ABSTRACT

AN IMPROVED RADIOIMMUNOLOGICAL METHOD
FOR SERUM FREE INSULIN AND
ANTIBODY-BOUND INSULIN

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A practical, sensitive, and specific method for the assay of serum free insulin and antibody-bound insulin in insulin-treated diabetic patients has been developed and clinically evaluated. Clinical evaluation was carried out at the Youngstown Hospital Association in cooperation with the Department of Laboratories and several staff physicians.

The method for free insulin involves the use of aqueous polyethylene glycol which causes the precipitation of the free antibody and the antibody-bound insulin with little or no precipitation of the free insulin. The free insulin in the upper phase was then measured in a simplified, but sensitive radioimmunological system.

Free insulin was found to be completely soluble in a 12% w/v final concentration of 6000 molecular weight polyethylene glycol. Two other polyethylene glycol solutions with average molecular weights of 1,600 and 20,000 were studied to determine their ability to extract the free insulin and precipitate the insulin antibody complex. Only the 6000 molecular weight compound was found to be suitable.

Particular attention was focused on the use of the polyethylene glycol extract containing the free insulin in the radioimmunoassay (RIA) system. The pH of the extract was found to have a range of 8.32 to 8.52; however when introduced into the RIA system, employing a phosphate-saline buffer, the pH was 7.40 $\pm$ 0.02. This pH range was considered optimum for the RIA system.

Studies were conducted on normal serum where insulin antibodies were absent to determine the correlation between the free insulin method and the "Phadebas Insulin" reference method. Results of the methods were compared at many different insulin levels. In 40 determinations, standard deviation, coefficients of variation, correlation coefficient, and t test values were determined and found to be within acceptable limits. The mean value for the Reference method at all insulin levels on these 40 samples was 61 µU/ml. In the same 40 determinations and for the same insulin levels, the mean values for the free method were 60 µU/ml.

The method for total insulin requires prior treatment of serum with 1 N HCl to break the conjugation of the insulin antibody complex. The total insulin is then extracted with polyethylene glycol and assayed in the RIA system. The antibody-bound insulin is the total insulin minus the free insulin.

The polyethylene glycol extract in the total insulin method after neutralization has a pH range of 8.20 to 8.80. When the extract is introduced into the RIA system employing the phosphate, saline buffer, its pH was 7.40 ± 0.02.

Studies were conducted on the non-insulin-treated patients to determine the correlation between the total insulin method and the "Phadebas Insulin" reference method. In addition, correlation experiments to compare the total method and the free insulin method were performed. In 40 determinations, standard deviation, coefficients of variation, correlation coefficient, and t test values were determined at all levels of insulin concentration. In the same series of 40 measurements described for the reference and free insulin methods, the total insulin method gave a mean value of 59 μU/ml.

Recovery studies of added insulin were performed for both the free and total insulin methods. The free method gave an average recovery of 85% at all levels with better recovery at the 50-100 uU/ml range. The total method gave an average recovery of 87% at all levels with better recovery at the 100-300 range.

Clinical studies on insulin-treated diabetic patients as well as non-insulin-treated diabetic patients were conducted. In the non-insulin-treated patients, good correlation was found by all three methods (reference, free, and total). Mean values for these patients by the three methods were 22.7, 21.8, and 22.1 μU/ml respectively.

In the insulin-treated patients, where insulin had been taken for 30 days or more, antibody-bound insulin levels were found to be present in varying amounts depending, in part, on length of insulin therapy and amount of insulin taken. The range of all insulin-treated patients studied for free insulin was 10 to 440 with a mean of 46.7 μU/ml. The range for the same patients for total insulin was 67 to 17,920 with a mean of 2,718 μU/ml.

Two patients studied with bound insulin levels of 4992 and 17,480 μU/ml and who were difficult to control, were immediately started on pork insulin therapy in an effort to reduce their immunological response to the exogenous insulin.

The methods provide a practical approach to following the dynamics of insulin therapy in the presence of insulin antibody and can be used to quantitate the level of antibody present in terms of bound insulin. In addition, the Free method could be used to determine the amount of endogenous insulin produced by the pancreas in insulin-treated diabetic children where remission in insulin requirements has occurred.

ACKNOWLEDGEMENTS

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SYMBOL	DEFINITION	UNITS
w/v	Weight per volume	g/100 ml
RIA	Radioimmunoassay	
μU/ml	Micro units per milliliter 1/1,000,000 part of the unit per milliliter	
N	Normal solution	equivalent weight in g/l
HCl	Hydrochloric Acid	
pH	Hydrogen ion potential	millivolts
^{131}I-Insulin	Radiolabeled iodine ^{131}-insulin	
I_f	Free insulin	
I_b	Bound insulin	
CPR	C-peptide immunoreactive assay	
ng/ml	Nanograms per milliliter 1/1000 part of a microgram per milliliter	
"Phadebas"	A registered tradename for the Pharmacia Laboratories Insulin test	
%	Units per 100 ml	
S-S	Refers to the sulfur-sulfur bonds of amino acids	
kg	Kilogram	one thousand grams
mgm/100 ml	Milligrams per 100 ml	
U	Unit of insulin	1/24 mgm of an international standard
USP	United States Pharmacopeia	
U-40	Preparation of insulin containing	40 units/ml

LIST OF SYMBOLS (CONT'D)

SYMBOL	DEFINITION	UNITS
U-80	Preparation of insulin containing	80 units/ml
U-100	Preparation of insulin containing	100 units/ml
NPH	Neutral protamine Hagedorn insulin	
COOH	Carboxyl group terminus of the amino acid chain	
NH_2	Amino group terminus of the amino acid chain	
PEG	Polyethylene	
rpm	Revolutions per minute	
LDM	Laboratory data manager	
CPU	Central processing unit	
NaOH	Sodium hydroxide	
m mol/liter	milli moles/liter	1/1000 of the molecular wt. per liter
NaCl	Sodium chloride	
μ Ci	Micro curies of radio labeled material	
^{125}I-insulin	Radiolabeled iodine^{125}-insulin	
μl	Microliters	1/1000 part of the liter
Ag/AgCl	Silver/silver chloride type junction	
NaI	Sodium iodide	
^{137}CsCl	Radiolabeled ^{137}cesium chloride	
K	Thousand	
RDOS	Real time disk operating system	

LIST OF SYMBOLS (CONT'D)

SYMBOL	DEFINITION	UNITS
SEM	Standard error of the mean = $\frac{SD}{\sqrt{n}}$	
SD	Standard deviation = $\sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$	
COV	Coefficient of variation = $\frac{SD}{mean} \times 100$	
$\approx$	Approximately	
Σ	Summation	
r	Correlation coefficient	
p	Probability	Per cent
t test	Student's t test for statistical comparison of 2 methods	
$\bar{x}$	x bar - Average value of all results	
∞	Infinity	
CSF	Cerebrospinal fluid	

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CHAPTER I

INTRODUCTION

A. The Use of Insulin

The discovery of insulin in 1921 by Frederick G. Banting and Charles H. Best, working in the laboratory of Professor J. J. R. Macleod in Toronto, revolutionized the outlook for the diabetic patient. Prior to this time, there was little hope for the diabetic patient, at best, he could be maintained through strict diet control. Aretaeus[1] of Cappodoca (A.D. 30-90) identified the disease in his "Of the Causes and Signs of Acute and Chronic Disease", where he outlined the prominant signs and symptoms: "Diabetes is a wasting of the flesh and limbs into urine from a cause similar to dropsy. The patient never ceases to make water. The discharge is as incessant as a sluice let off. The patient does not survive long, for the marasmus is rapid and death speedy. The thirst is ungovernable, the copious potations are more than equaled by the profuse urinary discharge". During the past half century, this terrible disease has been brought under control, though not cured, and those afflicted are able to enjoy full, productive lives through the use of insulin.

In 1923, only two years after the laboratory breakthrough, commercial insulin became available to all physicians in the treatment of diabetes mellitus. Suffering from the disease began to be alleviated, and the hidden nature of the disease began to be unfolded. The point in time, the discovery of insulin occupies, in the history of man's struggle to rule his internal environment is significant. Some thirty years later Dr. Elliott P. Joslin[1] said: "The finding of insulin by Banting and Best was unusual in that it was an anticipatory discovery. Then there were comparatively few diabetics in the world, because the average age at death of people was generally around forty years, whereas onset of two-thirds of all cases of diabetes occurs above the age of forty. Future generations therefore will rise up and call them blessed even more than those in their own generation".

Diabetes mellitus is a chronic systemic disease characterized by disorders in (1) metabolism of insulin and of carbohydrate, fat, and protein, and (2) the structure and function of blood vessels. Vascular degeneration is common in those who have been diabetics for 15 years or more and manifests itself in an unusually high incidence of arteriosclerotic disease of the heart, retina, kidney, and lower extremities. The primary defect in metabolism is decreased ability to utilize carbohydrates by oxidation, conversion to fat, or storage as glycogen, because of diminished output or lowered effectiveness of insulin.

Diabetes mellitus ordinarily appears as one of two recognized clinical syndromes - the juvenile (youth-onset) type or the more common maturity-onset type. The incidence of these two kinds of diabetes is shown in Figure 1, and their characteristics are listed and compared in Table 1.

The Unites States Public Health Service[3] estimates that in 1970 there were 4 million diagnosed diabetics in the United States, approximately 2% of the population. Even more startling, is the estimation that about 6 million people are undetected diabetics who now have or will develop clinical diabetes. Undetected diabetes occurs at a rate of 8.1 cases per 1000 people per year.

The frequency of occurrence for all age groups is shown in Figure 2, which includes both male and female. It is interesting to note that the incidence at all ages for females is 16.1 per 1000, and for males of all ages, 12.9 per 1000. It has been estimated that the number of people who now have or will develop clinical diabetes may exceed 5% of the American population.

Insulin therapy, as Table 1 indicates, is required in approximately 20 to 30% of maturity-onset diabetes, while the youth-onset diabetes requires insulin administered on a daily basis in every case. The amount of insulin required for those diabetics who take it is dependent on their ability to maintain relatively normal blood sugar levels and sugar-free urine on a diet adequate to maintain ideal weight and nutrition.

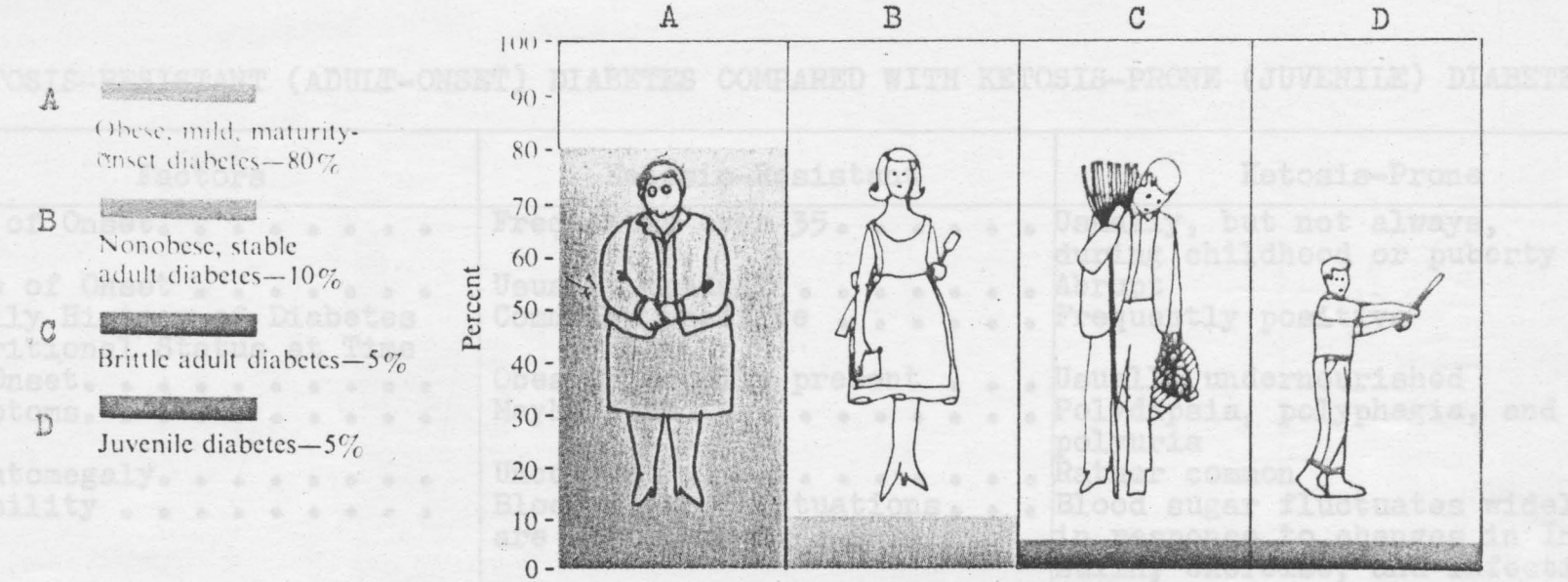

Figure 1. The Incidence of Various Types of Diabetes Among Children and Adults. These Figures Include both Male and Female in each of the Four Categories. Many Diabetics Show Overlapping Symptoms Which Make it Difficult to Classify their Illness into One of These Four Groups[2].

TABLE 1

KETOSIS-RESISTANT (ADULT-ONSET) DIABETES COMPARED WITH KETOSIS-PRONE (JUVENILE) DIABETES[2]

Factors	Ketosis-Resistant	Ketosis-Prone
Age of Onset	Frequently over 35	Usually, but not always, during childhood or puberty
Type of Onset	Usually gradual	Abrupt
Family History of Diabetes	Commonly positive	Frequently positive
Nutritional Status at Time of Onset	Obesity usually present	Usually undernourished
Symptoms	Maybe none	Polydipsia, polyphagia, and polyuria
Hepatomegaly	Uncommon	Rather common
Stability	Blood sugar fluctuations are less marked	Blood sugar fluctuates widely in response to changes in Insulin, exercise, and infection
Control of Diabetes	Easy, especially if patient adheres to proper diet	Difficult
Ketosis	Uncommon except in the presence of unusual stress or moderate-to-severe sepsis	Frequent, especially if treatment program is insufficient in food and/or insulin
Plasma Insulin (Endogenous)	Plasma-insulin response may be (1) adequate but delayed (2) diminished but not absent	Negligible to zero
Vascular Complications and Degenerative Changes	Frequent	Infrequent until diabetes has been present for about 5 years
Diet	If diet is utilized fully hypoglycemic therapy may not be needed	Mandatory in all patients
Insulin	Necessary for 20 to 30 percent of patients	Necessary for all patients
Oral Agents	Efficacious	Rarely efficacious

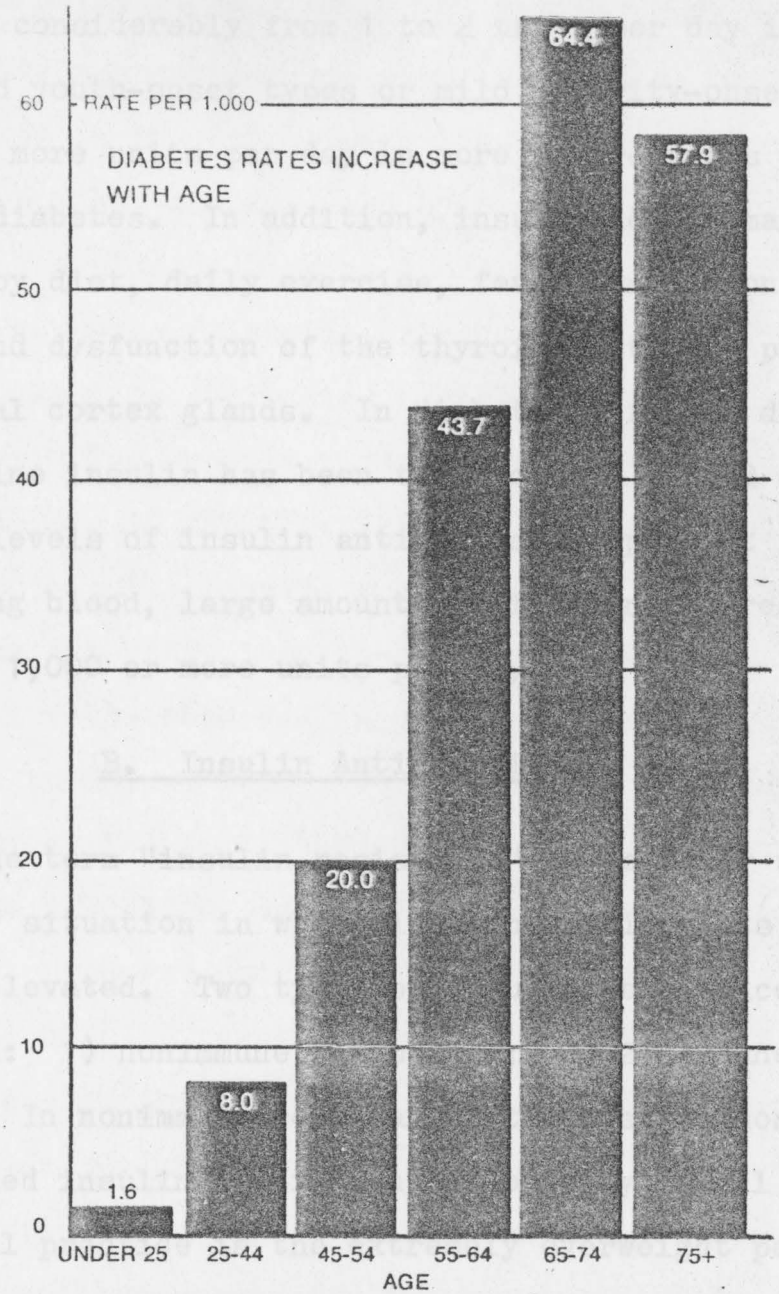

Figure 2. Diagnosed Diabetes Rates Per 1,000 Civilian Noninstitutional Population, by Age, United States, July 1965-June 1966.[3]

It is obvious from the above statement that insulin dosage will vary considerably from 1 to 2 units per day in recently discovered youth-onset types or mild maturity-onset types, to 100 or more units per day in more severe cases of both types of diabetes. In addition, insulin dosage may be affected by diet, daily exercise, fever, infection, severe trauma, and dysfunction of the thyroid, anterior pituitary and adrenal cortex glands. In diabetics of long duration, where bovine insulin has been taken over a period of years, and high levels of insulin antibodies are present in the circulating blood, large amounts of insulin are required, sometimes 1,000 or more units per day.

B. Insulin Antigenicity

The term "insulin resistance" is commonly used to denote any situation in which insulin requirements are abnormally elevated. Two types of insulin resistance is recognized: 1) nonimmune resistance; and 2) immune resistance. In nonimmune resistance, the most common cause of increased insulin requirement is obesity. Well known in clinical practice is the extremely overweight person who often requires insulin doses of 100 units or more per day. Upon losing weight, the same person may require very little insulin, in some cases may even be controlled by diet alone. Other nonimmune disorders sometimes associated with resistance to insulin are acromegaly, hyperthyroidism, Cushing's disease, hemochromatosis, chronic lymphocytic leukemia, and liver disease.

Immune insulin resistance results from the binding of exogenous insulin by antibodies in the serum. Berson and Yalow[4] first demonstrated that antibodies to insulin were present in the sera of insulin-treated diabetic patients. Furthermore, they demonstrated by electrophoretic and ultracentrifugal methods employing insulin-I^{131}, that in almost all human subjects (diabetic or nondiabetic) receiving exogenous insulin, antibodies capable of binding insulin soon appear. The quantitative agreement between insulin antibody levels in the circulating blood and insulin requirements in patients with immune insulin resistance, further suggests that insulin-binding antibodies are responsible for the observed insulin-resistance. It is reasonable to assume that insulin bound to antibody is not immediately available to the cells and insulin requirements in such cases would be expected to increase. The serum from these patients contains a mixture of free (I_f) and antibody-bound (I_b) insulin together with free antibody.

These antibodies which neutralize the hormonal action of insulin arise spontaneously as a response to the antigenic stimulus. The antibodies thus formed are termed "specific" antibodies and can react only with the substance which stimulated its production. Specificity depends upon the physiochemical structure of the reacting substances.

In immune insulin resistance patients where antibody levels are high, the use of pork insulin to replace beef insulin has proven to be effective in maintaining better control of the patient on 40-50% of the previous dosage.

The pork insulin will react with free insulin antibody present in human circulating blood; however, it may not bind to the same degree as beef insulin. Differences in the type and arrangement of amino acids in the "A" chain of pork insulin, more closely resemble human insulin, as will be shown later in Table 3. Thus, pork insulin does not stimulate the immunological antibody-producing tissues as much as beef insulin[5]. This amino acid difference may also account for the changes in binding to the antibody.

The half-life of insulin antibodies was studied by Sebriakova and Little[6] and found to be approximately 18 days. Others[7] have reported a half-life of twenty days, but both are of the same order as that given for human gamma globin, namely 13 days[8]. Table 2 shows the decrease in insulin antibody over a period of 320 days as determined by Sebriakova and Little[6].

Many factors contribute to the effect on insulin requirements in the presence of insulin-binding antibodies. Berson and Yalow[9] concluded that the important factors were: a) the rate at which insulin-antibody complexes are removed from the circulation; b) the ratio of bound to free insulin at the free insulin concentration necessary for control of diabetes; c) the extent of saturation of the antibody at this ratio; and d) the rate at which complexes form and dissociate. In addition, insulin requirements are dependent on factors which effect exogenous free insulin as it becomes available such as:

TABLE 2

DECREASE IN INSULIN ANTIBODY INDEX IN DIABETIC PATIENT, A.S., AFTER STOPPING BEEF LENTE INSULIN TREATMENT.[6]

Days Off Insulin	Binding Capacity%	Antibody Index* μU/ml	Blood Sugar 3 hr.pp.mg/100ml
1	94	76,464	149
4	95	71,300	169
7	94	56,943	110
11	97	53,363	120
14	94	53,797	80
18	90	36,450**	132
20	88	31,208	104
24	86	25,720	135
30	86	22,218	152
70	93	14,894	-
166	77	2,001	-
257	78	600	-
320	57	303	-

*Significant decrease during first thirty days, $p<.001$.
**Antibody half-life approximately eighteen days.

1) the solubility of subcutaneously injected insulin at the pH of body fluids; 2) the rate of hepatic degradation of insulin; 3) the distribution of insulin into extravascular spaces; 4) the rate of metabolic, non-hepatic degradation of insulin; and 5) the rate of uptake by peripheral tissues. All these factors contribute to the half-life of insulin which has been estimated by Yalow and Berson[10], to be approximately 35 minutes or degraded at the rate of about 2% per minute. Soeldner and Stone's[11] data, developed from intravenous glucose tolerance tests, indicate that the half-life of endogenous insulin may be as short as 8 to 10 minutes.

C. Statement of the Problem

Since the development of the competitive binding assay based on the use of radioisotope labeled ligands in 1959 by Yalow and Berson[12], this technique has found acceptance as the method of choice in the assay of insulin. The reasons for such widespread acceptance of this basic technique appears to be related to the chemical specificity and the high sensitivity permitting quite accurate estimations of minute amounts of insulin present in the circulating blood. Many modifications have been proposed since 1959 to the original RIA procedure for insulin and have led to greater simplification and more widespread use in clinical laboratories throughout the world.

The serum insulin levels in patients previously treated with insulin, however, cannot be determined by the original method or by most of the modifications because of the presence of human anti-insulin antibodies. These antibodies combine with the added radiolabeled insulin and interfere in the competitive reaction between radiolabeled insulin, endogenous insulin present in the serum, and guinea pig anti-insulin antibody.

Results of the RIA assay for insulin in the presence of insulin antibodies are completely unreliable and may, in fact, be misleading in interpretation of high patient insulin levels. The analyst cannot determine from the data that antibodies were present and are affecting the assay. The exception would be when high levels of insulin-antibody are encountered, and the limits of the system are exceded. This conceivably could alert the analyst to review the patient history for the possibility of previous insulin therapy.

The RIA method for insulin assay has been used primarily along with the glucose tolerance test to determine: 1) the degree of pancreatic insufficiency or damage in suspected diabetics; and 2) the importance of the metabolic factors as a cause of the decreased glucose tolerance. Through this measurement, the course of the disease can be better foreseen. The insulin response to a glucose load allows classification of diabetes into 3 major types:

1) latent diabetes associated with obesity where there is decreased glucose tolerance with full insulin response; 2) diabetes with partial insulin response where there is decreased glucose tolerance, but the rise of insulin is not proportional to the rise of blood glucose. Most cases of adult-onset diabetes are found in this catagory with varying degrees of response; and 3) diabetes without insulin response where there is significant absence of pancreatic response to glucose challenge. This type of diabetes is usually found in youth-onset or in severe forms of adult-onset diabetes.

The insulin - RIA is also of importance for the diagnosis of insulinomas, as the measurement of glucose levels alone does not allow one to differentiate them from other glucose-lowering processes. The use of insulin - RIA, together with the measurement of blood glucose, has made possible the diagnosis of most of the insulin-secreting tumors.

