FURTHER STUDIES ON THE ABNORMAL INSULIN OF DIABETES MELLITUS

by

Dennis James Shapcott

F.I.M.L.T., Institute of Medical Laboratory Technology, 1953

A Thesis submitted to the Faculty of the Graduate School of the University of Colorado in partial fulfillment of the requirements for the Degree

Master of Science

Department of Pediatrics

1966

This Thesis for the M.S. degree by

Dennis James Shapcott

has been approved for the

Department of

Pediatrics

by

C. H. Kempe

Donough O'Brien

Date May 8, 1966

Shapcott, Dennis James (M.S., Pediatrics)

Further Studies on the Abnormal Insulin of Diabetes Mellitus

Thesis directed by Professor Donough O'Brien

Insulin was extracted from pancreases obtained at autopsy from non-diabetic subjects (normal insulin) and from adult-onset diabetic subjects (diabetic insulin). Two pancreases were extracted with I M acetic acid, and the insulin containing fraction of the fluid portion of the extract was separated using Sephadex G 50. This fraction was lyophillised, then incubated with anti-insulin serum (guinea pig) and the insulin-anti-insulin complex separated using Sephadex G 50. Insulin was then dissociated from this complex using I M acetic acid, and separated from other protein with Sephadex G 50. This process yields only substances reacting with anti-insulin serum.

Other pancreases were extracted with a mixture containing ethanol, water and hydrochloric acid at pH $<$ 1.5. The fluid portion of this extract was neutralised with ammonia and centrifuged. Insulin was precipitated from the supernate on addition of ethanol and diethyl ether. Catecholamines and cortico-steroids were not precipitated.

A mixture of uniformly labelled ^{14}C glucose (2 µc) together with normal or diabetic insulin in a total volume of 2 ml was injected intraperitoneally into each of 10 male Wistar rats. The mixture contained 800-1100 µ units of insulin per 2 ml, as determined by immunoassay. After 2 hours the rats were killed and their diaphragms removed. The diaphragmatic glycogen was isolated by digestion with hot 30% potassium hydroxide, followed by precipitation with 66%

ethanol. The ^{14}C activity of this glycogen was used as the measure of glucose incorporation into glycogen.

Highly significant differences were found between the level of glucose in corporation following diabetic insulins and normal insulins.

This abstract of about 250 words is approved as to form and content. I recommend its publication.

Signed Roshough S Bonar
Instructor in charge of dissertation

TABLE OF CONTENTS

SECTION PAGE

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION.

By the beginning of the twentieth century experimental evidence had been gathered demonstrating that a disease state similar to human diabetes was produced by pancreatectomy in laboratory animals. This led to the proposal that an internal secretion of the pancreas was necessary for carbohydrate metabolism, and the name "insuline" was given to this substance by De Meyer in I909.

The successful isolation of this substance by Banting and Best together with the description of its' clinical effects appeared to establish diabetes mellitus as due to a deficiency of insulin.

To confirm such a hypothesis it was necessary to demonstrate quantitatively a difference in the circulating levels of insulin between normal and diabetic subjects.

It was early early recognised that insulin was a protein, and would be present in the blood in such low concentration that it could not be measured by chemical techniques (I).

Development of techniques for measuring insulin.

To measure the comparatively high concentrations of insulin used in therapeutic preparations, biological assays were introduced. These utilize the fall in blood sugar or the induction of hypoglycemic coma in laboratory animals, and while satisfactory for this purpose are much too crude to determine insulin levels in biological fluids where the insulin level may be one millionth of that obtained in a therapeutic preparation.

From observations that adrenalectomised animals were more sensitive to insulin, Hemmingsen (1938) attempted to measure blood insulin by the induction of coma in such animals (2). He found values of less than 0.002 unit/ml of blood.

Gellhorn (1941) introduced a much more sensitive preparation in hypophysectomised-adrenodemedullated mice (3). By measuring the fall of blood sugar following the injection of 1 ml of blood, he found levels of about 200 microunits/ml in normal subjects. With this technique he was able to detect gross alterations in blood insulin level.

These techniques using whole animals are impractical in that numerous animals are required to obtain statistically significant results and their preparation often entails considerable mortality.

Insulin bio-assay using rat tissue in vitro

Rat hemidiaphragms will continue to take up glucose from an incubating medium for several hours after removal from the animal.

Groen (1951) showed that such a preparation is sensitive to insulin, and could be used for measuring blood insulin (4). He reported that no insulin effect is obtained when blood from depancreatectomised animals, or from human diabetics in coma is used.

Although this assay, or refinements of it, is widely used, it is subject to criticism in that dilution of plasma results in a greater response than the original plasma (5). Pituitary hormones (oxytocin, vasopressin) also increase the rate of glucose uptake in this preparation.

Martin (1958) introduced the isolated epididymal fat pad assay where the fat pads are incubated with glucose containing ^{14}C at the 1 carbon position. The $^{14}CO_2$ produced is a measure of the rate of glucose oxidation and is increased by insulin (6). Amounts of insulin as low as 10 microunits/ml will give a measurable increase in $^{14}CO_2$ production.

