1.17	3.42	242	7	2.7	150	4	226	0.006	1.6
biotin		1.17	3.84	339*	9	3.9	219	6	226	0.006	1.5
9	1.6	0.86	5.14	225	6	1.4	580	16	∞	0	0
heparin		0.88	5.27	280*	8	3.1	673	18	∞	0	0
biotin		1.15	4.40	317*	9	3.7	151	4	276	0.005	1.2
10	1.6	1.32*	3.62	363*	10	2.3	124	3	139	0.01	2.6
heparin		1.12	4.13	438*	12	4.9	407	11	340	0.004	1.0
biotin		0.96	4.43	374*	10	4.3	610	16	∞	0	0
11	1.5	0.43*	12.02*	446	12	2.8	4,929	133	∞	0	0
heparin		0.65*	7.52*	224	6	2.5	2,111	57	∞	0	0
biotin		0.45*	9.98*	220	6	2.5	3,660	99	∞	0	0
12	1.5	0.58*	12.38*	237	6	1.5	3,273	88	∞	0	0
heparin		0.82	7.78*	369*	10	4.1	1,074	29	∞	0	0
biotin		0.54*	10.99*	298*	8	3.4	2,606	70	∞	0	0

TABLE XXIXB. (CONTINUED)

Fraction	Serum equiv. assayed (ml)	ν	r (mg/10^6·d)	ILA Total μU	ILA μU/ml serum	ILA % total	ILA_S Total μU	ILA_S μU/ml serum	t_D (hrs.)	FBS equiv. (ml/ml serum)	% total
13	1.5	0.64*	9.98*	417*	11	2.6	2,681	72	∞	0	0
heparin		0.69*	9.58*	292*	8	3.3	2,472	67	∞	0	0
biotin		0.81	8.28*	323*	9	3.7	1,771	48	∞	0	0
14	1.5	0.67*	12.05*	291*	8	1.8	1,887	51	∞	0	0
heparin		1.17	7.71*	342*	9	3.8	191	5	268	0.006	1.5
biotin		0.72	11.58*	256*	7	3.0	1,658	45	∞	0	0
15	1.4	1.23	9.83*	632*	17	4.0	1,126	30	203	0.008	2.1
heparin		1.05	8.72*	582*	16	6.5	676	18	861	0.002	0.5
biotin		1.35*	8.07*	645*	18	7.6	411	11	140	0.01	2.7
16	1.5	0.40*	11.58*	0	0	0	3,138	85	∞	0	0
heparin		0.46*	10.31*	84	2	0.9	2,513	68	∞	0	0
biotin		0.57*	8.61*	76	2	0.9	1.650	44	∞	0	0
17	1.4	0.06*	33.30*	0	0	0	16,039	432	∞	0	0
heparin		0.09*	19.71*	0	0	0	8,299	224	∞	0	0
biotin		0.11*	15.81*	0	0	0	6,088	164	∞	0	0
18	1.5	0.02*	0*	0	0	0	0	0	∞	0	0
heparin		0.05*	10.66*	0	0	0	4,209	113	∞	0	0
biotin		0.05*	1.85	0	0	0	0	0	∞	0	0

TABLE XXIXB. (CONTINUED)

Fraction	Serum equiv. assayed (ml)	ν	r (mg/10^6·d)	ILA Total μU	ILA μU/ml serum	ILA % total	ILA_s Total μU	ILA_s μU/ml serum	t_D (hrs.)	FBS equiv. (ml/ml serum)	% total
19	1.5	0.27*	13.29*	0	0	0	4,990	134	∞	0	0
heparin		0.08*	7.60*	0	0	0	1,688	46	∞	0	0
biotin		0.07*	2.16	0	0	0	0	0	∞	0	0
20	1.4	0.06*	8.52*	0	0	0	3,296	89	∞	0	0
heparin		0.01*	70.99*	0	0	0	48,481	1,307	∞	0	0
biotin		0.004*	0*	0	0	0	0	0	∞	0	0
21	1.0	0.02*	25.72*	0	0	0	12,210	329	∞	0	0
heparin		0.01*	98.77*	0	0	0	58,127	1,567	∞	0	0
biotin		0.07*	12.52*	0	0	0	4,089	110	∞	0	0
22	1.2	0.04*	15.09*	0	0	0	9,440	254	∞	0	0
heparin		0.01*	0*	0	0	0	0	0	∞	0	0
biotin		0.04*	1.77	0	0	0	0	0	∞	0	0
23	1.8	0.08*	13.30*	0	0	0	3,507	95	∞	0	0
heparin		0.02*	80.22*	0	0	0	32,487	876	∞	0	0
biotin		0*	0*	0	0	0	0	0	∞	0	0
24	1.3					TOXIC					

