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PURIFICATION AND PROPERTIES OF A DIABETOGENIC PEPTIDE FROM URINE OF DIABETIC PATIENTS

by
David Wing Chiu Law

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Biological Chemistry)
in The University of Michigan
1975

Doctoral Committee:

Professor Lawrence H. Louis, Chairman
Professor Billy E. Frye
Associate Professor Frederic L. Hoch
Associate Professor Merle Mason

To

Mom and Dad

ACKNOWLEDGEMENTS

I wish to extend my deepest gratitude to my thesis director, Professor Lawrence H. Louis for his advice and guidance which have been essential for the completion of this work, and for his constant interest in my development as a scientist.

I am grateful to Professors Billy E. Frye, Frederic L. Hoch and Merle Mason for their guidance, encouragement and instructive criticism.

To the Department of Biological Chemistry of The University of Michigan and the United States National Institute of Health thanks are due for their financial support.

I extend my everlasting appreciation to my wife Villie for her endless devotion, understanding and patience.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
Bovine PI	bovine pituitary diabetogenic peptide
C.P.M.	counts per minute
F	fasting
O.D.	optical density
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'tetramethylethylenediamine

INTRODUCTION

Knowledge of the symptoms and progress of the disease, diabetes mellitus, existed a long time ago. The classic description by Aretaeus in the second century B.C. is regarded as the first concise and accurate definition of the disease which includes thirst, body wasting and loss of body constituents via the urine. In the seventeenth century, Thomas Willis found the diabetic urine to be sweet by tasting it. In 1776, Dobson[1] proved that the sweet taste of diabetic urine was due to sugar. For a long time the disease was thought to originate in the kidneys or stomach. Glycosuria became a constant and diagnostic feature of diabetes, and Cullen[2] added the adjective mellitus to the term diabetes to distinguish this disorder from diabetes insipidus. The first discovery of special endocrine areas in the pancreas was the work of Langerhans[3] in 1869. He thought that the group of cells (islet cells) which finally bear his name had certain connections with the nervous system.

Von Mering and Minkowski[4] discovered in 1889, that experimental total pancreatectomy of a dog resulted

in a permanent condition resembling diabetes mellitus. This supported the view that the etiology of the disease was the result of pancreatic deficiency. The discovery of pathological changes in the islets of Langerhans of some diabetic patients by Opie[5] in 1901 provided further evidence to support this view. There was a hyalin degeneration of the islet cells. In 1902, Ssobelow[6] further found that after the ligation of the excretory ducts of the pancreas in an experimental animal, the acinous tissue gradually atrophied while the islet cells remained intact. McCallum[7] also noted that if the islet cells escaped damage during the ligation procedure, no diabetic symptoms developed. But if the islets were injured, classical diabetic symptoms appeared. In 1921, the epoch-making discovery of insulin by Banting and Best[8] lay to solid ground the unparalleled importance and usefulness of insulin as a therapeutic hypoglycemic agent. The etiology of diabetes was then taken to be a primary insulin insufficiency for some time.

In 1927, the first experimental production of diabetes by hormone action was accomplished by Johns,

O'Mulvenny, Potts and Laughton[9] who induced hyperglycemia in dogs by short-term injection of an extract of the anterior lobe of the pituitary. Houssay[10] also demonstrated the antagonistic relationship between the functions of the anterior pituitary and the islets of the Langerhans during 1930-42. In 1937, Young[11] observed that daily injections of crude anterior pituitary extracts in dogs over prolonged periods will produce permanent diabetes which persists indefinitely after cessation of the injection. A clear relation between the anterior pituitary and diabetes was thus established.

The various ways in which patients respond to insulin, though, presented another problem. In 1936, Falta[12] suggested that there are two types of diabetics-insulin sensitive and insulin resistant. This view was supported by Himsworth[13]. He suggested that the possible explanation of such a finding was that in those cases which gave a sensitive response to his test, the diabetic state was due to a deficiency, while in the cases which gave an insensitive response the diabetic state was due rather to some interference with the action of insulin.

Ricketts, Brunschwig and Knowlton[14] reported in 1945 a case of total pancreatectomy of a patient with carcinoma of the pancreas, the resultant diabetic state was controlled by a normal carbohydrate diet and a daily insulin dosage of only 40 units. In 1949, Lawrence[15] observed that the young insulin sensitive diabetic eventually comes to require a daily dosage of only 40-60 units of insulin, representing a state of absolute diabetes which would appear to result when all the pancreatic islets have ceased to function. Many diabetics, however, require much larger doses of insulin to control the blood sugar level and prevent ketosis. This strongly suggests that in some diabetic subjects there must be a factor or factors at work to antagonize the action of insulin and that the disease is not due to primary insulin deficiency. That began the era of search for the specific factor that could increase blood sugar as well as antagonize the action of insulin.

In 1949, Cotes, Reid and Young[16], and independently Houssay and Anderson[17] demonstrated that growth hormone was the active substance in the pituitary extract

which was responsible for the induction of diabetes in animals. Further evidence to support this view was the demonstration by Ikkos [18] that human growth hormone given to hypophysectomized non-diabetic patients induces a condition similar to the idiohypophysial diabetes of laboratory animals. He also found that after human growth hormone administration to normal subjects, the glucose tolerance test was abnormal and in some cases reached diabetic levels [19]. It was then thought that growth hormone was the primary factor in the development of diabetes. However, in acromegalics in whom plasma growth hormone concentrations are known to be elevated to much higher level (10-100 times) than in normal people, the incidence of frank diabetes is not over 20-25 %. Furthermore, Russell [20] in reviewing 218 cases of acromegaly found only thirteen instances of diabetes, or 6 %. There are conflicting reports as to whether there are higher blood levels of growth hormone in diabetic patients or abnormal growth hormone response to agents that are known to stimulate growth hormone release [21-23], leading to insulin antagonism. Knopf [24] demonstrated that fasting

growth hormone levels were higher in diabetic females with retinopathy than in female diabetics without retinopathy. Merimee[25] reported that growth hormone deficiency is accompanied by lower frequency and degree of angiopathy in diabetic subjects when compared with a matched group of diabetic patients without angiopathy. However, patients with longstanding acromegaly with concomittant diabetes are also relatively free of the typical signs of angiopathy[26]. Therefore, it is difficult to correlate the elevated level of growth hormone with the development of the diabetic vascular complications.

A new approach to support the old idea that growth hormone plays a role in diabetes came from the recent work of Bornstein[27, 28, 29]. By acid hydrolysis, he succeeded in isolating two fragments of the growth hormone molecule. These he called In-G and Ac-G, the inhibitory fragment and the accelerating fragment respectively. The activities of the two fragments were determined on glucose uptake in vitro. By enzymatic assay, he found that the action of In-G was linked to the inhibition of glyceraldehyde-3-phosphate dehydrogenase which could lead to the accumula-

tion of glucose-6-phosphate (G-6-P). The accumulated G-6-P would inhibit the hexokinase reaction and bring about the inhibition of glucose uptake. Insulin action would therefore become weakened and its rate of evoking hypoglycemia would be markedly diminished. The Ac-G fragment, on the other hand, was found to be capable of reversing the inhibiting action of In-G. Thus, the well known paradoxical effects of growth hormone upon carbohydrate metabolism (hypoglycemia and hyperglycemia) can now be explained. However, he was unable to show whether the body is capable of hydrolyzing the growth hormone molecule into fragments. Also, the effect of In-G upon carbohydrate metabolism in intact animals is yet to be demonstrated. It is too early to theorize on its relation to the etiology of the disease. Recently, Lewis [30] succeeded in isolating from human growth hormone a protein preparation which was closely related to, but was different from growth hormone. He based his conclusion on the molecular weight and electrophoretic behaviour. This seems to suggest that growth hormone preparations might be contaminated with this protein, and that the diabetogenic action of growth hormone

resides in this fraction.

The discovery of adrenalin and its ability to induce hyperglycemia led to the belief that the adrenal medulla might be involved in the pathogenesis of diabetes [31]. However, it was subsequently found that adrenal medullectomy did not influence the development of diabetes [32]. In 1936, Long and Luken [33], while studying the interrelationship between the pancreas and the adrenal glands in experimental diabetes, demonstrated that the severity of diabetes in adrenalectomized-depancreatized cats was dramatically less than in animals subjected to pancreatectomy alone. Since that time the role of the adrenal glucocorticoid, hydrocortisone, in carbohydrate metabolism has been clarified and its ability to induce hyperglycemia has been repeatedly demonstrated [34]. Conn and Louis [35] demonstrated that ACTH (30 mg per day for several days) administration to normal human subjects provoked an intolerance to glucose. They also showed that injection of hydrocortisone (300 mg per day for 3 days) into human subjects elicited only a slight glucose intolerance [36]. Administration of moderate doses of hydrocortisone (50 mg

per day) did not produce any significant glucose intolerance. It is now recognized that the adrenal glands play no major role in the pathogenesis of diabetes in man.

Since 1958, Vallance-Owen[37,38,39] published a series of reports that synalbumin, a substance residing in the albumin fraction, was capable of antagonizing the effect of insulin. Furthermore, he reported that the plasma level of synalbumin of many diabetic subjects and their close relatives was higher than in most non-diabetics. He thought that synalbumin is involved in the pathogenesis of human diabetes. However, he was unable to isolate, purify, or characterize the substance.

In 1964, Ensinck and co-workers[40] incubated I-131 labeled insulin with a partially purified hepatic enzyme, insulin-glutathione transhydrogenase and found an α-globulin and an albumin-bound radioactive component. These two components were subsequently separated by dialysis. Further, these investigators[41] reported that upon intravenous administration of I-131 insulin into rabbits they were able to identify an albumin peak which was radioactive. They identified this peak as I-131-labeled

B-chain of insulin, and found that the peptide was formed from degraded insulin. They also noted that the albumin bound B-chain of insulin was capable of producing most of the metabolic effects of synalbumin in vitro and in vivo. They concluded, therefore, that this peptide is the insulin antagonist which Vallance-Owen previously described as synalbumin.

Davidson and Poffenbarger[42] in a review article pointed out the various weaknesses of the Vallance-Owen synalbumin, B-chain theory. The most serious objection was the use of rat hemidiaphragm as an assay method. This tissue was known to be relatively insensitive and imprecise. Also, specificity was greatly lacking since albumins of other species as well as egg albumin and gelatin are indistinguishable from synalbumin in the assay system. If the B-chain of insulin is indeed the antagonist, its level in diabetic patients would be expected to be elevated. A- & B- chain immunoassay data of human sera have repeatedly failed to show elevation of B-chain in diabetic subjects[43,44]. Also, Mirsky[45] found that the continuous intravenous infusion of B-chain insulin to normal dogs with and without

insulin was without effect. Therefore, the role of synalbumin (B-chain of insulin) as an insulin antagonist should be accepted with extreme caution.

In 1960, Louis and co-workers[46] reported the isolation of a polypeptide preparation from urine of patients with lipoatrophic diabetes which, upon administration to men and dogs, provoked hyperglycemia and insulin resistance. This finding has been repeatedly confirmed[47-50]. They also isolated a similar peptide from pituitary glands of cattle[51,52], sheep, hog[53,54] and man[55]. The peptide from all these sources is diabetogenic and antagonizes the action of insulin in men and dogs. In 1963, a similar peptide was also found from the urine of an obese, maturity-onset diabetic patient. Louis reported that this peptide was highly diabetogenic when administered to a human subject[56]. Further, it was found that in one diabetic patient who underwent hypophysectomy, the diabetogenic peptide disappeared from the urine following the operation. This indicates that the peptide originates from the pituitary gland. Thus, it was thought that excessive secretion of this anti-insulin

peptide might be the cause of insulin resistance in this patient. Since then, Louis and Conn succeeded in the isolation of a similar peptide from the urine of many proteinuric diabetic patients[57]. However, they were unable to obtain this substance from the urine of normal people and proteinuric subjects without diabetes. This strongly suggests that the peptide is somehow etiologically related to diabetes mellitus. It is the purpose of this thesis to investigate the physicochemical and biological properties of this peptide, and its possible role in the pathogenesis of diabetes mellitus in man.

The program to achieve these objectives is the following:

1. Preparation of sufficient peptide from urine of proteinuric diabetes for subsequent study.
2. Studies of its diabetogenic effects in dogs.
3. Purification of the peptide to homogeneity.
4. Studies of its physicochemical properties.
5. Comparison of properties of the urinary peptide with the properties of the peptide obtained from human pituitary glands.

6. Study of its mechanism of action.

7. Development of a radioimmunoassay procedure for the peptide.

METHODS

I. Materials

Human urinary diabetogenic peptide was isolated from urine of patients with proteinuric diabetes, with and without lipoatrophy. Human pituitary diabetogenic peptide was isolated from frozen human pituitary glands. Bovine diabetogenic peptide was isolated from frozen bovine pituitary glands. Isolation procedures for the above materials were described by Louis[58], Louis and Conn[55], and Louis, Conn and Minick[51]. These materials were further purified by several liquid chromatography procedures to near homogeneity as listed in the section on Purification Studies.

II. Stability Studies of Bovine Diabetogenic Peptide

Stability studies were carried out to investigate whether the diabetogenic potency of the peptide might be altered by various conditions of treatment, some of which would be encountered during purification of the urinary peptide. This information is important because it will be of help in preventing or minimizing the inactivation of the active peptide.

Bovine diabetogenic peptide (designated as Bovine PI) was chosen for this study because bovine pituitary glands are readily available commercially. Human pituitary glands are extremely scarce because the National Pituitary Agency donated a very limited number of the frozen glands.

The bovine peptide was subjected to various conditions of treatment as indicated below. Its diabetogenic activity was determined by oral glucose tolerance test on a normal dog.

The bioassay protocol consisted of:

(a) a control glucose tolerance test done at 8 a.m. on day 1, and

(b) an injection of the peptide ten hours before the second glucose tolerance test which was done at 8 a.m. on day 2.

Methods and procedures for glucose tolerance tests were performed in the same manner as described by Louis [54].

The peptide was dissolved in 0.05 N hydrochloric acid and neutralized by careful dropwise addition of 0.02 N sodium hydroxide to pH 6.8 - 7.0. The dose was kept at

2 mg per kg of body weight. It was administered subcutaneously as a single dose. An interval of at least two weeks elapsed before the dog was given another bovine peptide preparation. The bovine PI was treated in the following manners:

A. Storage at Room Temperature. Bovine PI was kept in a desiccator for six months at room temperature before testing it for diabetogenic activity.

B. Exposure to 0.1 N Hydrochloric Acid. Bovine PI was dissolved in 25 ml of 0.1 N hydrochloric acid and maintained at 4°C for 72 hours. The solution was then dialysed against several changes of cold distilled water at 4°C. The peptide which precipitated on dialysis was lyophilized and assayed for activity.

C. Exposure to 3 % Acetic Acid at 37°C. Bovine PI was dissolved in 25 ml of 3 % acetic acid and kept at 37°C for 24 hours. The peptide was recovered by dialysis as in the previous experiment, lyophilized, and assayed for activity.

D. Exposure to 3 % Acetic Acid at 22.1°C. Bovine PI was dissolved in 25 ml of 3 % acetic acid and kept at

room temperature (22.1°C) for 24 hours. The peptide was recovered by dialysis, lyophilized, and assayed for activity.

E. Exposure to pH 12.5. Bovine PI was dissolved in 10 ml of 0.05 N hydrochloric acid and adjusted by dropwise addition of 0.02 N sodium hydroxide to pH 12.5. The solution was allowed to stand at room temperature (22.4°C) for one hour. It was then acidified to pH 4.1, the isoelectric point of the peptide. Upon dialysis only a small amount (15 - 20 % of the original weight) of a material was precipitated. The amount of the material was insufficient for bioassay.