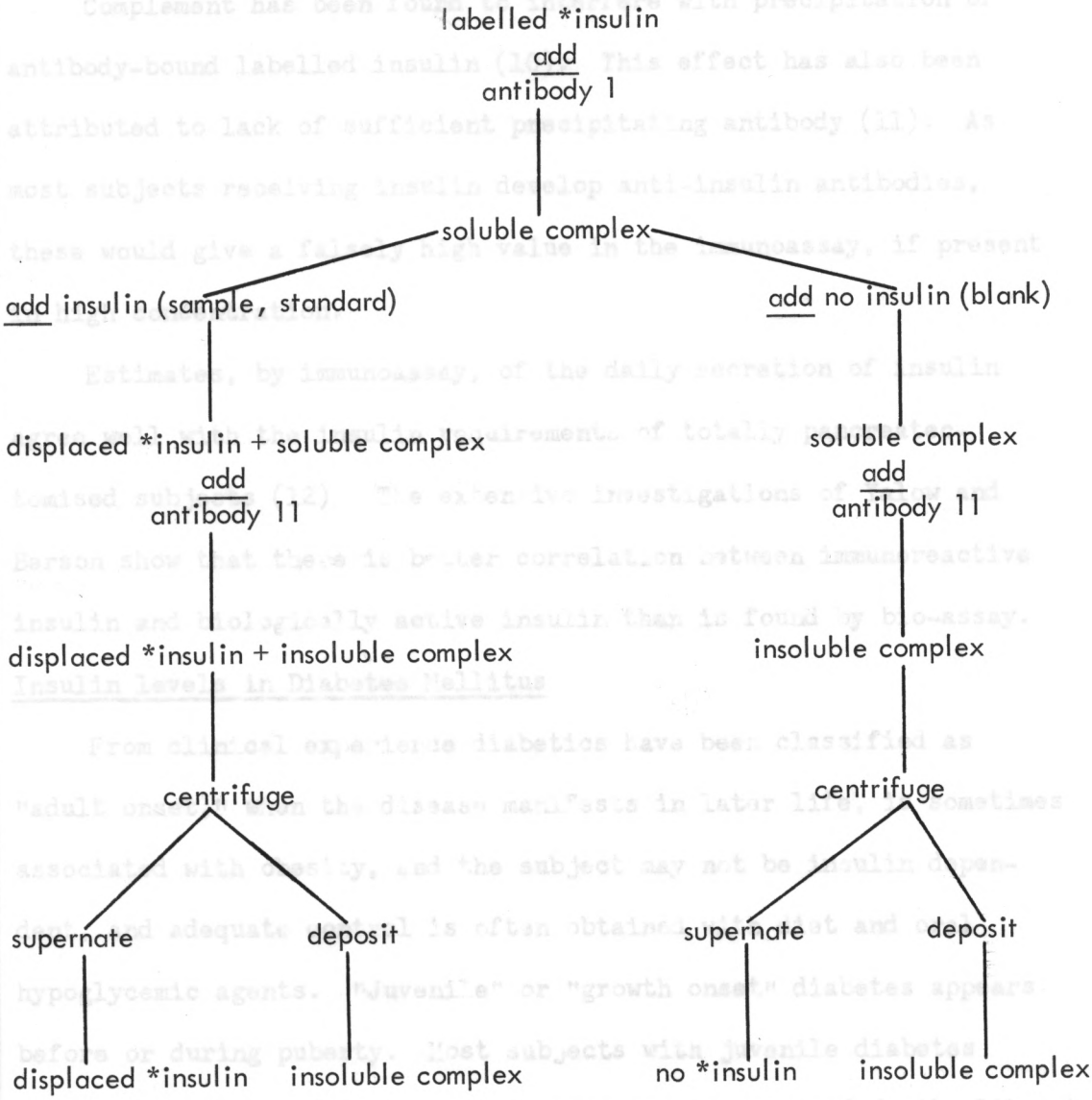
From the discordant values obtained for plasma insulin in normal subjects using these assays, it appears that they do not measure the same substance, and therefore comparisons of values between different investigators using different assay techniques is not valid (7).

Immunoassay of Insulin

The most recently introduced technique is the immunoassay (Fig. 1). Here, insulin displaces isotopically labelled insulin from a complex with anti-insulin serum. The amount of labelled insulin displaced is a measure of the unlabelled insulin added. The amount of bound labelled insulin remaining was measured in the original technique by chromatoelectrophoresis on paper (8). Subsequently a second antigen-antibody system was introduced with the use of an antiguinea pig globulin which precipitates the insulin bound to anti-insulin (9). The assumption inherent in this technique is that only substances reacting with anti-insulin antibody will be detected. This antibody is prepared by the immunization of guinea pigs with insulin extracted from pancreas. It is possible that not all forms of circulating endogenous insulin will react with this antibody.