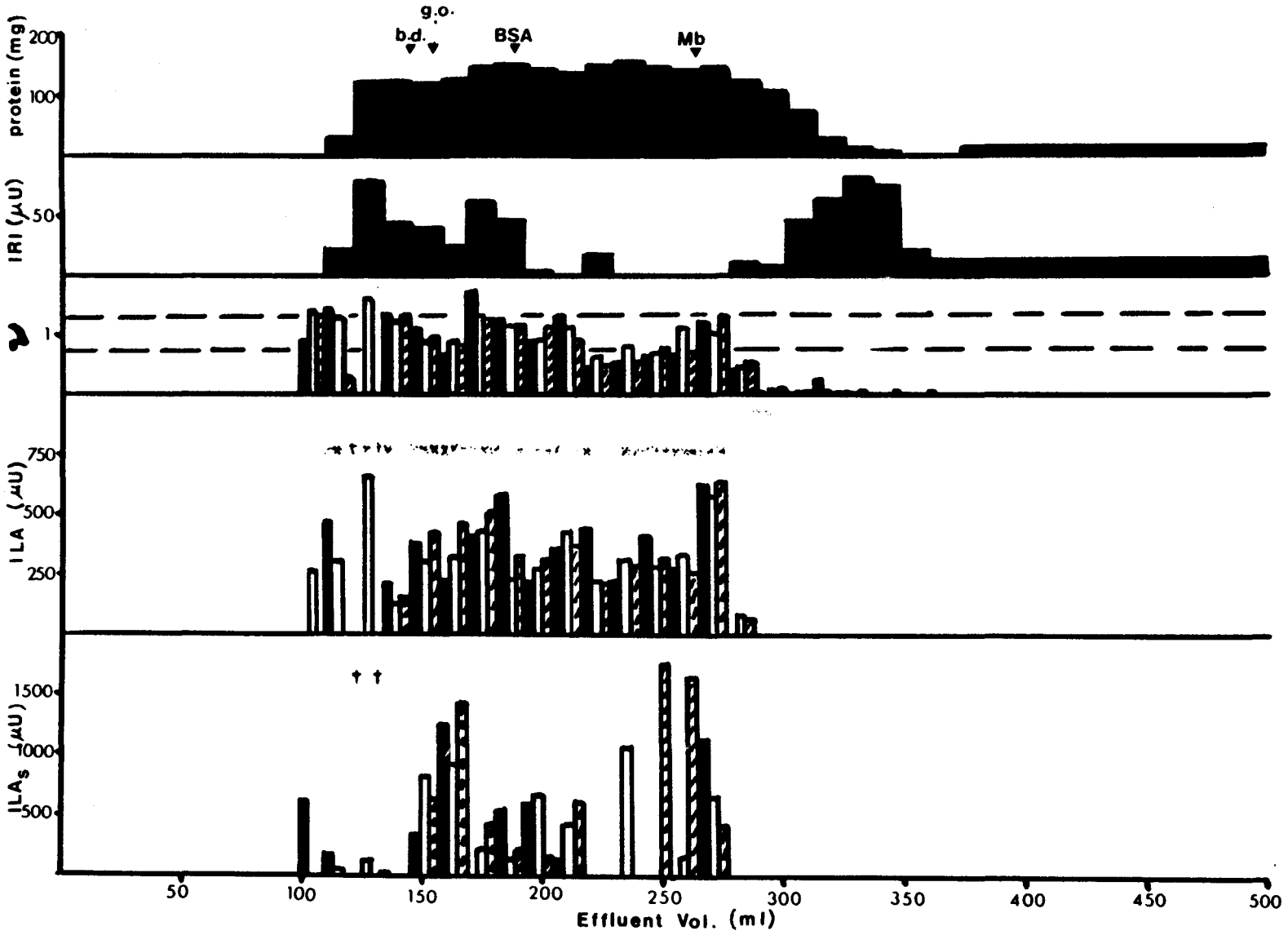
Fraction	Serum equiv. assayed (ml)	ν	r (mg/10^6·d)	ILA Total μU	ILA μU/ml serum	ILA % total	ILA_s Total μU	ILA_s μU/ml serum	t_D (hrs.)	FBS equiv. (ml/ml serum)	% total
Total				4,919	133	30.8				0.07	18.9
heparin				5,331	144	59.6				0.08	21.8
biotin				4,415	119	51.1				0.06	14.4
2	1.9	0.70*	0.85*	0	0	0	0	0	∞	0	0
	1.0	2.91*	5.90	88,065*	2,374	552	4,371	118	36.4	0.06	17.3
	0.2	1.66*	5.02	65,099*	1,755	408	15,386	415	76.7	0.15	41.0

* Significantly different from control population. (See legend to Fig. 34.)

FIGURE 35. SEPHACRYL S-200 CHROMATOGRAPHY OF HUMAN SERUM ACETONE POWDER.

See legend to Figure 34.

† Lost due to microbial contamination.


g.o.
b.d.
BSA
Mb
protein (mg)
200
100
IRI (μU)
50
1
ILA (μU)
750
500
250
ILA$_S$ (μU)
1500
1000
500
50
100
150
200
250
300
350
400
450
500
Effluent Vol. (ml)

(M.W. >250,000), 4 (M.W. 160,000), 5, 8, 11 and 13, and ILA_S in the acetone powder and Fractions 1, 2, 4, 6 (M.W. 79,000), 8 and 15. Biotin inhibited cell proliferation in Fraction 2, ILA in 11, 13 and 14 (M.W. 12,000), and ILA_S in the acetone powder and Fractions 1,2,4, 8, 9 and 15.

SUMMARY OF RESULTS PERTINENT TO SPECIFIC OBJECTIVES

Cation-exchange chromatography with Sephadex C-50 was carried out on human plasma as described by Guenther. Although about 5 μU IRI/ml plasma was found in the most cationic fraction eluted from the column, significant quantities of heparin-induced IRI were not found in any fraction, nor did heparin increase IRI in normal human serum and plasma. Thus, heparin was not used in subsequent experiments designed to elucidate a serum insulin-binding protein.

Sephadex G-200 gel chromatography of ^{125}I-insulin (6.2-70 μU/ml) added to normal human serum and plasma revealed that less than 5% of radioactivity was associated with a protein fraction that included albumin. This pattern was not changed by treatment of the mixture with biotin (10 g/ml) or a ten-fold excess of unlabeled insulin, or prolonged incubation (5 days, 4°C) of the ^{125}I-insulin-plasma solution. High molecular weight radioactivity was unaffected by rechromatography with or without excess unlabeled insulin (1 mU). Dextran-coated charcoal analysis of the high molecular weight I-125 activity revealed that radioactivity was not dissociated by a thirty-fold excess of unlabeled insulin or 8 M urea. Affinity chromatography of this material showed that more than 80% of radioactivity was associated with albumin and about 10% exhibited a molecular weight of less than 2,000 daltons.