F. Exposure of pH 9.5 at 4°C. Bovine PI was dissolved in 0.05 N hydrochloric acid, adjusted by dropwise addition of 0.02 N sodium hydroxide to pH 9.5. It was stored at 4°C for 6½ hours. The peptide was recovered by dialysis, lyophilized, and assayed for diabetogenic activity.

G. Exposure to pH 6.8 at 4°C for 3 days. Bovine PI was dissolved in 0.05 N hydrochloric acid and adjusted by dropwise addition of 0.02 N sodium hydroxide to pH 6.8. The solution was kept at 4°C for 3 days. The peptide was

recovered by dialysis, lyophilized, and assayed for activity.

H. Exposure to 100°C. Bovine PI was dissolved in 0.05 N hydrochloric acid and neutralized by dropwise addition of 0.02 N sodium hydroxide to pH 7.0. This solution was placed in a boiling water bath for one hour. The peptide was recovered by dialysis, lyophilized, and assayed for activity.

I. Exposure to ½ Saturation of Ammonium Sulfate. Bovine PI was dissolved in 20 ml of 0.05 N hydrochloric acid. 20 ml of saturated ammonium sulfate solution was added to the peptide solution. The resulting mixture was kept at 4°C for one hour. The peptide was recovered by dialysis, lyophilized, and assayed for activity.

Glucose tolerance tests for the above experiments are shown in the Results Section I.

III. Preparation of Diabetogenic Peptide from Urine of Proteinuric Diabetic Patients.

The diabetes-inducing substance was originally discovered in the urine of patients with lipoatrophic diabetes [46]. After the physicochemical properties of the

crude material had been established, such as its isoelectric point, solubility, molecular weight and biological activities, peptides of similar properties were isolated from proteinuric diabetic patients without lipoatrophy. Since lipoatrophic diabetes patients are rare, the urinary peptide was prepared from proteinuric diabetics without lipoatrophy. The isolation procedure presented here is an improved method over that of Louis and Conn[57]. It is of interest that patients with a trace amount of protein in the urine are the best source of the diabetogenic peptide. The reason for this is that the peptide is not contaminated much with other proteins as compared with severe proteinuric diabetic patients. Figure 1 outlines in detail the new procedure employed for the isolation of the diabetogenic peptide from urine.

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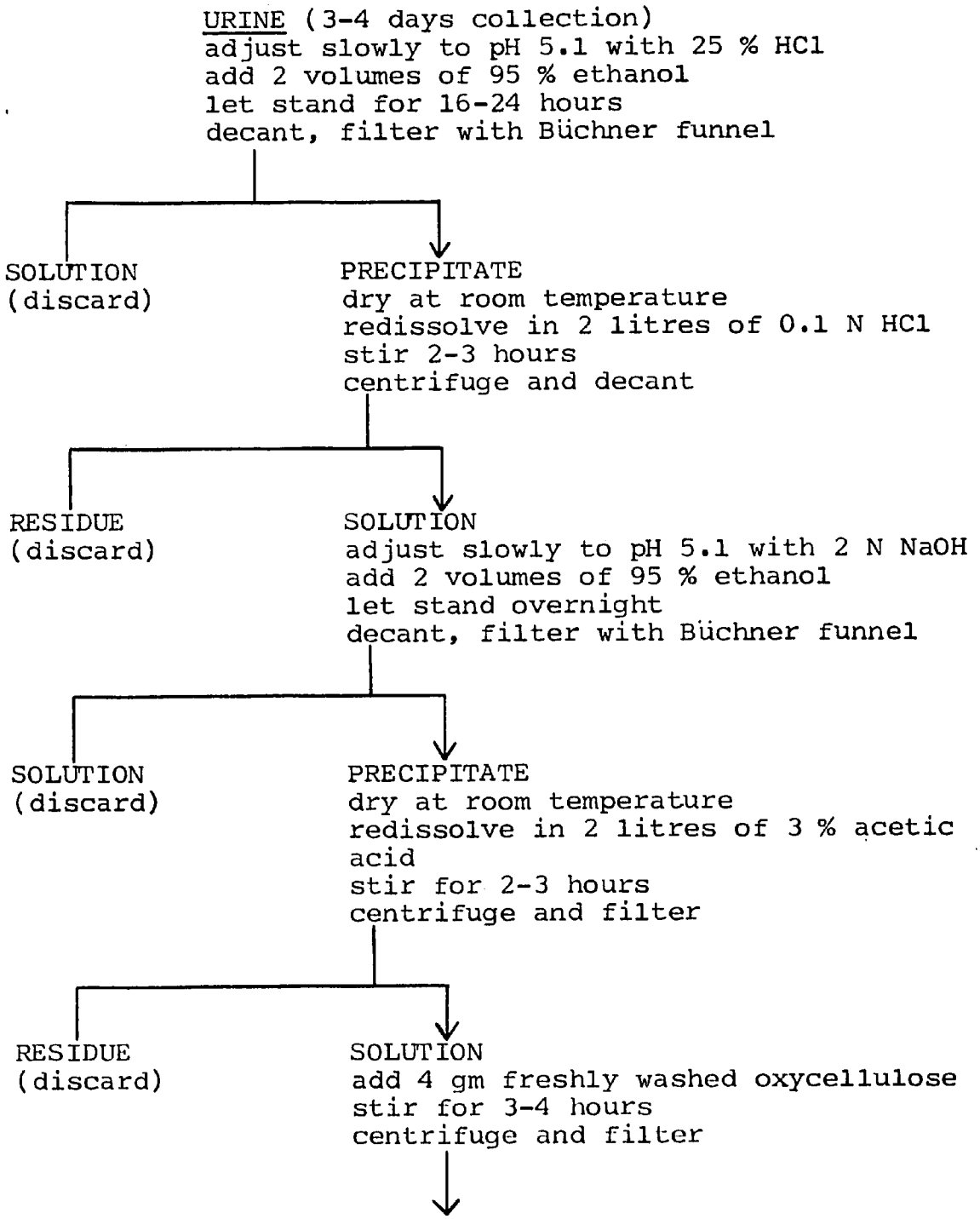
Figure 1.

Isolation of Diabetogenic Peptide from Urine of Proteinuric Diabetic Patients.



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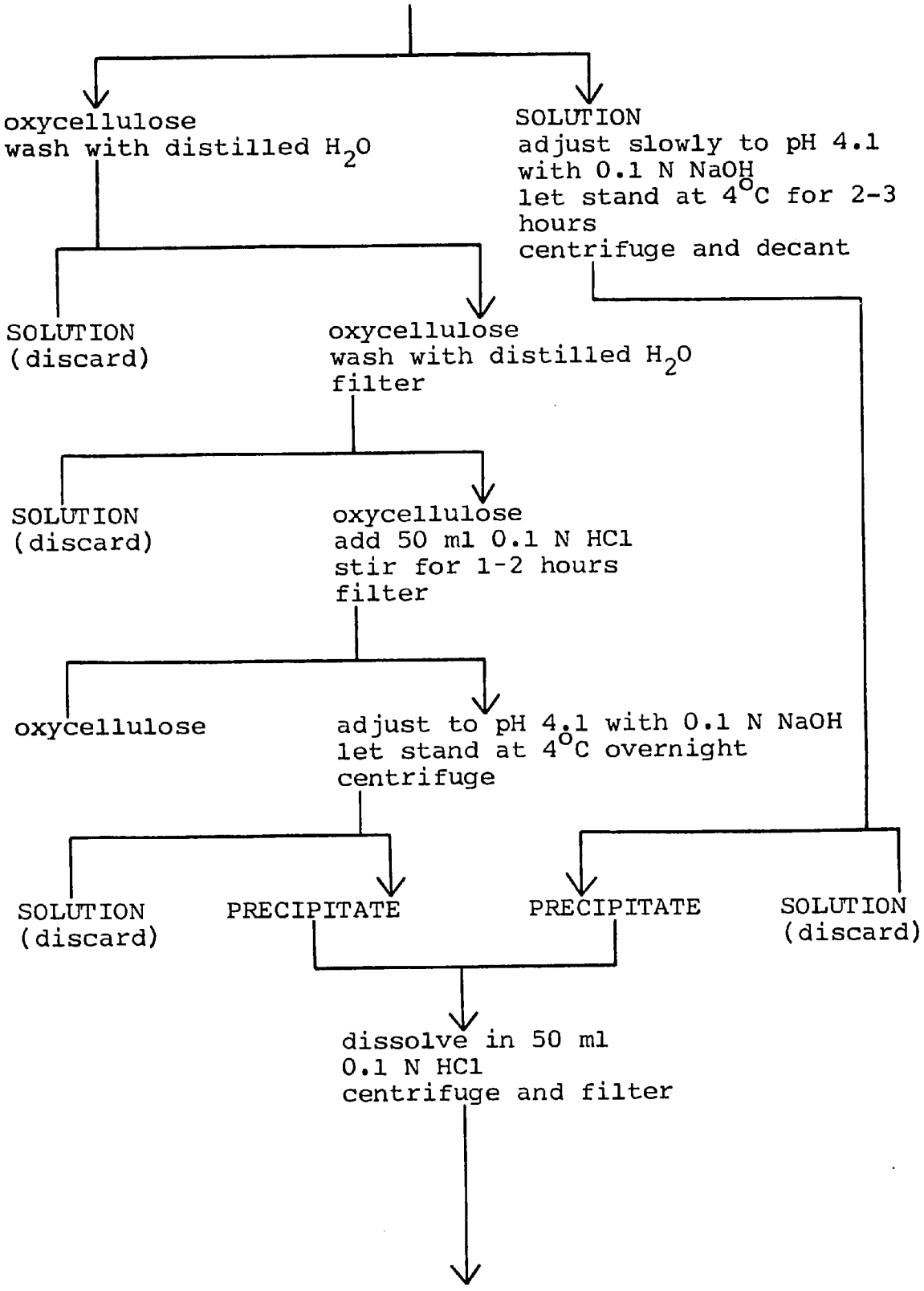
oxycellulose
wash with distilled H_2O
SOLUTION
adjust slowly to pH 4.1
with 0.1 N NaOH
let stand at 4°C for 2-3
hours
centrifuge and decant
SOLUTION
(discard)
oxycellulose
wash with distilled H_2O
filter
SOLUTION
(discard)
oxycellulose
add 50 ml 0.1 N HCl
stir for 1-2 hours
filter
oxycellulose
adjust to pH 4.1 with 0.1 N NaOH
let stand at 4°C overnight
centrifuge
SOLUTION
(discard)
PRECIPITATE
PRECIPITATE
SOLUTION
(discard)
dissolve in 50 ml
0.1 N HCl
centrifuge and filter

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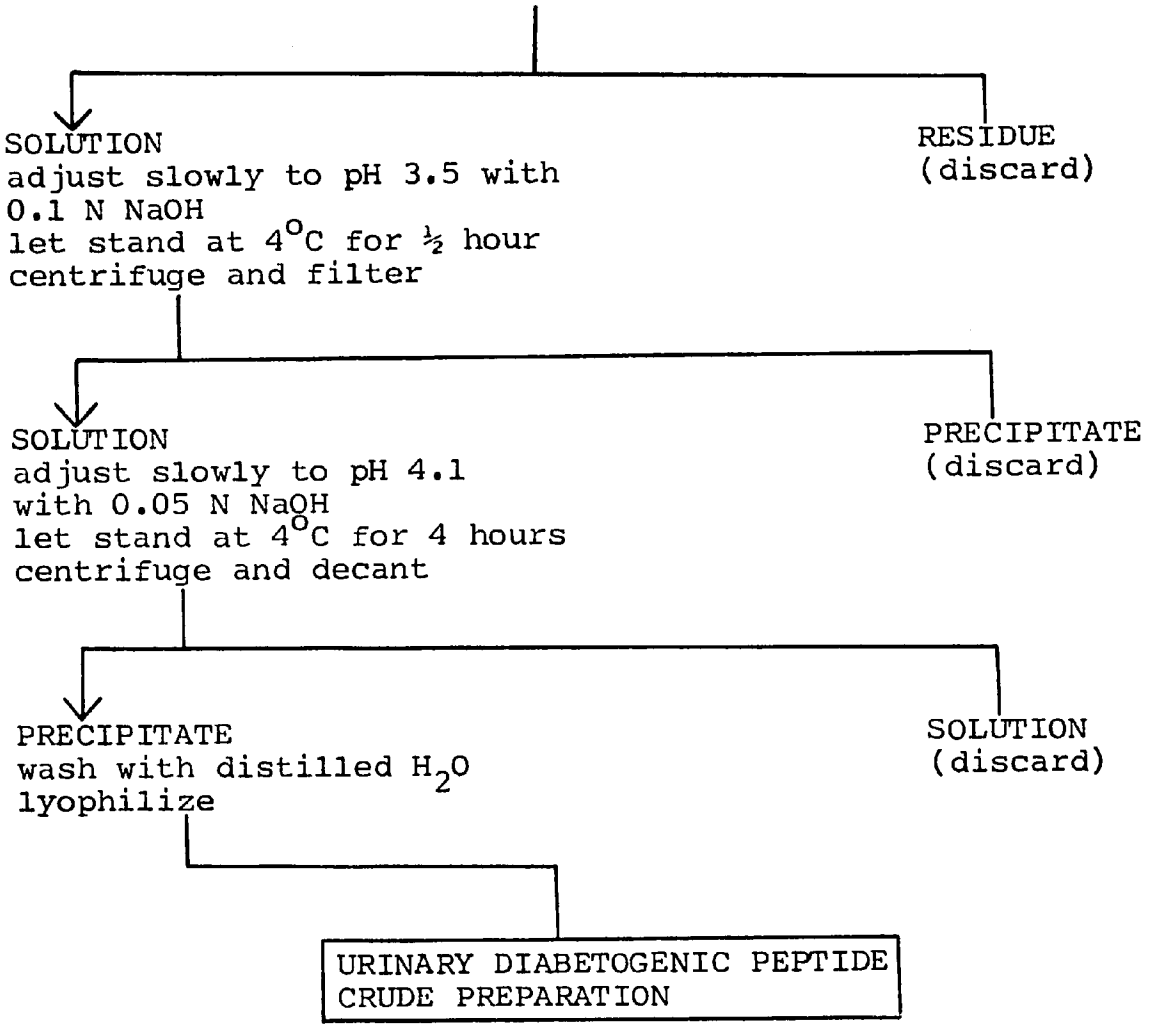


IV. Collection of Urine From Proteinuric Diabetic Patients.

Urine samples from proteinuric diabetic patients were collected and subjected to the procedure of isolation as described in the previous section. These patients were treated for diabetes control at The University Hospital. Specimens (24 hours, refrigerated) were collected daily for three to seven days or in one special case

several months. Proteins were detected by adding 2 drops of 20 % sulfosalicylic acid solution to 2 ml of the urine. The presence of protein was indicated by a white precipitate following the addition of the acid. Qualitative glucose test was done by testape (Eli-Lilly). A table of the findings was listed in the Results Section II.

V. Demonstration of Biological Activity of Diabetogenic Peptide Isolated from Urine of Proteinuric Diabetic Patients.

The crude peptide preparation from urine of proteinuric diabetic patients were tested for diabetogenic activity in a dog. The protocol for glucose tolerance test in dog was the same as that described on Methods Section II.

VI. Induction of Hyperglycemia and Hyperinsulinemia in Dogs by the Administration of Diabetogenic Peptide Isolated from Urine of Proteinuric Diabetic Patients.

The procedure consisted of:

(a) 2 control glucose tolerance tests done at 8 a.m. on day 1 and day 2,

(b) an injection of the peptide at 10 p.m. on day

2, ten hours before the third glucose tolerance test which was done at 8 a.m. on day 3,

(c) a second injection of peptide at 10 p.m. on day 3, ten hours before the fourth glucose tolerance test which was done at 8 a.m. on day 4,

(d) a fifth glucose tolerance test which was done at 8 a.m. on day 5,

(e) a follow-up glucose tolerance test 14 days after the second injection.