Furthermore, apparently high plasma insulin levels determined on undiluted plasma may show a sharp fall in calculated potency on

FIGURE 1



SIMPLIFIED SCHEME FOR THE DOUBLE ANTIBODY IMMUNOASSAY

dilution. This effect can be overcome by the use of diluted plasma in the assay.

Complement has been found to interfere with precipitation of antibody-bound labelled insulin (10). This effect has also been attributed to lack of sufficient precipitating antibody (11). As most subjects receiving insulin develop anti-insulin antibodies, these would give a falsely high value in the immunoassay, if present in high concentration.

Estimates, by immunoassay, of the daily secretion of insulin agree well with the insulin requirements of totally pancreatectomised subjects (12). The extensive investigations of Yalow and Berson show that there is better correlation between immunoreactive insulin and biologically active insulin than is found by bio-assay.

Insulin levels in Diabetes Mellitus

From clinical experience diabetics have been classified as "adult onset," when the disease manifests in later life, is sometimes associated with obesity, and the subject may not be insulin dependent, and adequate control is often obtained with diet and oral hypoglycemic agents. "Juvenile" or "growth onset" diabetes appears before or during puberty. Most subjects with juvenile diabetes require insulin, and if untreated, progress to coma and death although milder cases similar to the adult onset disease occur.

The necessity for insulin parallels the severity of the clinical condition, so it would be expected that, if diabetes is due to deficiency of insulin, plasma insulin levels would correspond to insulin therapy requirements.

Berson, studying a group of normal subjects and adult onset diabetes found no difference between the two groups as measured by

immunoassay(12). Vallance-Owen, using the rat hemidiaphragm technique, found insulin in the plasma of adult onset dibetes, but none in a group of juvenile diabetics (13).

Prolonged fasting in normal humans results in the absence of plasma insulin as determined by immunoassay, with an abrupt secretion into the plasma to above normal levels following glucose administration (14), (15).

To accomodate these and other findings, several theories have been promulgated, and those germane to this work will now be briefly reviewed.

(1) "Synalbumin"

Vallance-Owen observed that plasma from juvenile diabetics, in addition to a lack of stimulatory effect upon glucose uptake by the rat diaphragm, inhibits the effect of added endogenous insulin (16). Under similar conditions plasma from normal subjects is without such inhibitory action.

When plasma from juvenile diabetics is subjected to protein fractionation, the inhibitory effect was associated with the albumin moiety. As (a) the inhibition does not result in values lower than those found in untreated diaphragms, and (b) the addition of greater amounts of insulin overcomes the inhibition, Vallance-Owen assumes that the inhibitory effect is a competition for binding sites between insulin and the inhibitor. This inhibitor was found in plasma from normal subjects, but at lesser concentrations.

He has further demonstrated that this inhibitor--"Synalbumin"--can be seperated from albumin. From it's physicochemical and biological characteristics he concludes that Synalbumin is the isolated B chain of insulin (17). Synalbumin is decreased from the

plasma of hypophysectomised and adrenalectomised animals. Using the rat epididymal fat pad technique, Meade was unable to demonstrate an inhibitory action with albumin from severe diabetics (18). Also, there has been no report of an antagonistic effect of Synalbumin or B chain when given in vivo.

"Bound" insulin

Antoniades has reported a series of findings on the nature of circulating insulin. By passage of the serum over a cation exchange resin, a portion of the serum insulin ("bound" insulin) is retained on the resin, the rest ("free" insulin) is unabsorbed (19). This bound insulin has a high molecular weight, while free insulin appears to correspond to crystalline insulin, or insulin prepared by acid-ethanol extraction of pancreas. Bound insulin is converted to free insulin by treatment with an extract of adipose tissue, or by extraction with acid-ethanol. Free insulin stimulates both the rat diaphragm and epididymal fat pad, while bound insulin acts upon the fat pad only (20. Bound insulin is not inactivated by exposure to anti-insulin sera, and migrates electrophoretically with gamma globulins, while the free form has the mobility of albumin or alpha globulin (21). Bound insulin is not measured in the immunoassay.

Berson was unable to demonstrate an increase in immunoassayable insulin in plasma from either adult onset diabetics or normal subjects after the plasma had been treated with adipose tissue extract. (12)

Cell Membrane Permeability

(a) Levine first showed that insulin increased the rate of transport of a non-metabolite into cells, using galactose in eviscerated animals (22). Later he showed a similar effect with glucose (23).

With increased substrate thus made available, it appeared reasonable to attribute the anabolic stimulation by insulin to the increased production of ATP. However, it has since been shown that increased uptake of amino acids by cells in response to insulin is independent of glucose (24). Also, insulin will increase fat synthesis in cells without glucose as a source of energy (25).

(b) Krahl has proposed a mechanism which included all the effects known at that time (26). He considers that the action of insulin is due to interraction between the plasma membrane and insulin, resulting in a highly specific deformation of the membrane, rendering it more permeable.