In order to determine the nature of serum ILA, human cell lines were examined for their ability to serve as the basis for an in vitro bioassay capable of discriminating specific ILA and the pleiotypic effects of growth factors. I found that WI38 human fetal lung fibroblasts possess cell-surface insulin receptors. Physiologic concentrations of insulin added to replicate culture wells in medium containing 10% fetal bovine serum caused a significant increase in the rate of glucose uptake by these cells and inhibited traversal of the cells from lag phase to log phase. Both of these effects were found to be dose-dependent. The three of four malignant human cell lines that were found to possess insulin receptors also showed significant increases in glucose uptake rate and inhibition of cell proliferation in response to various concentrations of insulin. The one cell line negative for insulin receptors showed neither insulin response.

A cultured fibroblast bioassay for insulin was developed by maintaining WI38 cells in stationary phase in serum-free medium in multiple-well plastic cluster dishes for 4 days at 37°C, after which cells were counted in a hemacytometer and medium glucose was measured with a Beckman Glucose Analyzer. Insulin or other preparation to be tested was added to medium before addition to cells previously plated in wells. Cell number was not affected by physiologic concentrations (25-100 μU/ml) of insulin after 4 days. Total ILA measurements were based on the curvilinear increase of the parameter U (glucose uptake/control glucose uptake) with increasing insulin concentration, while measurements of specific ILA were based on the linear increase of the parameter R (rate of glucose uptake/control rate of glucose uptake) with increasing insulin concentration. Stimulation of cell proliferation was determined from the parameter ν(cell number/control cell number).

Concentration of normal human serum by ultrafiltration through a M.W. 10 kd cutoff membrane (PM 10) apparently caused the liberation of large amounts of IRI in the retentate (100 times the serum level). Less than 5% of serum IRI passed through the membrane, even in the presence of 8 M urea, but acid treatment caused two-thirds of serum IRI to pass through the membrane. Values obtained for both total ILA and specific ILA were similar to IRI in this fraction, but in serum these values were ten times the IRI. Less than 1% of serum ILA and three-fourths specific ILA passed through the membrane after treatment of a retentate fraction with 8 M urea.

On Sephacryl S-200 gel chromatography of a serum PM 10 retentate fraction, about 90% of the IRI apparently generated during serum concentration eluted at a position corresponding to proteins of M.W. <10 kd, but significant quantities of IRI eluted in discrete peaks at higher molecular weights. Dextran-coated charcoal analysis of elution fractions indicated a maximum serum insulin binding capacity of 4 μU/ml. Dextran-coated charcoal analysis also revealed no reversible binding of ^{125}I-insulin to acid-treated human serum and high molecular weight serum fractions.

Gel chromatography of a human serum acetone powder revealed that most serum ILA eluted at about 250 kd M.W., with virtually all of the serum's capacity to stimulate cell proliferation. Both biotin and heparin enhanced as well as decreased total ILA, specific ILA and cell number in various fractions. About half of serum IRI eluted at positions corresponding to proteins of M.W. >18 kd. Although only 0.5 μU IRI/ml serum was found at the column exclusion volume (Fraction 2), with large quantities of dilution-enhanced cell proliferation stimulation activity, dilution of this fraction also greatly enhanced specific ILA,

which attained a maximum of about 400 μU/ml serum at an equivalent of 0.2 ml serum/ml, representing about 20% of the total ILA in the fraction.

CHAPTER VIII

DISCUSSION

IS THERE A SERUM INSULIN-BINDING PROTEIN?

By 1965, two major lines of evidence supported the existence of an insulin-binding protein in human serum: 1) AIS-suppressible ATE-potentiated ILA in serum and BI fractions (390,511) and 2) apparent association of radiolabeled insulin with human serum α - γ globulins (520-522). However, Berson and Yalow and others were unable to enhance IRI of human serum and BI fractions by treatment that potentiated ILA measured by bioassays (523, 524). Furthermore, Berson and Yalow demonstrated that apparent association of radiolabeled insulin with globulins was, in fact, irreversible binding of damaged tracer to serum proteins, especially albumin (515). These investigators also refuted the existence of a serum insulin-binding protein on theoretical grounds, pointing out that the known effects of hormone-binding proteins are inconsistent with insulin decay kinetics *in vivo* and that insulin-binding antibodies in diabetics are highly disruptive of insulin-dependent carbohydrate homeostasis (528).

On the other hand, insulin-like growth factors (IGFs), such as NSILA-S (IGFs I and II) and the somatomedins, which show extensive homology with insulin and presumably derive from a common ancestral gene, have recently been shown to have serum binding proteins (557, 573, 585, 598, 602, 611, 612). Thus, an insulin-binding protein is a plausible expectation. Finally, Guenther's

demonstration of heparin-induced IRI in BI fractions (387) both confirmed Gundersen and Lin's observations with a rat diaphragm assay (391) and provided direct evidence for a human serum insulin-binding protein.

In the present study, Sephadex C-50 chromatography of human plasma using Guenther's protocol resulted in elution of 10 μU IRI/ml plasma retained on the cation exchanger, about half the IRI that Guenther found in the untreated BI fraction. About 43% of this IRI was eluted in the most cationic fraction, but the same quantity of heparin employed by Guenther did not significantly increase the IRI in this or any other fraction. Nevertheless, this experiment confirms the presence of cationic IRI in human plasma. The presence of an insulin-binding protein in human serum and plasma was also supported by loss of approximately 30% exogenous insulin added to CPD plasma and both plasma and serum after treatment with an anion-exchange resin (to remove insulin), measured by the dextran-charcoal RIA. However, these results could also be explained by potentiation of degradative enzyme activity. Again, heparin did not enhance IRI in any serum or plasma sample. Systematic examination of the ^{125}I-insulin binding capacity of human serum and plasma using gel chromatography and dextran-coated charcoal as employed by Zapf et al. (557) to elucidate the NSILA-S serum binding protein revealed that less than 5% of the I-125 activity was associated with material in the molecular weight range 50,000-160,000 daltons. This material was stable to dialysis and rechromatography and was apparently unaffected by treatment with biotin or large quantities of unlabeled insulin, suggesting irreversible binding of radiolabeled material.