Peptide administration was done according to procedure described in section II. The dose used was 3 mg/kg body weight. The second injection was 4 mg/kg body weight. The glucose load for each test was 1.75 gm/kg body weight. Glycosuria after glucose tolerance test was detected by testape (Eli-Lilly). Blood glucose was measured by the Somogyi-Nelson procedure [59]. Blood insulin was measured by radioimmunoassay for insulin. Campbell et al have shown that values obtained by the immunoassay procedure were actually representative of insulin in dog serum [60].

Induction of hyperglycemia in dogs was done sub-

sequently with diabetogenic peptide isolated from urine from other diabetic patients. In general, one single injection of the peptide ten hours before the test invariably resulted in an abnormal glucose tolerance curve.

VII. Purification Studies

The urinary diabetogenic peptide isolated according to the procedure described previously was examined for purity by disc-gel electrophoresis. Further purification steps were carried out by liquid column chromatography, using Sephadex gel G-100, G-100 superfine, and Bio-Gel P-100 -400 mesh. Gel electrophoresis was employed to monitor the degree of purification at each step. In the last step, gel electrophoreses were carried out at several pHs for detecting the homogeneity of the end product.

A. Sephadex liquid column chromotography

Descending liquid chromatography was carried out with a Sephadex G-100 column (0.9 x 60 cm). The column was equilibrated with 0.02 N HCl, containing 0.2 M KCl. Column runs were made at a flow rate of 0.2 ml per fraction per 20 minutes, with an operating pressure of 30 cm.

Protein in the eluate fractions was determined by the method of Lowry[61] and read in a Coleman Junior Sepectrophotometer at 660 mu. The various fractions were dialized against distilled water and lyophilized.

Sephadex G-100 superfine was substituted for G-100 for runs where a high degree of resolution was required. This was obtained at a sacrifice of flow rate.

B. Biogel P-100 Liquid Chromatography

When the separation of sephadex G-100 has reached its limit, Bio-Gel P-100 -400 mesh was employed. A solution of 0.02 N HCl containing 0.2 M KCl was used. A column of 0.9 x 60 cm was prepared. When the separation looked promising, smaller bore columns with increased length were employed. The small bore size has the advantage that a small amount of protein could be put in for chromatography. This was important especially during the final stages of purification when only a small amount of material was available. Columns of 0.5 x 60 cm and 0.5 x 120 cm were used; the latter provided a separation of protein that had never before been possible. Diabetogenic peptides from human pituitaries , urine and bovine pituitaries were purified

by this manner. The flow rate of the column (0.5 x 120 cm) was kept at 0.25 ml per fraction per 45 minutes. Protein of the eluants was measured as described in Section VII A. The various fractions were dialysed against distilled water and lyophilized. The homogeneity of various fractions was checked by gel electrophoreses at different pHs.

C. Gel electrophoresis

Acrylamide gel electrophoresis was used to monitor the degree of purity for each peptide preparation. For preliminary runs the standard disc-gel electrophoresis of Ornstein and Davis[62] was used.

a. pH 8.9 system

This system employed a 2.5 % acrylamide stacking gel of pH 8.9 and a 7½ % acrylamide separating gel of pH 9.5. Protein of 40-60 µg was used (gel tube 7.5 cm x 0.45 cm i.d.). The current was adjusted so that each gel tube would have a power consumption of 0.8 watt. This was to prevent overheating. Electrophoresis was terminated when the tracking dye moved 3/4 down the gel tube. The gel was taken out of the tube and stained in a 1 % Buffalo Black dye in 7 % acetic acid containing 45 %

methanol for 1 hour. It was destained by pre-soaking in 7½ % acetic acid for 1 hour before further destaining electrophoretically. Gels runs with 10 % and 15 % acrylamide were also used.

b. pH 6.0 system

The separating gel of 7½ % acrylamide had a composition of 1.9 M glycine and 0.06 M KOH, containing 3.5 % TEMED v/v. After polymerization by 0.07 % w/v ammonium persulfate, the gel was pre-run electrophoretically with a 0.05 M sodium phosphate buffer pH 6.0 for one hour at 4 milliamperes per tube. This step was to remove the ammonium persulfate. 40-60 µg of protein was dissolved in a minimal amount of 0.05 M sodium bicarbonate solution and mixed with one drop of glycerol before being layered onto the top of the gel for electrophoresis. The current was adjusted so that each tube would have a power consumption of 0.6 watt. Electrophoresis was carried out for 1 hour, after which the gel was stained and destained as in the pH 8.9 system.

c. pH 3.0 system

The separating gel of 7½ % acrylamide 0.2 % bisa-

crylamide had a composition of 27 % v/v acetic acid, 0.06 M KOH, containing 1.6 % TEMED v/v. After polymerization by 0.8 % w/v ammonium persulfate, the gel was pre-run electrophoretically for one hour with a 0.02 M glycine buffer adjusted to pH 3.0 by the addition of acetic acid. Protein of 40-60 µg was used, stained and destained as previously described in the pH 6 system.

VIII. Molecular Weight Determination

The molecular weight of the urinary diabetogenic peptide was determined by using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis procedure of Weber and Osborn[63]. Protein standards (ovalbumin, chymotrypsinogen A, ribonuclease A and cytochrome C) were included in each run. Electrophoresis was performed at a constant current of 8 milliamperes per gel for 4 hours in 10 % acrylamide gel containing 0.1 M sodium phosphate and 0.2 % SDS at pH 7.0. At the end of electrophoresis, the gel was taken out of the tube and stained with 0.25 % Coomassie Brilliant Blue dye for two hours. It was then soaked in an aqueous destaining solution containing 7½ % v/v acetic acid and 45 % v/v methanol for 2 hours before further destaining

electrophoretically. The relative migrations of standard proteins were correlated with their known molecular weights. The mobility, M, of each protein was calculated by:

$$\frac{\text{Migration of Protein}}{\text{Migration of tracking dye}} \times \frac{\text{Length of gel before staining}}{\text{Length of gel after destaining}}$$

The molecular weight of the diabetogenic peptide was obtained by comparing its mobility with a semi-logarithmic plot of the standard protein molecular weights versus mobilities. A plot of this experiment is shown in the Results Section V.

IX. Carbohydrate Content Study

Carbohydrate content of urinary diabetogenic peptide was determined by the method of Lehnhardt and Winzler[64]. Two miligrams of the peptide were subjected to acid hydrolysis (with 40 % w/v Dowex 50 x 2 H^+ 200-400 mesh resin) in 0.02 N HCl for 20 hours at 100°C. The mixture was neutralized by addition of 20 % w/v Dowex 1 HCO_3^- in water. An internal standard, β-CH_3-D-glucoside (0.1 µ mole) was added. The neutral hydrolysate (200 lambdas) was reduced with 20 lambdas 2.2 M $NaBH_4$ for 1 hour at room temperature. This step was to convert the neutral sugars to their

respective alditol acetate derivatives. Excess $NaBH_4$ was destroyed by the addition of 25 lambdas glacial acetic acid. One hundred lambdas HCl: methanol (1:1000) was added and the mixture was dried overnight in vacuo over P_2O_5. The alditol acetate derivatives were derivatized by the addition of 100 lambdas of anhydrous pyridine and 100 lambdas of acetic anhydride. The mixture was kept at 100°C for 45 minutes. Finally, the solution was evaporated to approximately 20 lambdas. One to three lambdas of the solution was injected into a Packard Gas Chromatograph Model 7401 for the quantitative determination of the various sugars. (I wish to thank Lillian Hsu for her help in this work.)

X. Immunological Studies

Immunological Studies were carried out in order to develop a radioimmunoassay procedure for the diabetogenic peptide. The goal was to use the method to measure the blood concentration of the peptide in health and disease - especially diabetes mellitus in man.

A. Preparation of antiserum

The antiserum to urinary diabetogenic peptide was

prepared by Dr. R. Knopf of the Metabolism Research Laboratory. The peptide was emulsified with complete Freund's adjuvant. It was injected by the interdermal multiple-sites technique to four albino rabbits. Immunization consisted of four separate injections over an 8 month period. The antisera from the four rabbits were tested for antibody titre.

B. Iodination

Five ug urinary diabetogenic peptide was iodinated with approximately 1 mCurie $Na^{131}I$ (Union Carbide). Exposure to the oxidant chloramine-T (20 µg in 50 µl) was for 45 seconds at room temperature after which the reaction was stopped by the addition of 72 µg sodium metabisulfide. All reagents were prepared in 0.5 M veronal buffer of pH 7.5. Approximately 25 % of the ^{131}I was found as bound ^{131}I on the peptide by paper electrophoresis. The ^{131}I-urinary peptide was purified by a P-60 Bio-Gel column filtration. The ^{131}I-urinary peptide was checked for unreacted ^{131}I and ^{131}I-damaged peptide by paper electrophoresis.

C. Paper electrophoresis

Paper electrophoresis was performed at a constant voltage (500 V) at $4^{o}C$ in 1.0 M barbital buffer, pH 8.6 for one hour. The chromatograms were scanned for radioactivity with a Packard Radiochromatogram Scanned.

D. Cross reactivity with human growth hormone.

Antisera from immunized rabbits were checked for cross reactivity with human growth hormone. (I wish to thank Dr. Knopf for his help and suggestions in this work.)

RESULTS

I. Stability Studies

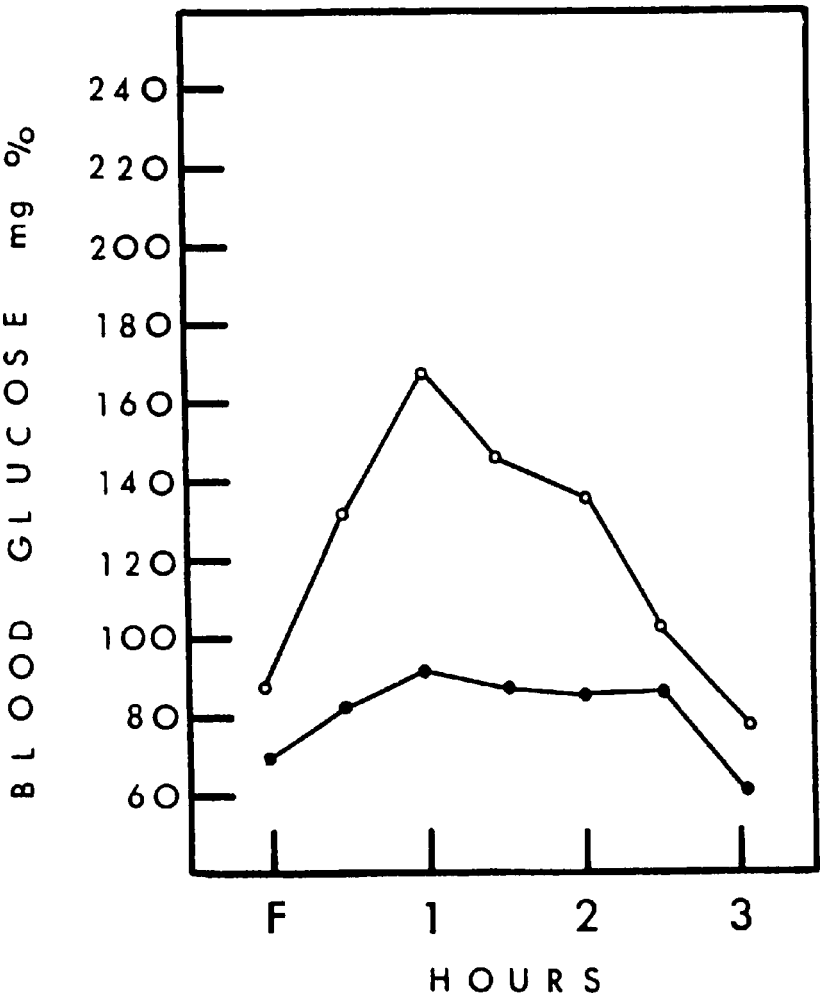


Figure 2. Effect of untreated bovine PI on glucose tolerance of dog W, 14.2 kg body weight. Glucose 1.75 g/kg (orally) o———o 10 hours after peptide injection, 2 mg/kg; •———• control.

The purpose of this experiment was to ascertain the diabetogenic activity of bovine PI which was subsequently used for all the stability studies. Two milligrams of

peptide per kilogram of dog body weight was used in all the experiments. Procedures for glucose tolerance tests and maintenance of dog were the same as previously described by Louis [52].

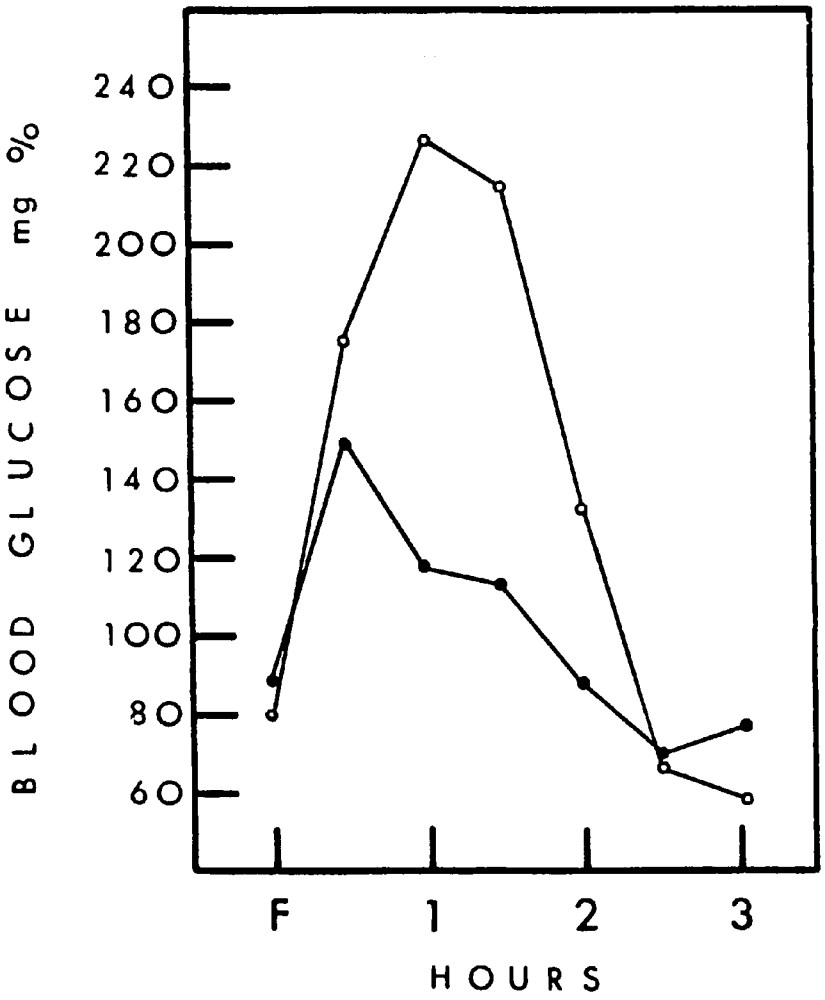


Figure 3. Effect of bovine PI after storage at room temperature for 6 months on glucose tolerance of dog W, 14.2 kg body weight. Glucose 1.75 g/kg body weight (orally). o——o 10 hrs after injection; •——• control.

The diabetogenic peptide did not diminish its activity after storage at room temperature for 6 months.