The need for measuring free insulin levels in insulin treated diabetics to follow the dynamics of insulin therapy may be advantageous and has led to development of several RIA methods. Berson and Yalow[13] described a method employing an ultracentrifuge in the separation of free insulin from the antibody-bound complexes. The ultracentrifuge, while providing an excellent method for separation, is not readily available in most clinical laboratories. Other methods have been proposed, including acid-ethanol and neutral ethanol extraction, talc absorption, dioxane extraction, and

ultrafiltration methods. None of these methods have found widespread use because of: 1) the difficulty in performance of the test or in obtaining materials and apparatus; 2) the unsatisfactory results obtained; and 3) the poor clinical correlation observed.

In 1971, Desbuquois and Aurbach[14] reported on the use of aqueous polyethylene glycol (PEG) as a means of separating heavier molecular weight antibody-bound peptide hormones with little or no precipitation of smaller molecular weight free hormones. Their preliminary investigations suggested that PEG could be applied advantageously to separation of phases and that the PEG hormone-rich upper phase could be used directly in various RIA systems.

Nakagawa et al.[15] reported a simplified method for determining free and total insulin levels in insulin-treated patients using PEG to separate the free hormone from insulin-antibody complexes and free antibody present in the serum of insulin-treated patients. The PEG extract was used directly in the RIA system as described by Desbuquois and Aurbach[14]. While the technique of this procedure for separating the free insulin from insulin-antibodies appears to be good, the RIA system is rather difficult and time consuming and does not lend itself to widespread clinical usage. In addition, the recovery of unlabeled insulin from normal serum for the free method was 75%, and by the method for total insulin, was 78% indicating that the analytical procedure may not be as sensitive as desired.

The purpose of this investigation therefore, was an attempt to utilize the PEG extraction technique as proposed in 1971 by Desbuquois and Aurbach[14], and study the possibility of using the PEG directly in a simplified, more practical RIA system. Development of the free insulin method as proposed would permit the measurement of free circulating insulin in insulin-treated diabetics to be simplified sufficiently so as to become useful to the clinical laboratory and the practicing physician in studying the dynamics of insulin therapy. Also, development of the total insulin method as proposed would allow the measurement of insulin-antibody levels to be followed in insulin therapy. The ability for the physician to distinguish between immune insulin resistance and other types of insulin resistance could be extremely desirable.

CHAPTER II

THEORY

A. Insulin Biosynthesis

Insulin belongs to that group of biological substances known as protein hormones. These hormones secreted by the pituitary, the pancreas, and the parathyroid glands, all are protein in nature. Hormones, in general, are defined as any substance normally produced by specialized cells in some part of the body and carried by the blood stream to another part of the body from which it affects the body as a whole.

Insulin, sometimes called the antidiabetic hormone is produced in the pancreas by the beta cells of the islets of Langerhans. It is known that the islets of Langerhans consists of at least two types of cells, the "A" or alpha cell and the "B" or beta cell and that these cells can be differentiated from each other in sectioned tissue by special stains. One method makes use of chrome alum hematoxylin phloxine which stains the granules of the beta cell a deep blue. In contrast, the alpha-cell granules stain red and appear to be much finer than the beta-cell granules. Beta cells outnumber all other islet cells.

It is generally agreed that insulin is formed from the proteolytic transformation of proinsulin into insulin and C-peptide. Proinsulin, then is considered an insulin precursor. This proteolytic transformation occurs within the beta cells of the islets of Langerhans, probably within the Golgi apparatus and in newly formed secretory granules as shown in Figure 3. Kemmler and Steiner[16], postulated the intracellular beta cell events associated with insulin production in 1970. The conversion of proinsulin to insulin and C-peptide appears to be a complex process that occurs in the Golgi apparatus and requires the participation of several enzymes. The products of transformation are then stored within the secretory sacs of the beta cell and discharged into the circulating blood in response to the metabolic signals that trigger insulin release.

Since insulin as stated earlier, is a hormone, it must be released by the beta cell into the circulating blood and carried to another site of the body where it can function to control certain metabolic activities. To reach these sites of action, it must first break out of its capsule and leave the beta cell. This process of insulin ejection from the beta cell is called "emiocytosis". The mechanism of emiocytosis is not completely understood, however it does appear to involve the fusion of the beta cell plasma membrane and the storage granule sac followed by rupture at the point of fusion. As a result, a passageway is provided for the insulin granule to escape from the beta cell.

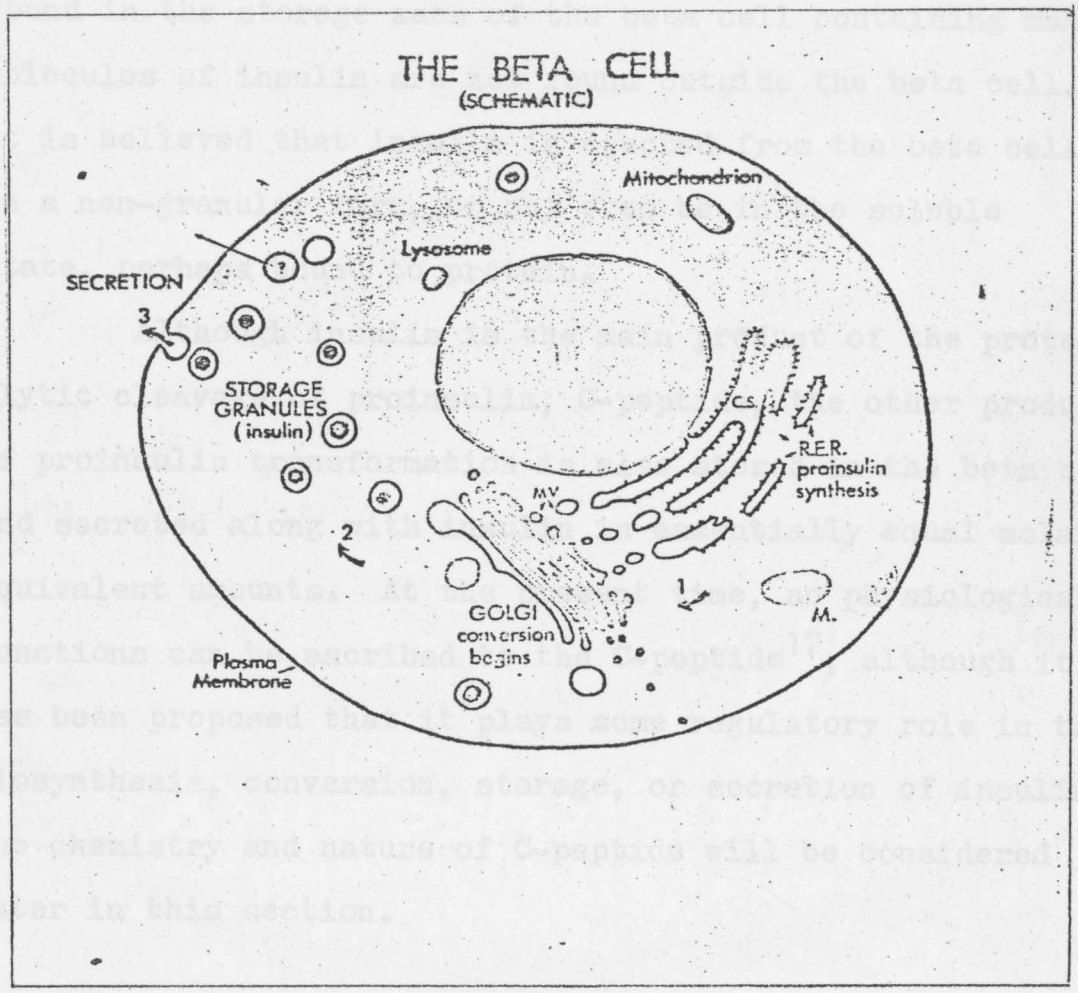

Figure 3. Schematic Representation of the Morphological Organization of the Biosynthetic and Secretory Apparatus of the Pancreatic Beta Cell.[17]

The insulin granules, at the time of emiocytosis appears to undergo a transformation. The free granules found in the storage sacs of the beta cell containing many molecules of insulin are not found outside the beta cell. it is believed that insulin is ejected from the beta cell in a non-granular form, or may even be in the soluble state, perhaps bound to protein.

Although insulin is the main product of the proteolytic cleavage of proinsulin, C-peptide, the other product of proinsulin transformation is also stored in the beta cells and secreted along with insulin in essentially equal molar equivalent amounts. At the present time, no physiological functions can be ascribed to the C-peptide[17], although it has been proposed that it plays some regulatory role in the biosynthesis, conversion, storage, or secretion of insulin. The chemistry and nature of C-peptide will be considered later in this section.

B. The Chemistry of Proinsulin

Proinsulin consists of three polypeptide chains, linked together and containing a total of 84 amino acids. The C chain forms a loop connecting the glycine residue at position A1 with the alanine residue at position B30. Two cysteines join the A and B chains by S-S bridges between positions A7 and B7 and between positions A20 and B19. In addition there is an internal S-S bridge between positions A6 and A11 of the A chain. See Figure 4.

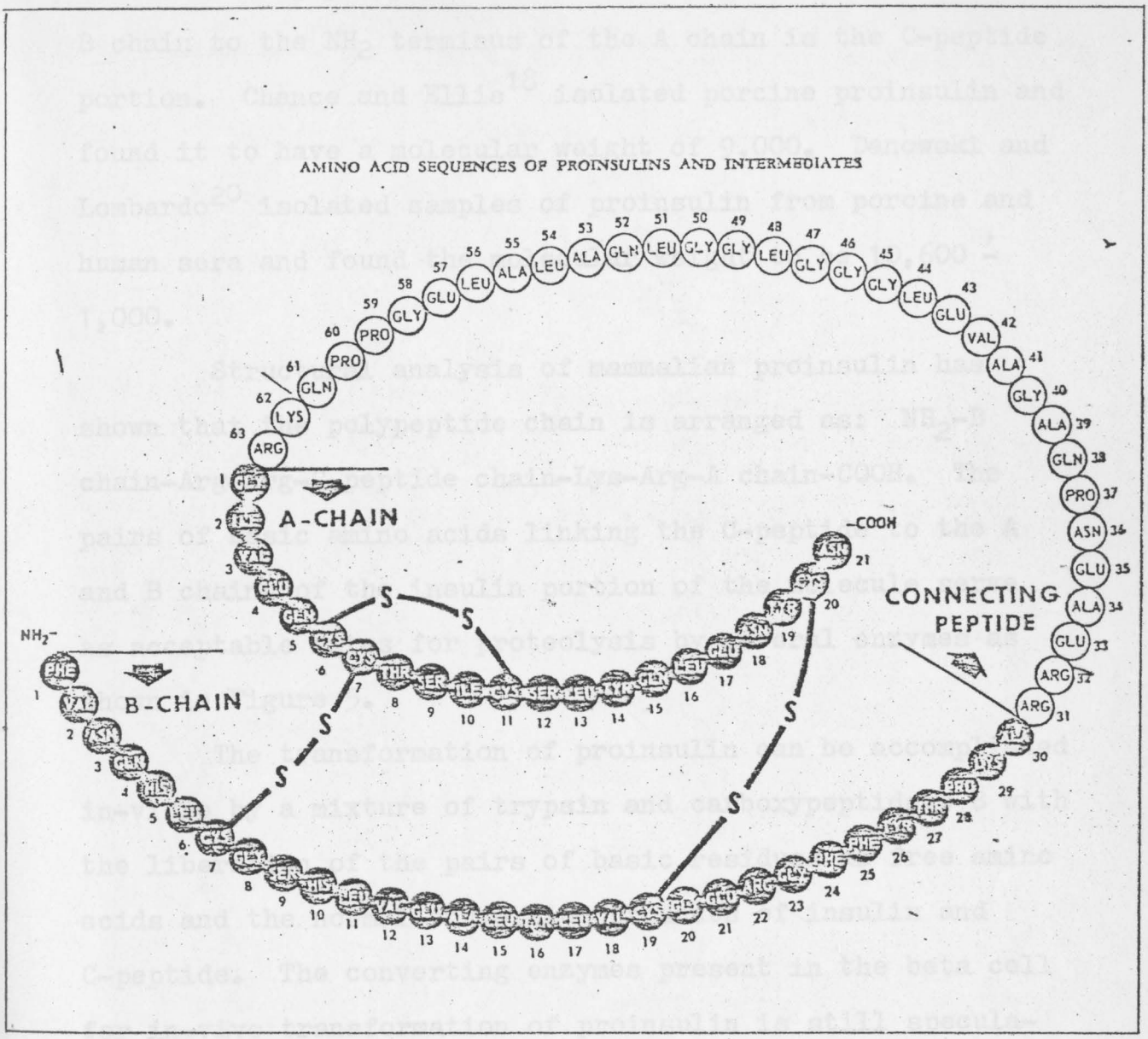

Figure 4. The Amino Acid Sequence of Porcine Proinsulin.[19]

The connecting polypeptide linking the COOH terminus of the B chain to the NH_2 terminus of the A chain is the C-peptide portion. Chance and Ellis[18] isolated porcine proinsulin and found it to have a molecular weight of 9,000. Danowski and Lombardo[20] isolated samples of proinsulin from porcine and human sera and found the molecular weight to be 10,600 $\pm$ 1,000.

Structural analysis of mammalian proinsulin has shown that the polypeptide chain is arranged as: NH_2-B chain-Arg-Arg-C-peptide chain-Lys-Arg-A chain-COOH. The pairs of basic amino acids linking the C-peptide to the A and B chains of the insulin portion of the molecule serve as acceptable sites for proteolysis by several enzymes as shown in Figure 5.

The transformation of proinsulin can be accomplished in-vitro by a mixture of trypsin and carboxypeptidase B with the liberation of the pairs of basic residues as free amino acids and the normal conversion products of insulin and C-peptide. The converting enzymes present in the beta cell for in-vivo transformation of proinsulin is still speculative and further study will be required to isolate these proteolytic enzymes.

Kemmler, et al.[17] proposed 2 partly cleaved forms of proinsulin, Figure 6, found to be present in crystalline preparations of bovine insulin. These intermediate forms I and II of bovine proinsulin could conceivably be possible

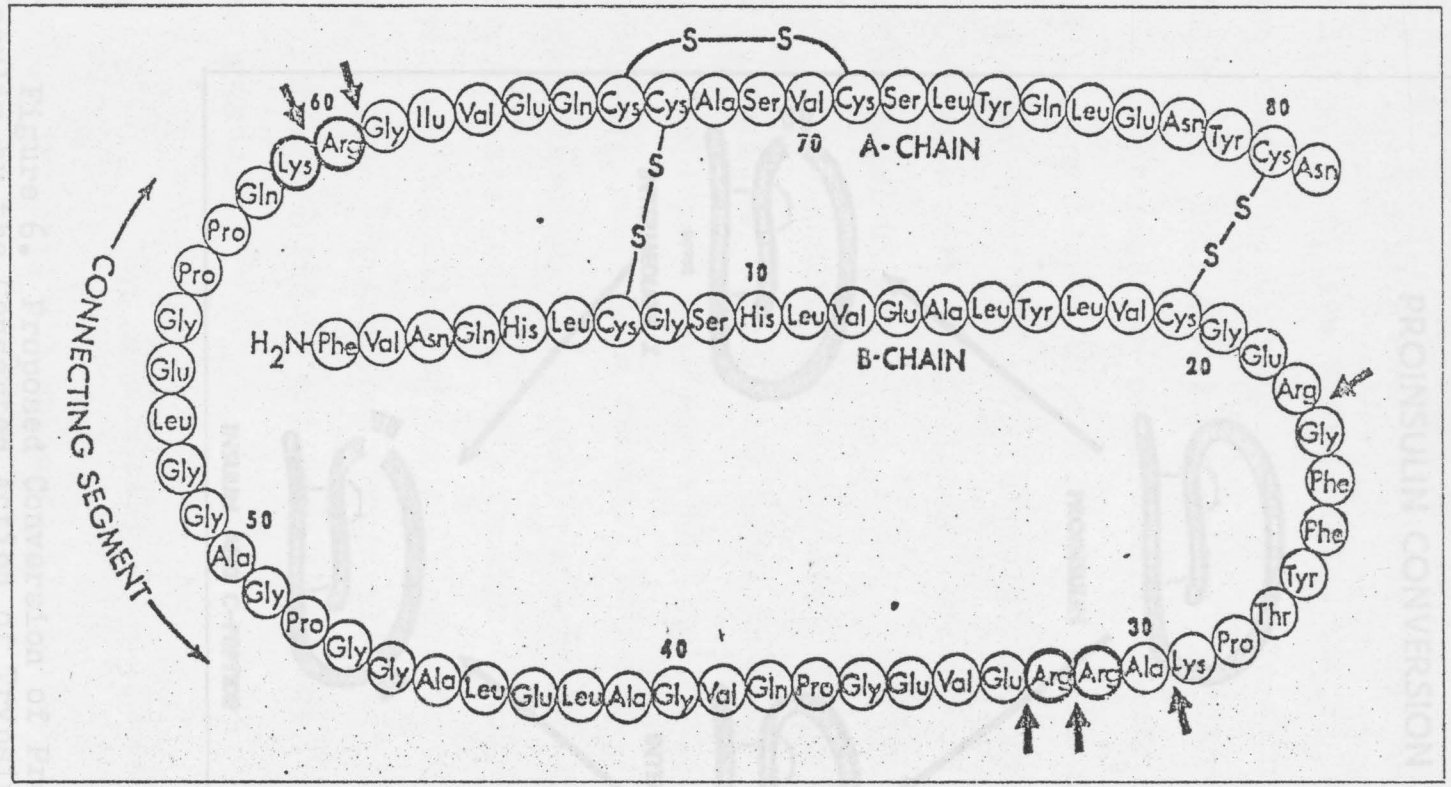

Figure 5. Structure of Bovine Proinsulin Showing Sites of Cleavage by Trypsin.[17]

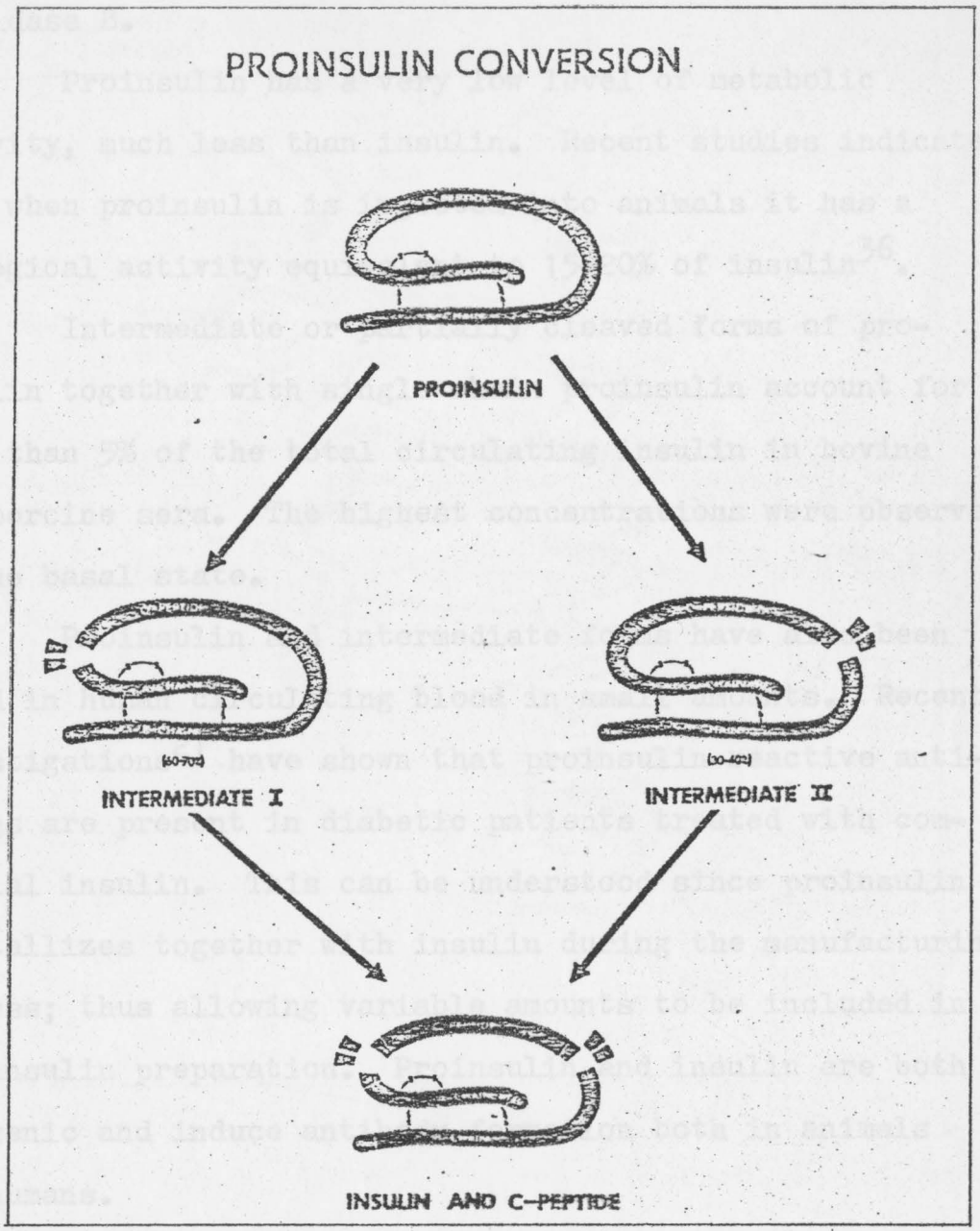

Figure 6. Proposed Conversion of Proinsulin to Insulin by the Concerted Action of Trypsin and Excess Carboxypeptidase B. Intermediates I and II are the Major Partly Cleaved Forms of Proinsulin Present in Crystalline Preparations of Bovine Insulin.[17]

in the proteolytic enzyme system using trypsin and carboxypeptidase B.

Proinsulin has a very low level of metabolic activity, much less than insulin. Recent studies indicate that when proinsulin is injected into animals it has a biological activity equivalent to 15-20% of insulin[36].

Intermediate or partially cleaved forms of proinsulin together with single chain proinsulin account for less than 5% of the total circulating insulin in bovine and porcine sera. The highest concentrations were observed in the basal state.

Proinsulin and intermediate forms have also been found in human circulating blood in small amounts. Recent investigations[21] have shown that proinsulin-reactive antibodies are present in diabetic patients treated with commercial insulin. This can be understood since proinsulin crystallizes together with insulin during the manufacturing process; thus allowing variable amounts to be included in the insulin preparation. Proinsulin and insulin are both antigenic and induce antibody formation both in animals and humans.

C. The Chemistry of Insulin

Insulin, the antidiabetic hormone is a polypeptide consisting of 51 amino acids and having a minimum molecular weight of 6,000. The insulin molecule is composed of 2 polypeptide chains designated "A" and "B" as depicted schematically in Figure 7.