Dextran-charcoal analysis of this high molecular weight radioactivity revealed no liberation of free I-125 activity by unlabeled insulin or 8 M urea,

confirming the irreversibility of the association. Subsequent dextran-charcoal analysis of Sephacryl S-200 fractions of a serum PM 10 retentate in which a large quantity of monomeric IRI was apparently liberated again revealed a maximum 6% binding of ^{125}I-insulin that could not be reversed by unlabeled insulin or 6 M urea. If the insulin had been dissociated from a serum carrier protein during ultrafiltration, one or more high molecular weight fractions would be expected to exhibit a large reversible binding capacity for ^{125}I-insulin.

Immunodiffusion analysis and SDS polyacrylamide gel electrophoresis revealed that the high molecular weight radioactivity (peak A) consistently eluted with the major fraction of albumin. Affinity chromatography of A-peak material in fact showed that five-sixths of the I-125 activity was bound to albumin and that about 10% activity exhibited a molecular weight of less than 2,000 daltons, confirming Berson and Yalow's contention that apparent association of radiolabeled insulin with serum components represents irreversible binding of damaged tracer to proteins, particularly albumin. Thus, it must be concluded that no physiologically significant insulin-binding protein exists in normal human serum or plasma.

WHAT IS ILA?

The reason that findings of atypical IRI were judged to be the most pertinent evidence for a serum insulin-binding protein despite extensive findings of atypical ILA measured by various bioassays relates to the subsequent discovery of numerous substances that mimic insulin's effects in bioassays, but possess structures and physiologic roles that differ from those of the hormone.

The extent of the discrepancy between typical insulin and atypical ILA is suggested by the fact that more than 90% of serum ILA is not suppressible by AIS (527, 546).

Much, if not all, of this NSILA is due to the effects of serum and plasma growth factors, several of which exhibit "specific" ILA (e.g., stimulation of glucose uptake and lipid and glycogen synthesis) at supraphysiologic concentrations. These IGFs include NSILA-S (IGFs I and II), which comprises about 10% of total serum NSILA, the somatomedins and MSA (546, 547, 550, 629). Poffenbarger (563) also showed that an antiserum raised against NSILP, which accounts for 70-80% human serum NSILA, reacts with MSA. In addition, non-IGF growth factors, such as FGF and EGF, may exert a pleiotypic effect on cells that could produce ILA in certain bioassays. For instance, BI may contain FGF as well as IGFs (717).

Clearly, a bioassay capable of discriminating specific ILA and growth potentiation would be a very useful tool in dissecting the nature of serum ILA. In this study, a cultured fibroblast bioassay apparently exhibiting this discrimination and a double-antibody RIA for insulin were used to characterize human serum fractionated according to molecular size.

The WI38 fetal lung fibroblast line is the only one of five human cell lines studied that was derived from a nonmalignant source. WI38 cells exhibit a diploid karyotype (740) and have a finite lifetime of 50 ±10 population doublings (741). The doubling time (t_D) of 30 hours reported by Houck and Cheng (716) is similar to the 22-hour t_D found in this study. Although these cells were used up to 54 generations, the consistency of t_D and rate of glucose uptake (r) indicates that they had not become senescent. Of the five cell lines studied, SK-HEP-1

was the only one that was negative for insulin receptors by both ^{125}I-insulin adsorption and PAP immunocytochemical staining. This cell line was also the only one that showed no significant increase of r in the presence of insulin.

Although the initial study of WI38 human fetal lung fibroblast growth in the presence of serum indicated a decrease of r at confluence, subsequent studies of this cell line demonstrated that r remains relatively constant at -3 to -4 mg/10^6·day in log-phase, stationary and confluent cultures. Thus, the rate of glucose uptake appears to be independent of cell number and medium glucose concentration, contrary to other reports (554, 724-726), suggesting a facilitated or active glucose transport mechanism. The rate of glucose uptake exhibited by the other cell lines studied indicates that r is apparently independent of rate of cell proliferation and malignant transformation.

Suprisingly, despite extensive documentation of insulin as a growth potentiator in pharmacologic doses, the hormone was found to inhibit proliferation of cultured cells positive for insulin receptors in this study when added at the time of plating in concentrations of 10 μ U/ml-10 mU/ml. Only SK-HEP-1 proliferation was apparently unaffected by insulin, suggesting that this effect, as well as stimulation of glucose uptake, is mediated through insulin receptors and thus is insulin-specific, rather than being due to an insulin contaminant. This contention is supported by the fact that these effects are elicited by both human and bovine insulin preparations and is confirmed for enhancement of r by reversal in the fibroblast bioassay by AIS. The absence of toxic contaminants in insulin preparations is further indicated by the normal appearence of insulin-inhibited cells, which did not exhibit excessive granulation or floating debris, and the lack of insulin effect on cell number when the

hormone was added during log phase, confluence or stationary phase. Therefore, the concentration-dependent insulin inhibition of cell proliferation appears to involve a hormone mechanism specific for lag-phase cells, in which cells are prevented from exiting lag phase or the subsequent log-phase cell cycle is somehow prolonged.

A possible mechanism is suggested by Shapiro and Lam's observation that intracellular Ca^{++} concentrations increase as human fibroblasts age in culture (742). Cultured fibroblast senescence is characterized by cessation of cell division (741, 743) and several investigators have provided evidence that insulin increases intracellular Ca^{++} concentrations (see Chapter III). Teng et al. (744) have found that 16 mU insulin/ml shortens G_1-phase and extends the S- and G_2-phase of CEF in culture, while Koontz and Iwahashi (745) showed that physiologic concentrations of insulin (5×10^{-11} M) cause rat hepatoma cells to exit G_1-phase. Thus, it is unlikely that lag-phase is G_1-phase. Inhibition of cell proliferation by insulin is also consistent with the hormone's physiologic role, which is to encourage energy storage through synthesis of macromolecules.