(c) Rodbell has suggested that the effects of insulin upon isolated fat cells are dependent upon an intact cell membrane (27). Phospholipase C (phosphatidylcholine esterase) and insulin both stimulate glucose transport, fatty acid synthesis and protein synthesis in such preparations. The degree of stimulation is proportional to the enzyme concentration to a maximum level. Insulin effect decreases with increasing enzyme effect. He suggests that insulin and phospholipase C both act upon the same parameter--membrane lipoprotein.

Blecher, using phospholipase A (phosphatidyl acyl hydrolase) demonstrated similar effects upon isolated fat cells (28). He also showed that, as with insulin, these effects of phospholipase A are inhibited by ethylmaleimide--a blocker of sulphydryl groups.

Encyme induction and inhibition

Weber describes a series of experiments concerning the effect of insulin and glucocorticoids upon the enzymes catalysing the four one-way reactions in gluconeogenesis (29). He shows that, in

rats, the activity of these enzymes is increased both by glucocorticoids and the induction of alloxan diabetes. Actinomycin D inhibits this rise in activity. Insulin reduces this activity in both diabetic and glucocorticoid treated rats to control levels.

He further reports that insulin is an inducer of pyruvate kinase, one of the key glycolytic enzymes (30). The level of pyruvate kinase falls sharply in rats made diabetic, returning to normal following insulin. This action of insulin is inhibited by actinomysin and ethionine.

Wool has investigated the stimulation by insulin of protein synthesis (31). Utilizing a preparation of rat heart muscle microsomes he found that insulin did not increase protein synthesis in vitro. However, when insulin was given to diabetic rats 1 hour before death a marked increase was found. He concludes that this action of insulin is consequential to the regulation of translation of messenger RNA.

Abnormal insulin

Stimmler and Elliot estimated the disappearance rate of insulin from a medium incubated with rat hemidiaphragms (32). They found that insulin from diabetics persisted longer in the medium than insulin from nondiabetics or crystalline porcine insulin. At the time this was attributed to an inhibitor, but further evidence suggests that the insulin molecule itself is abnormal (33). Insulin was extracted by a technique which ensures a product reactive only to anti-insulin serum. Insulin, extracted by this technique from human diabetic sera, was more resistant to the action of rat muscle insulinase than insulin similarly extracted from normal sera,

or porcine insulin. This suggests an alteration in the insulin molecule per se--an abnormal insulin.

This investigation was undertaken to extract insulin from pancreases of normal subjects and diabetics. The biological activity would be determined by measuring the increase in incorporation of labelled glucose into diaphragmatic glycogen in the intact rat. Significant differences in biological activity between normal and diabetic insulin would indicate that the diabetic insulin was different biologically, probably because of a different chemical structure.

EXPERIMENTAL

Except where otherwise indicated, all solutions are prepared using distilled water subsequently de-ionised by passage over Amberlite M.B.-3 resin. All chemicals are of "A.R." or "Reagent" quality.

APPARATUS AND MATERIALS

Borate Buffer: 0.1M boric acid-sodium hydroxide pH 8.5.

Phosphate Buffer: 0.1M sodium dihydrogen phosphate-disodium hydrogen phosphate pH 7.3.

Bovine Serum Albumin: 30% solution is diluted to 5% albumin with phosphate buffer (phosphate BSA) or borate buffer (borate-BSA).

^{125}I labelled Insulin: Diluted for use with borate-BSA to contain 5 μ units insulin in 50 μl. When newly received this amount gives 2.5-3.0 x 10^3 c.p.m. ^{125}I. Replaced after one half life.

Normal Guinea Pig Serum: Sterile filtered serum. Diluted for use in immunoassay 1:130 with borate-BSA (N.G.P.S.).

Anti-Insulin Serum--Guinea Pig: Adult guinea pigs, randomly selected, were injected subcutaneously at 3 weekly intervals with P.Z.I. insulin, starting with 5 units, increasing to 25.

To reduce mortality from hypoglycemia, 4 ml of 50% dextrose was given intraperitoneal concurrently. Two weeks after third injection, guinea pigs were bled by cardiac puncture.

Insulin injections were continued at three weekly intervals, bleeding two weeks after each injection.

Rabbit Anti-Guinea Pig Serum (RAGPS): Adult rabbits received normal guinea pig serum intravenously at two weekly intervals starting with 2 ml, subsequently 1 ml.

After the third injection they were bled by cardiac punture. Injections were continued at two weekly intervals, bleeding one week after each injection.

Glucose--U--^{14}C: Specific activity greater than 2 mc/mM. This ensured that no more than a tracer dose of glucose was received by the test animals. All solutions for injection with ^{14}C glucose were prepared from the freshly dissolved solid.

Liquid Scintillation Fluid (L.S.F.): 500 ml toluene
300 ml ethanol
500 ml dioxane
104 g naphthalene
130 mg 1.4 bis (5 phenoxazolyl) benzene
7.5 g 2:5 diphenyloxazole.