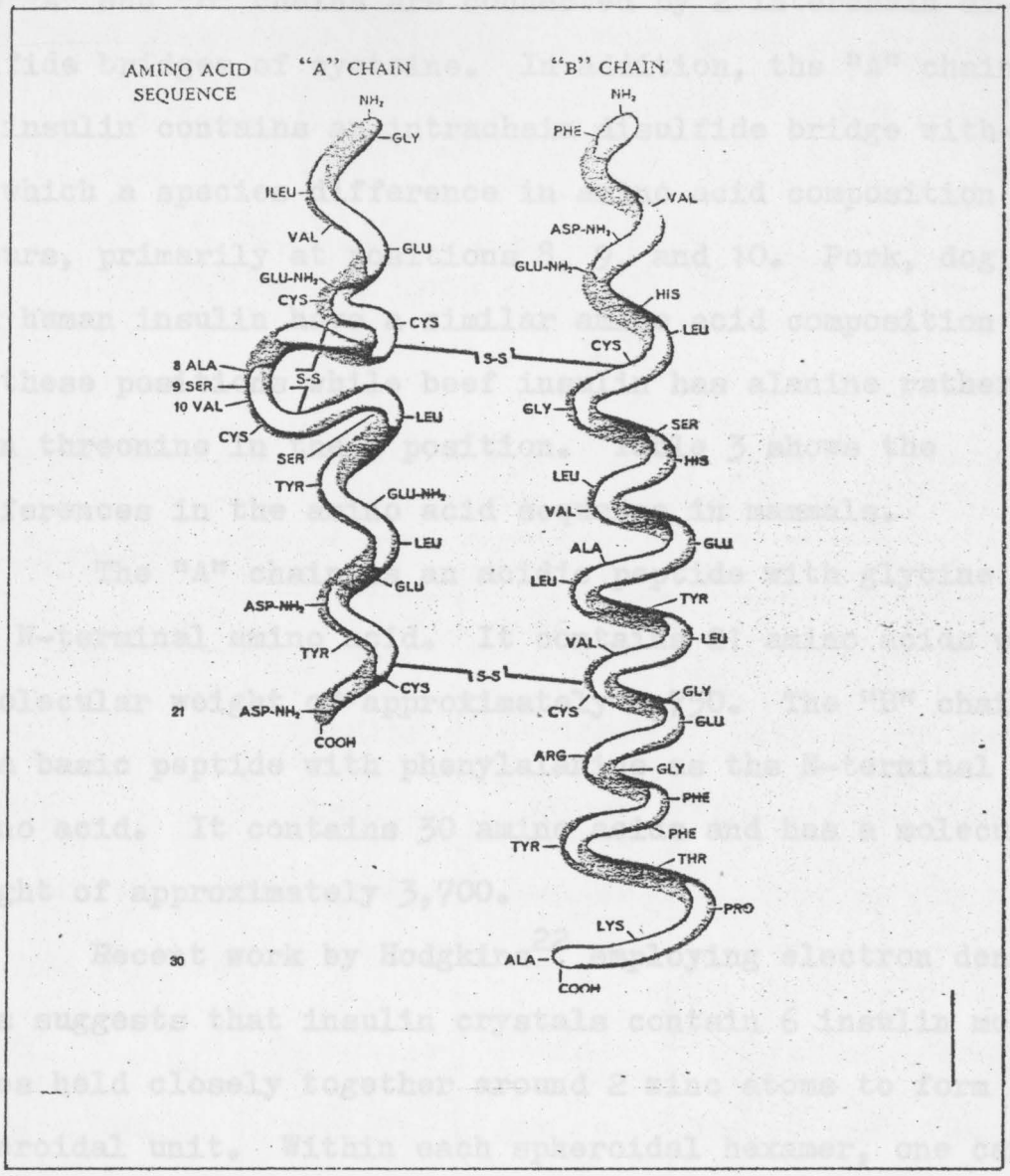

Figure 7. Schematic Representation of Beef Insulin Showing Two Polypeptide Chains Connected by Two Disulfide Bridges.[2]

The "A" and "B" chains are connected by 2 interchain disulfide bridges of cysteine. In addition, the "A" chain of insulin contains an intrachain disulfide bridge within which a species difference in amino acid composition occurs, primarily at positions 8, 9, and 10. Pork, dog, and human insulin have a similar amino acid composition at these positions while beef insulin has alanine rather than threonine in the 8 position. Table 3 shows the differences in the amino acid sequence in mammals.

The "A" chain is an acidic peptide with glycine as the N-terminal amino acid. It contains 21 amino acids with a molecular weight of approximately 2,750. The "B" chain is a basic peptide with phenylalanine as the N-terminal amino acid. It contains 30 amino acids and has a molecular weight of approximately 3,700.

Recent work by Hodgkins[22] employing electron density maps suggests that insulin crystals contain 6 insulin molecules held closely together around 2 zinc atoms to form a spheroidal unit. Within each spheroidal hexamer, one can see 3 individual molecules of insulin attached to each of the 2 zinc atoms, with no attachment between the zinc atoms. Zinc has been considered to form an integral portion of the insulin molecule, although neither the exact manner in which it is bound or the exact function is known with certainty. The zinc content of crystalline insulin has been shown to vary from 0.3 per cent to 0.6 per cent depending on the pH of crystallization.

TABLE 3

SPECIES DIFFERENCES IN AMINO ACID SEQUENCE OF MAMMALIAN INSULINS.[2]

Species	Positions			
	"A" Chain			"B" Chain
	8	9	10	30
Beef	Alanine	Serine	Valine	Alanine
Pork	Threonine	Serine	Isoleucine	Alanine
Human	Threonine	Serine	Isoleucine	Threonine
Other Species				
Dog	Threonine	Serine	Isoleucine	Alanine
Sperm Whale	Threonine	Serine	Isoleucine	Alanine
Rabbit	Threonine	Serine	Isoleucine	Serine
Horse	Threonine	Glycine	Isoleucine	Alanine
Sheep	Alanine	Glycine	Valine	Alanine
Sei Whale	Alanine	Serine	Threonine	Alanine

The zinc content of human pancreas ranges from 18.5 to 30.4 mg per kg of fresh gland[2].

The activity of insulin is destroyed by alteration of the free NH_2 groups as by reaction with formaldehyde, or by the reduction of the disulfide linkages between the "A" and "B" chains and within the "A" chain by such chemicals as cysteine, thioglycolic acid, or leucomethylene blue. Insulin is inactivated by pepsin and chymotrypsin, but is resistant to trypsin.digestion. Digestion of insulin with proteolytic enzymes also inactivates the hormone, and for this reason it cannot be administered orally. Insulin is comparatively resistant to denaturation by organic solvents and dilute acids, but may be irreversibly denatured with alkali. The activity of insulin is also destroyed in-vivo by an insulin-inactivating enzyme, insulinase. Insulinase is found primarily in the liver, but to a lesser degree in kidney and muscle and is responsible for the short half life of insulin, about 35 minutes. In plasma, insulin destruction proceeds very slowly since insulinase is not present or present in such small quantities that it has very little degrading effect.

Most commercial insulin preparations in this country are made from a combination of beef and pork insulin in approximate ratio of 70% beef and 30% pork. It has been estimated that 1 pound of pure zinc-insulin crystals can be produced from about 10,000 pounds of raw pancreas[23].

Insulin preparations are standardized by measuring their effect in the blood sugar of experimental animals, usually rabbits. One unit of insulin is the amount required to reduce the blood sugar level of a normal 2 kg rabbit after a 24 hour fast from 120 to 45 mg per 100 ml. More recently, an international standard preparation of zinc-insulin crystals has been maintained at the National Institute for Medical Research in London. One unit of insulin is 1/24 mg of this standard preparation. Originally the unit of insulin was based on the physiological reaction of rabbits, but now to insure greater uniformity among insulin manufacturers and researchers, it is based on an absolute weight of insulin prepared from a recrystallized sample. The USP standard and the International standard are the same.

Over the years, forms of insulin have been developed to obtain the desired effect in the treatment of diabetes. Fast and long acting preparations were developed to meet the needs of all diabetics and additionally, to reduce the number of injections required per day. The basic principle involved in the prolongation of the action of insulin is that of a material with low solubility at the pH of body fluids. Relative insolubility of different modifications has accounted for the various intermediate timing characteristics observed.

Five forms of insulin are commercially available in this country and other parts of the world: 1) Regular insulin made from zinc-insulin crystals is a clear solution and has rapid action. It is usually administered subcutaneously 1/2 to 2 hours before a meal so that its physiological effects will coincide with the absorption of glucose. If only regular insulin is used in controlling diabetes, it may be necessary to administer several injections because of its short duration as shown in Table 4. 2) Protamine-zinc insulin is a preparation made by mixing insulin, protamine, and zinc in a buffered solution. The resulting precipitate when injected subcutaneously has poor solubility at the pH of body fluids and is slowly absorbed from the site of injection. Thus, the depot of insulin is slowly available over a period of 36 hours with peak action at 14 to 24 hours. 3) Globin, another basic protein has been used to modify the solubility of insulin. It is available commercially, as Globin insulin, but has not found widespread use because of its failure to provide slow insulin release over a 24 hour or longer period. 4) NPH insulin was developed in an attempt to obtain the advantages of regular and protamine zinc insulin, yet retain the optimum timing of release so as to protect the patient from one day to the next with only one injection. At the Hagedorn laboratories in Copenhagen, it was discovered that careful control of the ratio of protamine and insulin made it possible to produce crystals in which the amounts of

TABLE 4

TYPES OF INSULIN AVAILABLE IN THE UNITED STATES.[24]

Type of Insulin	Appearance	Action	Duration (Hours)	Zinc Content (mgm/100 Units)	Buffer	Protein	
						Type	mgm%
Regular Crystalline	Clear	Rapid	5-7	0.016-0.04	None	None	-
Semilente	Turbid	Rapid	12-16	0.2-0.25	Acetate	None	-
Globin	Clear	Intermediate	18-24	0.25-0.35	None	Globin	3.8
NPH	Turbid	Intermediate	24-28	0.016-0.04	Phosphate	Protamine	0.5
Lente	Turbid	Intermediate	24-28	0.2-0.25	Acetate	None	-
Protamine Zinc	Turbid	Prolonged	36+	0.2-0.25	Phosphate	Protamine	1.25
Ultralente	Turbid	Prolonged	36+	0.2-0.25	Acetate	None	-

protamine and insulin approach stoichiometric proportion. 5) Lente insulin preparations were developed based on the concept of combining the insulin with increased amounts of zinc and eliminating the modifying protein such as globin or protamine. By using an acetate buffer, a higher concentration of zinc could be made to combine with insulin in such a way that the resulting product was very insoluble at pH 7.4. Two physical forms can be produced by careful adjustment of the pH. The crystalline form, Ultralente, is more insoluble and consequently very long acting. The amorphous form, Semilente, presents more surface area to the body fluids and therefore is more quickly absorbed. Clinical evaluation suggested that perhaps the best preparation could be achieved by mixing the Semilente and Ultralente together in ratio's of 30% and 70%. This mixture, designated Lente insulin has almost the same characteristics of NPH insulin except that Lente is free of a modifying protein.

During the past 10 to 15 years, the most commonly used concentrations of exogenous insulin of all forms were U-40 and U-80, indicating 40 and 80 units per ml. Syringes for injection were made and clearly labeled with either U-40 or U-80 to prevent patient error in prescribed dosage. Insulin dosage units are interchangeable providing the proper syringe is used for each type of insulin. The calibration of the U-80 syringe is such that 80 units of insulin is contained in one ml. It is important to use U-40

syringes with U-40 insulin and U-80 syringes with U-80 insulin. It has been estimated that patient errors in administration of insulin may be as high as 58 per cent. Much of the error is due to a misunderstanding of the amount and concentration the patient has been told to use. Some patients become confused by the availability of numerous types of color-coded insulin vials and the variety of single and double-scale syringes.

Recently a new concept in insulin therapy based on a standardized and simplified delivery system has been approved by the American Diabetes Association and is becoming the method of choice recommended by most physicians. The new concept is based on the development of U-100 insulin, a purer and more concentrated preparation of insulin. U-100 contains 100 units per ml and is the concentration most suitable for all insulin-dependent diabetics. It may soon replace U-40 and U-80 insulin preparations and may lead to a reduction in the frequency of mistakes in the patient administration of insulin.

U-100 is available in all formulations including Regular, Protamine-Zinc, NPH, Lente, Ultralente, and Semi-lente. To make it easy to use U-100, new syringes have been developed with improved design and markings. It is important for patients to use U-100 syringes with U-100 insulin to avoid dosage error which may result in hypoglycemia or hyperglycemia. Other benefits derived include:

1) less volume per injection because of increased potency; thus, less discomfort to the patient; 2) high dosage insulin users may not need additional injections during a 24 hour period; and 3) because of improved manufacturing processes, less impurities are found leading to reduced local reactions and systemic allergic reactions following administration.

In the early literature on insulin chemistry, it seemed unusual to many researchers that such powerful and biological effects should be controlled by a molecule containing only the common amino acids present in many other molecules. Today following 50 years of research and development and with our expanded knowledge of its structure and amino acid spatial configuration, it still seems unusual to many that we cannot understand its mode of action.

D. The Chemistry of C-Peptide

The products of proinsulin transformation as earlier described are insulin, C-peptide, and pairs of basic residues as free amino acids. C-peptide is the connecting peptide in the insulin precursor linking the COOH terminus of the "B" chain to the NH_2 terminus of the "A" chain. The connecting peptide of porcine proinsulin before cleavage contains 33 amino acids and is larger than the "A" or "B" chain of porcine insulin as shown in Figure 4. C-peptide has a molecular weight of approximately 3,000 and shows a species difference in number, type, and sequence

arrangement. The human C-peptide fragment after cleavage, is similar to that of the 26 residue bovine C-peptide and the 29 residue porcine C-peptide, except for the presence of 31 amino acids as shown in a comparison chart, Figure 8.

Recent work by Rubenstein[25] has confirmed the fact that C-peptide is stored in the beta cell and secreted along with insulin in essential molar equivalence. Circulating C-peptide was found present in small amounts in serum of humans and cows in the fasting state. After glucose administration in both humans and cows, the C-peptide level was significantly increased over fasting levels. The evidence appears to favor the concept that C-peptide is stored with insulin in the storage granules of the beta cell. Subsequent liberation of the entire granule by emiocytosis releases stoichimetric amounts of insulin and C-peptide, as well as small amounts of proinsulin and proinsulin intermediates.

Following the development of an immunoassay for human C-peptide, Rubenstein[25] found the level in healthy, fasting subjects to be 1.0 ± 0.23 ng/ml. In these same patients 60 minutes after oral glucose, he found a mean level of 3.1 ng/ml. The C-peptide immunoreactive assay (CPR) can be used to measure beta cell secretory capacity in insulin-treated patients where the presence of antibodies makes the RIA procedure for insulin unreliable.

Amino acid sequences of proinsulin C-peptides

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Human	NH_2-Glu	Ala	Glu	Asp	Leu	Gln	Val	Gly	Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro
Monkey	NH_2-Glu	Ala	Glu	Asp	Pro	Gln	Val	Gly	Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro
Pig	NH_2-Glu	Ala	Glu	Asn	Pro	Gln	Ala	Gly	Ala	Val	Glu	Leu	Gly	Gly	Gly	Leu
Cow, Sheep	NH_2-Glu	Val	Glu	Gly	Pro	Gln	Val	Gly	Ala	Leu	Glu	Leu	Ala	Gly	Gly	Pro
Dog	NH_2-Asp	Val	Glu	—	—	—	—	—	—	—	—	Leu	Ala	Gly	Ala	Pro

	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Human	Gly	Ala	Gly	Ser	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln-COOH
Monkey	Gly	Ala	Gly	Ser	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln-COOH
Pig	Gly	—	Gly	—	Leu	Gln	Ala	Leu	Ala	Leu	Glu	Gly	Pro	Pro	Gln-COOH
Cow, Sheep	Gly	Ala	Gly	—	—	—	—	—	Gly	Leu	Glu	Gly	Pro	Pro	Gln-COOH
Dog	Gly	Glu	Gly	Gly	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ala	Leu	Gln-COOH

Figure 8. Comparison of the Amino Acid Sequences of Proinsulin C-Peptides From Human, Monkey, Bovine, Ovine, Porcine and Canine Pancreas. All the Differences Shown Can be Accounted For by Single Nucleotide Replacements Except For Positions 4 (Asn-Gly), 9 (Gln-Ala) and 29 (Ser-Pro-Ala), Where at Least Two Nucleotide Replacements Have Occurred.[17]

At the present time, no physiological function has been ascribed to the C-peptide. It has been proposed that it may play some regulatory role in the biosynthesis, conversion, storage or secretion of insulin. Yet, the most important role of C-peptide may be in its ability to serve the function of holding the "A" and "B" chains of proinsulin together and thereby promote the correct S-S bonding between the two. It is clear that more information is needed to clarify the physiological role of C-peptide and also the function it serves in its role as a connecting polypeptide.

E. The Chemistry of Polyethylene Glycol

Polyethylene glycols, of the general formula $HOCH_2(CH_2OCH_2)_xCH_2OH$ are readily available as polymers of different molecular weight. These compounds that are available and have been studied for their protein precipitation properties are polymers with average molecular weights of 1,500, 4,000, 6,000, and 20,000. All are soft, white, waxy solids with good storage characteristics. Polyethylene glycol (PEG) compounds do not hydrolyze or deteriorate easily on storage and will not support mold or bacterial growth. PEG compounds show good solubility in H_2O at 20° C with values ranging from 50% for the PEG (20,000) to 70% for PEG (1,500).

Many investigators[26,27] have demonstrated the ability of high molecular weight linear polymers such as polyethylene glycol, dextran, polyvinyl alcohol, and

polyvinyl pyrrolidone to precipitate proteins. Of those studied, Polson[26], found that polyethylene glycol (mol.wt. 6,000) appeared to be the most suitable protein precipitant because its solutions were less viscous and caused virtually no denaturation at room temperature. Further, it was found that quantitative precipitation of a protein will occur over a wide range of concentration when the low molecular weight polymers are used.(less than 6,000 mol.wt.) with a resultant increased overlapping between the different fractions.

Many investigators have studied the interaction between a protein and a polymer and have proposed several explanations; 1) that the protein and polymer form a complex which leads to precipitation; 2) that the polymer sterically excludes the protein from part of the solvent and that this brings the protein solution to its solubility limit. Ceska[27], in 1969, concluded that the precipitation of protein by polyethylene glycol polymers may be due to their strong hydrogen-bonding affinities. A competition for H_2O molecules among proteins and polyethylene glycol polymers may be the important feature. The absence of highly charged groups in polyethylene glycol polymers suggest that their interactions with protein molecules occurs through hydrogen bonding.

In the course of a study by Debuquois and Aurbach[14], they found that aqueous PEG causes precipitation of higher molecular weight antibody-bound hormones but that smaller molecular weight free hormones remain in solution.

Several factors were found to affect the solubilities of free and antibody-bound hormones: 1) The minimum concentration of PEG required to assure complete precipitation of the antibody-bound hormone was 10% w/v.; 2) Solubility of the free hormone in aqueous PEG varies somewhat depending on the nature and concentration of the proteins. As the protein concentration increases, there is a slight increase in the precipitability of free hormone. This observation may be due to physical trapping of the hormone in the protein-precipitate; 3) There is no precipitation of antibody-bound hormone in serum albumin. Serum albumin is not precipitable by PEG at pH 7 or higher; 4) The nature of the hormone contributed to its behavior in PEG. It was found that precipitation of antibody-bound hormone was complete irrespective of the complex involved, but that solubility of the free hormone varied. Insulin and vasopressin remained completely soluble, whereas growth hormone and parathyroid hormone showed some precipitation; 5) The effect of pH on insulin solubility was negligible from pH 7 to 9. Precipitation of the antibody-bound hormone was not affected over the 7 to 9 pH range.

The use of PEG appears to offer advantages to separation of phases in RIA. It can be used to precipitate antibody-bound ligands as well as gamma-globulin. It can be used to determine bound to free ratios for many hormones present in the circulating blood.

The use of PEG in the determination of free and antibody-bound insulin levels appears to provide a simple approach to measurement of insulin in the presence of insulin-antibodies. In addition, the polymer provides a practical method for clinical laboratories to measure insulin antibody levels

CHAPTER III

Materials and Apparatus

A. Materials

All chemicals used were reagent grade or best available from the manufacturer as in the case of polyethylene glycol and the insulin RIA reagents.

HCl and NaOH solutions were prepared from reagent grade chemicals, then titrated and adjusted against primary chemical standards to exactly 1.0 N concentration. The 1.0 N reagents were then titrated against each other and adjusted if necessary to insure proper neutralization in the test system.

Polyethylene glycol, of the general formula $HOCH_2(CH_2OCH_2)_xCH_2OH$ in 3 different molecular weight compounds were obtained from J. T. Baker Company, Philipsburg, New Jersey. The PEG polymers had average molecular weights of 1,500, 6,000, and 20,000 with molecular weight ranges of 1,300-1,600, 6,000-7,500, and 15,000-20,000, respectively. All 3 molecular weight PEG polymers were made to 25% (w/v) aqueous solutions. All 3 materials require little mixing to dissolve since all show good solubility in H_2O.

Solubilities range from 50% at 20° C for the 20,000 molecular weight PEG, to 70% for the 1500 PEG. The solutions were stored at refrigerator temperature (2° to 8°C)

until needed. The stability of PEG solutions is exceptionally good at either room temperature or refrigerator temperature. These linear polymers do not support mold or bacterial growth and show very little deterioration from oxidation or hydrolysis.

The insulin reagents were obtained from Pharmacia Laboratories, Inc., Piscataway, New Jersey as the "Phadebas Insulin Test" kit. Each kit contains the following reagents, sufficient for 100 insulin determinations: 1) phosphate-saline buffer, pH 7.4, 50 m.mol/liter: 1.52 g of disodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$), 0.2 g of potassium di-hydrogen phosphate (KH_2PO_4), 1.8 g of NaCl, and 0.6 g of human serum albumin; 2) sephadex-anti-insulin complex, lyophilized; 3) insulin standard, porcine (320 microunits/ml after reconstitution), lyophilized; and 4) ^{125}I-labeled insulin, porcine, 8 ng, 3 μ Ci (at date of manufacture).

Preparation of the reagents involves very little time and can be accomplished in 15 to 20 minutes prior to setting-up the test as follows: 1) buffer solution: dissolve the buffer powder in 200 ml of redistilled H_2O. This provides a buffer solution of pH 7.4; 2) sephadex-anti-insulin complex: transfer this material quantitatively to a 200 ml erlenmeyer flask containing a magnetic stirring rod using 100 ml of buffer solution in portions; 3) ^{125}I-labeled porcine insulin: reconstitute the lyophilized material by adding 10 ml volumetric pipette; and 4) insulin

standard: reconstitute the lyophilized material by adding 4 ml of redistilled H_2O with a volumetric pipette. From this stock solution, working standards are prepared by diluting with the buffer to final concentrations of 0, 5, 10, 20, 40, 80, 160, and 320 microunits/ml. The doubling dilution technique as shown in Table 5 may be used to obtain the above concentrations.

The recommended storage temperature for the unopened reagents is between 2° C and 8° C with shelf life approximately 4 months. For storage conditions and shelf life of reconstituted reagents, see Table 6. The reagents are carefully matched for optimal efficiency and lyophilized to promote good stability. The stability of the labeled insulin limits the shelf life of the entire reagent system, but good results can be obtained for up to 4 months from date of manufacture providing a gamma-counter with high efficiency and low background is used.

B. Apparatus

All solutions were prepared from crystalline reagents using a Gram-atic Mettler balance available from Mettler Instrument Corporation, Hightstown, New Jersey. The readability of the balance was 0.0001 grams. Volumetric flasks and pipettes were employed to prepare all solutions to final volume.

TABLE 5

DOUBLING DILUTION TECHNIQUE FOR PREPARING

WORKING STANDARD CURVE

Insulin Standards	Content		Final Insulin Concentration (μU/ml)	Final Volume (ml)
	ml Buffer	ml Insulin		
A	-	1.0	320	0.5
B	0.5	0.5 of A	160	0.5
C	0.5	0.5 of B	80	0.5
D	0.5	0.5 of C	40	0.5
E	0.5	0.5 of D	20	0.5
F	0.5	0.5 of E	10	0.5
G	0.5	0.5 of F	5	1.0
H	0.5	-	0	0.5

TABLE 6

SHELF LIFE AND STORAGE TEMPERATURE OF

(PHADEBAS) RECONSTITUTED REAGENTS

Reagents	Shelf Life/Temp.
Sephadex-Anti-Insulin Complex	4 months at 2-8°C
Buffer	4 months at 2-8°C
Insulin Standard	one week at 2-8°C
Insulin-^{125}I	4 months at -20°C one week at 2-8°C

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A	-	1.0	320	0.5
B	0.5	0.5 of A	160	0.5
C	0.5	0.5 of B	80	0.5
D	0.5	0.5 of C	40	0.5
E	0.5	0.5 of D	20	0.5
F	0.5	0.5 of E	10	0.5
G	0.5	0.5 of F	5	1.0
H	0.5	-	0	0.5

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Insulin-^{125}I	4 months at -20°C one week at 2-8°C

In the clinical test phase of the project, all blood samples were drawn using B-D Multiple Sample Vacutainer Systems with color-coded, red stoppered, plain tubes for obtaining serum. These vacutainer tubes were 16 X 100 mm and have a 10 ml capacity. All B-D equipment is available from Becton-Dickinson Company, Rutherford, New Jersey.

In addition to the "Phadebas Insulin" kit, the RIA system required plastic or siliconized glass centrifuge tubes with round bottoms. In these experiments, polystyrene, 12 X 75 mm, disposable tubes were used. All tubes were obtained from Scientific Products Company, McGaw Park, Illinois.

The small volume of sample and labeled insulin needed in the test procedure required a micro system of pipetting with good accuracy and precision. In addition, because of insulin adsorption on the walls of glass pipettes, the use of semi-automatic micropipettes with dispisable plastic tips is recommended. All pipetting employing 0.2 ml sample or less was done using a 50, 100, or 200 µl MLA precision pipette with disposable tips. These units are manufactured by Medical Laboratory Automation, Inc., Mount Vernon, New York and distributed by Scientific Products Company, McGaw Park, Illinois.

Centrifuging was accomplished by using an International Model UV, non-refrigerated centrifuge and an International Model PR-2 refrigerated centrifuge. Both were obtained from International Equipment Company of Needham Heights, Mass. Speeds employed were between 2,500 and 3,000

RPM depending on number of carriers, tubes, and type of head employed. The temperature of the refrigerated centrifuge was maintained between 2° and 5° C while the Model UV centrifuge was operated in an air conditioned laboratory maintained between 25° to 27° C.