Use of cultured WI38 human fetal lung fibroblasts for an insulin bioassay is well-suited to examination of the nature and effect of human serum ILA on normal human cells since this cell line is diploid and well-characterized. Artifacts that could be encountered using malignant or non-human cells are exemplified by the evident loss of insulin receptors by SK-HEP-1 hepatoma cells, production of a growth factor (MSA) by Coon rat liver cells, rendering them serum-independent (629), and the fact that insulin is a much more potent mitogen for avian fibroblasts than for mammalian fibroblasts (616).

In this study, a significant, specific (AIS-reversible), consistent, dose-

dependent insulin-induced increase in rate of glucose uptake by cultured WI38 fibroblasts is well documented. In addition, the use of stationary medium according to the method of Morell and Froesch (615) enables discrimination between specific ILA (ILA_S) and the pleiotypic effects of growth factors. The insulin dose-dependent increase in the parameter U (glucose uptake/glucose uptake of control) is appropriate for measurement of total ILA since it is independent of cell number and does not discriminate between increments of U due to the stimulation of cell proliferation and those due to increase of r. ILA_S is best measured by determination of the parameter R (r/control r), which also increases with increasing insulin concentration. However, because this parameter is dependent upon cell number, factors that cause loss of cells can yield artifactual enhancement of ILA_S. Therefore, ILA_S values were ignored where ν (cell number/control cell number) fell below the control population (< 0.71). The validity of these assay parameters is indicated by the fact that both the ILA (177 μU/ml) and ILA_S(208 μU/ml) found for normal human serum are very close to the mean fat-pad assayable serum ILA (180 μU/ml) reported by Froesch et al. (527).

Similar quantities of ILA were recovered in the total of Sephacryl S-200 chromatography fractions (each assayed in 1-2 ml serum equivalents) from a human serum PM 10 retentate (PM10r1, M.W. >10,000, ILA recovered =242 μU/ml serum) and from a human serum acetone powder (133 μU/ml serum). However, total ILA recovered from the PM10r1 fractions represents only 14% of the 1,735 μU ILA/ml serum found when PM10r1 was assayed at a 0.035 ml serum equivalent, a value similar to ILA concentrations obtained by Gundersen and Lin (391) for dilute human sera using the fat pad assay. No ILA_S was

detected in the tenfold diluted PM10r1 preparation, suggesting the dilution-enhanced ILA is due to attenuation of inhibitors of one of more serum growth factors. This possibility is supported by dilution enhancement of PM10r1 stimulation of WI38 cell proliferation, to 5 and 40 times the rate expected with equivalent amounts of FBS and human serum, respectively, based on cell proliferation rates in the presence of 10% concentrations of these sera. Furthermore, numerous S-200 fractions caused decreases in cell number relative to controls and several exhibited frank toxicity, evidenced by disintigration of cells. The possibility that high molecular weight lipidic material constitutes an important class of growth factor inhibitors is supported by abolition of toxicity in exclusion fractions by extraction of fractions or serum with acetone, enabling measurement of cell growth stimulation by these fractions.

About 5% recovered PM10r1 ILA eluted at 6,800-7,800 daltons, where free NSILA-S would be expected. Only 5% recovered serum acetone powder ILA eluted around 70,000 daltons, where NSILA-S associated with its carrier protein should be found. However, nearly 20% ILA, coincident with an ILA_S peak, eluted from both columns between 30,000 and 50,000 daltons. Zapf et al. (557) reported that considerable amounts of serum NSILA-S-binding activity was also detected at molecular weight 30,000-40,000 daltons, indicating that the binding protein may be present in serum partly as oligomers. The results of this study support the contention that a 35,000-dalton protein represents the primary NSILA-S -binding component of human serum. About 10% ILA eluted from both columns between 12,500 and 17,500 daltons, suggesting a NSILA-S dimer. All these putative NSILA-S fractions contained concomitant ILA_S and did not stimulate significant cell proliferation. The ability of the cultured fibroblast assay to

detect serum protein-bound NSILA-S is supported by passage of about one-third PM10r2 ILA through a PM 10 membrane after treatment of the fraction with 8 M urea. The acetone powder ILA peak at about 10,000 daltons may be due to MSA. This assay, however, would not be expected to detect EGF or FGF.

About 20% of acetone powder ILA eluted at the column exclusion volume with a peak of cell proliferation potentiation. This activity is very concentration-dependent, however. When Fraction 2 was assayed at a 1.9-ml serum equivalent, complete suppression of both ILA and cell growth potentiation was seen, but both these parameters increased markedly as the fraction was diluted, until at 0.2 ml serum equivalent it exhibited 1,755 μU ILA/ml and 41% total acetone powder growth potentiation. In addition, 1.5-1.8 ml serum equivalent of Fractions 3 and 4 caused WI38 fibroblasts to form multilayered foci in which the cells grew in a random orientation, satisfying a morphologic criterion of malignant transformation (746). Thus, a serum growth factor of approximately 240,000 daltons molecular weight not only accounts for the entire potentiation of WI38 cell proliferation by human serum, but also is the apparent cause of serum dilution-potentiated ILA. The dilution enhancement of ILA and ν may be due to attenuation of an inhibitory substance or a concentration optimum of factor-receptor interaction. This growth factor may be an aggregate form of NSILA-P or the WI38 growth factor, or an as-yet uncharacterized growth factor. However, only 8% of acetone powder ILA, with concomitant ILA_S and without growth stimulation, eluted between 92,000 and 130,000 daltons, where NSILA-P and WI38 growth factor would be expected, but a major peak of cell proliferation, containing 8% ILA and no ILA_S, eluted at half the molecular weight, 49,000-65,000 daltons.