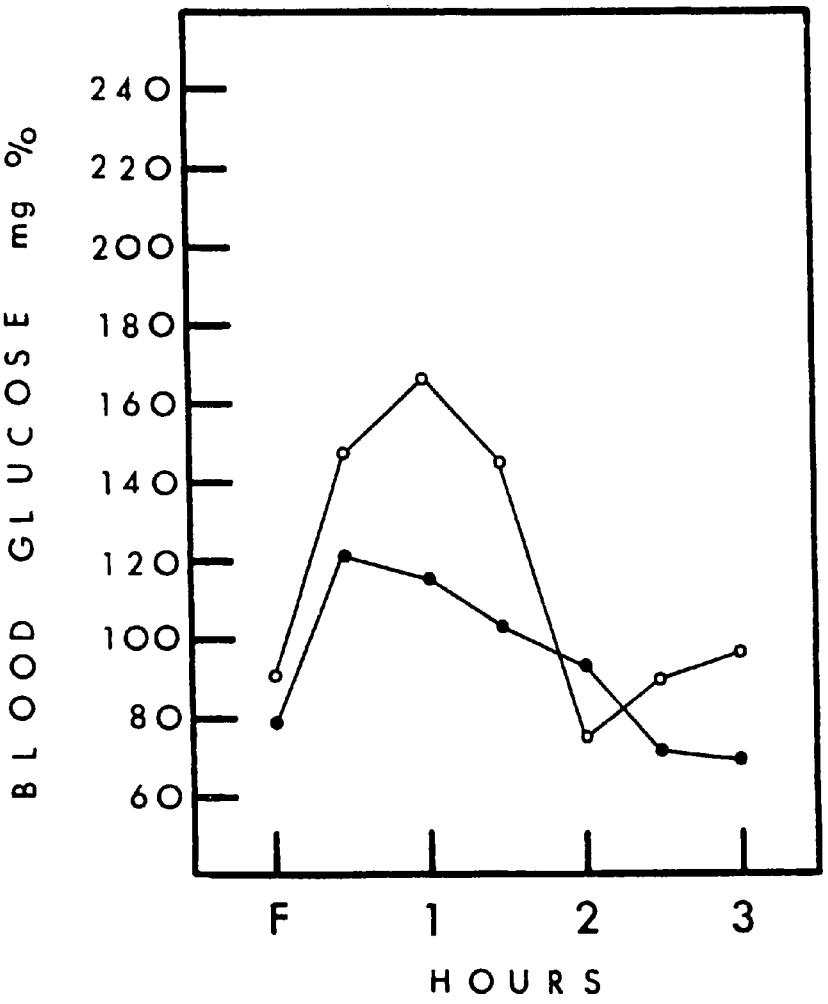


Figure 4. Effect of bovine PI after exposure to 0.1 N HCl on glucose tolerance of dog W, 14.2 kg body weight. Glucose 1.75 g/kg body weight (orally).
o———o 10 hours after injection;
●———● control.

Diabetogenic activity was somewhat diminished after exposure to 0.1 N HCl at 4^{o}C for 72 hours.

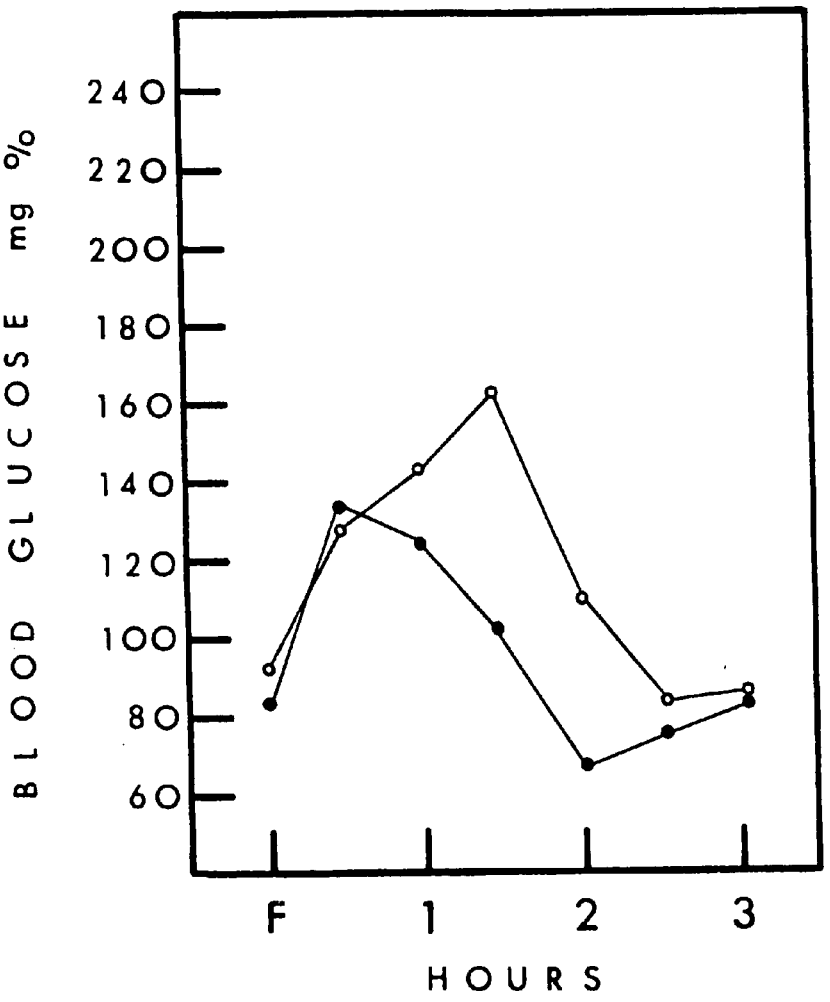


Figure 5. Effect of bovine PI after exposure to 3 % acetic acid at 37°C on glucose tolerance of dog W, 14.2 kg body weight. Glucose 1.75 g/kg body weight (orally). o———o10 hours after injection; •———•control.

Diabetogenic activity of bovine peptide was slightly reduced after exposure to 3 % acetic acid at 37°C for 24 hours.

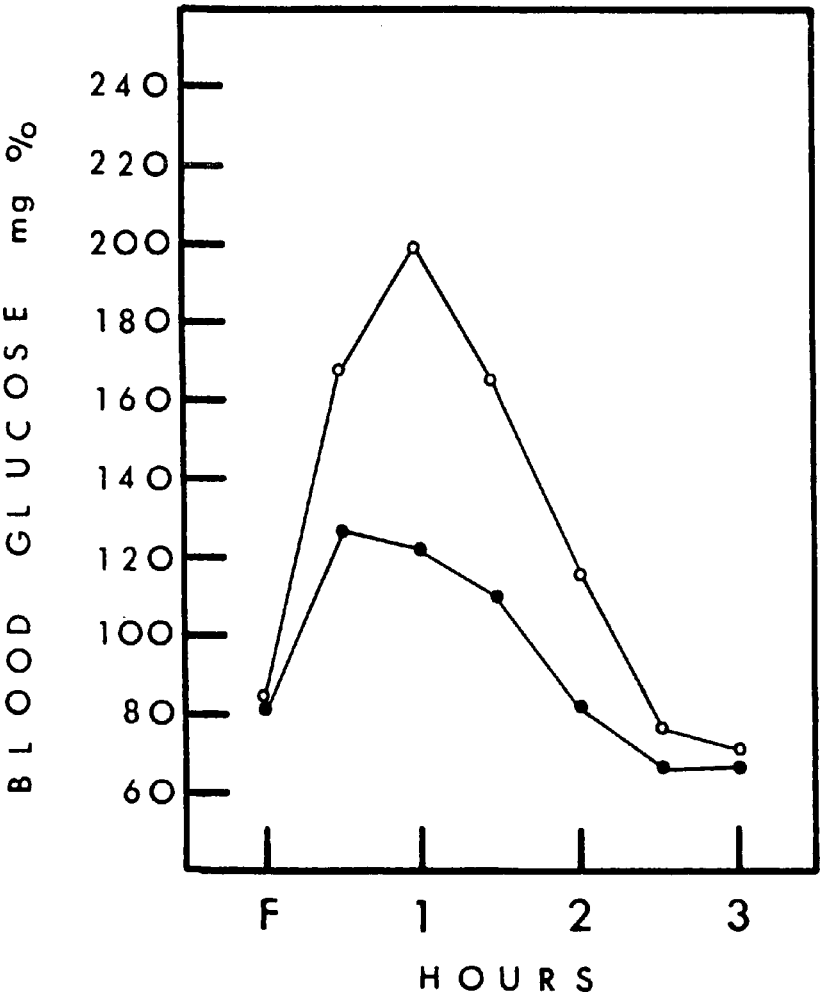


Figure 6. Effect of bovine PI after exposure to 3 % acetic acid at 22.1°C on glucose tolerance of dog W, 14.2 kg body weight. Glucose 1.75 g/kg body weight (orally). o——o 10 hours after injection; •——• control.

Bovine peptide solution in 3 % acetic acid at 22.1°C for 24 hours did not impair its diabetogenic activity.

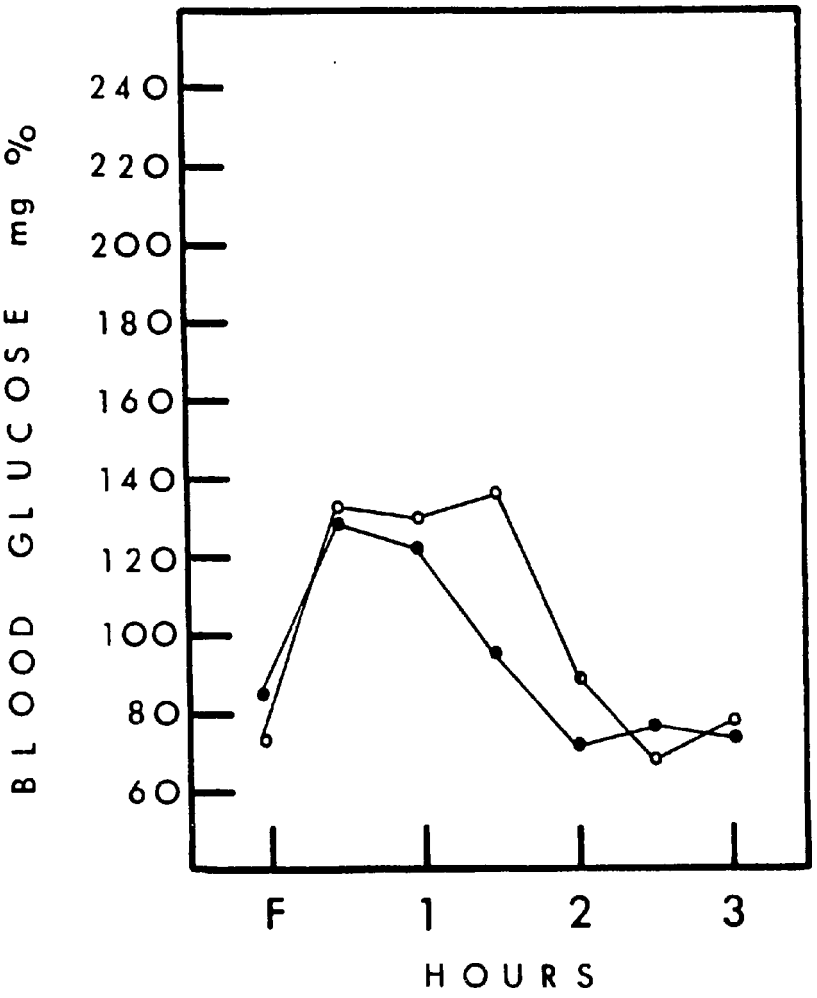


Figure 7. Effect of bovine PI after exposure to pH 9.5 on glucose tolerance of dog W, 14.2 kg body weight. Glucose 1.75 g/kg body weight (orally). o———o 10 hours after injection; ●———● control.

Bovine peptide solution kept at pH 9.5 at 4°C for 6½ hours lost its diabetogenic activity completely.

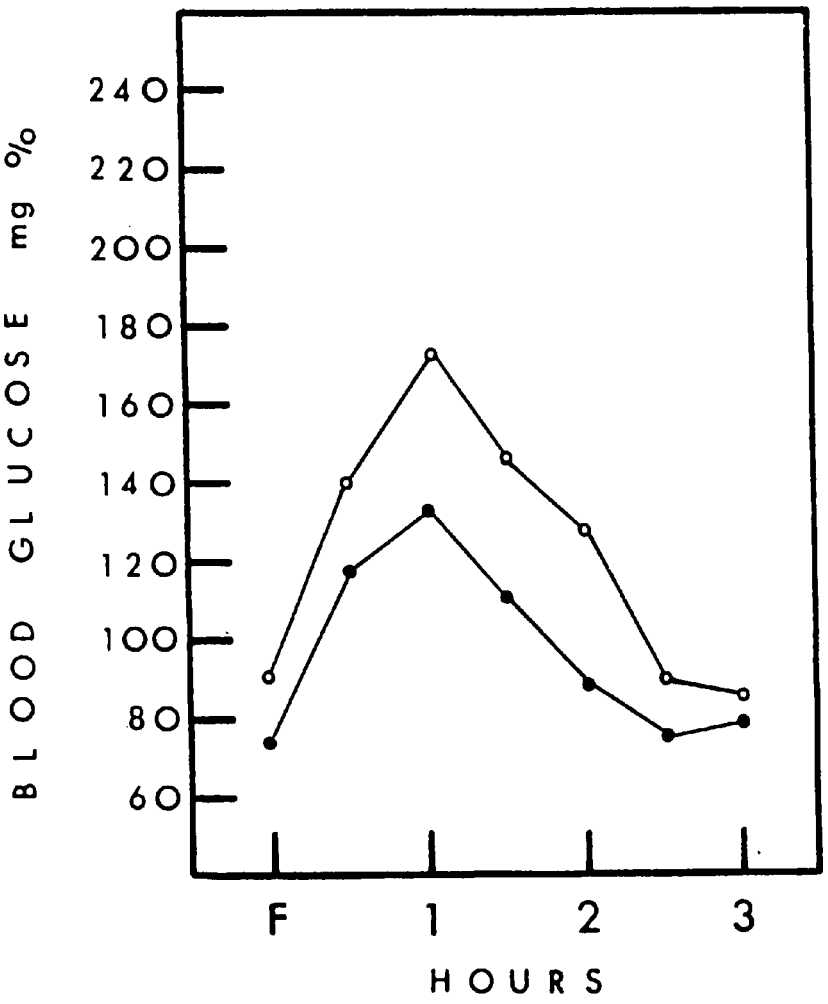


Figure 8. Effect of bovine PI after exposure to pH 6.8 at 4°C for 3 days on glucose tolerance of dog W, 14.2 kg body weight. Glucose 1.75 g/kg body weight (orally). o——o 10 hours after injection; •——• control.

Bovine peptide solution kept at pH 6.8 at 4°C for 3 days did not lose its diabetogenic activity.