A Beckman Model 76 Expandomatic pH meter was used with a Corning semi-micro combination electrode for measuring pH of all samples. The Model 76 pH meter was obtained from Beckman Instruments, Inc., Fullerton, California. The meter has a pH range from 0 to 14 or expandable over any 2 pH range. Stated accuracy, relative to buffer solution is ± 0.05 pH for any value in the standard 0-14 range and ± 0.01 pH for values in the expanded 2 pH range. All pH values measured in this project were obtained on the 0-14 standard pH range. The combination semi-micro electrode with Ag/AgCl internals is manufactured by Corning Glass Works, Scientific Instruments Division, Medfield, Mass. The use of this electrode allowed minimum sample volume, often as little as 0.3 ml. The triple purpose glass membrane employed permits measurements over the full 0-14 pH range with fast response time. The combination electrode is said to result in more accurate readings due to minimized polarization effects and electrical pick-up noise.

Two different gamma counters were used during the course of this study. Initially, a Baird-Atomic, Model 530 Spectrometer in conjunction with a Baird-Atomic Model 810 C Well Scintillation Detector was employed. The Baird equipment

was obtained from Baird-Atomic, Cambridge, Mass. The well detector has a 1-3/4 inch diameter X 2 inch thick, solid NaI crystal and photomultiplier tube. The standard source for calibration of the Baird System is ^{137}CsCl. The single well was satisfactory for initial test development; however, during the experimental and clinical phase, where many samples were being processed, it became necessary to use an instrument which permitted automatic counting at preset times.

In the later phase of this project, a Packard Model 5320 Auto-Gamma Spectrophotometer System was used with 300 sample capacity. Other features included controlled temperature, automatic operation, and teletype output. This unit was obtained from Packard Instrument Company, Downers Grove, Illinois.

Results were comparable using either instrument, thus no basic technique changes were necessary. Both instruments provided differential or integral windows, a low-level discriminator and window and low level reject and background subtract. Background counts were approximately 20X higher for the Baird Instrument and could not be reduced; however, since background subtract was automatically employed, no problems were encountered by this relatively high background.

Laboratory Data Manager (LDM) computer manufactured by T & T Technology of Madison, Wisconsin was used for statistical computation. The LDM employs a Nova 1200 CPU

with 24 K of 16 bit memory. In addition a 0.5 megabyte disk and a 120 column, 300 line per minute printer interacting with the Nova 1200 CPU were utilized. The statistical programs for determining mean values, standard deviation, coefficient of variation, regression data, and regression coefficient were written in real time disk operating system (RDOS) assembler language.

CHAPTER IV

RESULTS AND DISCUSSION

A. Experimental Organization

The efforts of this investigation were directed towards utilizing previously reported methods[15] for free and total insulin extraction with PEG and using these extracts in the "Phadebas" insulin RIA system.

The "Phadebas" method was used as the reference procedure in evaluation of methods and data for the free and total insulin. Slight modification of the reference method was made and evaluated and found to improve the reproducibility of the procedure. Studies were conducted to determine precision of the reference method at 3 different concentration levels of insulin. In addition, within-run precision, between-run reproducibility and insulin recovery studies were conducted. The standard insulin curves run with each test batch were compared to establish mean values, standard deviation, and coefficient of variation.

Procedures for the free and total methods were developed and results compared to the "Phadebas" reference method. Studies were conducted for the free and total methods to determine correlation at 3 different concentration levels of insulin with the reference method.

In addition, studies to establish within-run precision, between-run reducibility and insulin recovery for both the free and total methods were performed. Finally, the affect of the PEG on the "Phadebas" reference method was investigated to establish the influence of PEG on all phases of the RIA system.

Clinical evaluation of the 3 methods using normal subjects, non-insulin-treated diabetics and insulin-treated diabetics was carried out to a limited extent. Patients just starting insulin therapy as well as patients of long duration therapy were selected to determine and compare insulin-antibody levels. Several patients were selected to follow the dynamics of exogenous insulin release from intramuscular depots throughout a 16 hr. period in the absence of and in the presence of insulin binding antibodies.

B. Methods

1. Blood Sample Collection

Blood samples were drawn in 10 ml B-D vacutainer tubes and allowed to clot for 1 hr. at room temperature. After centrifugation, the serum was separated from the clot and placed in either glass test tubes or polystyrene tubes and stored until assayed. The serum harvested from the clot was sufficient to test by all three methods described. Short term storage, less than 24 hrs., was at refrigerator

temperature (2-8°C). Long term storage, where the assay could not be started for 24 hrs. or longer was at -20°C.

Feldman and Chapman[28] have reported good stability of insulin in serum at room temperature for up to 4 hrs., however, their experimental data did not extend beyond this time period. They also reported good stability of insulin in serum stored at -15°C for up to 30 days and suggested that long term storage exceeding this period may result in significant insulin loss, as much as 59% in 18 months.

The use of polystyrene tubes for storage of serum is said to avoid insulin adsorption to the walls of glass tubes. This phenomenon is observed with pure aqueous insulin standards, but not observed to any significant degree in serum containing protein. Reference[29] is made to the use of plastic tubes for serum storage in several reports, but no data is given as to actual studies conducted.

2. "Phadebas" Reference Method

The analytical procedure was routinely performed using the following technique:

a) Pipette 0.1 ml of standards or unknown samples in duplicate into 12X75 mm polystyrene tubes.
b) Pipette 0.1 ml of ^{125}I-labeled insulin into each tube.
c) Pipette 1.0 ml of Sephadex-anti-insulin immunosorbant. Adequate mixing of the suspension while dispensing is necessary.

d) Stopper the tubes and incubate for 16 hrs. (overnight) at room temperature, keeping the particles in suspension by rotation of the tubes in a vertical rotator.

e) Centrifuge the tubes at 2,500 to 3,000 rpm for 10 minutes, then carefully remove the stoppers and aspirate the supernatant down to within 8 mm of the bottom where the Sephadex particles are packed. An adjustable collared suction nozzle attached to a laboratory vacuum line or a water aspirator may be used.

f) Pipette 2.0 ml of 0.9% (w/v) NaCl into each tube without splattering and centrifuge for 10 minutes.

g) The washing procedure should be repeated 3 times to remove all unbound ^{125}I-labeled insulin. Extreme care must be used in the washing procedures to avoid removal of the particles. Loss of particles will result in lower counts and give falsely elevated insulin levels.

h) After the last washing, withdraw the supernatant and re-stopper the tubes. Determine the radioactivity in each of the tubes using a gamma-counter with high efficiency and low background. Counting times of between 2-5 minutes may be used.

i) Calculation: Express the count rates (CR) for each of the standards and unknowns as a percentage of the mean count-rates (MCR) of the "zero" samples. If automatic background subtract is not a feature of the gamma-counter, significant background counts should be subtracted manually.

$$Y = \frac{\text{(CR) of standards or unknowns}}{\text{(MCR) of "zeros"}} \times 100 \qquad (1)$$

Plot the percentage values obtained for the insulin standards against the logarithm of insulin concentration on lin-log paper and construct a standard curve (Figure 9). The percentage values (Y) obtained for the insulin standards may also be plotted vs. insulin concentration on linear graph paper and a standard curve drawn (Figure 10). As an alternative to lin-log and linear plots, the percentage values (Y) for the insulin standards can be plotted on logit-log paper which gives a virtually straight line curve (Figure 11).

Read the concentration of insulin directly from the curve (in microunits/ml) for each of the unknown samples.

3. Free Insulin Method

The extraction of free insulin from serum with PEG was performed routinely as follows:

a) Pipette 1.0 ml of serum into a 16X100 mm glass test tube.
b) Pipette 1.0 ml of cold 25% (w/v) polyethylene glycol.
c) The mixture is immediately mixed on a vortex mixer for one minute.
d) Centrifuge the tubes at 2,500 to 3,000 rpm for 30 minutes in either a refrigerated or non-refrigerated centrifuge.
e) The upper phase is then used directly in the "Phadebas" RIA system.

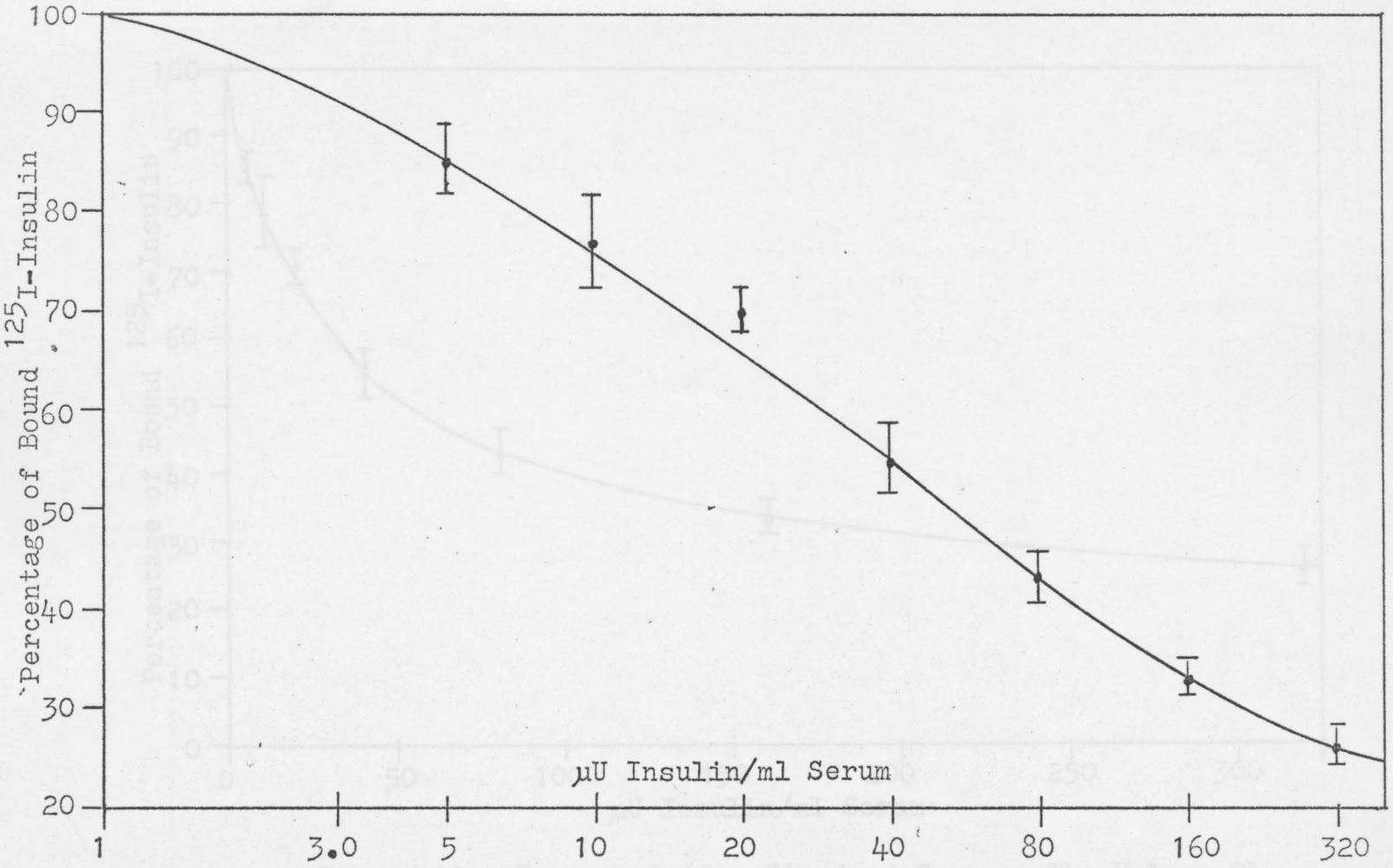

Figure 9. Representative Standard Curve. The Values Obtained For the Insulin Standards (Expressed as Percentages) are Plotted vs. the Logarithm of Insulin Concentration on Lin-Log Paper. Each Point Represents 10 Determinations, and the Standard Error of the Mean is Shown.

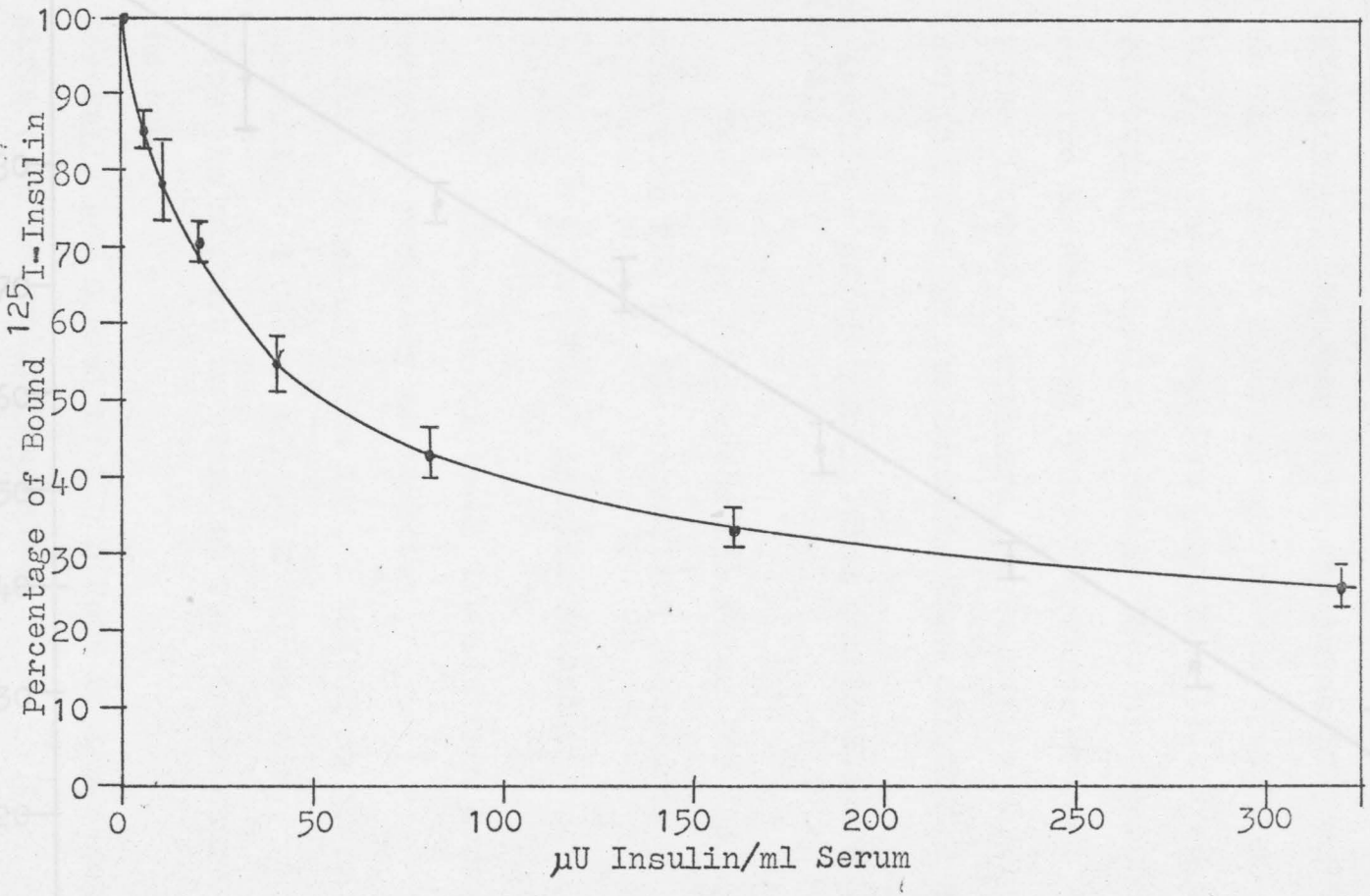

Figure 10. Representative Standard Curve. The Values Obtained For the Insulin Standards (Expressed as Percentages) Are Plotted vs. Insulin Concentration on Linear Paper. Each Point Represents 10 Determinations, and the Standard Error of the Mean is Shown.

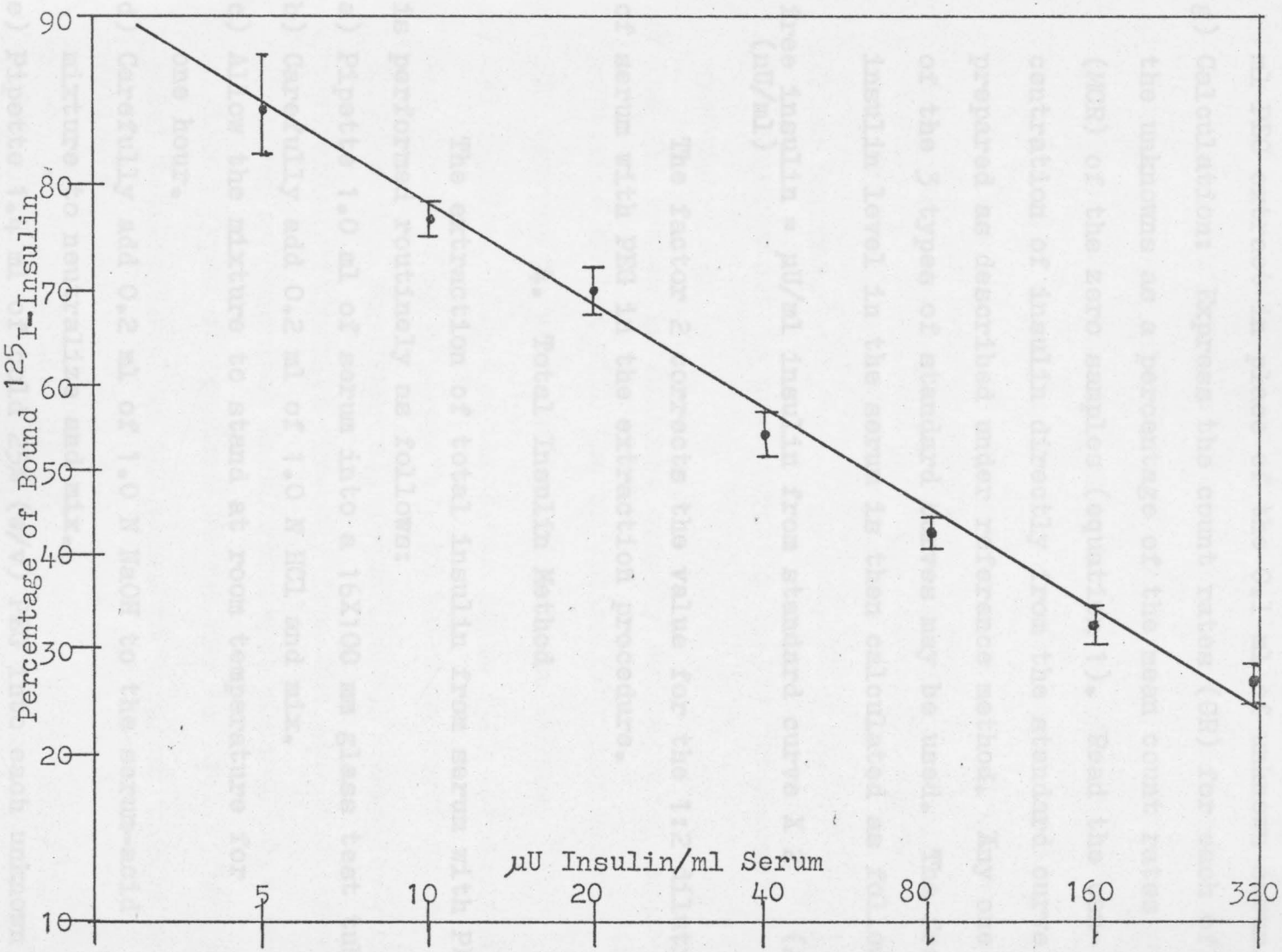

Figure 11. Representative Standard Curve. The Values Obtained For the Insulin Standards (Expressed as Percentages) are Plotted vs. Insulin Concentration on Logit-Log Paper. This Type of Curve Gives an Essentially Straight Line. Each Point Represents 10 Determinations, and the Standard Error of the Mean is Shown.

f) The RIA system is followed exactly as described under reference method with the exception of using the 0.1 ml PEG extract in place of the 0.1 ml of unknown serum.

g) Calculation: Express the count rates (CR) for each of the unknowns as a percentage of the mean count rates (MCR) of the zero samples (equation 1). Read the concentration of insulin directly from the standard curve prepared as described under reference method. Any one of the 3 types of standard curves may be used. The free insulin level in the serum is then calculated as follows:

$$\text{free insulin } (\mu U/ml) = \mu U/ml \text{ insulin from standard curve} \times 2 \quad (2)$$

The factor 2 corrects the value for the 1:2 dilution of serum with PEG in the extraction procedure.

4. Total Insulin Method

The extraction of total insulin from serum with PEG is performed routinely as follows:

a) Pipette 1.0 ml of serum into a 16X100 mm glass test tube.

b) Carefully add 0.2 ml of 1.0 N HCl and mix.

c) Allow the mixture to stand at room temperature for one hour.

d) Carefully add 0.2 ml of 1.0 N NaOH to the serum-acid mixture to neutralize and mix.

e) Pipette 1.4 ml of cold 25% (w/v) PEG into each unknown tube.

f) The mixture is immediately mixed on a vortex mixer for one minute.

g) Centrifuge the tubes at 2,500 to 3,000 rpm for 30 minutes in either a refrigerated or non-refrigerated centrifuge.

h) The supernatant should be clear, free of globulin type proteins. If turbidity is noticed, slight adjustment of pH to 8.5 ± 0.3 is necessary. One small drop of 0.5 N HCl (less than 0.05 ml) will adjust the pH to the desired range for clearing of the material. Remixing and recentrifuging are again necessary to obtain the final clear extract.

i) The supernatant is used directly in the "Phadebas" RIA system.

j) The RIA system is followed exactly as described under reference method with the exception of using 0.1 ml PEG extract in place of 0.1 ml unknown serum.

k) Calculation: Express the count rates (CR) for each of the unknowns as a percentage of the mean count rates (MCR) of the zero samples, (equation 1). Read the concentration of insulin directly from the standard curve prepared as described under reference method. Any one of the 3 types of standard curves may be used. The total insulin level in the serum is then calculated as follows:

$$\text{total insulin } (\mu U/ml) = \mu U/ml \text{ insulin from standard curve} \times 2.8 \qquad (3)$$

The factor 2.8 corrects the value for the 1:2.8 dilution of serum with HCl, NaOH, and PEG in the extraction procedure.

1) The antibody-bound insulin level may then be calculated as follows:

$$\underset{(\mu U/ml)}{\text{bound-insulin}} = \underset{(\mu U/ml}{\text{total insulin}} - \underset{(\mu U/ml)}{\text{free insulin}} \qquad (4)$$

The bound insulin reflects the amount of human insulin antibody present in circulating blood. Berson and Yalow[9] conclude from their experimental investigations that insulin is univalent in its reaction with antibody and that binding of more than a single antibody molecule at different possible antigenic sites is conceivably prohibited by steric hindrance.

C. Results

1. The Effects of PEG on the RIA System

Studies were conducted to determine any interference in the RIA system by PEG.

The effects of PEG on ^{125}I-insulin solution was determined by comparing duplicate counts of 0.1 ml labeled insulin (R-tube) and duplicate counts of tubes containing 0.1 ml labeled insulin and 0.1 ml PEG (RP-tubes). The R-tube mean count was 24,909 counts/2 min. (SEM = 141) and the RP-tubes mean was 24,983 counts/2 min. (SEM = 186). The difference between the 2 mean counts is 74 and falls well

within the 95% confidence limits for the standard error of the mean for the reference method.

The effect of PEG on the "zero" tube (H) was determined by comparing duplicate counts on the H-tubes containing 0.1 ml buffer, 0.1 ml labeled isotope, and 1.0 ml of antibody-Sephadex complex with duplicate counts on the "zero" tubes (HP) containing 0.1 ml PEG, 0.1 ml labeled isotope, and 1.0 ml antibody-Sephadex complex. The H-tube mean was 4,602 counts/2 min. (SEM = 80) and the HP-tubes mean was 4,737 (SEM = 60). The difference between the 2 mean values is 135 and falls well within the 95% confidence limits for the standard error of the mean for the reference method.

The effect of PEG on the RIA system in the presence of unlabeled insulin is reflected by the degree of correlation between the reference method and both the free and total insulin procedures in terms of final insulin concentration. These figures will be presented under "Correlation of Methods". The PEG could not be added to the reference method tubes in which 0.1 ml serum was added since the PEG precipitated the globulin fraction of proteins present and made the test unreliable. In view of this, mean counts could not be compared on an equal basis as was the case with the R and H tubes. It then became obvious that correlation of methods in terms of recovered insulin could be used to demonstrate any interference by PEG on the RIA assay.

On the basis of the 3 studies conducted and the data obtained, it was concluded that PEG in the volume and concentration added did not interfere in the "Phadebas" reference method. In addition, the use of PEG to extract free insulin from serum with use of this extract in the reference method proved to have a reasonable degree of correlation.