On the other hand, the exclusion-volume cell proliferation stimulating activity may represent aggregate forms of transforming growth factors (TGFs) and platelet-derived growth factors (PDGFs) bound to serum carrier proteins. However, only a biotin-potentiated peak of cell proliferation was found in a PM10rl S-200 fraction corresponding to molecular weight ≈37,000 daltons, within the range of 30-40 kd reported for PDGF (747), and no stimulation of cell proliferation was found in the molecular weight range 10-20 kd, where TGFs would be expected to elute (748).

IS THERE OCCULT INSULIN?

Although the results of this study indicate that serum ILA could be due totally to IGFs and other growth factors, there also remains evidence for the existence of an occult form of insulin in human blood. Like Guenther, I found a significant amount of IRI (about 4 μU/ml plasma) in highly cationic plasma fractions. Furthermore, more than half the IRI in a serum acetone powder eluted from Sephacryl S-200 at positions corresponding to proteins in excess of 18,000 daltons molecular weight.

If occult insulin cannot be due to an as-yet uncharacterized specific serum insulin-binding protein, then it most likely is due to the one known insulin-binding protein: insulin. Elution of serum IRI peaks from Sephacryl roughly corresponds to what would be expected for the dimer, tetramer, hexamer, di-hexamer and hexa-hexamer and, in fact, the ILA ascribed to IGFs could also be due to insulin. Since insulin aggregates, especially the hexamer and polyhexamers, are expected to exhibit only a small fraction of monomeric IRI,

this high molecular weight serum IRI could represent a much greater absolute amount of insulin —as much as 2 mU/ml or more, which was apparently "liberated" in PM10r1. However, this phenomenon could not be reproduced (although the circumstances of the reproduction were not identical to the original conditions) and comparable quantities of IRI were not found in PM10r2 and UM2r2, the fractions resulting from dilution and reconcentration of one-half PM10r1. Unfortunately, the possibility that this IRI was an artifact due to contamination of the fraction with porcine insulin was unable to be tested since the RIAs employed were unable to distinguish between human and porcine insulins. The existence of insulin aggregates in human serum is also supported by a 2- to 3-fold enhancement of IRI and passage of two-thirds to three-fourths of this IRI through a PM 10 membrane after treatment of human serum or PM10r2 with acid, whereas $\leq 1\%$ untreated serum or PM10r2 IRI passes through the membrane.

A specific ILA of 415 μU/ml serum was found at a 0.2 ml serum equivalent of acetone powder S-200 Fraction 2, which exhibited an IRI of only 0.5 μU/ml serum. The ILA_S in the assay well at this dilution thus was about 80 μU, nearly the optimum insulin concentration for this assay. This result, therefore, indicates that this fraction actually contains about 1,000 times more insulin than the IRI (consistent with insulin aggregates, which would be expected to exhibit only a small fraction of the IRI of monomeric insulin) and that circulating insulin aggregates may account for 20-25% total serum ILA.

The exceedingly small proportion of aggregates found in neutral aqueous insulin solutions at physiologic concentrations of the hormone (and of Zn^{++}) and calculations based on equilibrium association constants determined for insulin

aggregates has led to the general conclusion that significant quantities of insulin aggregates cannot exist in systemic circulation. However, these studies involved measurement of insulin association at equilibrium after crystalline insulin was dissolved at acid pH. In vivo, crystalline insulin is released into the circulation at pH 7.4. Therefore, a more appropriate model of insulin aggregates in vivo would be measurement of crystalline insulin dissociation in dilute neutral solution. An example of such an experiment is measurement of the appearance with time of monomeric IRI in a stirred suspension of 80 μg crystalline zinc-porcine insulin in one liter 5% BSA/pH 7.4 PBS at 37°C.

The possibility that the equilibrium association constants observed for the formation of insulin aggregates results from rates of dissociation far slower than relatively slow association rates, to the extent that insulin aggregate dissociation in vivo is far from equilibrium is supported by examination of insulin aggregate characteristics iterated in Chapter V. Whereas hydrogen bond formation between amino acid substituents cannot be expected to contribute to subunit association (and, in fact, should add an unfavorable ΔG increment since organic nitrogen and oxygen form stronger hydrogen bonds with water than with each other (749)), the 4 hydrogen bonds per dimer and 6 additional hydrogen bonds per hexamer contribute a ΔG of about +2.4 kcal/mole each to dissociation of these aggregates (749). The net ΔG of about +0.75 kcal/mole required to break amino acid-water hydrogen bonds and form internal hydrogen bonds during subunit association is reversed upon dissociation. Likewise, the ΔG involved in displacement of water coordinated to Zn^{++} by coordination of amino acid substituents to Zn^{++} during formation of the Zn^{++} tetramer and hexamer is reversed during dissociation of these species.

Therefore, the - ΔG underlying the positive equilibrium association constants observed for insulin aggregation is probably due primarily to transfer of apolar amino acid side chains from water to an apolar environment. However, if the 12 apolar side chains that appear to make contacts in the dimer and the 42 additional apolar side chains that have been implicated in the hydrophobic interactions of hexamer formation were completely buried, yielding a ΔG of about -2.5 kcal/mole each (750, 751), the resultant equilibrium association constants would be much higher than those observed, suggesting that these side chains are not completely buried. This contention is supported by the fact that the A14 tyrosines and B25 phenylalanines are exposed at the surface of the hexamer (464).