Optical Density: Measurements were made with a Beckman D.U. spectrophotometer with #220 Gilford Absorbance Indicator. Quartz cells with 10 mm light path used throughout.

pH: Leeds and Northrup #7401 direct reading pH meter with micro-glass electrode.

^{125}I activity: #314 E AutoGamma counter.

^{14}C and ^{3}H activity: #3000 Tri-Carb liquid scintillation counter.

Centrifuges: (a) International SBVI--used in cold room at 4°.
(b) Sorval RC2 refrigerated high speed centrifuge.

Syringe Microburettes: were used to dispense 50 µl and 100 µl aliquots of reagents in the immunoassay.

METHODS

Insulin Immunoassay (modified from Morgan and Lazarow) (9)

(a) Standardization of reagents.

(1) A.I.G.P. Guinea pig antiserum was diluted serially from 1:250 to 1:5000 with borate--BSA.

Aliquots (50 µl) of these dilutions were added to tubes containing 0.9 ml borate--BSA. Each dilution was set up in triplicate. ^{125}I insulin--5 µ units in 50 µl of borate--BSA was added to each tube, mixed, and stored at 4° for 18-24 hours.

RAGP (50-100 µl as indicated) was added, mixed, then 50 µl NGPS added, mixed, and stored at 4° for 18-24 hours. Tubes were centrifuged at 3000 rpm for 15 minutes (SBVI). The supernates were decanted, and the walls of the tubes washed down with 0.5 ml of 1% bovine albumin in borate buffer.

Re-centrifuge and decant supernates.

Measure ^{125}I activity of precipitates.

The dilution of AIGP which complexed 60-65% of the ^{125}I insulin, as shown by 60-65% of the ^{125}I activity added appearing in the deposit, is used in the immunoassay.

RAGP The volume of undiluted serum used in the immunoassay was the smallest which precipitated 50-70% of the radioactivity from 5 µ units of ^{125}I insulin complexed with 50 µl of AIGP and 50 µl of NGPS.

Procedure:

(a) 0.1 ml of serum or each standard solution is pipetted into 0.1 ml of 5% bovine albumin reagent. Each is done in triplicate.

(b) Add 0.05 ml labelled insulin reagent

(c) Add 0.05 ml anti-insulin guinea pig serum reagent

(d) Mix and allow to stand in the cold (4°) for 20-40 hours

(e) Add rabbit anti-guinea pig serum (amount added to be determined as described above under reagents)

(f) Add 0.05 ml normal guinea pig serum and mix

(g) Allow to stand at least 24 hours in the cold. Centrifuge for 15 minutes in the cold

(h) Pour off supernatant fluid into another labelled tube

(i) Wash precipitate with 0.5 ml of 1% bovine albumin reagent, centrifuge for 15 minutes and puur supernatant fluid into the same tube as in step (h)

(j) Count the radioactivity of the precipitate and the supernatant fluid separately.

(k) Calculate the ration of the total count (i.e. precipitate and SNF) to the count on the precipitate alone for each standard and test. Plot this ratio for the standards on linear graph paper. The calibration has been repeatedly shown to be a straight line up to 500/μ u/ml with a coefficient of variation of ± 12%.

(l) The concentration for each test is determined.

Blood glucose determined by glucose oxidase technique (34).

ISOLATION OF PANCREATIC INSULIN

(a) Antibody complexed--gel filtered. Modified from Elliot (33).

Principle. Pancreas is extracted with acid. The liquid extract is gel filtered to separate insulin from larger proteins. The crude insulin thus obtained is complexed with anti-insulin and this complex separated by gel filtration. The complex is dissociated with acid and gel filtered to separate insulin from antibodies.

Technique.

1). The pancreas is weighed, coarsely sliced, and extracted in a Waring blendor for 5 minutes, using 6 ml of I M acetic acid per gram of tissue.

2). The pH of the extract is checked, if necessary adjusted to pH 3.0-3.2 with glacial acetic acid.

3). The mixture is kept overnight at -18°.

4). After thawing, centrifuge at 12000 x g for 30 minutes. (RC2)

5). The intermediate fluid layer below the surface fatty layer is removed and kept. The deposit is re-extracted with I M acetic acid, using the same volume as in the initial extraction.

6). Re-centrifuge at 12000 x g for 30 minutes. Remove the intermediate layer, combine with the other intermediate layer and measure volume.

7). Add G 25 coarse Sephadex to the combined liquids to form a thin slurry.

8). Pour slurry into a Buchner funnel and allow liquid portion to drain and discard.

9). Wash the Sephadex 3 times with I M acetic acid, the

volumes used being each 2/3 the volume of the combined liquid fractions (Stage 6).

10). Suck the Sephadex to dryness, combine all washings and bring to dryness in freeze drier.

11). Redissolve the powder in 50 ml of 0.5 M acetic acid, gel filter in a 3.8 x 50 cm column of G 50 coarse Sephadex, suspended in 0.5 M acetic acid.