2. Recovery Studies

Studies to determine the recovery of added standard insulin to serum were carried out by all three methods. Recovery was attempted at 3 different levels of insulin for the ranges normally encountered in clinical testing.

A pool of serum was prepared by mixing low level insulin serums from patients previously tested. The pool was assayed by the reference method in triplicate and found to contain 16 μU/ml of insulin. Further, the pool was tested for insulin-binding antibodies and found free of these antibodies. The pool was then divided into 3 parts and standard porcine insulin (320 μU/ml) was added to adjust the total value of insulin to: Pool #1) 44μU/ml; Pool #2) 89 μU/ml; and Pool #3) 169 μU/ml. The base pool serum and the pool mixtures were then assayed by each method and results compared to determine percent recovery of theoretical value and correlation between recovery of all 3 procedures. Table 7 shows the volumes of serum and standard used in preparing the mixtures and Table 8 shows the results of the recovery study in terms of μU/ml insulin for each method and mixture and percent recovery based on theoretical value.

TABLE 7

SHOWN ARE THE VOLUMES OF BASE SERUM AND STANDARD INSULIN USED IN PREPARING RECOVERY STUDY SPECIMENS AND THE THEORETICAL VALUE OF EACH ONE.

	Base Pool	Pool #1	Pool #2	Pool #3
ml of serum		6.0	4.5	5.0
ml of 320 µU/ml standard		1.0	2.5	0
ml of 640 µU/ml standard		0	0	2.0
µU/ml insulin from serum	16	14	10	11
µU/ml insulin from standard	0	45.7	114.3	183.0
Theoretical value µU/ml	16	59.7	124.3	194.0

TABLE 8

RESULTS OF RECOVERY STUDY SHOWING μU/ml INSULIN RECOVERED AND % RECOVERY OF THEORETICAL VALUE.

Specimen	Theoretical Value	Reference		Free Insulin		Total Insulin	
		μU/ml	% Recov.	μU/ml	% Recov.	μU/ml	% Recov.
Base serum	16	16	100%	12	75%	13	81%
Pool #1	59.7	53	89%	37	62%	41	69%
Pool #2	124.3	99	80%	127	102%	117	94%
Pool #3	194.0	180	93%	193	99%	197	102%

The mean percent recovery at all levels for each method was: reference, 90.5%; free insulin, 84.5%; and total insulin, 86.5%. As shown in the chart, the recovery for the reference method was 80% or more at all levels with the lowest recovery at the 124 µU/ml level. The free insulin method had good recovery at the intermediate and high levels, but fell off at the lower level of insulin concentration. The total insulin method had a better overall percent recovery than did the free method, but also showed a drop in recovery at the lower level.

The percent recovery obtained for the "Phadebas" reference method agrees well with studies published by Velasco and Oppermann[29], where they report recovery on ten different concentrations of crystalline human insulin dissolved in a solution of bovine serum albumin and analytically determined by the "Phadebas" method. They report 60 to 102% recovery of insulin in concentrations ranging from 37 µU/ml to 235 µU/ml and show their lowest recovery at levels of below 40 µU/ml. Velasco and Oppermann[29] also report a mean percent recovery of 94 at all concentration levels with a standard deviation of 12.31 and the standard error of the mean (SEM) of 3.89. Thus, in their evaluation of the "Phadebas" method, 95% confidence limits would mean that the average percent recovery could vary between 86.22 and 101.78.

Results obtained in this project for percent recovery for the "Phadebas" method (Table 8) give a mean percent recovery at all levels of 91, standard deviation of 8.4 and SEM of 4.19. Thus, the mean value of percent recovery could be expected to vary between 82.62 and 99.38 for 95% confidence limits. It appears reasonable to conclude that performance of the "Phadebas" method in terms of percent recovery of added insulin to protein material can be shown to be reproducible in the hands of different users and that the method appears to be quite specific for insulin.

The free insulin and total insulin method were then compared to the "Phadebas" reference method in terms of percent recovery . The free method for the same recovery specimens (Table 7) had a mean percent recovery at all levels of 85, a standard deviation of 19.3 and SEM of 9.65. Thus, in the free method, the mean value for percent recovery could be expected to range between 66 and 104 for 95% confidence limits. The SEM for the free method is approximately 2X the value obtained for the reference method and indicates somewhat less precision and accuracy when comparing all concentration levels. If one compares the higher levels, the percent recovery improves significantly.

Studies conducted by Nakagawa, et al[15], on the use of PEG to measure free insulin show an average percent recovery at all concentration levels of 75. In the present study, with the procedures described, the percent recovery

has been improved to 85%.

The total insulin method for the same recovery specimens (Table 7) had a mean percent recovery at all concentration levels of 87, standard deviation of 14.5 and SEM of 7.25. The mean percent recovery could be expected to vary between 72.5 and 101.5 for 95% convidence limits. The total method shows closer agreement with the reference method in terms of percent recovery and also a lower SD and SEM than does the free method. Nakagawa, et al[15], reported percent recovery for their total insulin method of 78% at all concentration levels with lowest recovery at 15 μU/ml insulin concentration. Thus, the proposed method, appears to offer better overall recovery throughout the range of 0-320 μU/ml than does former methods for PEG extraction of insulin.

3. Reproducibility Studies

To investigate the reproducibility of repeated assays on aliquots of the same material, between-run precision studies as well as with-in run assays by all 3 methods; reference, free, and total were carried out.

The between-run study was determined using a pool of normal serum. Aliquots of 0.1 ml of fresh pooled serum were pipetted into polystyrene tubes and immediately stored at -20°C until used. These tubes were then removed, thawed prior to assay, and used in the reference method. Duplicate extracts of the same pool were prepared for the free and

total methods. These extracts were then aliquoted into 0.1 ml quantities and stored in polystyrene tubes at -20°C until used.

The results for the reference method ranged from 22 to 50 µU/ml with a mean value of 35.5. One standard deviation is 8.66 and the COV is 24.4%. For the free method, the values ranged from 26 to 44 µU/ml with a mean value of 33.6. One standard deviation is 5.71 and the COV is 16.9%. The total method results ranged from 20 to 39 µU/ml with a mean value of 29.6. One standard deviation is 6.06 and the COV is 20.49%. All the data is shown in Table 9 for each method and for each of the 15 between-run assays completed.

The high COV obtained for all methods did not correlate well with published data by Pharmacia Laboratories, manufacturers of the "Phadebas" kit or with Velasco and Oppermann[29]. Pharmacia believes that one can obtain precision throughout the entire range of 0-320 µU/ml with a COV of approximately 10%. Velasco and Opperman[29] at low level ranges of concentration show a COV of 5.7% which is in line with Pharmacia. Other studies conducted in this investigation show better precision than this between-run study. While the free method showed less variability than the reference and total methods, all 3 methods show more variability than is desirable for insulin assays. In view of the data for this between-run reproducibility study, further studies should be carried out with other serum specimens, preferably at several concentration levels,

TABLE 9

BETWEEN-RUN REPRODUCIBILITY STUDIES ON HUMAN POOLED SERUM BY REFERENCE, FREE, AND TOTAL INSULIN METHODS.

	μU/ml Reference	μU/ml Free Method	μU/ml Total Method
	36	34	34
	22	38	39
	27	44	39
	50	42	20
	38	30	28
	34	34	22
	31	34	31
	41	26	27
	39	28	27
	36	30	28
	48	30	30
	24	38	32
	34	40	33
	36	33	33
	30	32	31
Mean	35.5	33.6	29.6
1 SD	8.6	5.7	6.0
2 SD	17.32	11.4	12.1
C.O.V.	24.39%	16.98%	20.49%

to better establish the between-run precision.

The limited data of this between-run precision study does not suggest less precision when using the PEG extracts than when using serum in the reference method. In fact, the precision seems to be slightly better in both the free and total methods. The preparation of the PEG extract for both the free and total methods with subsequent aliquoting, freezing, and thawing, does not appear to introduce any significant loss of precision over that of the reference method where serum is prepared and used as described above. It may be that the precision of all 3 methods is adversely affected by the freezing, storing, and thawing and should be considered in future studies.

The within-run precision studies were performed using samples of the same serum for the reference method and aliquots from the same extract for both the free and total methods. The purpose of this experiment was to eliminate multiple extraction samples for both free and total methods and determine precision on repeated assays from the same free extract or total extract at different insulin concentration ranges.

The results, Table 10, show decreased precision for all three methods as one increases insulin concentration. The reference method gives expected precision of ($\approx$ 10%) at levels up to 50 µU/ml but decreases in precision at 50-100 µU/ml to an average COV of 17.9%. If one compares the between-run data with the within-run results for the

TABLE 10

TABLE SHOWING WITHIN-RUN PRECISION AT DIFFERENT CONCENTRATION LEVELS. ALL ASSAYS WERE FROM SAME SERUM OR SAME EXTRACT IN EACH METHOD.

Insulin Range	Reference Method			Free Method			Total Method		
	No.	SD	COV	No.	SD	COV	No.	SD	COV
0-30	5	2.4	12.9%	5	1.2	6.0	5	2.1	16.8
31-50	12	4.1	10.6	5	6.1	18.3	5	6.2	15.2
51-80	5	15.4	19.4	5	9.0	13.5	6	18.2	23.8
81-100	5	16.4	16.5	6	14.8	15.4	7	23.0	23.8
101-250	-	-	-	13	20.7	16.5	6	20.7	18.1

reference method, the COV percentage differs by 6.4% and indicates somewhat less variability in the within-run results which would be the expected trend.

The within-run data shown in Table 10 for the free method shows comparable precision at all ranges with the reference method except for the 31-50 µU/ml range. Here the COV increased to 18.3% which appears to be out of line with data at the range of 51-80 µU/ml. It is obvious that further within-run studies should be made at this range to better establish the correct precision. The average over-all COV for the free method is 13.9% which is approximately the same average value as found for the reference method.

The within-run data shown in Table 10 for the total method shows somewhat more variability than does either the reference or free method. The average over-all COV is 19.5% with greatest variability between the 50 and 100 µU/ml range. The lowest COV which gives least variability is seen at the 31-50 µU/ml range which is in contrast to the free method where the highest COV was observed.

If one compares the between-run and the within-run COV for the free and total methods, the expected lower COV of the within-run study is observed for both methods. The between-run COV in the free method is 3.0% higher than the within-run COV and in the total method, the difference is 1% higher.

4. Comparison of Methods

The three methods were evaluated by several statistical techniques to determine the degree of correlation.

Scatter diagrams were drawn showing the relationship between the reference and free methods, the reference and total methods, and the free and total methods. Figure 12 shows the scatter of results around the slope derived from the linear regression equation where: $Y = 1.03\ (X) - 3.01$. The equation is derived from 40 serum samples at all concentration levels analyzed both by the reference and free insulin methods.

The regression equation[30] was calculated using the relationship:

$$Y = a + bX \tag{5}$$

where: a = the intercept with the Y axis

b = the slope of the line or the change in Y per unit change in X

The slope (b) is then found using the equation:

$$b = \frac{N\Sigma XY - (\Sigma X)(\Sigma Y)}{N\Sigma X^2 - (\Sigma X)^2} \tag{6}$$

The intercept (a) is then calculated by the expression:

$$a = \bar{Y} - b\bar{X} \tag{7}$$

Finally substitute the values for (a) and (b) in equation (1) and this gives the linear regression equation which is the best-fit relationship between the 40 serum samples assayed by both methods.

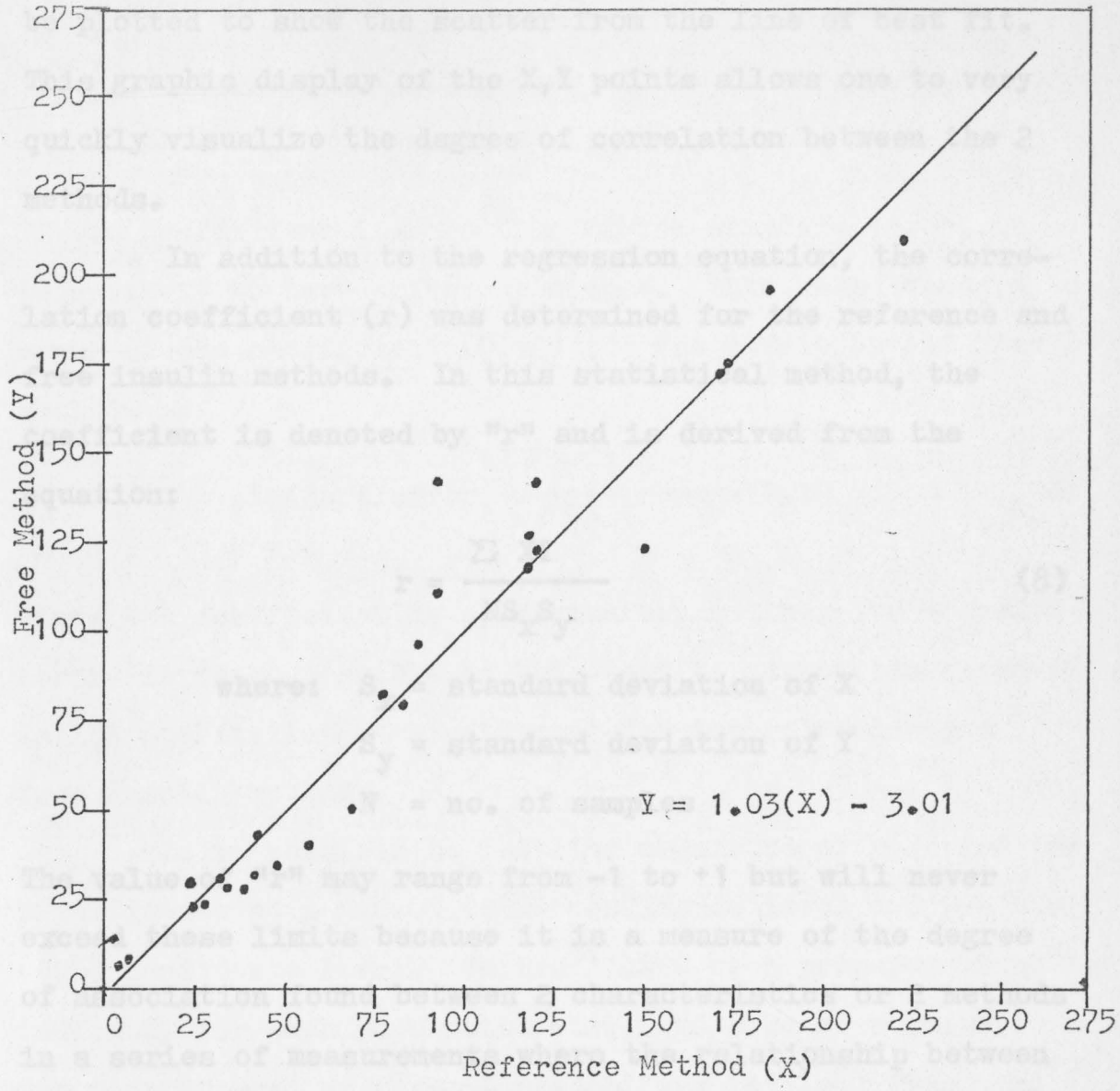

Figure 12. Scatter Diagram Showing the Correlation Between the Reference and Free Insulin Methods. Evaluation of 40 Serum Samples by Both Methods Yields the Linear Regression Equation, Y = 1.03(X) - 3.01.

In the scatter diagram, the X,Y points can then be plotted to show the scatter from the line of best fit. This graphic display of the X,Y points allows one to very quickly visualize the degree of correlation between the 2 methods.

In addition to the regression equation, the correlation coefficient (r) was determined for the reference and free insulin methods. In this statistical method, the coefficient is denoted by "r" and is derived from the equation:

$$r = \frac{\Sigma XY}{NS_xS_y} \qquad (8)$$

where: S_x = standard deviation of X

S_y = standard deviation of Y

N = no. of samples

The value of "r" may range from -1 to +1 but will never exceed these limits because it is a measure of the degree of association found between 2 characteristics or 2 methods in a series of measurements where the relationship between the 2 methods is described by a straight line. The value of $r = \pm 1$, denotes perfect correlation, either in the positive or negative direction, whereas an r value of 0.00 indicates that the 2 methods are uncorrelated.

The correlation coefficient for the reference and free methods is 0.97 for the 40 serum samples assayed. The test of significance for the coefficient is to test whether the value might have arisen by chance. The standard error used for this test of significance is:

$$\text{standard error} = \frac{1}{\sqrt{n-1}} \qquad (9)$$

where n = the number of samples assayed by both methods

If the coefficient is more than 2X the standard error, it is unlikely to have arisen by chance. Thus, the standard error of the coefficient (0.97) for the reference and free methods is:

$$\text{standard error} = \frac{1}{\sqrt{40-1}} = 0.16 \qquad (10)$$

Since the coefficient is approximately 6 times its standard error, it seems possible by this test to accept the correlation coefficient as significant for the reference and free insulin methods.

It is possible to test the magnitude of r needed for significance at p = 0.05 (95%) confidence level and p = 0.01 (99%) confidence level. Tables[31] have been prepared which indicate, for each population size, the size of r required for significance at these 2 probability levels. Thus, the magnitude of r needed for significance where p = 0.05 is 0.312 and where p = 0.01 for 40 samples is 0.401. The correlation coefficient of 0.97 for the reference vs free methods is well within 95% confidence limits given by the probability tables for r values. The value of 0.312 is called the critical value of r at the level of p = 0.05 and if the sample correlation coefficient exceeds this

critical value, then the sample value is significantly different from 0. It may also be said that if the sample value exceeds the critical value for r, it is too great to have occurred by chance from an uncorrelated population. Thus, it can be concluded that there is significant relationship between the reference and free insulin methods.

Similar method comparison was performed between the reference and total insulin procedures. Figure 13 shows the scatter diagram of the X, Y points plotted in relationship to the linear regression curve determined as in equation (1). The regression equation which describes the curve is:

$$Y = 0.98(X) + 1.27 \qquad (11)$$

and the correlation coefficient is 0.945. It should be mentioned that the distance the X, Y points lie from the best-fit line of the scatter diagram affects the size of the correlation coefficient. If there is a large amount of scatter among the points, the distances of the points from the best-fit line are greater; thus, the size of the coefficient is smaller. On the other hand, if there is a small amount of deviation from the best-fit line, the coefficient is large. If there is no deviation from the best-fit line, the coefficient would be 1.00 or -1.00 and the correlation of the 2 methods would be perfect.

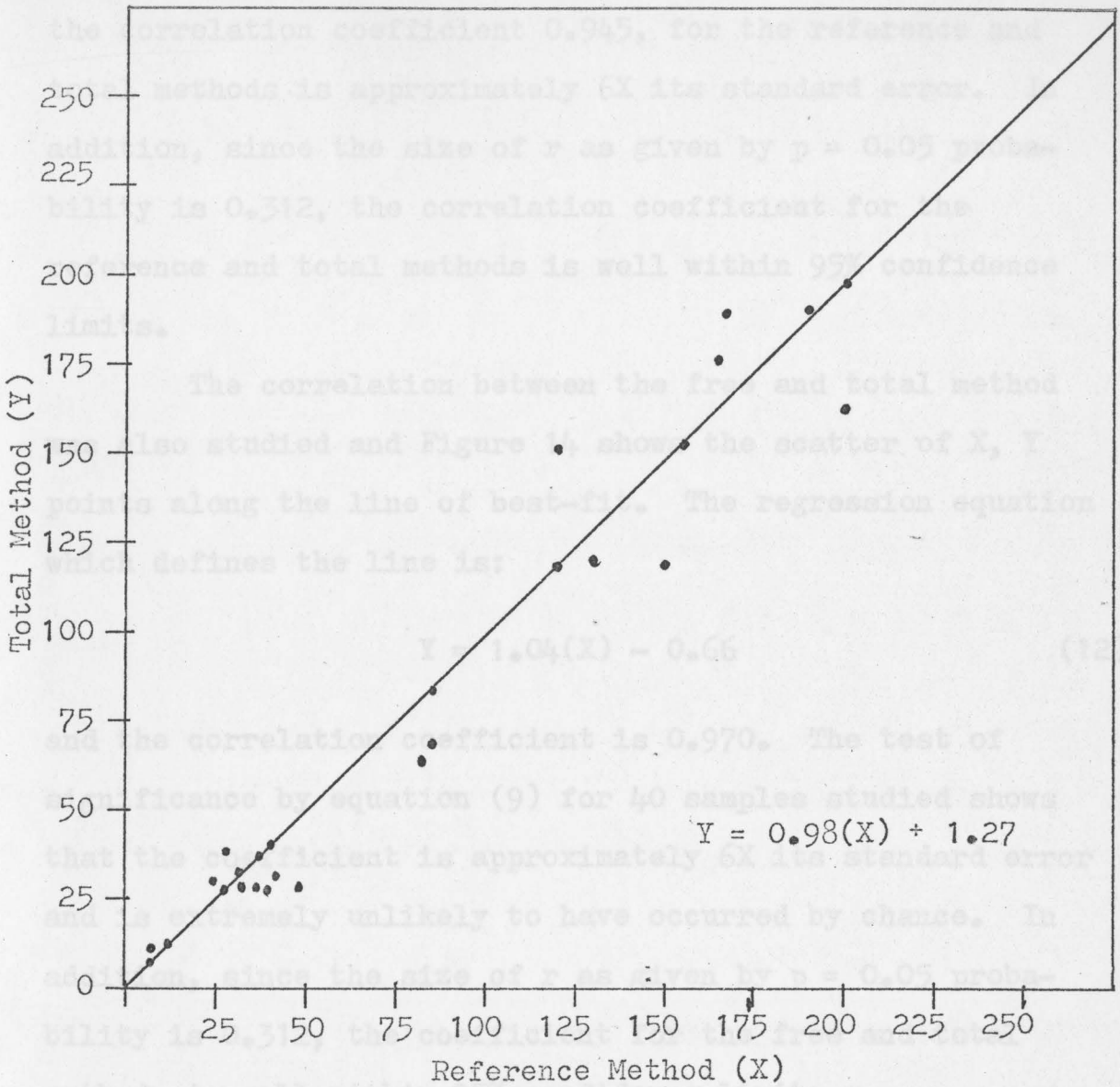

Figure 13. Scatter Diagram Showing the Correlation Between the Reference and Total Insulin Methods. Evaluation of 40 Serum Samples By Both Methods Yields the Linear Regression Equation, Y = 0.98(X) + 1.27

The test of significance by equation (9) shows that the correlation coefficient 0.945, for the reference and total methods is approximately 6X its standard error. In addition, since the size of r as given by p = 0.05 probability is 0.312, the correlation coefficient for the reference and total methods is well within 95% confidence limits.

The correlation between the free and total method was also studied and Figure 14 shows the scatter of X, Y points along the line of best-fit. The regression equation which defines the line is:

$$Y = 1.04(X) - 0.66 \qquad (12)$$

and the correlation coefficient is 0.970. The test of significance by equation (9) for 40 samples studied shows that the coefficient is approximately 6X its standard error and is extremely unlikely to have occurred by chance. In addition, since the size of r as given by p = 0.05 probability is 0.312, the coefficient for the free and total methods is well within 95% confidence limits.