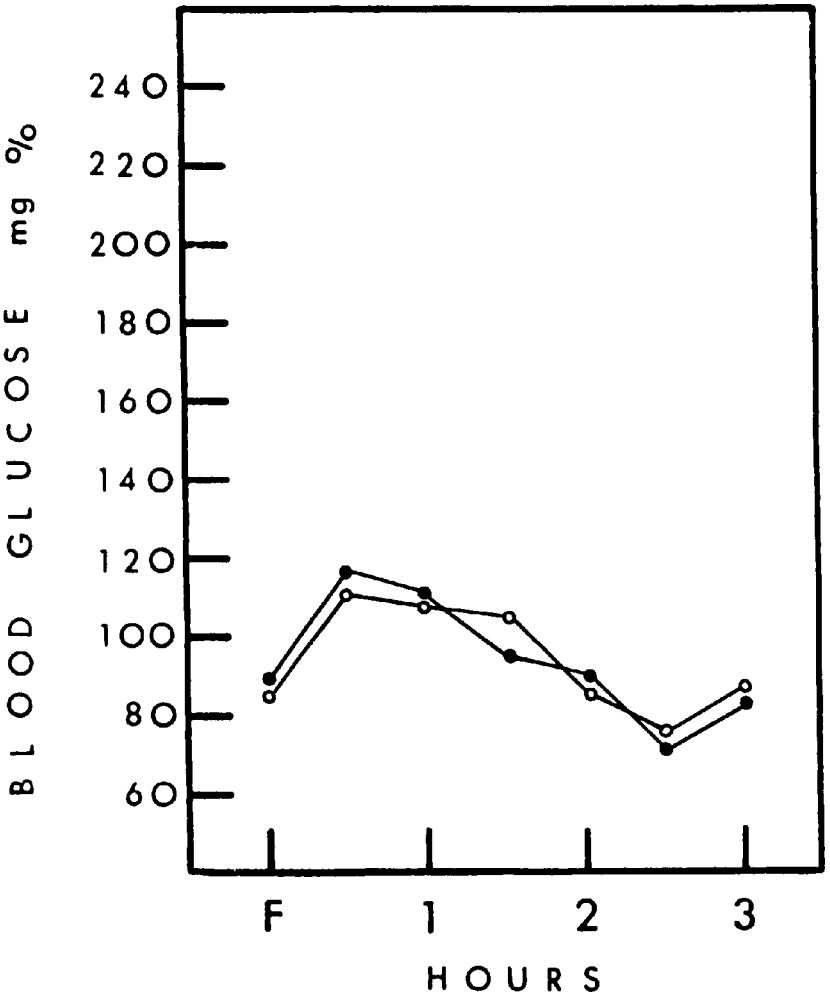


Figure 9. Effect of bovine PI after exposure to 100°C on glucose tolerance of dog W, 14.2 kg body weight. Glucose 1.75 g/kg body weight (orally). o———o 10 hours after injection; ●———● control.

Neutral peptide solution boiled at 100°C for 1 hour completely lost its diabetogenic activity.

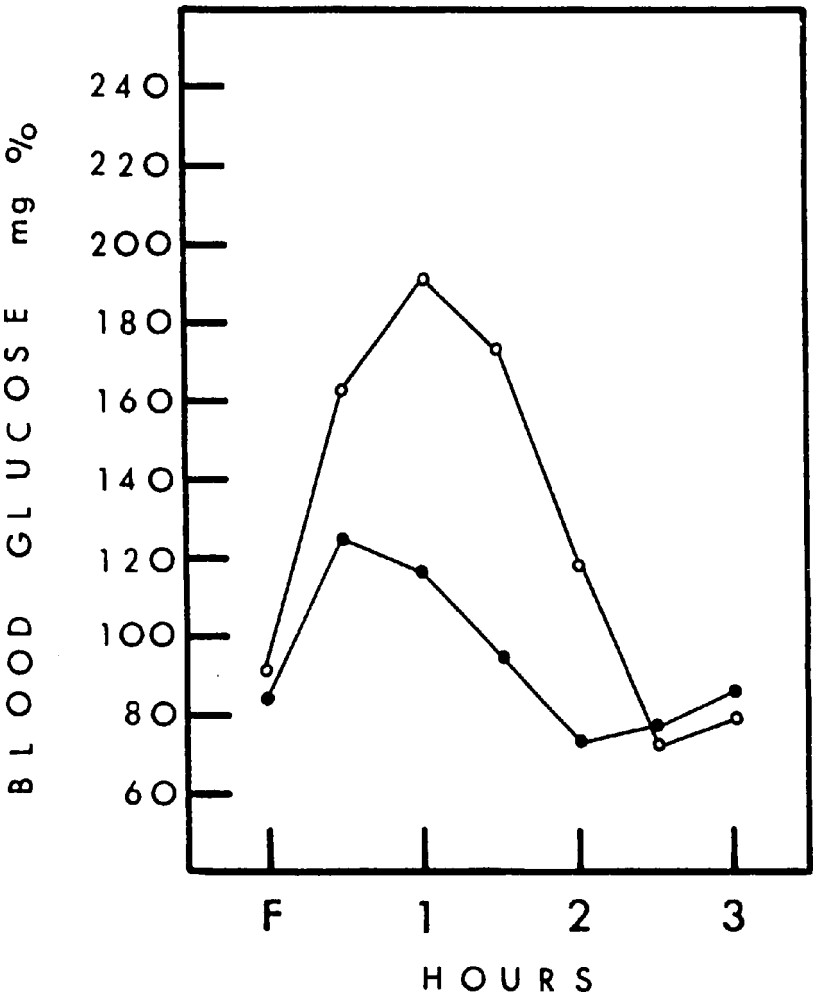


Figure 10. Effect of bovine PI after exposure to ½ saturation of ammonium sulfate on glucose tolerance of dog W, 14.2 kg body weight. Glucose 1.75 g/kg body weight (orally). o——o 10 hours after injection; •——• control.

Figure 10 showed that ½ saturation ammonium sulfate solution at 4°C for 1 hour did not impair the diabetogenic activity of the bovine peptide.

II. Isolation of Diabetogenic Peptide from Urine of Diabetic Patients.

Urine from proteinuric diabetic patients was subjected to the isolation procedure as previously described. The results are shown in Table I. The diabetogenic peptide was invariably present in the urine of all proteinuric diabetic patients. However, there was no correlation between the severity of proteinuria and the quantity of diabetogenic peptide excreted. As mentioned earlier, urine samples which showed the least amount of protein (trace to 1+) were somewhat easier to work with. This may be due to the smaller amount of unwanted protein in the urine.

Table I.

ISOLATION OF DIABETOGENIC PEPTIDE FROM PROTEINURIC DIABETIC PATIENTS.[1]

Patient	Glucose in urine	Protein in urine	Diabetogenic Peptide mg/day
J.H.	+++	++++	5.5
H.H.L.	++++	++	3.0
F.M.	+++	Trace	4.1
C.F.	++++	Trace	2.1
R.G.	++++	+	5.2
H.C.	++++	Trace	7.0
C.L.	++++	+	1.1
R.B.	+++	+	6.2
C.S.K.	Trace	0	0
H.W.	Trace	0	0
S.B.	+	Trace	2.8
W.B.B.[2]	+	+	2.6
E.I.F.	++++	+	2.5
M.K.[3]	+	+	0.3
V.A.	+++	++	2.8
C.L.	++++	++	1
L.L.	++	+	1.6
A.P.[4]	++++	0	0
G.G.	++++	+++	4.2
C.L.	++++	++	1.7
T.H.	0	+	amount not determined
G.E.	++++	+	"
R.K.	+	Trace	"
F.H.	++++	+++	"
C.W.	++++	++++	"
G.B.	++++	Trace	"
M.M.	++++	++++	"
T.S.	+	+	"

1. Patients of University Hospital, Ann Arbor, except otherwise noted.
2. From Phoenix, Arizona.
3. From Nebraska Medical School.
4. From Eli Lilly Laboratory.

III. Demonstration of Biological Activity of Diabetogenic Peptide Isolated from Urine of Proteinuric Diabetic Patients.

Two batches of urinary diabetogenic peptide preparations (pooled #1 and #2) were tested for biological activity in a normal dog (Dog P). The results which demonstrated glucose intolerance induced by the 2 peptide preparations are shown in Figures 11 and 12. It is evident that both preparations are biologically active in this dog. The result of glucose tolerance test after administration of a peptide isolated from the urine of a lipoatrophic diabetic patient is shown in Figure 13. This preparation is also capable of inducing glucose intolerance in this dog (Dog P).

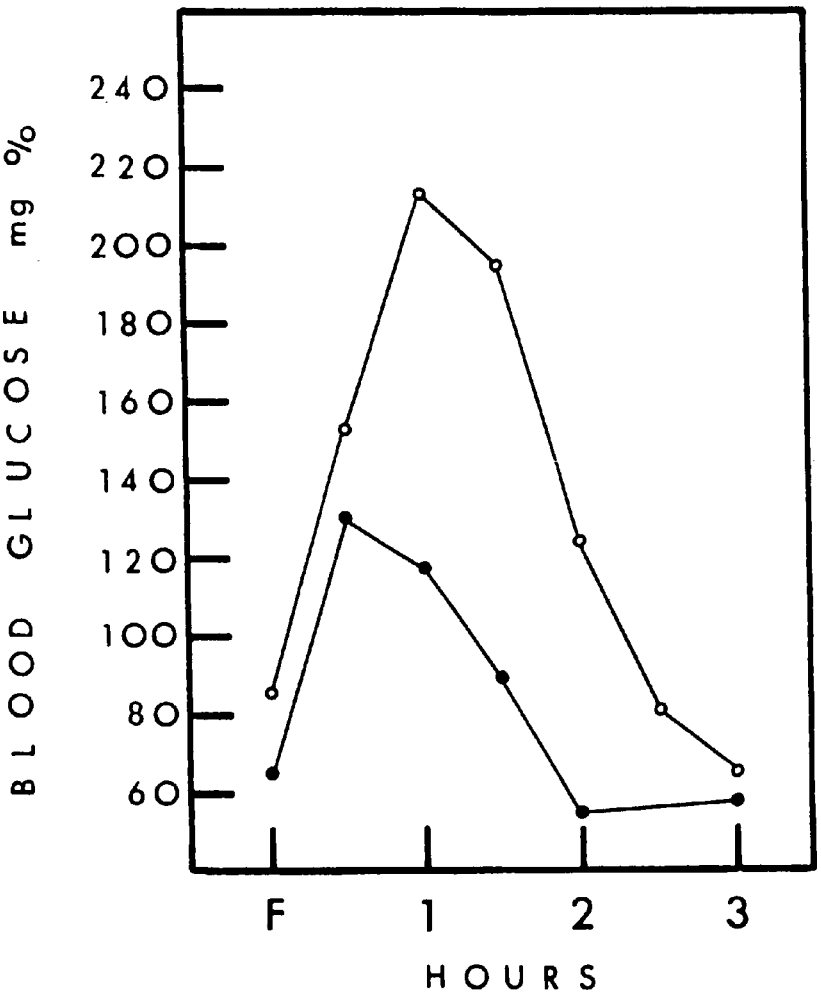


Figure 11. Effect of diabetogenic peptide from urine of 15 diabetic patients (pooled # 1) on glucose tolerance of dog P, female, 8.0 kg. Glucose 1.75 g/kg body weight (orally). o——o 10 hours after injection of 14 mg peptide; ●——● control.

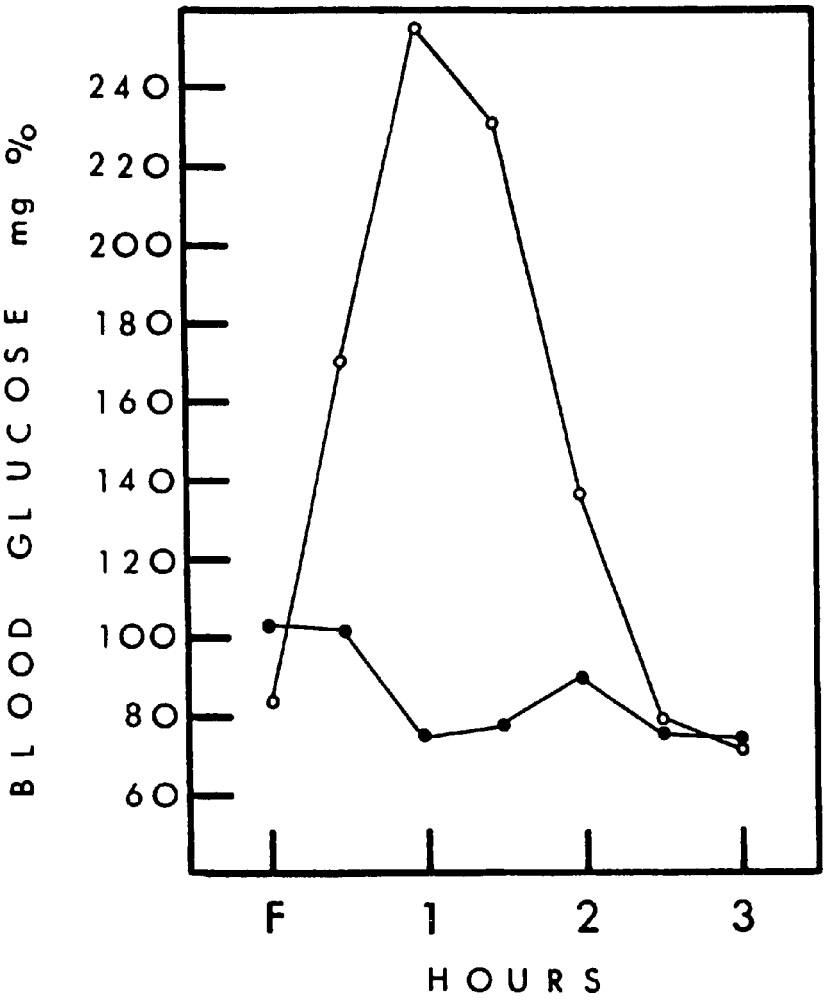


Figure 12. Effect of diabetogenic peptide from urine of 13 diabetic patients (pooled # 2) on glucose tolerance of dog P, female, 8.0 kg. Glucose 1.75 g/kg body weight (orally). o——o 10 hours after injection of 14 mg peptide; •——• control.

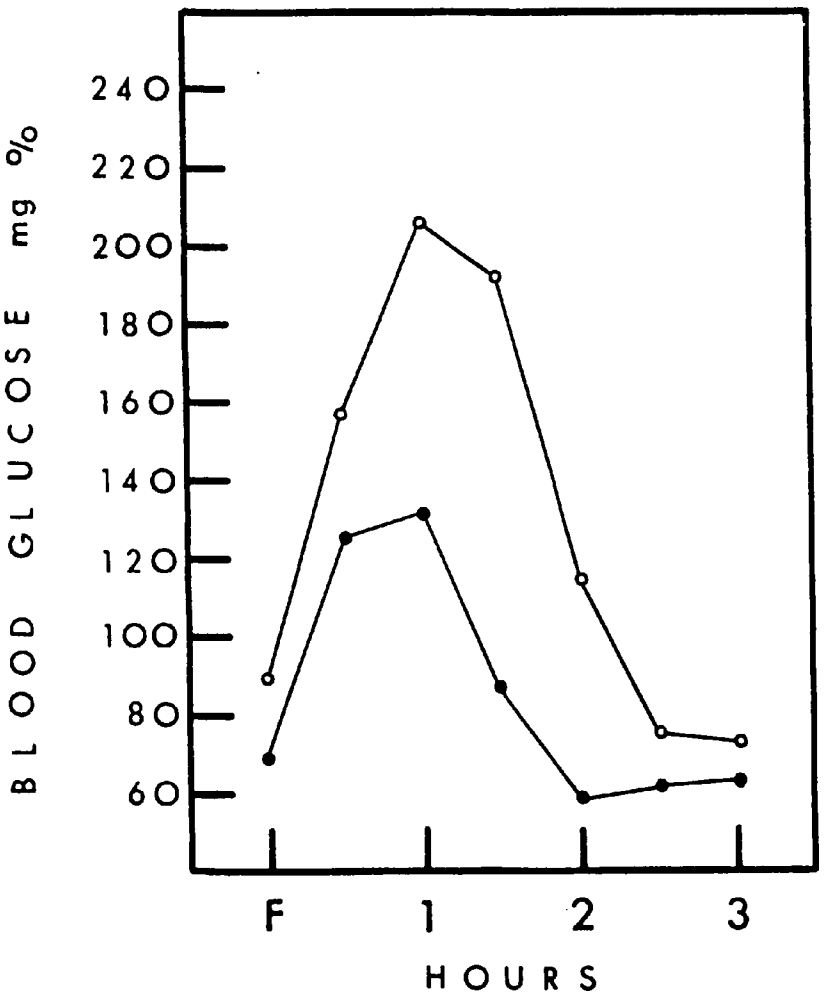


Figure 13. Effect of diabetogenic peptide from urine of lipoatrophic diabetic patient (M. Dunham) on glucose tolerance of dog P, female, 8.0 kg body weight. Glucose 1.75 g/kg body weight (orally). o———o 10 hours after injection of 14 mg peptide; •———• control.