12). Elute at 4° with 0.5 M acetic acid, collected in 10 ml fractions at 80-100 ml/hr. Measure the absorbance at 278 mu and plot readings against tube number.

13). Two distinct peaks are found. The contents of the tubes corresponding to the inter-peak space are combined and freeze-dried.

14). The dried material is dissolved in 50 ml of borate buffer and 3 ml of anti-insulin serum added, mixed, and stored at 4° for 48 hrs.

15). Repeat gel filtration at 4° with this mixture, using Sephadex G 50 suspended in borate buffer with borate buffer as eluent.

16). Collect the material in the tubes giving peak at 278 mu, combine and freeze dry.

17). Dissolve the powder in 0.5 M acetic acid, gel filter at 4° using G 50 Sephadex coarse suspended in 0.5 M. acetic acid, with 0.5 M acetic acid as eluent.

18). Combine contents of the ten tubes following the protein peak and freeze dry.

19). Redissolve the powder in phosphate--BSA to ensure that no AIGP has been carried through into the final product, immunoassay is

performed using the product as sample, but without the addition of AIGP.

In the absence of AIGP no more than 1% of the ^{125}I insulin activity is found in the precipitate.

(b) Acid-ethanol Extraction--Best et al (35)

Principle. Pancreas is extracted with acid-ethanol. The fluid portion is brought to neutrality, precipitating proteins--but not insulin. Ethanol and ether are added, and hydrophillic compounds--including insulin--are precipitated.

1). The weighed pancreas is extracted for 5 minutes in a Waring blendor with acid-ethanol-water (15 ml HCl, 750 ml ethanol, 250 ml water) using 6 ml mixture per gram of tissue.

2). Check pH, adjust if necessary to (2.0 keep overnight at 4°.

3). Centrifuge at 6000 x g (RC2) for 30 minutes.

4). Collect supernate, re-extract deposit using the same volume of acid-ethanol-water as in stage (1), recentrifuge and combine supernates.

5). With continuous stirring, bring pH of combined supernates to 7.5 using concentrated ammonia solution.

6). Allow 20 minutes for precipitate to flocculate, then centrifuge at 6000 x g for 15 minutes. Collect supernate and measure.

7). For each 9 ml of supernate, add, with continuous stirring 15 ml of ethanol, then 25 ml of diethyl ether per 9 ml of supernate.

8). Keep overnight at 4°, then gently detach the precipitate from walls of flask, allow to settle, aspirate and discard

supernate. Dry the deposit in a stream of air at room temperature.

9). Dissolve the deposit in 25 ml of 0.5 M acetic acid, bring pH to (3.5 if necessary with glacial acetic acid. Centrifuge at 6000 x g for 10 minutes, collect supernate and freeze dry.

10). Dissolve the powder in phosphate--BSA.

All pancreas extracts were stored at -18°.

THE TEST OF BIOLOGICAL ACTIVITY from Rafaelson (36).

In a series of preliminary experiments designed to ascertain the optimum technique for this assay, the following protocol was established and was rigidly adhered to in all the tests performed. Male Wistor rats, aged three months and weighing 200 ± 5 grams were used unless otherwise noted. In all but the firsttwo experiments they were obtained from Simonsen Laboratories, Minneapolis. The rats are used within three days of receipt, usually the day following arrival, and are allowed laboratory animal chow ad libitum until 16-18 hrs. before test, when food is removed. Water is allowed at all times. To minimise the disturbance of metabolism due to stress, animals are kept overnight in the room in which the experiment is carried out. All tests were performed during the morning. No anaesthetic is used.

Preparation of insulin-glucose injections.

The pancreas extract to be used is diluted with phosphate--BSA to give a concentration of 800-1000 μ units/ml by immunoassay. This solution is assayed and frozen.

Both diabetic and normal pancreas extracts are treated identically. Immediately before the test a solution of ^{14}C glucose in phosphate--BSA is prepared by dissolving the contents of a 50 μc ampoule in 25 ml of phosphate--BSA. Twelve millilitres of ^{14}C glucose solution is mixed with twelve millilitres of the pancreas extract, and aliquots are removed for immunoassay and ^{14}C activity measurement.

Animal inoculation and diaphragm removal.

Using a blunted, smooth tipped #21 needle, two millilitres of insulin--^{14}C glucose solution is injected intraperitoneally, slightly to the left of mid line and about halfway between the

sternum and pubis. Best results are obtained when the needle is directed about 1 cm laterally before penetration of the muscle. Ater two hours the animal is killed with a blow to the head, and the abdomen opened longitudinally.

A careful search is made for evidence of needle perforation of the bowel, if present, results are not included.

The diaphragm in a segment of rib cage is removed and washed with running tap water at 8-10° or in a large volume of iced tap water. It is then placed on an ice cube, and the diaphragm muscle is dissected out, leaving the crura. This muscle is then washed twice in ice cold 0.1% sodium fluoride in 0.9% NaCl solution and blotted dry, then weighed to the nearest milligram on a torsion balance. The second fluoride wash fluid is kept.