As another statistical method for comparing two procedures, the "t" test[30] was used. The t test is a means of comparing 2 methods using the variance and mean of differences between pairs of results. The Student's t test is given by the equation:

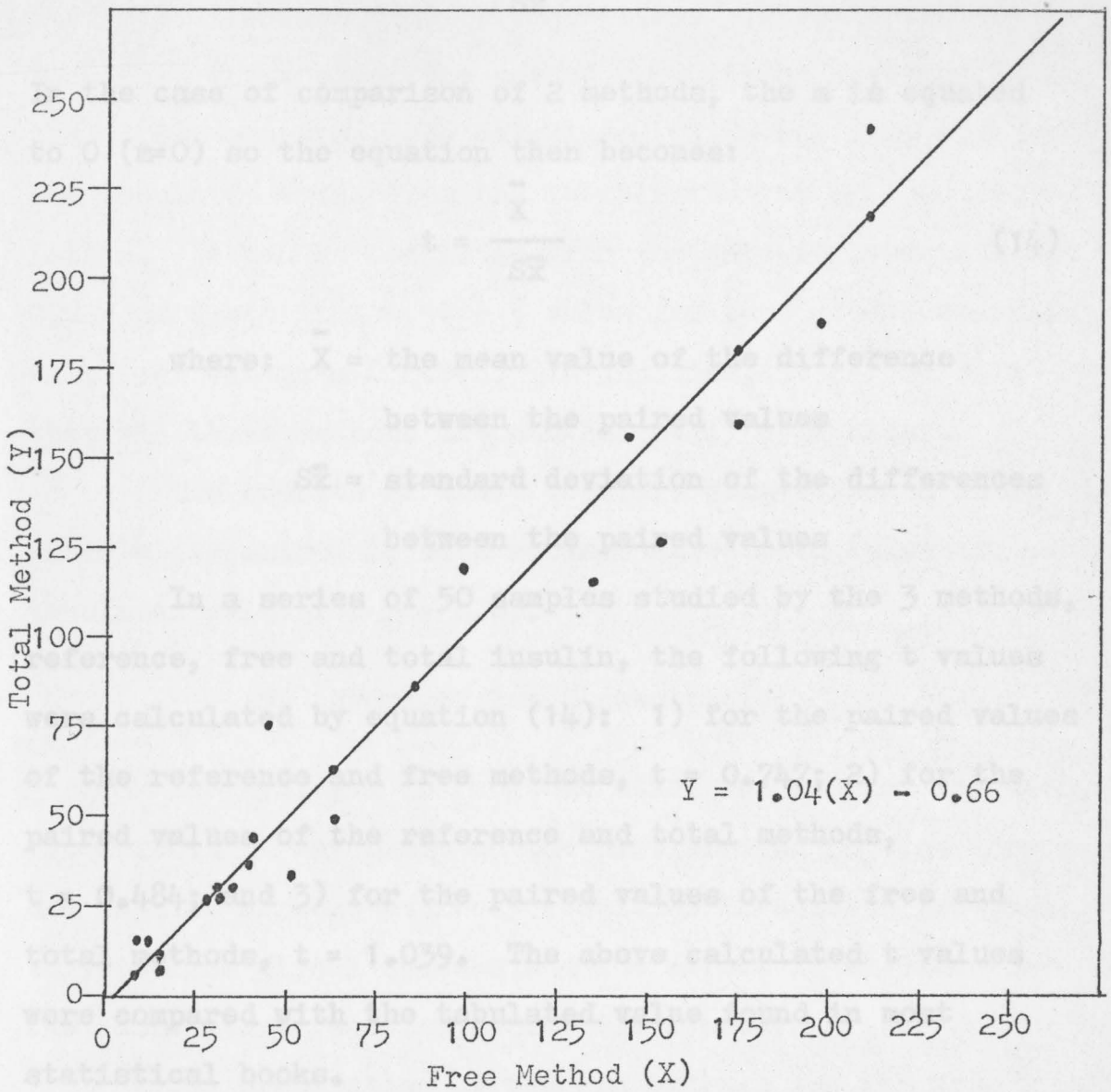

Figure 14. Scatter Diagram Showing the Correlation Between the Free and Total Insulin Methods. Evaluation of 40 Serum Samples by Both Methods Yields the Linear Regression Equation, Y = 1.04(X) - 0.66.

$$t = \frac{(\bar{X}-m)}{S\bar{x}} \qquad (13)$$

In the case of comparison of 2 methods, the m is equated to 0 (m=0) so the equation then becomes:

$$t = \frac{\bar{X}}{S\bar{x}} \qquad (14)$$

where: $\bar{X}$ = the mean value of the difference between the paired values

$S\bar{x}$ = standard deviation of the differences between the paired values

In a series of 50 samples studied by the 3 methods, reference, free and total insulin, the following t values were calculated by equation (14): 1) for the paired values of the reference and free methods, t = 0.747; 2) for the paired values of the reference and total methods, t = 0.484; and 3) for the paired values of the free and total methods, t = 1.039. The above calculated t values were compared with the tabulated value found in most statistical books.

The t value for 50 degrees of freedom where p = 0.05 is ± 2.018. It should be mentioned that when using the Student's t test, the degrees of freedom is equal to the number of paired results minus one (n-1); however, no table could be found listing the tabulated value for 49 degrees of freedom. The t value for 50 paired samples

gives a probability that 95% of such samples should have Student's t values of ± 2.018 or less. Thus, it can be seen that for each of the method comparisons analyzed by the t test equation, the calculated value was less than the tabulated value from the t table indicating good correlation. It can be concluded from the data derived that at 95% confidence limits, the t value for each method compared will fall within a ± 2.018. In addition, it can be said that all three methods are statistically equivalent.

The results of the t test are summerized in Table 11 where a comparison is made between calculated value and tabulated value for p = 0.05.

5. Hydrogen Ion Concentration

The hydrogen ion concentration was studied for both the effects it may exert on the partition of peptide hormones and peptide hormone-antibody complexes in aqueous two-phase polyethylene glycol systems and the effect it may have on the competitive reaction in the RIA system.

The preparation of the PEG extract for the free insulin method was investigated for hydrogen ion concentration and its affects on the RIA system in altering the pH from that of the reference method. The pH of the PEG solution (25% w/v) was found to be 5.5 ± 0.1. When 1.0 ml of human serum (pH range 7.40 to 7.50) was mixed with 1.0 ml of 25% PEG, the resulting pH was found to be 8.42 ± 0.10. This pH range compares favorably with data reported by

TABLE 11

THE CALCULATED t VALUES FOR COMPARISON OF THE REFERENCE, FREE, AND TOTAL INSULIN METHODS. ALSO THE TABULATED t VALUE FOR p = 0.05 IS SHOWN. ALL RESULTS ARE FOR 50 DEGREES OF FREEDOM.

Method	Calculated t Value	Tabulated Value at p=0.05
Reference Vs Free Method	± 0.747	± 2.018
Reference Vs Total Method	± 0.484	± 2.018
Free Vs Total Method	± 1.039	± 2.018

Desbuquois and Aurbach[14] who studied the solubility of the free hormone insulin in PEG solutions. They found that as the pH was lowered from 9 to 7 there was no decrease in solubility of the insulin hormone. Precipitability of the heavy molecular weight antibody-bound hormone was not affected by pH over the 9 to 7 range. The free insulin hormone is not coprecipitated with other serum proteins until the pH drops below 7, with maximal precipitation effect at pH 4 to 6. Polson[26] reported that between the pH range of 4.4 to 5.8, all fibrinogen and globulin could be precipitated. Thus, since insulin is bound to the globulin fraction, it would not be precipitated in the pH range 9-7.

The optimum pH of 7.40 is maintained in the RIA system by a phosphate-saline buffer. The addition of 0.1 ml of free insulin PEG extract to the reaction tube which contained 0.1 ml of labeled insulin and 1.0 ml of antibody-Sephadex dissolved in phosphate-saline buffer did not stress the buffer system and pH was maintained at 7.40 ± 0.02.

The hydrogen ion concentration was also studied in the total insulin extraction procedure where prior action by 1N HCl was necessary to break the binding between the insulin and the insulin antibody. The use of 1N HCl was necessary to insure adequate hydrogen ion concentration, yet not dilute the sample excessively. It was found that with the addition of 0.2 ml of 1N HCl to 1ml of serum, the pH dropped to 2.5 ± 0.10.

The serum-acid mixture was permitted to stand at room temperature for 1 hr. to allow cleavage at the antigen-antibody binding sites. Again the pH was measured and found to be 2.5 ± 0.10. Upon the addition of 0.2 ml of 1N NaOH, the pH adjusted to 8.5 ± 0.3. Finally, with the addition of 1.4 ml of 25% w/v PEG, the pH stabilized at pH 8.4 ± 0.3. Centrifugation for 30 minutes at 2,800 rpm produced a clear to slightly turbid supernatant with a precipitated layer of protein packed at the bottom of the tube. The addition of 0.1 ml of this extract to the RIA system reaction tube which contained 0.1 ml labeled insulin and 1.0 ml antibody-Sephadex dissolved in phosphate-saline buffer did not stress the buffer system so that pH was ideally maintained at pH 7.40 ± 0.02.

Occasionally in the preparation of the total method extract, a serum was encountered which produced an extract with considerable turbidity and difficult to separate into the 2 phases. Further study of the pH on these specimens revealed that after addition of the 0.2 ml of 1N NaOH, the pH ranged between 9.3 and 9.5 and upon addition of the PEG remained at a pH well above 9.0. This high hydroxyl ion concentration does not permit the PEG to properly precipitate the heavy molecular weight protein molecules and after centrifugation shows incomplete separation of the 2 phases with gross turbidity of the upper liquid portion. These specimens can be salvaged by the addition of 1 small

drop (0.05 ml) of 0.5N HCl, to adjust the pH between 8.0 to 9.0 where precipitation of the heavy molecular weight compounds is ideal and solubility of the lower molecular weight peptide hormones is not affected.

The use of 1N HCl and 1N NaOH in the preparation of the total insulin extract requires extreme care in pipetting these two reagents to insure proper hydrogen ion concentration. It can easily be seen how one small drop, more or less, of either the 1N acid or the 1N base can tremendously affect the hydrogen ion concentration. This difficulty in controlling the 0.2 ml of added HCl or NaOH is responsible for the occasional non-separation of the 2 phases in the PEG extraction rather than the particular serum sample being at fault.

6. The Influence of Time on Precipitation in the RIA System

In the RIA system, the competitive reaction between the antibody-Sephadex, labeled insulin, and free insulin present in the serum or PEG extract results in insulin-antibody complex formation or precipitation. It has been reported that incubation times of from 3 hrs. to 24 hrs. can be used to accomplish this precipitation. Velasco and Opperman[29] investigated the minimum time required to obtain the greatest insulin-antibody precipitate in the reference method using serum. They concluded that there was an increase in insulin-antibody complex formed up to 5 hrs. incubation time and then remained practically constant for

24 hrs.

In this study, it was desirable to determine optimum incubation time since PEG extracts were used in place of serum and conceivably there might be some differences.

Twenty-four tubes of zero standard were prepared by adding 0.1 ml of buffer, 0.1 ml of labeled insulin, and 1.0 ml of antibody-Sephadex dissolved in phosphate-saline buffer. At hourly intervals up to 10 hrs. duplicate tubes were removed from the rotator and centrifuged. The precipitates were washed in saline three times as required in the procedure and then counted for radio-activity. In addition to the hourly samples up to 10 hrs., 16 and 24 hr. samples were determined. The results of these time studies are shown in Figure 15 where counts per 2 minutes are plotted versus hours for each of the 3 methods.

The results of the reference method show that at 5 hrs. approximately 82% of the radioactivity found in 24 hrs. had been achieved. In 10 hrs., the percentage had increased to 96%, and in 16 hrs. showed some decrease in radioactivity, but within the SEM of $\pm$ 52 counts.

The results of the free and total methods which have the PEG material in the reaction tubes show somewhat similar results in 10 hrs. and 16 hrs. compared to the reference method, but show 12% less radioactivity at the 5 hr. interval of incubation.

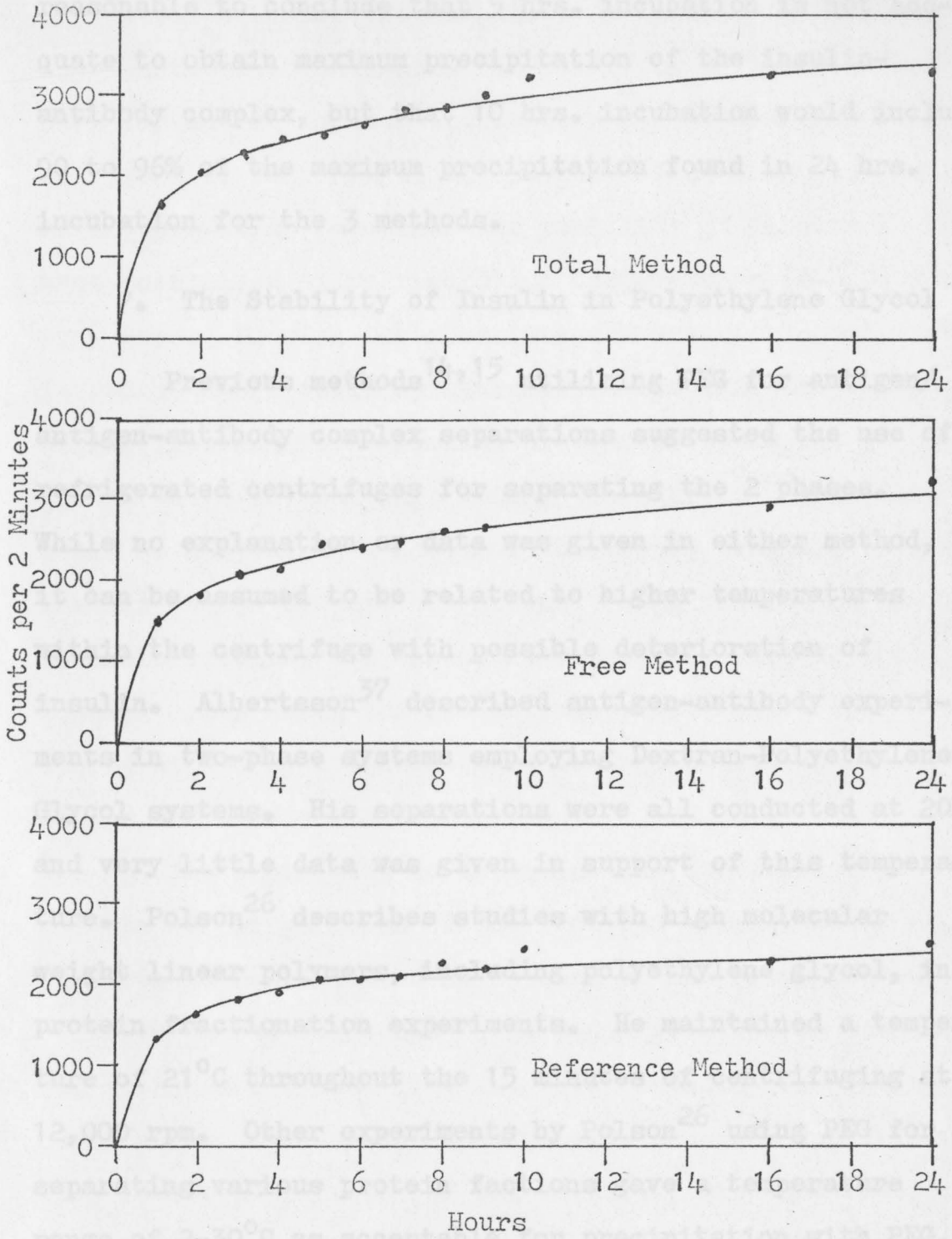

Figure 15. Effect of Incubation Time on Binding of Labeled Insulin (0.08 ng of ^{125}I-Insulin) to the Antibody-Sephadex Complex in the Reference, Free, and Total Insulin Methods.

From the experimental work of this study, it appears reasonable to conclude that 5 hrs. incubation is not adequate to obtain maximum precipitation of the insulin-antibody complex, but that 10 hrs. incubation would include 90 to 96% of the maximum precipitation found in 24 hrs. incubation for the 3 methods.

7. The Stability of Insulin in Polyethylene Glycol

Previous methods[14,15] utilizing PEG for antigen/antigen-antibody complex separations suggested the use of refrigerated centrifuges for separating the 2 phases. While no explanation or data was given in either method, it can be assumed to be related to higher temperatures within the centrifuge with possible deterioration of insulin. Albertsson[37] described antigen-antibody experiments in two-phase systems employing Dextran-Polyethylene Glycol systems. His separations were all conducted at 20°C and very little data was given in support of this temperature. Polson[26] describes studies with high molecular weight linear polymers, including polyethylene glycol, in protein fractionation experiments. He maintained a temperature of 21°C throughout the 15 minutes of centrifuging at 12,000 rpm. Other experiments by Polson[26] using PEG for separating various protein factions gave a temperature range of 2-30°C as acceptable for precipitation with PEG. In fact, Polson lists as one of the advantages of PEG, the fact that it can be used to fractionate proteins at room temperature.

In this study, two different experiments were conducted to determine the stability of insulin in PEG: 1) An experiment to determine the stability of insulin in PEG when exposed to ambient temperature or temperatures of non-refrigerated centrifuges for periods of 30 minutes; and 2) an experiment to determine the stability of insulin in PEG when maintained at refrigeration temperatures (2-8°C) for many days. Knowing the stability characteristics of insulin in PEG at 2-8°C would be extremely helpful if the RIA portion of the free and total insulin procedures could not be performed until several days later. In addition, this data could be helpful when necessary to repeat free and total insulin assays because of extremely high results or unreliable results. If one could repeat the free or total insulin assay using the PEG extract previously prepared and stored at 2-8°C, a considerable savings in both serum sample and time would be realized.

The experiment to determine the correlation of results for preparation of the free and total insulin extracts in refrigerated as opposed to non-refrigerated centrifuges was conducted using 4 different serum samples. The extracts for both free and total methods were set up in duplicate on each of the 4 samples. Procedure was followed exactly for preparation of the extracts, with the exception that 1 free extract and 1 total extract for each serum sample was centrifuged in a refrigerated centrifuge maintained at 0-4°C and in a non-refrigerated centrifuge maintained at approximately 27 to 30°C. Centrifuging in both

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machines was carried out for 30 minutes at 2,700 rpm ± 200.

The results at 3 different levels of insulin concentration show no appreciable difference between centrifuging at room temperature and using a refrigerated centrifuge maintained at 0-4°C. Results for the free method for non-refrigerated centrifuging were 2, 144, 30, and 24 μU/ml of insulin as opposed to 4, 142, 22, and 20 μU/ml of insulin centrifuged in the refrigerated unit. Results for the total method gave values of 8, 86, 28, 34 for non-refrigerated samples and values of 3, 92, 25, 25 for refrigerated samples. It is apparent in this limited study that the use of a refrigerated centrifuge does not appear to improve results.

The experiment to determine the stability of free insulin in PEG was carried out using 3 different serums at different concentration levels. PEG extracts for both free and total methods were prepared on day one and assayed for insulin concentration. The extracts were placed in the refrigerator at 2 to 8°C and analyzed on subsequent days for insulin.

The results as shown in Figures 16 and 17 show a similar pattern for both the free and total extracts. There is a gradual increase in concentration from day one to day five, and then a gradual decrease in insulin concentration. By the third day, there appears to be an average increase in concentration of approximately 25%. On the fifth day an

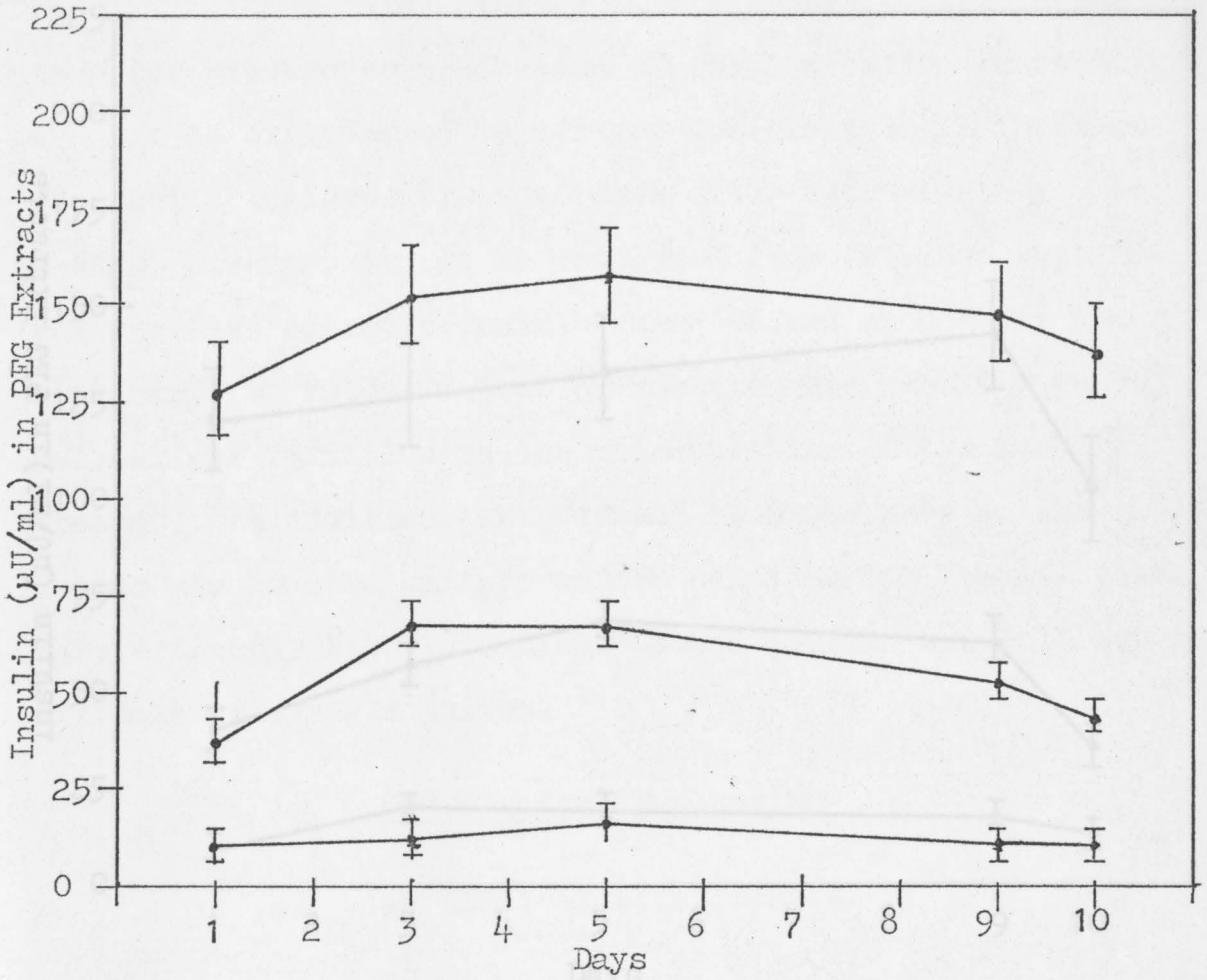

Figure 16. Data Showing the Stability at 2 to 8° C. of Insulin in Polyethylene Glycol Extracts for the Free Insulin Method. Three Serums Were Used Each at Different Concentration Levels. The S.E.M. is Shown for Each Point Plotted as I.

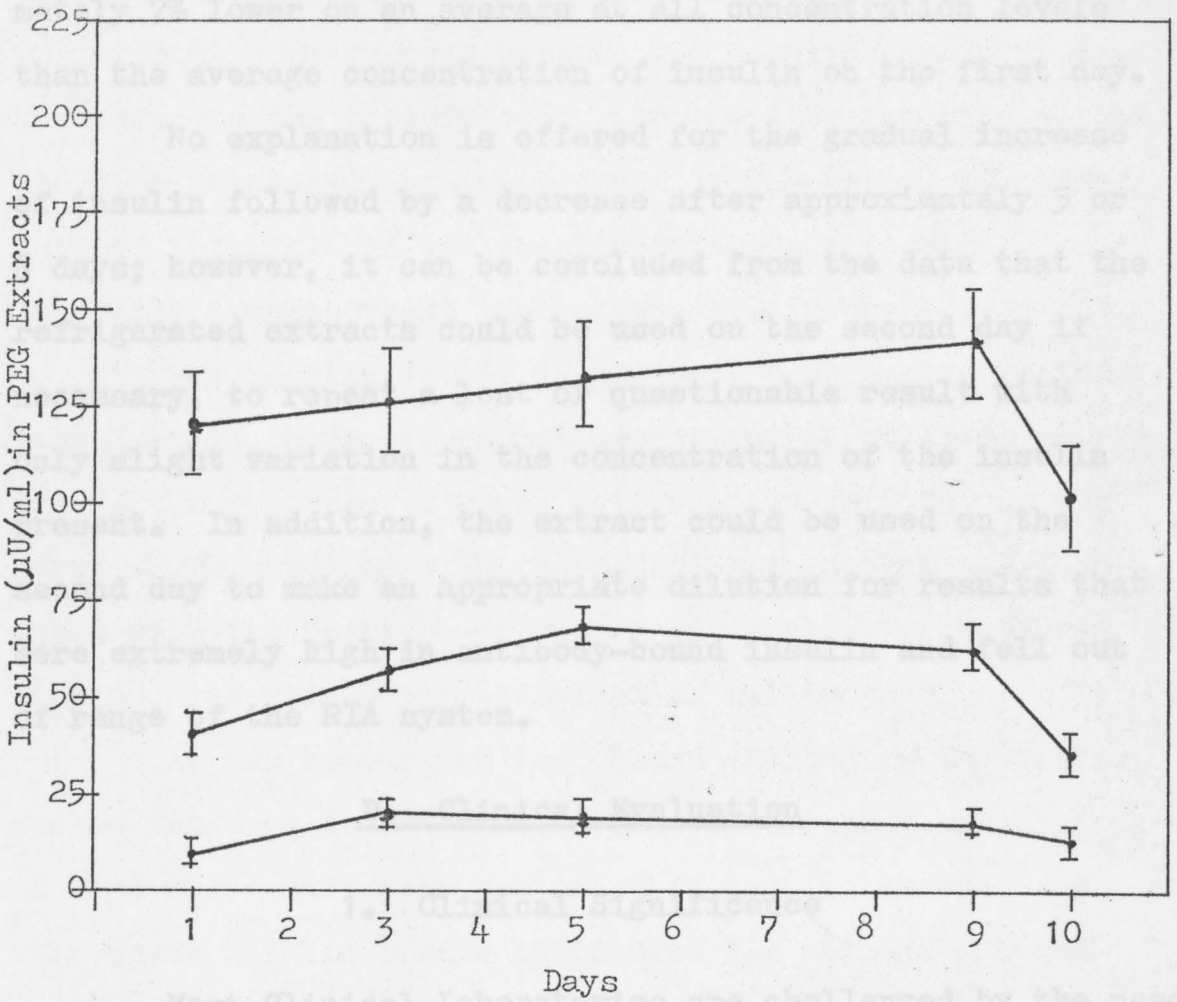

Figure 17. Results of Studies Conducted to Determine the Stability of Insulin in PEG Extract for the Total Insulin Method. Three Serums Were Used, Each at Different Concentration Levels. The S.E.M. is Shown For Each Point Plotted as I .

average increase of 32% is seen for both the free and total extracts. The decrease as seen by the tenth day is approximately 7% lower on an average at all concentration levels than the average concentration of insulin on the first day.