IV. Induction of Hyperglycemia and Hyperinsulinemia by Administration of Urinary Diabetogenic Peptide in Dogs.

Experiments on the effect of urinary diabetogenic peptide (pooled) on glucose tolerance were performed on two normal dogs. Blood glucose and insulin were measured. The results are shown in Table II and III. The data showed that the diabetogenic peptide was capable of inducing simultaneously hyperglycemia and hyperinsulinemia. It should be noted that blood glucose was significantly elevated in spite of the very high level of insulin. This was particularly noticeable after the second peptide administration. This phenomenon was similar to that in some diabetic patients, whose glucose intolerances were accompanied by a high level of insulin (Berson and Yalow [65]).

Induction of Hyperglycemia and Hyperinsulinemia by Urinary Diabetogenic Peptide in Dog O, female, 12.1 kg. body weight. Glucose Load 1.75 gm/kg.

Table II.

GLUCOSE mg %							
	F	½	1	1½	2	2½	3
Control (1)	76	80	99	82	66	73	80
Control (2)	86	116	99	101	76	76	75
10 hrs after 1st injection[1]	90	99	110	114	116	100	82*
10 hrs after 2nd injection[2]	118	213	252	245	162	108	92**
34 hrs after 2nd injection	88	126	145	147	103	73	72
14 days after 2nd injection	95	105	107	106	76	85	89
INSULIN µU/ml							
	F	½	1	1½	2	2½	3
Control (1)	10	35	46	8	7	4	7
Control (2)	18	75	49	31	16	14	14
10 hrs after 1st injection[1]	21	108	127	64	49	24	17
10 hrs after 2nd injection[2]	25	135	155	45	40	29	28
34 hrs after 2nd injection	30	65	32	59	25	23	29
14 days after 2nd injection	19	78	--	36	29	23	20

1. 3 mg/kg body weight ;
2. 4 mg/kg body weight ;

* ++ } Glycosuria at end
** ++++ } Glycosuria of test

Induction of Hyperglycemia and Hyperinsulinemia by Urinary Diabetogenic Peptide in Dog K, male, 14.6 kg. body weight. Glucose Load 1.75 gm/kg.

Table III.

GLUCOSE mg %							
	F	½	1	1½	2	2½	3
Control (1)	72	102	99	81	84	80	81
Control (2)	79	99	101	76	80	82	82
10 hrs after 1st injection[1]	98	164	152	104	101	105	102*
10 hrs after 2nd injection[2]	113	204	198	128	109	106	101**
34 hrs after 2nd injection	96	122	131	78	84	89	91
14 days after 2nd injection	90	120	123	80	93	96	96
INSULIN μU/ml							
	F	½	1	1½	2	2½	3
Control (1)	4	35	87	8	10	12	13
Control (2)	2	37	28	4	3	2	3
10 hrs after 1st injection[1]	6	44	74	16	12	15	6
10 hrs after 2nd injection[2]	16	104	168	32	17	16	12
34 hrs after 2nd injection	6	55	80	12	7	6	5
14 days after 2nd injection	4	32	39	2	3	3	4

1. 3 mg/kg body weight ;
2. 4 mg/kg body weight ;

* ++ Glycosuria } at end
** ++++ Glycosuria } of test

V. Purification Studies

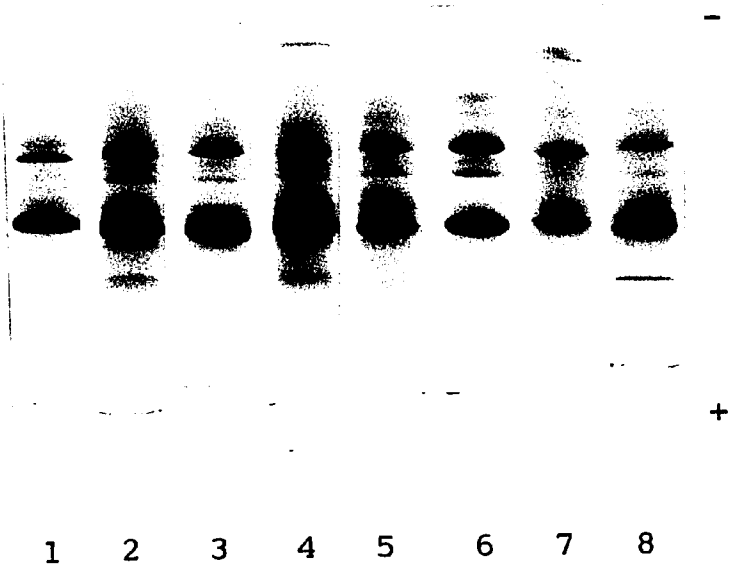


Figure 14. Disc-gel electrophoresis patterns (pH 8.9 system) of diabetogenic peptides isolated from urine of diabetic patients. 1. (Ross), 2. (Berryman), 3. (Kruska), 4. (Washington), 5. (Pancyzk), 6. (Lyons), 7. (pooled # 1), 8. peptide isolated from human pituitary glands.

The patterns demonstrate the similarity of the materials from urine of different patients and human pituitary glands. The urinary peptide was then purified by Sephadex G-100 superfine column filtration. A typical run is shown in Figure 15.

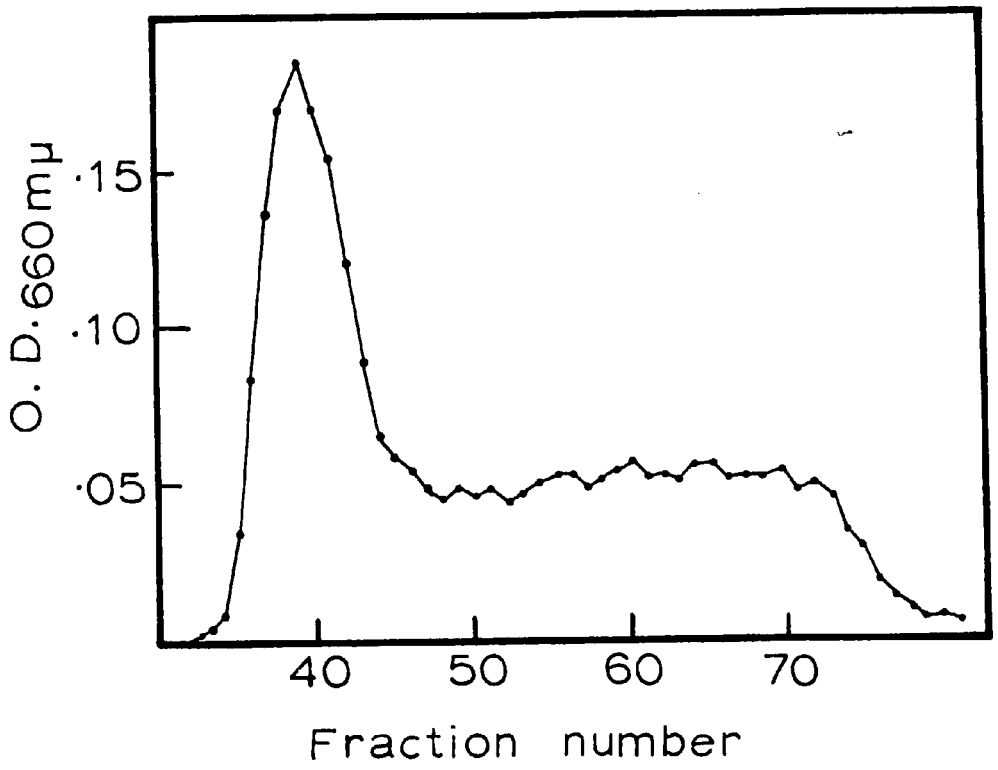


Figure 15. Elution profile of crude urinary diabetogenic peptide from a Sephadex G-100 superfine column (0.9 x 60 cm), flow rate 0.2 ml/20 min. operative pressure 20 cm.

The elution profile revealed only one major peak and an indefinite tailing. However, when the crude protein preparation was run through a Bio-Gel P-100 -400 mesh column, a second peak emerged. This is shown in Figure 16.

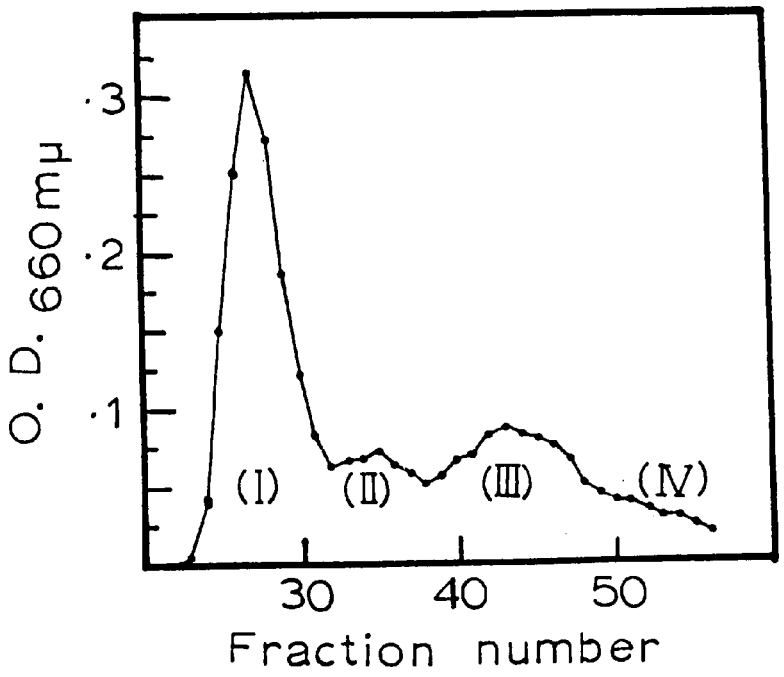


Figure 16. Elution profile of crude urinary diabetogenic peptide from a Bio-Gel P-100 -400 mesh column (0.9 x 60 cm), flow rate 0.2 ml/20 min. and operative pressure 25 cm.

The second peak was designated as Fraction III. Urinary diabetogenic peptide from a diabetic patient (Ross) was semipurified by pooling Fraction III from several runs. This material was then put on a long and narrow Bio-Gel P-100 -400 mesh column (0.5 x 120 cm). The elution profile is shown in Figure 17. The disc gel electrophoresis patterns (pH 8.9 system) of Fractions I, II, III and IV are shown in Figure 18.

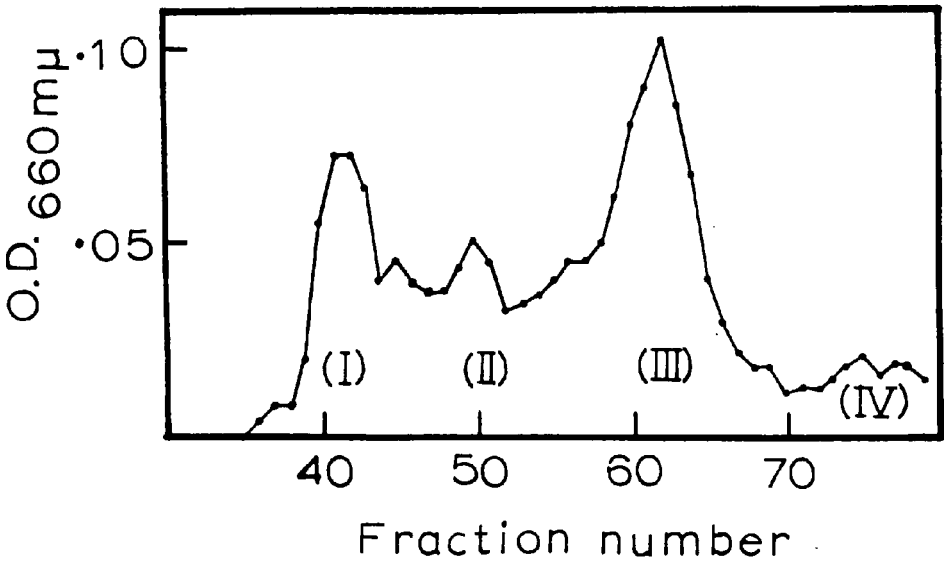


Figure 17. Elution profile of semi-purified diabetogenic peptide (Ross) from a Bio-Gel P-100 -400 mesh column (0.5 x 120 cm). Flow rate 0.3 ml/45 min. and operative pressure 35 cm.

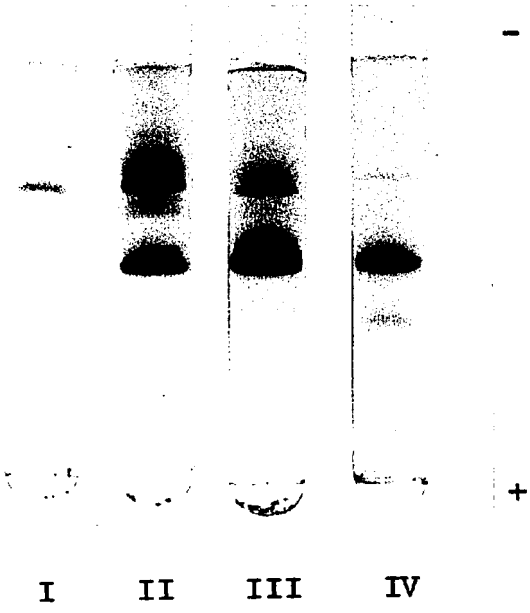
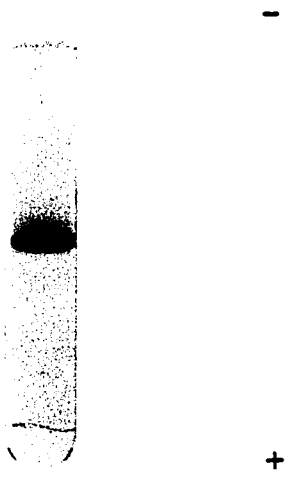


Figure 18.

Disc-gel electrophoresis patterns (pH 8.9 system) of diabetogenic peptide (Ross) after Bio-Gel column (0.5 x 120 cm) chromatography. Fractions I,II,III and IV correspond to that of Figure 17.

Fractions I to IV are in decreasing molecular weights, as shown on disc-gel electrophoresis patterns in Figure 18. Lower molecular weight materials migrated faster toward the anode. When fractions corresponding to only the tip of peak III of Figure 17 were pooled together, only one major protein band was obtained. This is shown in Figure 19.



Frac. III

Figure 19. Disc-gel electrophoresis pattern (pH 8.9 system) of a diabetogenic peptide (Ross).

Fraction III of diabetogenic peptide (Ross) was subjected to gel electrophoreses at pHs 8.9, 6.0, and 3.0 in order to establish homogeneity. The results are shown in Figure 20.

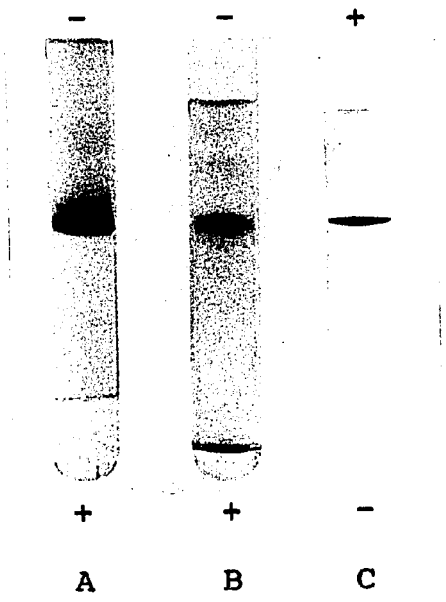


Figure 20. Gel electrophoresis patterns of Fraction III of diabetogenic peptide (Ross) at (A) pH 8.9, (B) pH 6.0, (C) pH 3.0

Fraction III reveals a single band pattern during electrophoreses at 3 different pHs. From these results, it appears that the peptide is homogeneous. So far, this is the purest material ever prepared in this laboratory. A similar result is obtained from purification of diabetogenic peptide pooled from a group of patients. This is shown in Figure 21.