The muscle is placed in a tube containing 2 ml of 30% KOH solution at 93°.

The whole procedure from the blow to the head to the placing of the diaphragm in hot KOH takes from 3 to 3½ minutes. The usual bio-assay involves two groups of ten animals, and initially the injections were performed at five-minute intervals, but this may be safely reduced to four minutes.

To ensure that there is no external contamination of the muscle the second wash fluid is assayed for radioactivity, if the ^{14}C content is more than twice background the results would be discarded. This did not happen with the technique described.

<u>The isolation and measurement of ^{14}C glycogen</u>.

(1) The diaphragm tissue is kept in the KOH solution at 93° for one hour, with occasional mixing to disperse the digested protein.

(2) Cool to room temperature, add 0.8 ml of anhydrous sodium sulfate solution, mix, add 6.4 ml of ethanol and mix again.

(3) Cool to -18°, and store at that temperature for one hour.

(4) Centrifuge at 9000 x g for 15 minutes.

(5) Decant supernate, drain over filter paper for 5 minutes.

(6) Wash walls of tube with 2 ml of 66% ethanol, add 3 small glass beads and resuspend the precipitate.

(7) Centrifuge at 9000 x g for 15 minutes.

(8) Decant supernate, drain over filter paper for 5 minutes.

(9) Add 0.5 ml of water, mix thoroughly.

(10) Wash contents of tube into liquid scintillation counting vial, using 3 x 5 ml aliquots of LSF.

(11) Add Cab-o-Sil to the top of vial, shake vigorously, measure ^{14}C activity.

RESULTS

The following control experiments were performed to confirm the validity of the experimental procedures used in the test of biological activity.

1). To confirm that the precipitate washing removed all soluble radioactivity:

Three rats received 2 ml phosphate--BSA containing 2 μc ^{14}C glucose intraperitoneally.

After two hours, the diaphragms were removed and treated as in the glycogen assay,--

A sample of: diaphragm digest after heating,
the first supernate, and finally
the second supernate, were removed and their ^{14}C activity determined.

CPM ^{14}C

	0.1 ml digest	0.5 ml 1st supernate	0.5 ml second supernate
Rat 1	137	83	38
Rat 2	493	72	44
Rat 3	522	86	53

Injected dose = 1,791,000 CPM/ml

Average background = 44 CPM

This washing procedure is adequate for removing soluble radioactivity.

2). While the washing procedure is adequate in removing soluble radioactivity, the possibility that labelled glucose was bound to the precipitate was tested for:

Two rats were killed, diaphragms removed and placed in hot 30% KOH. To each diaphragm was added immediately 0.1 ml of a ^{14}C glucose solution containing about 1 μc in 0.1 ml.

The diaphragms were processed for glycogen.

^{14}C glucose = 1,550,000 CPM/0.1 ml.

Diaphragm	Weight in mg	CPM/diaphragm	% of added ^{14}C
A	435 mg	1548	<0.1
B	387	2130	<0.1

^{14}C glucose is not carried through into the glycogen/L.S.F. mixture.

Liquid scintillation counting may yield falsely high values from chemiluminescence, but as the tissue was digested immediately and the reagents throughout were identical this was not investigated.

Incorrectly low values may be obtained from quenching due to color or opacity.

This was investigated as follows:

Three rats were killed and their diaphragms removed and processed. To each glycogen/L.S.F. mixture was added 0.1 ml of a ^{14}C glucose solution, mixed, and the ^{14}C activity measured immediately after mixing, and after 30 minutes.

Then, to one vial (Rat I) was added enough Cab-o-Sil to fill the vial, and well mixed.

To the second vial (Rat II) was added about twice the amount of Cab-o-Sil, and to the third vial (Rat III) about 3 times the original quantity.

All 3 vials were checked for ^{14}C level after 30 minutes. As control, 15 ml of L.S.F. plus 0.1 ml of the ^{14}C solution were mixed and the activity determined immediately and after 30 minutes.

	^{14}C CPM			% Quench
	Immediate Mixing	After 30 Mins.	After Cab-o-Sil	After Cab-o-Sil
Rat I	35,993	28,642	36,013	2.3
Rat II	36,338	26,491	36,226	1.7
Rat III	36,015	26,997	36,319	1.7
Control	36,699	36,845		

The results obtained show that quenching due to color is negligible, while that due to turbidity can be eliminated with Cab-o-Sil. The quantity of Cab-o-Sil is not critical.

Catecholamine recovery experiment:

10 grams of a normal pancreas were extracted with 60 ml of acid-ethanol-water, and 500 µl of a solution of ^{14}C ring labelled norepinephrine. The mixture was processed and the activity of the final extract determined.

CPM ^{14}C added = 1.836.800

CPM ^{14}C in extract = 47.320

% of label in final extract = 2.5%

When 50 µl aliquots of the ^{14}C norepinephrine solution were chromatographed on paper using a Butanol/acetic acid/water system, from 1-2% of the activity did not travel with unlabelled norepinephrine. Therefore, it may be assumed that less than 1% of the norepinephrine is extracted.