Nevertheless, amino acid-water hydrogen bonds and Zn^{++} - water coordinate bonds must first be broken before hydrophobic interactions can occur during subunit association, while not only internal hydrogen and coordinate bonds must be broken, but energy must also be expended to transfer apolar side chains to water before the - ΔG of water-bonding can be realized during aggregate dissociation, indicating that the rate of dissociation must be much slower than the rate of association. Studies of insulin aggregation in neutral solution have indicated that the 2-Zn^{++} hexamer is essentially completely dissociated at concentrations below 10^{-7} M (472). Therefore, if hexamers exist in the systemic circulation at a concentration of 10^{-9} M, they are less than 5% dissociated. In other words, if significant quantities of insulin aggregates exist in the systemic circulation—and the results of this study indicate that they do—it can only be due to an extremely low rate of dissociation, much lower than the rate of systemic degradation of monomeric insulin so that insulin aggregate dissociation

is extremely limiting. Furthermore, since examination of the primary structure of insulin reveals that the hormone possesses a net charge of -1 per monomer at neutral pH, cationic IRI could represent > 2 Zn^{++} tetramers and hexamers.

The disruption of glucose homeostasis by insulin-binding proteins postulated by Berson and Yalow would not apply to insulin aggregates because of a fundamental difference between the nature of competitive protein binding, such as that which occurs with antibodies, carriers and receptors, and insulin aggregation. The former would be expected to involve high-avidity interactions, ie. a very rapid association and a relatively rapid dissociation, enabling displacement by additional ligand or dilution, while the latter apparently involves a very slow dissociation and a relatively slow association. This supposition is supported by the fact that 8 M urea caused no generation of monomeric IRI in PM10r2, while acid treatment of this fraction resulted in passage of 75% IRI through a PM 10 membrane. Provided the systemic insulin degradative mechanism remains intact, monomeric insulin provided by aggregate dissociation will be destroyed nearly as quickly as it is generated.

What, then, is the purpose of circulating insulin aggregates? One possibility is that it is the harmless consequence of an elegant mechanism of acute secretion of a hormone that causes the rapid utilization of nutrients, like the proinsulin coincidentally secreted with insulin. The other possibility is that circulating insulin aggregates serve a physiologic function. One possible function is suggested by insulin enhancement of glucose transport across the blood-brain barrier (752). An acute requirement for glucose by the central nervous system may arise during a prolonged fast, when serum insulin and glucose levels are basal. Although nervous stimulation of pancreatic insulin release has been

demonstrated, the physiologic role of this phenomenon is unclear (753). Certainly, neural induction of pancreatic insulin secretion during a prolonged fast would appear to be both wasteful and dangerous, since most of the serum glucose would be absorbed by peripheral tissues and a severe hypoglycemia would ensue before a glucagon-induced release of glucose by the liver could occur. If brain capillary endothelium, for instance, possessed an inducible enzyme system capable of dissociating insulin aggregates *in situ* however, insulin-stimulated glucose uptake would occur where needed and serum glucose levels would fall slowly enough to enable compensation by liver glucose release. In addition, a precedent for enzyme-mediated dissociation of macromolecular aggregates stabilized by hydrogen bonds and hydrophobic interactions exists within the most ancient forms of life: DNA polymerases.

Besides the *in vitro* study of crystalline insulin dissociation mentioned above, the following experiments would be helpful in ascertaining the existence and role of circulating insulin aggregates.

1. The existence of insulin aggregates in blood should be verified by further purification of gel chromatography fractions containing high molecular weight serum IRI, using ion-exchange chromatography; purified IRI should then be subjected to gel chromatography after acid treatment to determine whether monomeric IRI is generated.

2. The hypothesis that insulin aggregates dissociate slowly *in vivo* should be tested by monitoring blood IRI and glucose in fasted animals infused with specific inhibitors of the insulinases, such as antibodies raised against the enzymes and biologically inactive insulin analogs (*eg.*, A- and B-chains and DOP-insulin).

3. The existence of an insulin aggregate dissociating enzyme should be ascertained by testing for generation of monomeric IRI in human serum during prolonged incubation after concentration and in the presence of tissue homogenates, especially of brain.

THE EFFECTS OF HEPARIN AND BIOTIN ON SERUM ILA

Heparin did not enhance the IRI of human serum or plasma, even after treatment with an anion-exchange resin, and it did not cause significant decrease in recovery of IRI from serum or plasma. Neither did it significantly affect the IRI of plasma cation exchange fractions and it did not cause IRI to be passed through a PM 10 membrane, indicating that heparin does not potentiate dissociation of insulin aggregates or a putative hormone-protein complex. Heparin also did not increase serum ILA or ILA_S and did not appear to dissociate NSILA-S from its carrier protein, since, where it increased ILA (eg., in dilute PM10r1), it did not increase ILA_S. On the other hand, several instances of heparin enhancement of ILA_S without increase of ILA are probably artifactual. The possibility that heparin enhances serum ILA by potentiating the effects of one or more growth factors is supported by heparin-stimulated WI38 cell proliferation with PM10r1, PM10r2, PM10r1 S-200 Fraction 10 (M.W. 33-42 kd), and human serum acetone powder S-200 Fraction 1 (M.W. >240 kd). Generally, it is expected that heparin, as a polyanion, will potentiate anionic effecters by complexing cationic inhibitors and will inhibit cationic effecters. Therefore, heparin would be expected to potentiate the effects of NSILP, SMB, MSA and WI38 growth factor, and to inhibit NSILA-S and SMC. The expected effects on

ILA and/or cell proliferation were seen in fractions corresponding to free NSILA-S and NSILA-S bound to a carrier protein of about 30 kd M.W., NSILP and SMC, but not in fractions where SMB, MSA or WI38 growth factor would be expected to elute.