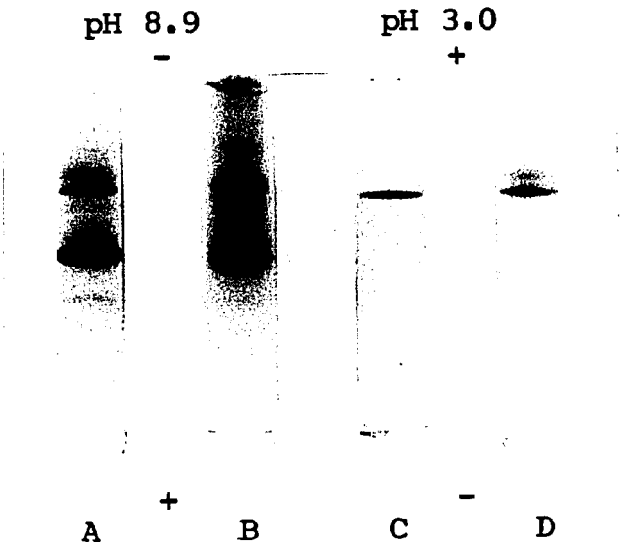


Figure 21. Gel electrophoresis patterns of Ross and pooled urinary diabetogenic peptide. (A) and (C)—Ross; (B) and (D)—pooled. (A) and (B)—initial fraction; (C) and (D) —fraction IIIs.

VI. Molecular Weight Determination

The molecular weight of the urinary diabetogenic peptide was determined by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis method of Weber and Osborn [63]. The binding of dodecyl sulfate ions to proteins rendered them negatively charged. Consequently, their electrophoretic migration rates were dependent on their molecular sizes. This is the basis of the method. A typical run is shown in Figure 22. The molecular weight of a urinary peptide sample (patient Sher) was found to be 20,800 ± 300 (average of 3 runs).

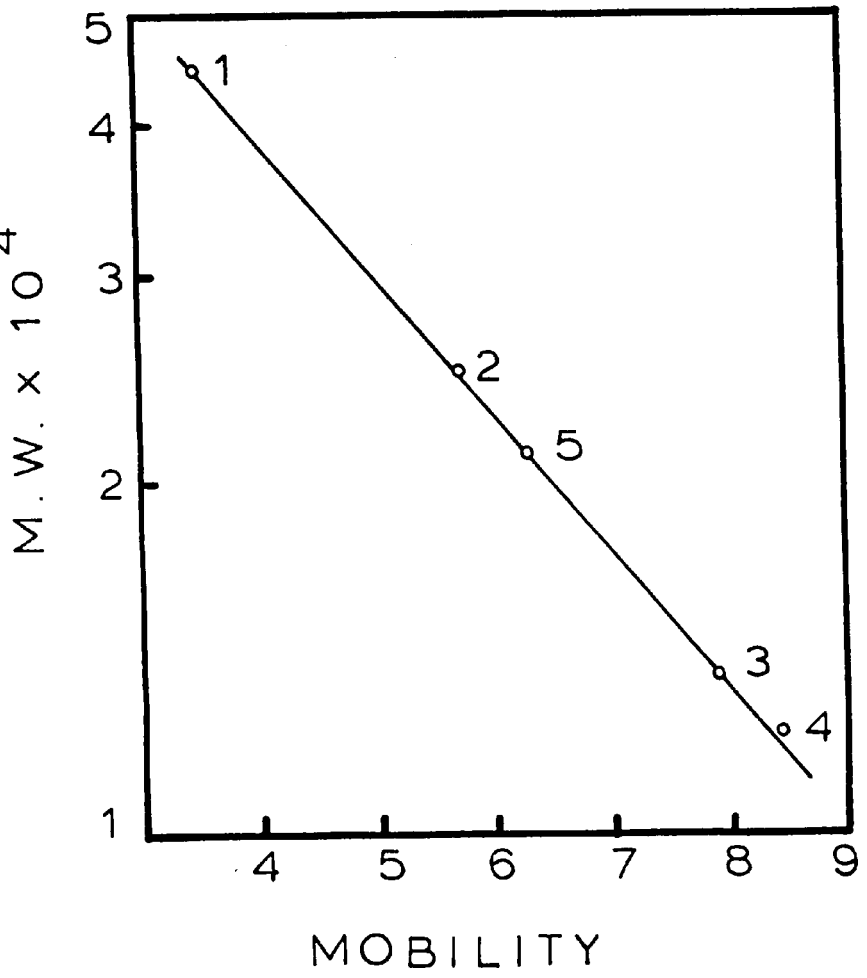


Figure 22. A plot of molecular weights versus mobilities in the SDS polyacrylamide electrophoresis for the determination of molecular weight of urinary diabetogenic peptide (Sher).

1. Ovalbumin	M.W.	45,000
2. Chymotrypsinogen A		25,000
3. Ribonuclease A		13,700
4. Cytochrome C		12,400
5. Urinary peptide (Sher)		20,500

VII. Carbohydrate Content Study

Glucose, galactose and mannose were found to be present in less than 0.01 μM in the peptide sample (2 mg). Since this particular peptide preparation had gone through a Sephadex column during its purification, the small amount of sugars found (less than 1 %) could possibly be attributed to contamination. Therefore, it was concluded that the urinary diabetogenic peptide was not a glycoprotein.

VIII. Immunological Studies

Four rabbits were used in this study. After the first injection with the crude urinary diabetogenic peptide (250 μg/injection), booster shots were given at an interval of two, five and eight months (200-300 μg/injection). The animals were bled two, three, six and ten months after the first injection for the detection of antibodies. Unfortunately, none of the animals produce any antibody that bind ^{131}I-urinary peptide to any significant extent. At present, four rabbits are employed for another immunization study.

A. Iodination

Iodination of the urinary diabetogenic peptide

by $Na^{131}I$ was successful. The yield of the iodinated compound was over 90 % with a small amount of damaged product (<5 %). The iodinated peptide was then purified by Bio-Gel P-60. Scanning of the paper electrophoresis chromatogram revealed a single peak. This is shown in Figure 23.

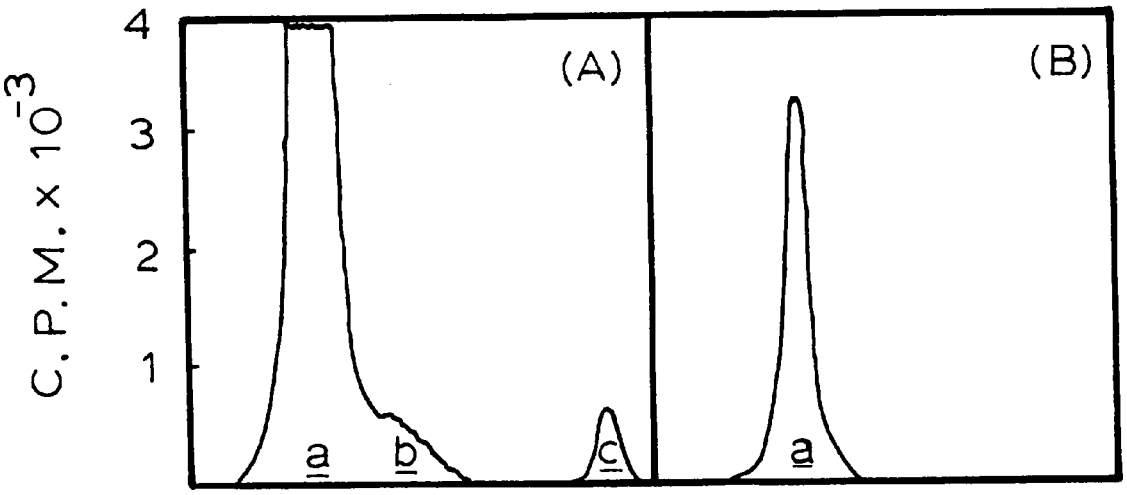


Figure 23. Radioactivity scans of paper electrophoresis chromatograms (A) before and (B) after Bio-Gel P-60 column chromatography. a ^{131}I-urinary peptide, b ^{131}I-damaged peptide, c free ^{131}I.

B. Cross reactivities

The urinary diabetogenic peptide was tested for cross reactivities in the following systems.

1. Anti-human pituitary diabetogenic peptide antiserum + ^{131}I-human pituitary diabetogenic peptide as trace.

2. Anti-human growth hormone antiserum + ^{131}I-human growth hormone as trace.

The displacement of bound traces by the urinary peptide, pituitary peptide and growth hormone for the two systems are shown in Figure 24 and Figure 25.

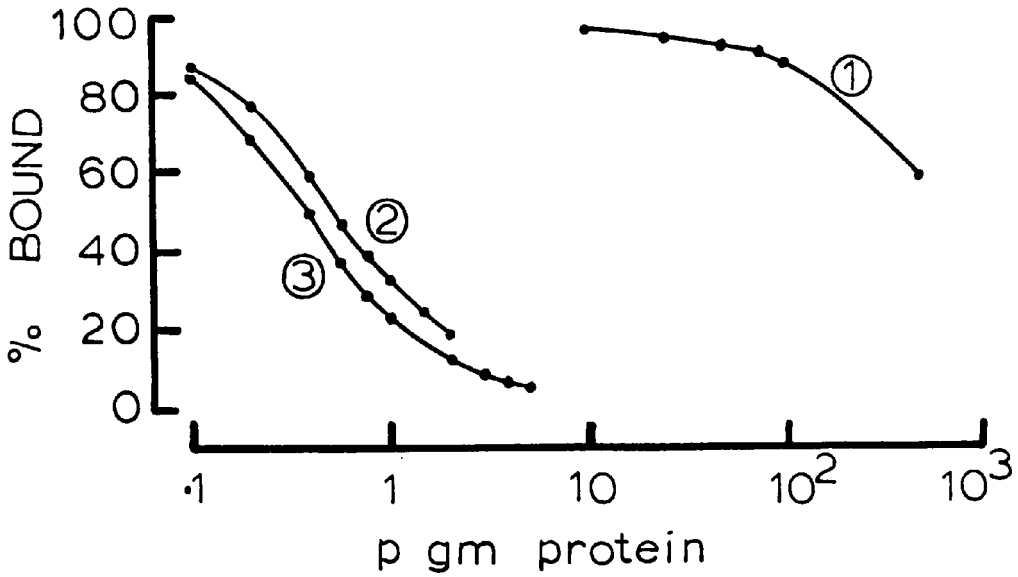


Figure 24. Displacement of bound ^{131}I-pituitary peptide from pituitary peptide antibody by (1) urinary peptide, (2) pituitary peptide, and (3) human growth hormone.

In the pituitary diabetogenic peptide radioimmunoassay system, the urinary peptide cross reacts with the pituitary peptide to 0.08 %, and with growth hormone to 0.06 %.

In the human growth hormone radioimmunoassay system, the urinary peptide cross reacts with the pituitary

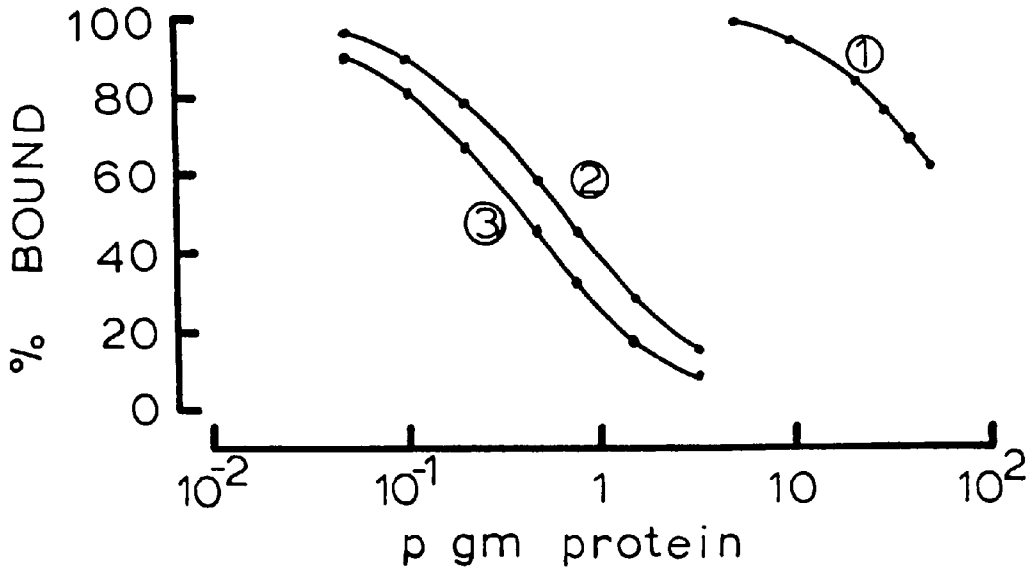


Figure 25. Displacement of bound ^{131}I-human growth hormone from human growth hormone antibody by (1) urinary peptide, (2) pituitary peptide, and (3) human growth hormone.

peptide to 0.7 %, and with human growth hormone to 0.4 %. Cross reactivity between pituitary peptide and human growth hormone was quite high. There was 53 % cross reactivity in the first system and 63 % in the second. In the pituitary peptide system, human growth hormone is immunologically more reactive to the pituitary diabetogenic peptide antiserum than the peptide itself. This inconsistency is not clear at the present time.

In these immunological studies, the similarity between the pituitary diabetogenic peptide and the urinary diabetogenic peptide could not be demonstrated.

DISCUSSION

I. Stability Studies

Since some of the properties of bovine diabetogenic peptide have been shown to be similar to those of the urinary peptide (isoelectric point, solubility, diabetogenicity)it is reasonable to assume that the information obtained from one may be applied to the other. This would be important in the purification process.

The first important characteristic of the bovine peptide is its extreme sensitivity to alkaline pH. In solution at pH 9.5 at $4^{o}C$ for 6½ hours the diabetogenic activity of the peptide is completely destroyed. However, solution at pH 6.8 at $4^{o}C$ for three days did not impair its biological activity. Thus, it is apparent that if high potency of the peptide is to be retained, it is of paramount importance to maintain rigorously the pH of the solution below 7 throughout the isolation and purification procedures. Adjustment of pH with sodium hydroxide must be carried out with extreme caution. The protein solution should be stirred vigorously with each dropwise addition of alkali. Also the pH of the solution should be checked

frequently.

The second characteristic of the peptide is its moderate insensitivity to acid pH. Treatment of the peptide with 3 % acetic acid at 22-37°C for 24 hours did not significantly alter its diabetogenic potency. However, treatment with 0.1 N HCl at 4°C for 72 hours diminished the activity slightly. It is important, therefore, to avoid unnecessary delay in carrying out the isolation step where 0.1 N HCl is required. If these precautions are not observed, loss of potency or complete inactivation will result.

An interesting side product from this study is the finding that the stability of bovine growth hormone and bovine diabetogenic peptide are very different in acid and alkaline solutions. Li and Papkoff [66] demonstrated that bovine growth hormone is unstable in 0.1 N acetic acid (0.6 %) kept at 0°C or 25°C for 24 hours. Both treatments resulted in a significant loss of growth-promoting activity. Reid [67] showed that at an acidic pH, the diabetogenic activity of bovine growth hormone is markedly diminished. Also, Li and Papkoff [66] demonstrated that bovine

growth hormone did not suffer any significant loss of activity after exposure to 0.1 N NaOH at 25°C for 3 hours. Growth hormone solution at pH 9.5 at 25°C for 24 hours also did not elicit any loss of biological activity. In contrast, bovine diabetogenic peptide is unstable in alkaline solution and stable in acid solution. Urinary diabetogenic peptide is also very sensitive to alkaline pH. Its diabetogenic activity was completely destroyed in cases when solutions of the peptide were over-titrated with alkali. (Dr. L. Louis - personal communication).