No explanation is offered for the gradual increase of insulin followed by a decrease after approximately 5 or 6 days; however, it can be concluded from the data that the refrigerated extracts could be used on the second day if necessary, to repeat a lost or questionable result with only slight variation in the concentration of the insulin present. In addition, the extract could be used on the second day to make an appropriate dilution for results that were extremely high in antibody-bound insulin and fell out of range of the RIA system.

D. Clinical Evaluation

1. Clinical Significance

Most Clinical Laboratories are challenged by the need to provide a full complement of easily performed, accurate, and reproducible tests for the diagnosis and control of diabetes. These tests include glucose in blood, urine, and CSF, glucose tolerance and tolbutamide tolerance procedures, insulin assays, and other related tests as required by the situation.

Insulin assays by RIA have recently become available to the Clinical Laboratory and provide very helpful information in the diagnosis of diabetes and its sometimes puzzling variations. The current methods for insulin assay by RIA, are based on the principle of a competitive reaction between the free unlabeled insulin present in the serum, the added labeled insulin, and the insulin antibody attached to an insoluble immunosorbant (Sephadex), see Figure 18.

The insulin levels in patients previously treated with insulin, however, cannot be determined by these methods, due to the presence of human anti-insulin antibody in the serum which combines with the added radioinsulin and interferes with the reaction between radioinsulin, endogenous insulin, and guinea pig anti-insulin antibody.

It has been shown that human antibodies to insulin can be measured in the circulating blood approximately four to five weeks after the beginning of insulin therapy and subsequent insulin assays by present RIA methods are completely unreliable for reasons cited[32].

The clinical evaluation of the free insulin method was directed toward 3 types of subjects: 1) normal subjects to establish a "normal-range"; 2) non-insulin-treated diabetics; and 3) insulin-treated diabetics where some level of insulin antibody was present in the circulating blood.

Methods for determining the insulin antibody level present in insulin-treated diabetics have been available but because of their complexity, have not found wide use in

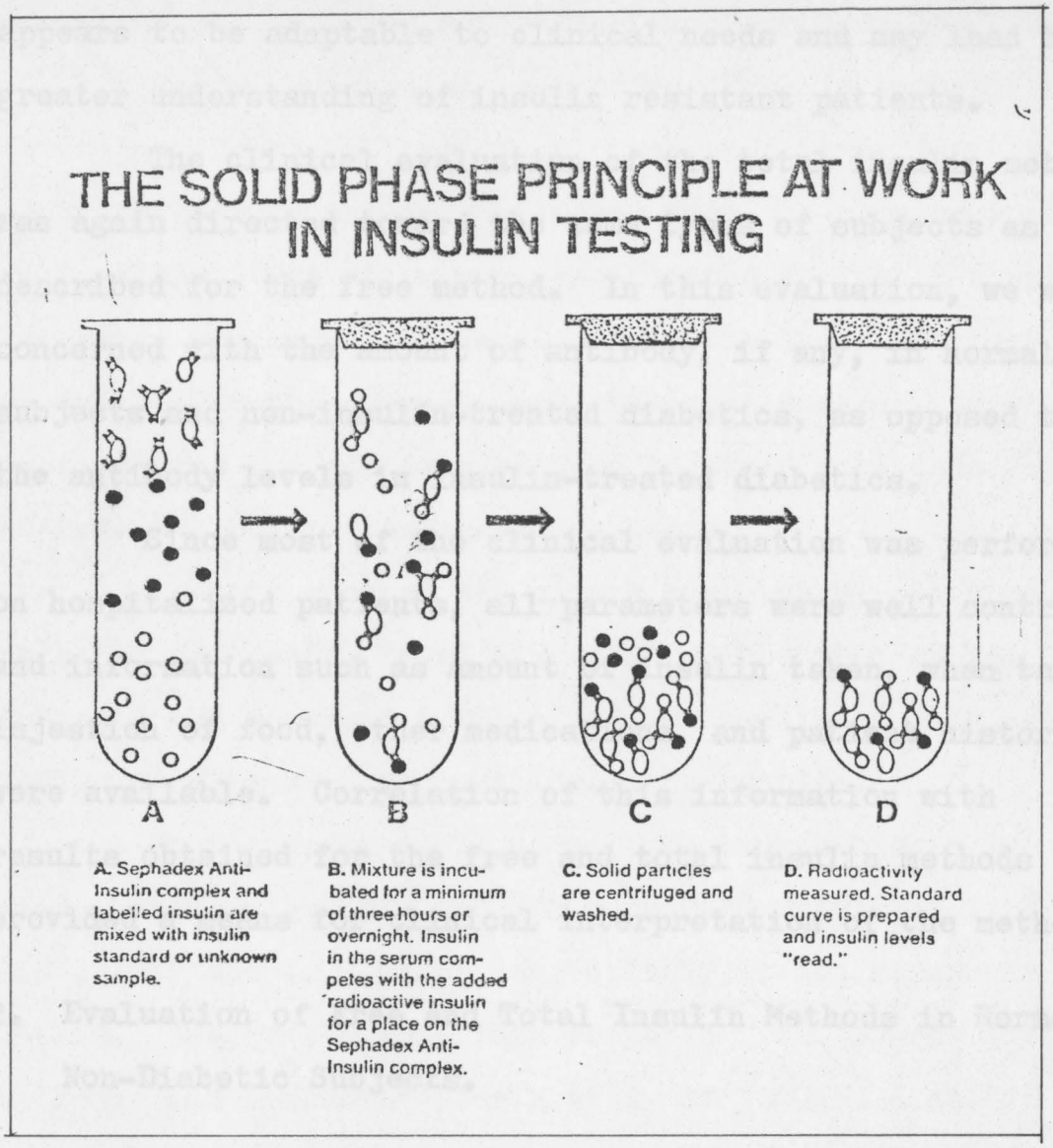

Figure 18. Schematic Showing the Solid Phase Principle in the Competitive Reaction of Labeled and Unlabeled Insulin for Binding Sites on the Anti-Body-Sephadex Complex. O Sephadex, M Insulin-Anti-Body, • Labeled Insulin, o Unlabeled Insulin.[33]

Clinical Laboratories. The method proposed in this study, appears to be adaptable to clinical needs and may lead to greater understanding of insulin resistant patients.

The clinical evaluation of the total insulin method was again directed toward the same types of subjects as described for the free method. In this evaluation, we were concerned with the amount of antibody, if any, in normal subjects and non-insulin-treated diabetics, as opposed to the antibody levels in insulin-treated diabetics.

Since most of the clinical evaluation was performed on hospitalized patients, all parameters were well controlled and information such as amount of insulin taken, when taken, injestion of food, other medications, and patient history were available. Correlation of this information with results obtained for the free and total insulin methods provided a means for clinical interpretation of the methods.

2. Evaluation of Free and Total Insulin Methods in Normal Non-Diabetic Subjects.

Blood samples were drawn from a control group of 15 non-hospitalized, apparently normal people. These subjects were screened for diabetes, but no other restriction was considered. All subjects had not eaten for at least 4 hrs. and were considered in a fasting state.

Blood samples were drawn from another group of 17 non-diabetic subjects. These patients were all hospitalized and were being treated for problems other than diabetes.

The specimens in each case were considered as non-fasting since the patients had eaten within a four hour period.

The blood samples were drawn in plain, stoppered Vacutainer tubes, allowed to clot at room temperature, centrifuged, and the serum removed from the clot as soon as possible. The serum was refrigerated at 2 to 8°C until the assay was begun. In most cases, the assay was performed on the day the specimen was obtained; however, in no instance was the specimen allowed to stand more than 24 hrs. at 2 to 8°C before the assay was begun.

In both groups, the assays were run by all three methods; reference, free, and total, since none of the subjects had received insulin and no insulin-binding antibodies were present to invalidate the results.

In the group of 15 normal, fasting subjects the range, mean value, SD, and SEM were 7-28 μU/ml, 17.1 μU/ml, 6.8 μU/ml, and 1.77 μU/ml respectively by the reference method. See Table 12. This compares with figures given by Boone and Crabtree[34] for a study of 500 non-diabetic adults. The statistics given for their group of fasting subjects ranged from 6 to 24 μU/ml with a mean of 11.3, an SD of 3.1 and an SEM of 0.86. Other studies[29] reported for normal, fasting adults give a mean free insulin level of 19.7 μU/ml with an SD of ± 10.3 μU/ml.

The data from the non-fasting group of 17 subjects studied in this experiment is summarized in Table 13. It can clearly be seen that the mean values for each of the

TABLE 12

THE DATA OF 15 FASTING, NORMAL SUBJECTS USED AS A CONTROL GROUP. RESULTS ARE SHOWN FOR EACH OF THE THREE METHODS.

Method	Range μU/ml	Mean μU/ml	SD μU/ml	SEM
Reference	7-28	17.1	6.8	1.77
Free	8-30	16.3	6.1	1.59
Total	11-25	18.0	4.3	1.11

TABLE 13

THE DATA OF 17 NON-FASTING, NORMAL SUBJECTS USED AS A CONTROL GROUP. RESULTS ARE SHOWN FOR EACH OF THE THREE METHODS.

Method	Range μU/ml	Mean μU/ml	SD μU/ml	SEM
Reference	7-37	26.5	9.2	2.23
Free	12-44	26.6	10.6	2.57
Total	11-45	25.2	8.5	2.06

3 methods are elevated over that of the fasting group as would be expected, since each of the subjects had been challenged by food and were showing increased circulating insulin as a result.

Since no control was maintained on the time of drawing the specimen in relation to when the patient ate, no particular level of increase could be expected over the mean value of the fasting group. The fact that the mean value of the free and total methods was almost identical to the reference method in both control groups confirms once again, the correlation of these methods. The data is shown in Table 14. In addition, the fact that all three methods showed an approximate 7 μU/ml average increase in the non-fasting control group over the fasting control group indicates the sensitivity of the 3 methods.

In this study of 32 non-diabetic subjects, it is clear that the "Phadebas" RIA assay for insulin can be used with human serum or with the PEG extracts from either the free or total insulin methods with almost identical results. No particular advantage exists, however, in preparing a PEG extract in non-diabetic patients since no antibodies are present to interfere in the antigen-antibody reactions of the RIA. The insulin present in the circulating blood of non-insulin-treated subjects is considered "free" or "unbound" and is not complexed by insulin-binding antibodies.

TABLE 14

RESULTS SHOWING THE CORRELATION STATISTICS OF THE REFERENCE, FREE, AND TOTAL INSULIN METHODS IN 27 NORMAL SUBJECTS. THIS GROUP INCLUDES FASTING AND NON-FASTING SUBJECTS.

Method	Regression Equation	Correlation[a] Coefficient (r)	(t) Test[b]
Reference Vs. Free	Y = 0.90(X) + 1.84	0.834	0.343
Reference Vs. Total	Y = 0.56(X) + 9.43	0.682	0.228
Free Vs. Total	Y = 0.48(X) +11.22	0.640	0.044

[a]The significant value of r at p = 0.05 is 0.3809
[b]The significant value of t at p = 0.05 is 2.060

3. Evaluation of Free and Total Insulin Methods in Non-Insulin-Treated Diabetics.

In some diabetics, especially of the "adult-onset" type, good control can be maintained by diet and oral hypoglycemic agents such as tolbutamide. The use of these hypoglycemic agents allows the diabetic the luxury of not having to inject insulin into the arm or leg tissues once or twice daily.

The reference, free and total insulin methods were evaluated in a group of 16 hospitalized non-insulin-treated diabetic patients to determine: 1) if any insulin-binding antibodies were present in these patients; and 2) to determine if factors present in diabetes, other than insulin-binding antibodies, may affect the correlation of the 3 methods.

Table 15 shows the range, mean, and SD for this group of 16 patients. The blood specimens were not fasting, thus the results should be somewhat higher than the normal range for fasting subjects. The mean value for this group shows almost the identical mean value as for the non-diabetic, non-fasting control group shown in Table 13. The correlation data for the 3 methods, shown in Table 16, are all significant and show correlation at $p = 0.05$ (95% confidence limits).

As expected, the total method shows no antibodies present in this group of diabetic patients and further establishes the fact that circulating insulin is in the free

TABLE 15

THE DATA OF 16 NON-FASTING, NON-INSULIN-TREATED DIABETIC PATIENTS, ALL OF WHOM WERE BEING TREATED WITH ORAL HYPOGLYCEMIC AGENTS. RESULTS ARE SHOWN FOR EACH OF THE THREE METHODS.

Method	Range μU/ml	Mean μU/ml	SD μU/ml	SEM
Reference	7-83	26.3	21.7	5.4
Free Insulin	12-80	25.9	18.4	4.59
Total Insulin	8-84	25.4	18.3	4.59

TABLE 16

CORRELATION DATA FOR THE REFERENCE, FREE, AND TOTAL INSULIN METHODS IN A GROUP OF 16 NON-FASTING, NON-INSULIN-TREATED DIABETICS. ALL PATIENTS IN THIS GROUP WERE ON ORAL HYPOGLYCEMIC AGENTS.

Method	Regression Equation	Correlation Coefficient(r)	t test
Reference Vs. Free	Y=0.80(X)+4.73	0.947	0.274
Reference Vs. Total	Y=0.78(X)+4.75	0.923	0.471
Free Vs. Total	Y=0.94(X)+1.00	0.945	0.289

form and can be measured by all three methods.

The use of the oral hypoglycemic agent, tolbutamide, does not appear to affect the results of the 3 methods as would be expected. Its role is to stimulate the production of insulin by the beta cells of the pancreas; further, since tolbutamide is not a protein, it does not stimulate production of antibodies which bind the circulating insulin.

4. Evaluation of Free and Total Insulin Methods in Insulin-Treated Diabetic Patients.

Evaluation of the 2 methods was performed on a group of 21 insulin-treated diabetic patients. The patients ranged in age from 9 to 82 yrs. with a mean age of 49 years. The group included patients that had been on exogenous insulin for as little as 1 year and as long as 30-35 years. Patients that had just started insulin therapy, less than 4-5 weeks, were included in another group and will be discussed later. The insulin dosage of these 21 patients ranged from 3 to 80 units of either U-80 or U-100 insulin with a mean dosage of 36 units. As discussed earlier, the units of either U-80 or U-100 insulin are equivalent since the increased or decreased volume required is compensated for in the syringe used to administer the insulin. Thus, it is important to always use the proper syringe with each of the different insulin concentrations available.

It is evident from the data for the total method, that all 21 patients in this group have developed some level

of insulin-binding antibody. In spite of the inaccuracy of the reference method because of the antibodies present in these patients, all specimens were assayed by the three methods to more dramatically demonstrate the non-correlation of each of the methods; and further, to show that the results of the reference method when performed in the presence of human antibodies to insulin can be extremely misleading.

Table 17 shows the results of the 21 insulin-treated diabetic patients by all three methods. As indicated above, the results of the reference method should not be considered accurate and no statistical analysis has been attempted to compare the free and total methods to it.

The free insulin method shows a range of 10 to 440 μU/ml of insulin with a mean value of 47. The blood specimen procurement time was variable, ranging from 8:00 AM to 3:00 PM and would have some influence on the level of free circulating insulin as measured by the free method.

The insulin measured in this group of 21 diabetics, unlike the other 3 groups studied, is primarily all exogenous insulin which is derived from the intramuscular injection taken some time during the day, usually early A.M. These patients, especially the juvenile-onset type, are not capable of producing endogenous insulin which is the insulin derived from the beta cells of the pancreas and released into the circulating blood. Thus, the free insulin measured in

TABLE 17

CHART SHOWING RESULTS OF REFERENCE, FREE, AND TOTAL INSULIN METHODS IN 21 INSULIN TREATED DIABETIC PATIENTS WITH LIMITED PATIENT HISTORY.

Pt.	Age/Sex	Units Insulin/ Yrs. Taking	Reference μU/ml	Free μU/ml	Total μU/ml
S.S.	17/F	3U/3	90	10	263
J.D.	16/F	65U/2	210	28	1500
J.G.	9/M	27U/6	280	24	740
D.M.	65/M	40U/10	>400	50	4090
M.C.	55/F	35U/2	75	12	340
J.F.	62/M	25U/2	97	14	238
A.R.	46/F	30U/15	>400	40	2968
C.R.	55/F	40U/10	>400	26	6450
A.S.	49/F	35U/14	270	24	630
W.R.	18/F	20U/10	33	28	154
J.M.	56/F	30U/5	160	10	150
G.G.	59/M	38U/6	>400	12	1333
J.V.	31/M	80U/10	>400	72	9520
M.S.	55/F	70U/35	>400	48	5040
E.B.	82/M	70U/15	>400	440	17020
C.A.	45/F	40U/3	250	30	1610
L.T.	23/F	25U/6	>400	12	2228
B.D.	70/F	5U/3	75	44	75
N.S.	77/F	35U/7	55	26	67
W.G.	62/F	25U/10	135	18	476
L.R.	79/F	25U/12	185	14	1300

this group by the free method would be exogenous insulin primarily, with some small amount of endogenous insulin, depending on the patient. The concentration of exogenous, circulating free insulin measured at any given time is dependent on a combination of factors which include: 1) the amount and solubility of the exogenous insulin and the rate of release from the depot at the site of injection; 2) the level of insulin-binding antibody present in the circulating blood as opposed to the concentration of free insulin present. It is known that the binding of insulin to antibody is a reversible process; 3) the rate of degradation of insulin by the liver; and 4) the requirements of the body to supply energy from carbohydrate and fat metabolism.

The free insulin levels obtained in these 21 patients does not appear to correlate with the amount of insulin taken. There does appear to be some correlation between elderly patients and higher free insulin levels, but in this small number of samples, the conclusion can not be made as other factors may be responsible. The data also suggests some correlation between high levels of insulin antibody and increased amounts of free circulating insulin. This may be possible since the binding of insulin to antibody is a reversible process and the law of mass action is applicable to these reactions. Berson and Yalow[9] described transient state studies where association reactions to determine the rate of formation of insulin-antibody complexes were per-

formed and the reverse reaction, dissociation, where the ^{125}I-Insulin was dissociated from the preformed insulin-antibody complex. This transient nature of the insulin-antibody complex has been observed by Berson and Yalow in cases where acute increases in insulin requirements occur, as during infections, and when after a short period of time of increased insulin therapy there follows a period of repeated insulin reactions where the patient is in a hypoglycemic state due to the sudden increases in circulating free insulin. The increase in free insulin has been interpreted as due to gradual release of insulin from dissociating insulin-antibody complexes following the accumulation of high concentrations of insulin in these complexes.

In spite of the fact, that no correlation seems evident between the free insulin level and exogenous insulin dosage, there may be some correlation of free insulin with dosage when interpreted in light of the insulin-antibody present. In 11 patients where the total insulin was over 1,000 μU/ml indicating a high level of insulin-binding antibodies, the free insulin was elevated in 8 cases, while 3 patients had normal free insulin levels. These 8 patients were all on rather high insulin dosage, ranging from 30 units to 80 units per day. In terms of normal, fasting, circulating insulin concentration of 0-20 μU/ml, the 30 to 80 units of exogenous insulin would be equivalent to 30,000,000 to 80,000,000 μU. If one assumes an apparent 6 liter volume

distribution of circulating blood, the dosage then becomes 5,000 to 13,333 μU/ml if all the exogenous insulin were released at one time. Because of its low solubility at the pH of body fluids, the insulin is slowly released into the circulating blood and the peak of exogenous insulin in this group probably never exceeds 208 μU/ml to 555 μU/ml if one assumes that the insulin is released equally over a 24 hr. period from the depot at the site of injection. Remembering that the insulin antibody complex formation is reversible, if this large concentration of free insulin were present, the equilibrium would probably favor the association reaction where the complex would be formed, thus binding the free insulin. When the complex level is high, the equilibrium would favor the dissociation reaction, thus increasing the free insulin. In addition one would have to consider other factors such as insulin degradation, the rate of antibody synthesis as well as the rate of antibody destruction, and the rate at which complexes form and dissociate. It is evident that many factors contribute to the level of free insulin present in insulin-treated diabetics where insulin-binding antibodies are present.

The free insulin in the 21 insulin-treated diabetic patients ranged from 10 to 440 μU/ml. If the one result of 440 μU/ml obtained on patient E.B. were discarded, the range then would be 10 to 72 μU/ml with a mean value of 27 μU/ml. These specimens were obtained primarily at 11 A.M. and 3 P.M.

therefore they cannot be considered as fasting. The mean value and range certainly will be influenced by the variables discussed earlier and cannot be compared to published results for free insulin on fasting patients. In a small group of 5 fasting, insulin-treated diabetics studied, the mean value was 17.6 with a range of 10-28 uU/ml. This is somewhat higher than the fasting free insulin levels in insulin-treated diabetics given by Nakagawa, et al.[15], as 0 to 20 μU/ml range with a mean value of 7.3 μU/ml.

The one case of free insulin found to be extremely high (440 μU/ml) was associated with a high titer of insulin antibody, 17,480 μU/ml in terms of μU of insulin bound. A number of specimens were taken on this patient on different days with the following results for free insulin: 1) result of 6/1/74 taken at 3 P.M. was 286 μU/ml; 2) result of 6/4/74 taken at 3 P.M. was 202 μU/ml; 3) result of 6/9/74 taken at 3 P.M. was 230 μU/ml; and 4) result of 6/10/74 taken at 3 P.M. was 440 μU/ml. The free insulin values over a period of 10 days on this patient remained high ranging from 202 to 440 μU/ml with a mean value of 278 μU/ml. Table 17 shows that this patient (E.B.) was an 82 year old male, taking 70 units of insulin daily for a long period of time and in whom a high level of insulin-binding antibody was found. All of the above facts contribute to the high level of free insulin as discussed earlier in this section.

It has been suggested by some investigators[15] that the determination of free insulin in insulin-treated diabetics may be useful in studying the dynamics of insulin throughout the period of hospitalization when the patient is being regulated. To determine the usefulness of this suggestion, 2 patients were selected, one normal subject and one insulin-treated diabetic in which a rather high level of antibody titer was found. The results of the study are shown in Figure 19 and in Figure 20. The normal subject, (W.G.) was not a hospitalized patient and had no symptoms of diabetes. He was used only as a normal control in the experiment. The insulin-treated diabetic, (J.V.) was a 31 year old male who had been a known diabetic for 10-12 years. He was hospitalized by his doctor in an attempt to better regulate his diabetic condition. At the time of this study, patient J.V. was taking between 70 to 80 units of U-100 NPH insulin.