Biotin appeared to cause a significant decrease in the recovery of IRI from serum. However, S-200 chromatography of exogenous radiolabeled insulin in human serum after treatment with biotin showed no difference in magnitude or position of eluted radioactive and protein peaks relative to the control, indicating that biotin causes neither dissociation of an insulin-protein complex nor enhancement of serum proteolysis. Biotin also did not significantly stimulate cell proliferation or ILA of human serum or bulk serum fractions, but did increase these parameters in specific serum S-200 fractions. The possibility that biotin enhancement of ILA in serum fractions is due to a synergistic potentiation of growth factor effects by the substance is supported by a report that biotin stimulates guanylate cyclase activity in rat tissues (754). The resultant increase in intracellular concentrations of cGMP is known to stimulate cell proliferation in many systems (296). In this study, however, biotin alone did not affect the RIA and neither heparin nor biotin alone affected the fibroblast bioassay.

Finally, there remains the question of heparin-induced IRI. I found that heparin interferes with the RIA, causing a partial dissociation of antigen-antibody complexes that would result in false increments of IRI, but not at the concentration employed by Guenther. Neither did Guenther find any effect of this amount (100 μg/ml) of heparin on his RIA (387). For his study, however, Guenther employed a pool of patient sera prepared by the Foster G. McGaw Hospital Clinical Chemistry Laboratory, reasoning that this pool "should thus

represent a good cross-section of sera". There is also an excellent possibility that this pool included sera from diabetics whose blood often contains large quantities of exogenous insulin complexed by anti-insulin antibodies (409). Although high-affinity guinea pig anti-insulin antibodies are unaffected by 100 μgheparin/ml, relatively low affinity human anti-insulin antibodies (449) may be partially dissociated from antigen by this concentration of heparin, yielding concentrations of free insulin that could produce the increments of IRI observed by Guenther.

In conclusion, the results of this study indicate that there is no serum insulin-binding protein, that most (≥ 75%) of the serum ILA observed using in vitro bioassays is due to the effects of growth factors, and that significant quantities of circulating insulin aggregates may exist in vivo and play a physiologic role in carbohydrate homeostasis.

SUMMARY

With the advent of in vitro bioassays for insulin, human blood components that mimic the effects of insulin in these assays, yet do not possess the physicochemical properties of insulin were discovered. Specifically, serum substances retained by cation-exchange resins were found to enhance glucose uptake and utilization by rat diaphragms and epididymal fat pads, but did not react with anti-insulin antibodies. This atypical insulin-like activity (ILA) led to speculation that a fraction of circulating insulin is complexed to a specific carrier protein. In 1974, H. Guenther (Loyola University, Chicago, Ph.D. Dissertation) lent support to this contention by demonstrating that insulin concentrations measured by radioimmunoassay (RIA; immunoreactive insulin, IRI) in cationic human serum fractions were increased by heparin, a polyanion, and that ILA measured by in vitro bioassays in these fractions was increased by heparin and biotin. The present study was undertaken to test the reproducibility of these results and to ascertain more specifically the nature of atypical ILA in human serum and plasma using a cultured fibroblast bioassay capable of discriminating specific ILA and the pleiotypic effects of growth factors.

Four hundred fifty milliliters normal human plasma was mixed with ten grams Sephadex C-50 cation exchanger after hydration of the gel, which was then transferred to a 2.5 x 100 cm column and eluted with a 0.005-1.5 M potassium chloride gradient. Although about 5 μU IRI/ml plasma was found in the most cationic fraction eluting from this column, heparin did not significantly affect the IRI of this or any other fraction. Heparin also did not increase the IRI

of normal human serum or plasma, or bulk serum fractions prepared by ultrafiltration, nor did it significantly decrease the recovery of exogenous IRI from serum or plasma.

A systematic examination of the ability of human serum and plasma components to bind exogenous radiolabeled insulin specifically and reversibly, using Sephadex G-200 gel chromatography and dextran-coated charcoal, revealed that less than 5% of the I-125 activity eluted with albumin (A-peak) and less than 1% radioactivity eluted at the column exclusion volume (C-peak, molecular weight >360,000 daltons), while about 90% of the added radioactivity eluted at positions corresponding to molecular weights less than 40,000 daltons (B-peak, M.W. <40 kd) and was immunochemically identical to free ^{125}I-insulin. The size and relative positions of the high molecular weight radioactive peaks were unaffected by rechromatography, biotin or 1-2.25 mU unlabeled insulin. A thirtyfold excess of unlabeled insulin and 8 M urea also failed to liberate free insulin from A-peak material, as determined by dextran-charcoal adsorption of I-125 activity. Affinity chromatography of A-peak material through columns of anti-albumin antibodies covalently coupled to Sepharose 4B revealed that 84% of A-peak radioactivity was bound to albumin and an additional 11% radioactivity exhibited a molecular weight of less than 2,000 daltons, confirming Berson and Yalow's contention that exogenous radiolabeled insulin apparently associated with serum components represents "damaged" label irreversibly bound to serum proteins, particularly albumin, and that a physiologically significant insulin-binding protein does not exist in human serum.

Cultured human fibroblasts maintained in stationary phase in the absence

of serum were found to offer a means for discriminating the effects of growth factors from specific ILA since the rate of glucose uptake by these cells is significantly stimulated by insulin in a dose-dependent manner. The use of 1-ml cultures in multi-well plates affords a convenience of replicate determinations hitherto lacking in in vitro bioassays. Use of this bioassay to examine human serum fractionated by molecular size revealed that virtually all of the capacity of human serum to stimulate cell proliferation and about 95% potential serum ILA reside in one or more serum components of M.W. ≈ 240 kd. Most of the remaining ILA was consistent with well-characterized insulin-like growth factors. Half of serum IRI eluted at M.W. >18 kd, suggesting that appreciable amounts of insulin aggregates exist in the peripheral circulation. Both biotin and heparin enhanced ILA in certain serum fractions concomitant with potentiation of cell proliferation, but without stimulation of glucose uptake rate, indicating that previously reported increase of ILA by these agents is due to potentiation of serum growth factors.

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

January 3, 1984
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