II. Isolation of Diabetogenic Peptide from Urine.

One of the difficulties in this research is the lack of sufficient material for investigation. Since not all diabetic patients are proteinuric, one has to wait for weeks to obtain urine from a suitable patient. In general, most patients are not hospitalized for more than ten days. Furthermore, the amount of peptide excreted per day is small (0.5 - 8 mg crude material). Another problem is the lack of a good, reliable biological assay procedure which would require micrograms of the peptide. Glucose and insulin tolerance tests in normal dogs are excellent and

reliable but the amount of material require for a single assay is moderately large (1 mg per kilogram body weight). Clearly, a reliable micro bioassay procedure is indicated.

III. Induction of Hyperglycemia and Hyperinsulinemia in Dogs.

This study shows that administration of the partially purified urinary diabetogenic peptide to dogs results in hyperinsulinemia together with impairment of glucose tolerance. The hyperinsulinism is not the consequence of hyperglycemia since the fasting blood glucose (except after the second injection of peptide) is not elevated. Thirty-four hours after peptide administration, the blood concentrations of glucose and insulin still have not returned to the pre-injection level. Thus, the half-life of the active principle is of very long duration. This finding is in accord with the studies of Louis and Conn who used diabetogenic peptides obtained from beef [51, 52] hog, sheep [53, 54] and human pituitary glands [55].

Perley and Kipnis [68] first reported that serum insulin level is higher in maturity-onset diabetic patients (obese or non-obese) than in normal people during a glucose

load. Sabeh[69] found that intravenous glucose administration to newly diagnosed diabetic women evoked an excessive output of insulin. The fact that maturity-onset diabetic patients either obese or non-obese have higher serum insulin level than normal people during the glucose tolerance test has been well documented and accepted by most diabetes investigators. The reason for this finding is not known. However, Shen[70] thought that there might be an antagonistic factor which is responsible for the endogenous insulin resistance. This implies that the islet cells of some diabetics remain functional to deliver insulin for physiologic needs and counterbalance the antagonist. If the amount of insulin is not sufficient to completely overwhelm the antagonist the net result is hyperinsulinism and diabetes. The exact nature and site of origin of this factor is not known but it is believed to be a low molecular weight compound and possibly a polypeptide. This view is also shared by Gordon[71].

The experimental results of this study seem to support the insulin antagonist theory and that the hypothetical factor might indeed be the peptide excreted in the

urine of diabetes. Further evidence to strengthen this view are the reports of Louis and Conn[57] which showed that the urinary diabetogenic peptide is capable of antagonizing the hypoglycemic effects of exogenous insulin in normal dogs and man.

IV. Immunological Studies

Up to the present time, it has not been possible to produce an anti-serum that could specifically bind to the urinary peptide. In general, a protein of this size (20,800) should have little difficulty in evoking an antibody response. There seems to be no explanation as to why it does not in this case. Perhaps one should use more animals or other animal species. In contrast, the human pituitary diabetogenic peptide presented no difficulty in the production of antibodies.

Since the urinary diabetogenic peptide is similar to that of the human pituitary peptide (isoelectric point, molecular weight, diabetogenicity) it is reasonable to assume that the substance is unaltered or slightly altered when excreted in the urine. The fact that the substance is found only in the urine of proteinuric diabetics but not

in normal people suggests that it is related to diabetes mellitus. Thus, it is important to develop an immunoassay procedure for this "marker" as an adjunct for the diagnosis of diabetes mellitus in man. Unfortunately this has not as yet been accomplished.

V. Diabetes-inducing and Insulin-antagonizing Peptide: Relation to Diabetes Mellitus in Man.

The phenomena of insulin resistance and diabetes has attracted the attention of a large number of clinical investigators. Many atempts have been made to study its mechanism and to identify a factor or factors responsible for insulin resistance. Pituitary extracts, growth hormone, "modified" growth hormone, synalbumin, B-chain insulin, ACTH, hydrocortisone, epinephrine, glucagon and insulin antibody have all been proposed as candidates for the role. However, since hormonal antagonism to insulin seems centered in the anterior lobe of the pituitary gland, it is not surprising that growth hormone emerges as the strongest endocrine candidate of opponent of insulin action. However, Levine and Luft [72] presented arguments against the concept that excessive secretion of growth hormone is

responsible for for the pathogenesis of diabetes mellitus in man. Also, the various evidence against this hypothesis have already been discussed in the introduction. It would be superfluous to discuss this topic further.

In 1970, Davidson and Poffenbarger[42] proposed a set of conditions which a substance must fulfil if it is to be accepted as a diabetes inducing substance and as having a possible role in the pathogenesis of diabetes mellities in man. It is presented here in a modified and expanded form.

1. The plasma concentration of the antagonist should be at a higher level in diabetics than in non-diabetics.
2. The action of the antagonist should be consistent with the qualitative and quantitative metabolic alterations found in diabetes.
3. The antagonist should be capable of decreasing sensitivity to endogenous and exogenous insulin in vivo.
4. Glucose intolerance and insulin insensitivity should be reversed if the antagonist can either

be neutralized in vivo or its source removed (assuming that B-cell function is not irreversibly altered).

5. The vascular complications of diabetes should be lessened or reversed after the antagonist is neutralized, or its activity reduced by removal of its source or other means.
6. Chronic administration of the antagonist into intact animals should produce all the complications of diabetes.

No agent discovered so far has yet been able to satisfy all these conditions. The urinary peptide isolated by Louis and Conn[57] appears to be qualified but still handicapped by lack of an accurate method for its measurement. Consequently, its concentration in blood of diabetics and normal people is not known. However, the fact that it is excreted in the urine of diabetics but not in non-diabetics is the strongest argument that it is somehow involved in diabetes. Further evidence to support this view is its ability to induce hyperglycemia and hyperinsulinemia in normal dogs, a condition similar to that of maturity-onset

diabetic patients during glucose tolerance test. The peptide is also capable of counteracting the hypoglycemic action of exogenous insulin in vitro and in vivo. Conn and Louis [57] reported some years ago that in one diabetic patient the peptide disappeared from the urine after hypophysectomy. Subsequently, the insulin requirement of this patient was greatly reduced. This strongly suggests that its site of origin is the pituitary gland and more importantly is that the insensitivity to insulin has been reversed. This seems to indicate that in some diabetics at least, the primary fault is excessive secretion of the antagonist, presumably in amounts too great to be overcome by insulin. There is still much work to be done to establish this peptide as a diabetes inducing agent and to clarify its possible role in the pathogenesis of diabetes mellitus in man. At present, the diabetogenic effect of the purified peptide has not been demonstrated because of insufficient material. However, its electrophoretic behavior has been shown to be very similar to the active peptide isolated from human pituitary glands.

CONCLUSION

The existence of a diabetogenic peptide in the urine of proteinuric diabetic patients is now firmly established. Over 90 % of proteinuric diabetics so far studied excreted this substance. It is not an abnormal peptide characteristic of diabetes since the compound is similar in its electrophoretic pattern, isoelectric point, molecular weight and column chromatography elution profile to that of the peptide isolated from human pituitary glands. Also, the peptide from both sources is capable of inducing hyperglycemia and hyperinsulinemia during glucose tolerance test in dogs. This indicates that site of origin of the peptide is the pituitary gland. Since the peptide is found only in the urine of proteinuric diabetics but not in proteinuric non-diabetics, this strongly suggests that it is somehow involved in the disease of diabetes mellitus. The phenomena of hyperglycemia and hyperinsulinemia during glucose tolerance test of some maturity-onset diabetes patients can now be explained by the probability of an excessive secretion of the diabetogenic peptide by the pituitary gland. Of importance is that the peptide isolated

from proteinuric diabetics has now been purified and partially characterized.

BIBLIOGRAPHY

1. Dobson, M., In Experiments & Observations on the Urine in a Diabetes, Medical Observer and Inquirer, 5:298 (1776).
2. Cullen, W., In The First Lines of the Practice of Physician, Edinburgh (1778).
3. Langerhans, P., In Beitrage zur Mikroskopischen Anatomie die Bauchspeicheldruse, Inaugural Dissertation, Berlin, G. Lange (1869).
4. von Mering, J., and Minkowske, O., Arch. Exper. Path. u. Pharmakologie, 26:371 (1890).
5. Opie, E. L., J. Exp. Med., 5:527 (1901).
6. Ssobelow, L., Virchow Arch., 168:91 (1902).
7. McCallum, W. G., Bull. John Hopkins Hosp., 20:265 (1909).
8. Banting, F. G., and Best, C. H., J. Lab. Clin. Med., 7:251 (1922).
9. Johns, W. S., O'Mulvenny, T. O., Potts, E. G., and Laughton, N. B., Amer. J. Physiol., 80:100 (1927).
10. Houssay, B. A., Endocrinol., 30:884 (1942).

11. Young, F. G., Lancet, 2:372 (1937).

12. Falta, W., In Die Zuckerkrankheit, Urban and Schwarzenberg, Berlin (1936).

13. Himsworth, H. P., Lancet, 1:127 (1936).

14. Ricketts, H. T., Brunschwig, A., and Knowlton, K., Proc. Soc. Exp. Biol. N. Y., 68:254 (1945).

15. Lawrence, R. D., Lancet, 2:401 (1949).

16. Cotes, P. M., Reid, E. and Young, F. G., Nature, 164:209 (1949).

17. Houssay, B. A., and Anderson, E., Endocrinol., 45: 627 (1949).

18. Ikkos, D., and Luft, R., Lancet, 11:897 (1960).

19. Ikkos, D., Luft, R., Gemzell, C. A., and Almqvist, S., Acta Endocrinol., (Kbh) 39:547 (1962).

20. Russell, J. A., Amer. J. Clin. Nutr., 5:404 (1957).

21. Molnar, G. D., Ackerman, E., Rosewear, J. W., Gatewood, L. C., and Moxness, K. E., Mayo Clinic Proc., 43:833 (1968).

22. Tchobroutsky, G., Assan, R., Rosselin, G., and Derot, M., Ann. Endocrinol., (Paris) 27:766 (1966).

23. Burday, S. Z., Fine, P. H., and Schalch, D. S., J.

Lab. Clin. Med., 71:897 (1968).

24. Knopf. R. F., Fajans, S. S., Floyd, J. C., Pek, S., and Conn, J. W., Diabetes, 21:322 (1972).

25. Merimee, T. J., Fineberg, S. E., and Hollander, W., Diabetes, 22:813 (1973).

26. Luft, R., and Guillemin, R., Diabetes, 23:783 (1974).

27. Bornstein, J., Armstrong, J. McD., and Jones, M. D., Biochim. Biophys. Acta, 156:38 (1968).

28. Bornstein, J., Armstrong, J. McD., Gould, M. K., Harcourt, J. A., and Jones, M. D., Biochim. Biophys. Acta, 192:265 (1969).

29. Bornstein, J., Taylor, W. M., Marshall, L. B., Armstrong, J. McD., and Gould, M. K., Biochim. Biophys. Acta, 192:271 (1969).

30. Lewis, U. J., Singh, R. N. P., Lindsey, T. T., Seavey, B. K., and Lambert, T. H., In Adv. in Human Growth Hormone Research, Raiti, S. (ed.) DHEW Publ. No. NIH 74-612, U. S. Gov't Print. Office (Publ.)

31. Blum. F., Deut. Arch. Klin. Med., 71:146 (1901).

32. Grollman, A., In The Adrenals, The Williams and

Wilkins Co., Baltimore, (1936).

33. Long, C. N. H., and Lukens, F. D. W., J. Exp. Med., 63:465 (1936).

34. Lazarus, S. S., and Bencosme, S. A., Proc. Soc. Exp. Biol. Med., 89:114 (1955).

35. Conn, J. W., Louis, L. H., and Wheeler, C. E., J. Lab. Clin. Med., 33:651 (1948).

36. Conn, J. W., Louis, L. H., and Johnson, M. W., Proc. Amer. Diabetes Assoc., 8:3 (1948).

37. Vallance-Owen, J., Dennes, E., and Campbell, P. N., Lancet, 2:336 (1958).

38. Vallance-Owen, J., Diabetes, 13:241 (1964).

39. Vallance-Owen, J., Ann. N. Y. Acad. Sci., 131:315 (1968).

40. Einsinck, J. W., Coombs, G. J., Williams, R. H., and Vallance-Owen, J., J. Biol. Chem., 239:3377 (1964).

41. Einsinck, J. W., Mahler, R. J., and Vallance-Owen. J., Biochem. J., 94:150 (1965).

42. Davidson, M. B., and Poffenbarger, P. L., Metabolism, 19:668 (1970).

43. Meek, J. C., Doffing, K. M., and Bolinger, R. E.,

Diabetes, 17:61 (1968).

44. Varandani, P. T., Diabetes, 17:547 (1968).

45. Mirsky A., quoted in Berson, S. A., and Yalow, R. S., Diabetes vol. 14, p568 (1965).

46. Louis, L. H., Minick, M. C., and Conn, J. W., J. Lab. and Clin. Med., (Abstract), 56:924 (1960).

47. Hipolito-Reis, C., Sobrinko-Simoes, M., Ferraz, A. JR., Harguaves, M. P., and Cerqueira-Magro, F., Rev. Franc. Endocr. Clin., 9:373 (1968).

48. Taton, J., Malezewski, B., and Wisniewska, A., Diabetologia, 8:315 (1972).

49. Okuyama, M., Gasuda, K., Demura, R., Torikai, T., and Challoner, D. R., Clin. Res., 20:634 (1973).

50. Wilkinson, J. S., and Bonar, J. R., Diabetes, 23:385 (suppl. 1) (1974).

51. Louis, L. H., Conn, J. W., and Minick, M. C., Diabetes, 14:445 (1965).

52. Louis, L. H., Conn, J. W., and Minick, M. C., Metabolism, 15:308 (1966).

53. Louis, L. H., and Conn, J. W., J. Lab. Clin. Med., 70:1016 (1967).

54. Louis. L. H., and Conn, J. W., Metabolism, 17:475 (1968).

55. Louis, L. H., and Conn, J. W., Metabolism, 21:1 (1972).

56. Louis, L. H., Conn, J. W., and Minick, M. C., Metabolism, 12:867 (1963).

57. Louis, L. H., and Conn, J. W., Metabolism, 18:556 (1969).

58. Louis, L. H., Metabolism, 18:545 (1969).

59. Somoygi, J., J. Biol. Chem., 195:19 (1952).

60. Campbell, J., and Rastogi, K. S., Diabetes, 15:749 (1966).

61. Lowry, O. H., Rosebrough, J., Farr, A., and Randall, R., J. Biol, Chem., 193:265 (1951).

62. Davis, B. J., Ann. N. Y. Acad. Sci., 121:404 (1964).

63. Weber, K., and Osborn, M., J. Biol. Chem. 244:4406 (1969).

64. Lehnhardt, W. F., and Winzler, R. J., J. Chromatography, 34:471 (1968).

65. Berson, S. A., and Yalow, R. S., Daibetes, 14:549 (1965)

66. Li, C. H., and Papkoff, H., J. Biol. Chem., 204:391 (1953).

67. Reid, E., J. Endocrinol., 9:210 (1953).

68. Perley, M., and Kipnis, D. M., Diabetes, 15:867 (1966).

69. Sabeh, G., Corredor, D. G., Mendelsohn, L. V., Morgan, C. R., Sieracke, J. C., Sunder, J. H., Wingert, J. P., and Danowske, T. S., Metabolism, 18:741 (1969).

70. Shen, S. W., Reaven, G. M., and Farquhar, J. W., J. Clin. Invest., 49:2151 (1970).

71. Gordon, E. S., Metabolism, 11:819 (1962).

72. Levine, R., and Luft, R., Diabetes, 13:651 (1964).