Figure 20 graphically shows the fluctuation in both subjects as to free insulin measured by the free insulin method and blood glucose measured by the copper neocuproine method. It should be pointed out that the interconnecting lines are drawn from point to point when in fact there may be considerable variation up or down within the time frame of these points. Table 18 shows the comparison for both subjects by the 3 methods, reference, free, and total insulin. The reference method for patient J.V. cannot be

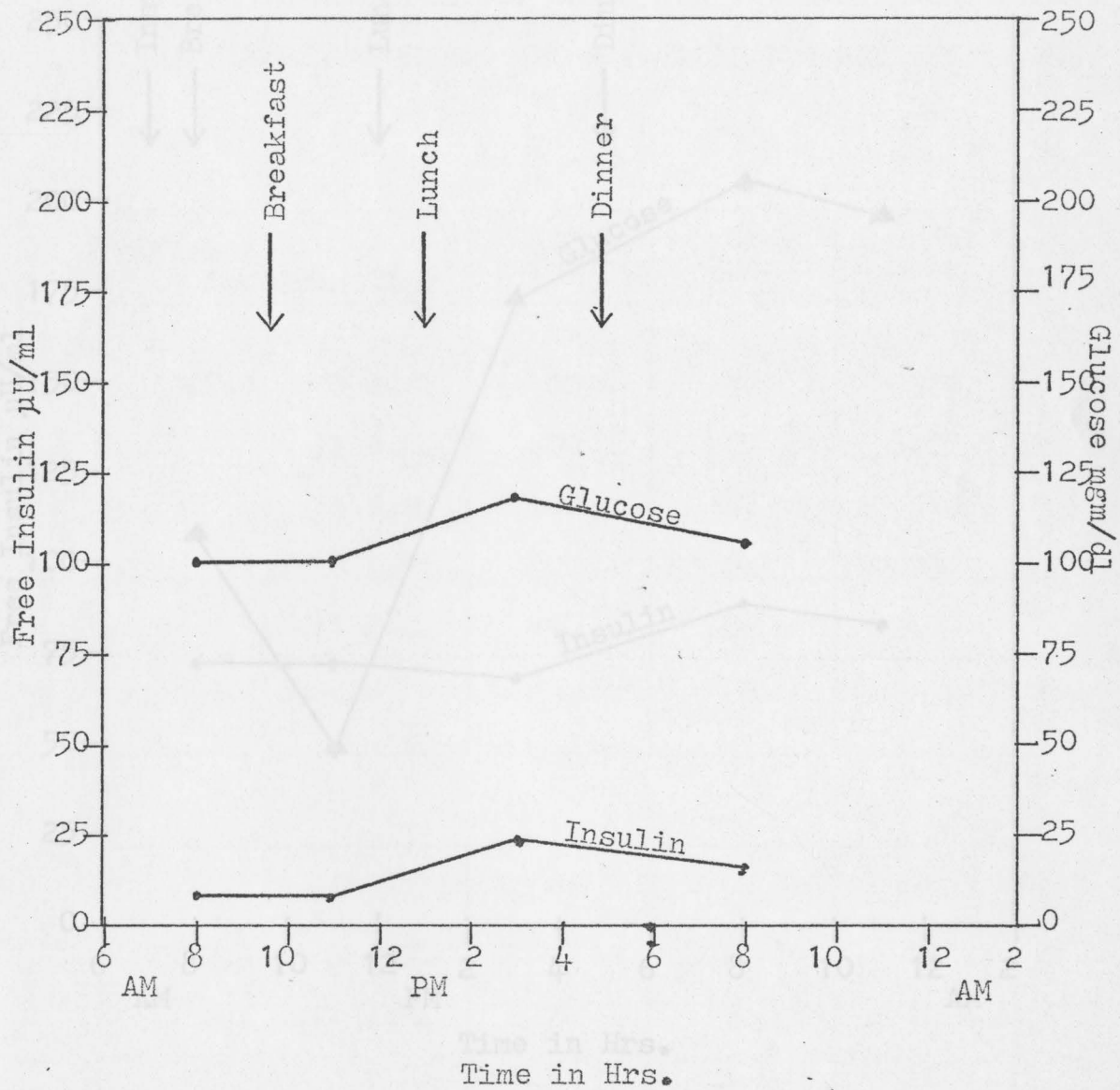

Figure 19. Changes of Free Insulin and Glucose Levels at Various Time Intervals in Relationship to Ingestion of Food. Data is From a Normal Subject.

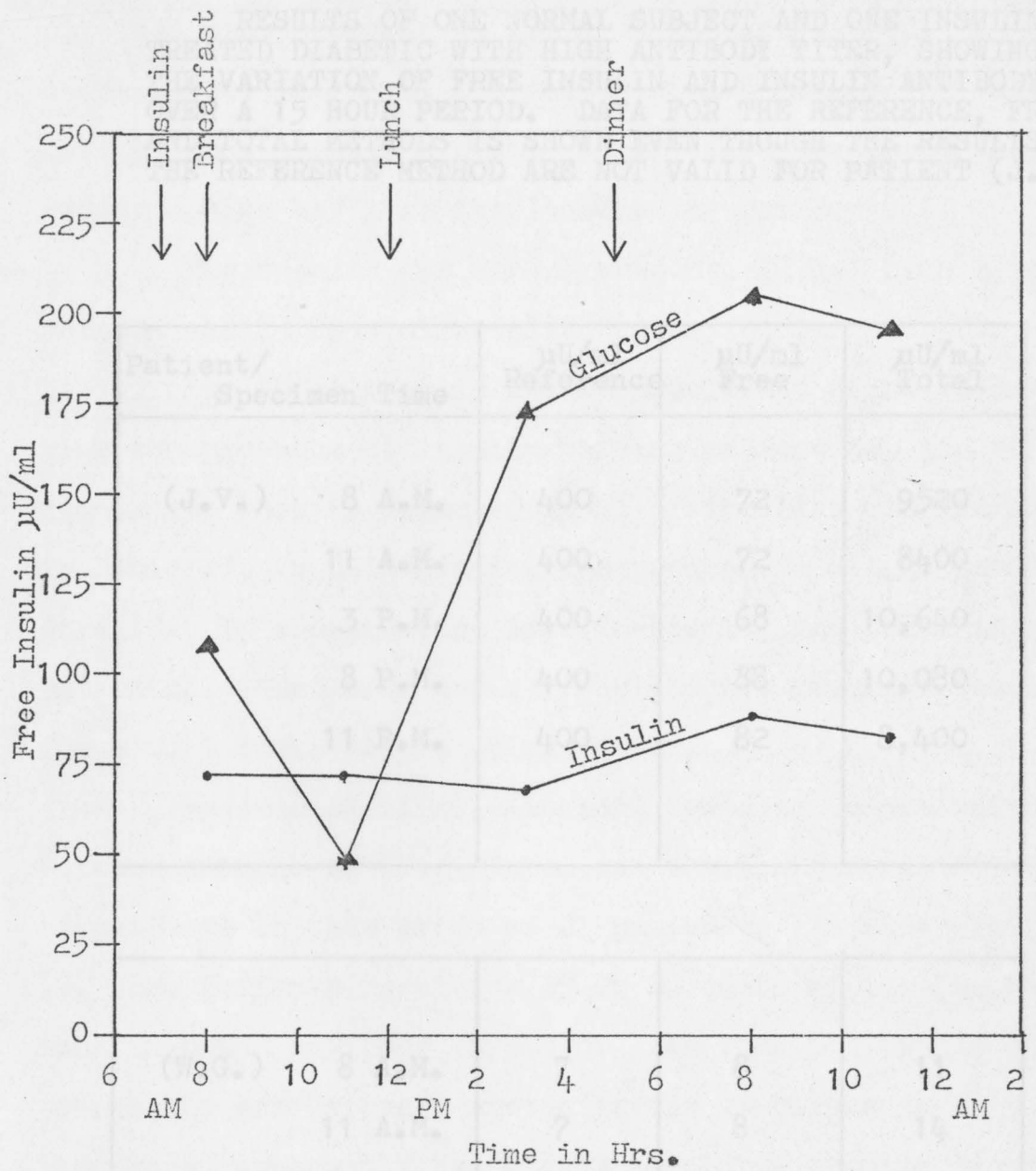

Figure 20. Changes of Free Insulin and Glucose Levels at Various Time Intervals in Relationship to Insulin Dosage and Food Ingestion. The Data is From a 31 Year Old Male Patient Taking 70-80 Units of Insulin With an Insulin Antibody Level of 10,000 µU/ml of Bound Insulin.

TABLE 18

RESULTS OF ONE NORMAL SUBJECT AND ONE INSULIN-TREATED DIABETIC WITH HIGH ANTIBODY TITER, SHOWING THE VARIATION OF FREE INSULIN AND INSULIN ANTIBODY OVER A 15 HOUR PERIOD. DATA FOR THE REFERENCE, FREE, AND TOTAL METHODS IS SHOWN EVEN THOUGH THE RESULTS OF THE REFERENCE METHOD ARE NOT VALID FOR PATIENT (J.V.).

Patient/ Specimen	Time	µU/ml Reference	µU/ml Free	µU/ml Total
(J.V.)	8 A.M.	400	72	9520
	11 A.M.	400	72	8400
	3 P.M.	400	68	10,640
	8 P.M.	400	88	10,080
	11 P.M.	400	82	8,400
(W.G.)	8 A.M.	7	8	11
	11 A.M.	7	8	14
	3 P.M.	27	24	22
	8 P.M.	18	16	18

considered valid since insulin-binding antibodies were present and was used only to show the unreliable results obtained when compared to the free insulin method. As can be seen from Table 18, the total insulin method for patient J.V. is consistently elevated in all five specimens indicating a high level of insulin-binding antibody.

The results for normal subject, (W.G.) by all three methods should be similar since no antibodies are present.

The total insulin method provides a means to determine the presence of insulin-binding antibodies, and to quantitate them in terms of μU/ml of insulin. As indicated in Table 17, insulin-binding was present in all 21 patients studied. In addition, no insulin-binding was found in patients, diabetic or normal, who had not received insulin for 4 to 5 weeks before being tested.

No consistent relationship could be demonstrated between insulin antibody level and the insulin requirements of patients in this group of 21 patients. As shown in Table 17, some patients receiving 35 or 40 units of insulin had insulin-bound antibody levels of less than 500, while others taking the same amount, showed levels as high as 4 or 5 thousand. Apparently the higher levels of insulin required in patients with low to moderate antibody levels is related more to the needs of the patient in controlling carbohydrate metabolism than to binding by insulin antibodies.

The data does suggest correlation between years of taking insulin and the level of antibody. As shown in Table 17, with the exception of 2 patients, all others having values above 1,000 μU/ml had been taking insulin for 6 years or more. On the other hand, not all patients taking insulin 6 years or more had high insulin-antibody levels above 1,000 μU/ml. Thus it appears that all patients show some antibody response to the introduction of animal insulin in their body; in addition, some patients develop more antigenic response than others over a given time span and some even to the point of insulin resistance.

As discussed earlier in Chapter II of this thesis, the ability to easily measure the insulin antibody level in difficult-to-control or insulin resistant patients may provide the doctor the necessary information to properly distinquish between immune and non-immune insulin resistance. In addition, the periodic measurement of these antibodies in insulin-treated diabetics may be justified in following the progress of each patient throughout their life span. The need for larger and larger doses of insulin, or the sudden periods of hypoglycemia may be better understood if the year to year antibody response were known.

It has been established by many investigators, including the data of this clinical evaluation that human antibodies to insulin are not present in persons not taking insulin. Further, that circulating antibodies are first

detected in insulin-treated patients approximately 5 to 7 weeks after insulin therapy is begun. Sebriakova and Little[6] found an average time of 7 weeks when they first detected circulating antibodies to insulin. Skom and Talmage[35] report the figure of 4 weeks as the minimum time before circulating antibodies are detected. In a very limited study conducted as part of this project, 4 patients just started on insulin therapy were evaluated by the reference, free, and total insulin methods to determine if antibodies were present. The time ranged from 2 days to 2 weeks from the beginning of insulin therapy and the dosage varied from 15 to 40 units for the 4 patients. No significant increase in the total method over that of the reference and free methods was observed in any of the 4 patients. Unfortunately, these patients were discharged from the hospital and follow-up studies could not be completed in determining the minimum time when antibodies could be detected.

It has been suggested that the difference between insulin resistance and non-resistance in insulin-treated patients is primarily a quantitative one based on the level of insulin-binding antibody. Berson and Yalow[9] further suggest that antibody levels from diabetic non-resistant patients are less than 10 units/liter plasma (10,000 μU/ml), whereas binding capacities of 500 units/liter (500,000 μU/ml) and greater have been observed in serum from insulin-resistant patients.

In the group of 21 insulin-treated patients studied in this project, only 2 patients fell into the insulin-resistant category as defined by Berson and Yalow. Both of these patients were indeed somewhat difficult to control and required 70 to 80 units of insulin. Both patients could be classified as slightly to moderately resistant to insulin based on their clinical history as supplied by their physician. Two other patients in this same group, (D.M.) and (M.S.) had total insulin levels of 4,090 and 5,040 μU/ml respectively, well below the 10 unit per liter level. These patients were difficult to control and exhibited consistently high glucose levels even though increased dosages of insulin was given. Thus, in spite of their somewhat lower level of antibody, these patients were classified as insulin resistant by their physicians and were switched to pork insulin as a means of better control. It should be understood that the level of 10 units per liter of insulin antibody serves as an average value to classify immune resistance or non-resistance and not as an absolute value.

The use of porcine insulin in insulin-resistant patients has been recommended as a means of better control and in most cases with less dosage. The porcine insulin because of its amino acid composition and structure more closely resembles human insulin and is less antigenic than beef insulin which is most commonly used. Palumbo, et al.[32] described several patients whose insulin requirements were

100 to 300 units per day prior to the use of porcine insulin. Upon treatment with porcine insulin, the daily insulin requirements were reduced 20 to 58% of the original dosage with apparently good control. In addition, they reported significant reduction in insulin binding by one patient but no apparent reduction in 2 other patients.

In the group of 21 insulin-treated diabetic patients studied in this project, 3 patients who were taking standard insulin preparations, (70% beef, 30% pork) were started on pork insulin. In all 3 patients, dosage was reduced to approximately 50% of previous requirements to maintain good control. Unfortunately, the patients were released from the hospital within 10 days, and no detectable change in insulin-binding antibody was observed in this time span. Further studies on these patients will be made at various intervals as a follow-up in evaluating the use of porcine insulin and in the reduction of high levels of insulin antibodies after beginning porcine insulin therapy.

In the course of this clinical evaluation, 2 subjects not included in the 21 insulin-treated diabetic group were found to be taking porcine insulin and volunteered to have samples of their blood assayed for free insulin and insulin antibodies.

Subject (D.M.), a 26 year old male had been an insulin-treated, juvenile-onset type diabetic for approximately 13 years. The first 10 years, he had taken in-

creasingly larger doses of standard mixed insulin (70% beef/ 30% pork), but during the past 3 years, had been put on pork insulin as a means of better controlling his diabetes. The exact details of insulin resistance due to high antibody levels could not be obtained in his history; thus, it was impossible to establish the change in antibody level during the period of porcine insulin therapy. It can be assumed, that after 10 years of beef insulin, a significant amount of circulating antibody was present and especially so, if his physician felt porcine insulin was required.

The serum was assayed by the reference, free and total methods, and gave insulin concentrations of 25, 14, and 22 μU/ml respectively. If one considers the average COV for the 3 methods (see Table 9) as 20%, the data indicated that the results are not significantly different. The variation of the 3 results are within the limits of precision. This apparent lack of insulin-binding antibodies in the serum of this person is indicative of: 1) complete loss of antibodies during a 3 year interval while maintained on porcine insulin; and 2) no further development of antibodies to the porcine insulin. The other subject studied was an 18 year old female, juvenile-onset type diabetic. She had taken lente type insulin (mixture of 70% beef and 30% pork) for 9 years and required approximately 70 units daily. During the past year, her doctor on the basis of clinical history, loss of good control in spite of high dosages of insulin, and

evaluation of the insulin-binding antibody level by the Clinical Research Division of Eli Lilly Company, placed her on porcine insulin as a means of controlling her apparent insulin-resistance. The value of her insulin-binding antibody level was reported as 3,400 μU/ml. Porcine insulin therapy was started August 10, 1973 and continued until the present study. Blood samples were taken March 3, 1974 and assayed by the free and total methods to determine free insulin and insulin-binding antibodies. The free insulin was 28 μU/ml while the insulin-binding antibody level was 126 μU/ml. In 7 months, the antibody level was lowered by 3,274 μU/ml to 126 uU/ml. Since only one test was done 7 months after porcine insulin therapy began, it can not be said with certainty that the antibody level was still falling. It is conceivable that a minimum level was reached and the level was rising again in response to the porcine antigenicity.

The half-life of insulin antibodies has been reported by Sebriakova and Little[6] to be approximately 18 days and is of the same order as that given for human gamma globulin, approximately 13 days.[8]

CHAPTER V

Conclusions

The use of polyethylene glycol to selectively extract insulin from serum in the presence of heavier molecular weight insulin-antibody complexes appears to be a simplified, yet effective approach to this difficult problem.

Other methods have been proposed for the determination of free insulin in insulin-treated diabetics, such as the ultracentrifuge method, extraction techniques employing neutral ethanol, the talc absorption method, the dioxane extraction method, and the ultrafiltration method, but none have been found to be clinically useful in the measurement of free insulin and insulin-binding antibodies.

The use of PEG to prepare the free insulin containing extracts has been found to be extremely simple and does not require specialized equipment unavailable to an average Clinical Laboratory. In addition the 6,000 mw PEG is easily obtained, inexpensive, extremely soluble in water, and unusually stable both in solid form and in aqueous solutions.

Further, it has been established that PEG in final concentration of 12% w/v causes precipitation of the insulin-antibody complex, but that the smaller molecular weight free insulin remains in the upper aqueous phase with little or no precipitation loss.

The use of PEG to measure insulin-binding antibodies involves prior hydrolysis with 1 N HCl to release the insulin hormone from the antibody, followed by neutralization and finally extraction of the total insulin (free plus bound) in PEG with simultaneous precipitation of the antibody. The insulin-binding antibody level is the total insulin minus the free insulin measured in μU/ml of insulin.

It has been shown in this thesis study that the PEG extract for both the free and total methods can be introduced into a simplified radioimmunoassay system with no adverse effects. The Phadebas insulin method, obtainable from the Pharmacia Company in the form of a 100 test "kit" contains all the necessary reagents including the ^{125}I-Insulin, the antibody attached to an insoluble immunosorbant, and the pure insulin standard necessary for proper calibration. The ability to obtain lyophilized reagents which are difficult to prepare and standardize insures the Clinical Laboratory of a higher degree of reliability in performance of this very sensitive procedure.

The classical, RIA is based on the competition for antibody between radiolabeled and unlabeled antigen. Insulin determination can be performed quite accurately in circulating blood in non-insulin-treated persons by RIA. The presence of insulin-binding antibodies in serum renders the results of the classical RIA unreliable because the human insulin antibody present in the serum also competes for the radiolabeled antigen.

The use of PEG extracts in the RIA system replaces the serum, but allows all other reagents to be used in the same volume and concentration. The same concentration of standards can be used to establish the calibration curve. PEG was included in the standard tubes, the zero concentration tubes, and the radiolabeled insulin tubes to determine its effect on the RIA system. No significant change was observed in comparison to the same tubes without PEG. The phosphate-saline buffer employed in the RIA maintained the pH at 7.40 ± 0.02 throughout the incubation period for optimum competitive reaction between the labeled and unlabeled insulin for binding sites on the antibody attached to the immunosorbant (Sephadex). The incubation time employed for all assays in this study was 16 hrs. In most cases, incubation was started at 4 P.M. the day the sample was obtained and allowed to continue overnight. Final washing and counting was done beginning at 8 A.M. the second day. This overnight incubation period was found to be convenient and did not cause undue delay in obtaining the results.

Time studies were conducted to determine minimum incubation periods to facilitate the use of this procedure in clinical applications. It was found that 90 to 96% of the gamma counts obtained in a 24 hr. incubation time could be obtained when using a 10 hr. period. Thus the test could be done in one day if necessary by shortening the incubation period with little loss in total counts.

The basic Phadebas RIA procedure was studied for other factors which could either influence or be influenced by the PEG material. Included in this evaluation was: 1) pipetting of the PEG extract. Since the PEG has a rather high viscosity, measuring the 0.1 ml volume becomes rather critical. The use of a semi-automatic pipetting unit with disposable plastic tips is recommended; 2) the centrifuging and washing with saline. Centrifuging initially after incubation, and between each of the 3 saline washes was carried out for 10 minutes. The 3 minute period recommended in the Phadebas method for the regular method was not found to be adequate when using the PEG extracts. Variable results and loss of accuracy were attributed to loss of the radiolabeled insulin bound to the antibody-Sephadex complex. The washing of the precipitate for all assays in this study was performed 3 times to insure maximum accuracy and precision. It was determined that 1 washing gave variable results and could not be relied on but 2 washings gave consistent, acceptable results. The specimens washed twice gave slightly higher counts since all the unbound labeled insulin had not been removed; however, the results were consistent from one batch to the next.

Recovery, between-run, and within run precision and accuracy studies were performed at different levels of insulin concentration throughout the range of 0 to 320 μU/ml. Recovery of added insulin to antibody-free human serum was

determined at 3 different concentration levels, 44, 89, and 169 μU/ml, and by each of the 3 methods. The reference method gave an average recovery at all levels of 90.5% with better recovery at low and medium levels than at high levels. The free method gave an average recovery at all levels of 84.5% with better recovery at medium and high levels than at low levels. The total method gave an average value of 86.5% recovery which was somewhat better than the free method, but also showed decreased recovery at the low levels.

The between-run precision was determined using a pool of normal serum. Aliquots were frozen and assayed by the three methods on 15 different days to determine between-run precision. The reference method gave a mean value of 35.5, an SD of 8.66 with a COV of 24.4%. The free method gave a mean value of 33.6 μU/ml, an SD of 5.71 and a COV of 16.9%. Finally, the total method gave a mean value of 29.6, an SD of 6.06 and a COV of 20.5%.

The within-run studies were conducted for all three methods using the same serum for the reference method and the same free and total extracts for the other methods. The reference method gave a 10% COV at levels up to 50 μU/ml, but an average COV of 17.9% at all concentration levels studied. The free method gave an average COV of 13.9% at all levels and the total method gave an average COV of 19.5%. As was expected, the COV for all methods is higher in the between-run study than in the within-run study.

The free and total insulin methods were statistically compared to the reference method by the use of scatter diagrams and the regression equation. The regression equation for the line comparing the reference method vs. the free method is $Y = 1.03(X) - 3.01$. The equation for the line comparing the reference method vs. the total method is $Y = 0.98(X) + 1.27$. The regression equation was also generated for the comparison of the free and total methods and found to be $Y = 1.04(X) - 0.66$.

Statistical correlation was also made by use of the correlation coefficient and the Student's "t" test. The correlation coefficient was derived for the comparison between the: 1) reference and free method and found to be 0.97; 2) reference and total method and found to be 0.95; 3) free and total methods and found to be 0.97. In all comparisons, the value obtained for r was significant and exceeded the value for $p = 0.05$ indicating that statistically the methods give comparable results.

The Student's "t" test was used as another method of comparing the 3 tests. The "t" value derived for the comparison between the methods is: 1) the reference vs. free method, $t = 0.747$; 2) the reference vs. total method, $t = 0.484$; and 3) the free vs. total methods, $t = 1.039$. The "t" value from the probability tables is $\pm$ 2.018 for 50 degrees of freedom, where $p = 0.05$. Since the calculated "t" values in each case were less than $\pm$ 2.018, the correlation of the methods was considered significant.

Clinical evaluation was performed on four different groups of normal and diabetic subjects. The normal control group consisted of 15 fasting subjects. These subjects showed a range of 7-28 μU/ml of insulin with a mean value of 17.1. The other normal control group consisted of 17 non-fasting patients. The range for these patients was 7-37 μU/ml of insulin with a mean value of 26.5. All specimens were assayed by the reference, free, and total insulin methods to determine correlation since no antibodies were present to invalidate the reference method. The correlation was determined by the correlation coefficient and the Student's "t" test and found to be significant for 95% confidence limits.

The 2 abnormal groups studied were all diabetic patients who had been admitted to the hospital and were undergoing tests. One group consisted of 16 non-insulin-treated patients who were either controlled by diet, oral hypoglycemic agents, or both. These patients were evaluated by the reference, free, and total methods to determine the free insulin present and to establish the fact that no insulin-binding antibodies were present. The range of free insulin in these patients was 7-83 with a mean value of 26.3 μU/ml. These patients were not considered fasting; thus, the mean value is influenced by food ingestion and the endogenous insulin whose production and release from the beta cell of the pancreas has been enhanced by the oral hypoglycemic agent.

The other group consisted of 21 insulin-treated diabetics with an average age of 49 years. Since each member of this group had some level of insulin-binding antibody present in their serum, the reference method was not accurate and could not be used in evaluation. The free and total methods were not comparable in this group of patients since the total method measures the free insulin present plus the insulin-bound to antibody which was released by the action of HCl in the total extraction procedure. The free insulin in this group of insulin-treated diabetics ranged from 10 to 440 μU/ml with a mean of 47. The total insulin in this group of patients ranged from 67 to 17,920 μU/ml. Little correlation could be shown between the level of insulin-binding antibody and the free insulin present. In cases of extremely high antibody levels, the free insulin was elevated, but the amount was variable.

Finally, several cases of porcine insulin-treated diabetics were studied and found to be relatively free of insulin-binding antibodies. One patient, after 3 years of porcine insulin, showed no significant level of antibodies as measured by the reference, free, and total methods. The other patient, after approximately 7 months of porcine insulin had a reduction in insulin-binding antibody of 96.3%.

An improved radioimmunoassay for serum free insulin in the presence of insulin-binding antibodies and a simplified method for quantitating insulin antibodies in terms of bound insulin has been presented. The technique of PEG extraction of the free hormone with simultaneous precipitation of the antigen-antibody complex has been coupled with an easily performed RIA system. The methods have been shown to compare statistically with the basic Phadebas insulin method.

Studies for between-run and within-run precision were performed for both methods and found to be of the same order as the reference method. Clinical evaluation on normal and diabetic patients gave expected results, within the limits of precision and accuracy, for both groups. Insulin-binding antibodies were found to be present in all insulin-treated diabetics.

The two methods appear to be useful in following insulin therapy in long-term insulin-treated patients and to quantitate insulin antibody levels in immune insulin resistance. Both methods are easily performed and can be routinely used in the Clinical Laboratory.

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