Cortisol-recovery experiment

10 grams of a normal pancreas were extracted with 60 ml of acid/ethanol/water after adding 250 µl of a solution of ring labelled ^{3}H cortisol. The mixture was processed and the activity of the final extract determined.

CPM ^{3}H added = 1.366.650

CPM ^{3}H in extract = 5.670

% of label in final extract = 0.4%

Blood glucose

Blood glucose levels were measured on rats receiving no insulin, and on rats receiving normal or diabetic insulin. Blood was drawn at injection and death.

BLOOD SUGAR

(a) At Injection (b) At Death

(1) Control Rats		(2) Normal Insulin (850 µu)		(3) Diabetic Insulin (800 µu)	
a	b	a	b	a	b
75	93	64	73	59	69
77	94	62	73	62	76
64	71	60	78	65	78
78	96	58	76	62	66
57	60	51	69	68	73
53	66	52	65	58	65
62	76	50	68	51	70
64	70	64	76	56	61
56	84	65	71	61	66
60	73	62	75	62	63
63	69	63	68	58	66
62	67	62	75	59	64
Mean Blood Sugars		a	b	52	64
Control		64	77	63	76
Normal Ins.		59	72	43	56
Diabetic Ins.		59	68		

Mean rise in blood sugar Control 19%

Normal Ins. 21%

Diabetic Ins. 19%

The rise in blood sugar is attributed to handling. No discernable effect on blood glucose from either normal or diabetic insulin.

GLUCAGON IMMUNO-ASSAY OF PANCREATIC EXTRACTS

EXTRACTS WERE DILUTED AS FOR ASSAY.

EXTRACT	GLUCAGON mμg/ml
#2 NORMAL	0
#1 DIABETIC	15
#2 DIABETIC	0
#3 DIABETIC	18
#4 DIABETIC	174
#5 DIABETIC	10

This assay kindly performed by Dr. R. H. Unger.

Normal Insulin Extracts

I). Antibody complexed--Gel filtered insulin

Normal #1 pancreas

Patient was 55 years old, male with no history or family history of diabetes mellitus.

Insulin concentration in final extract = 11,100 μ units/ml in 50 ml.

II). Acid ethanol extracted insulin

Normal #2 pancreas

Patient was 68 years old, male, no history of diabetes mellitus or family history.

Insulin concentration in final extract = 500.000 μ units/ml in 25 ml.

ENDOGENOUS INSULIN ACTIVITY IN RATS

(TABLE I)

These rats received ^{14}C glucose--(2 μc in 2 ml of phosphate--BSA) only.

Rat #	Weight	Weight Diaphragm	Total ^{14}C/ Diaphragm	^{14}C CPM/mg Diaphragm
1	190	535	1665	3.11
2	200 g	542	820	1.51
3		462	202	0.44
4		408	119	0.29
5		457	356	0.78
6		405	1330	3.28
7		393	264	0.67
8		396	86	0.22
9		441	78	0.18
10		495	373	0.75
11		426	443	1.04
12		393	477	1.21
13		523	271	0.52
14		479	1279	2.67

^{14}C in 2 ml injectate = 1,798,000 CPM

$n = 14$

$\bar{x} = 1.11$

$\Sigma \partial^2 = 14.87$

$S = 1.02$

The rats' endogenous insulin converts only a minimal of ^{14}C glucose to glycogen ^{14}C.

NORMAL INSULIN AT 700,1000 and 1500 MICROUNITS/DOSE

(TABLE II)

Three groups of rats taken.

Gp I received approximately 700 μ units normal human insulin

Gp II " " 1000 " " " "

Gp III " " 1500 " " " "

All received 2 μc ^{14}C glucose.

INSULIN ASSAY	EXTRACT	DOSE INJECTED	^{14}C GLUCOSE IN DOSE
I	695		1.417.660 CPM
II	1030	1120	1.378.830 CPM
III	1455	1310	1.485.960 CPM

Results

1	n	$\bar{x}$	S.D.	Σa^2
I	10	9.198	2.70	72.89
II	10	19.38	6.38	406.81
III	10	20.68	7.27	526.70

S_1 (I&II) = 5.16

S_2 (II&III) = 7.21

t_1 (I&II = 4.41

t_2 (II&III) = 0.276

p_1 (I&II) $<$ 0.001

p_2 (II&III) $<$ 0.80 $>$ 0.70

At levels above 1100 μ units/dose, diaphragm is saturated with insulin.

TABLE II.

I. Diaphragm Weight	^{14}C/ Diaphragm	^{14}C/mg Diaphragm	II. Diaphragm Weight	^{14}C/ Diaphragm	^{14}C/mg Diaphragm
354	2749	7.76	389	12,959	33.31
335	3004	8.97	371	6,838	18.43
339	2818	8.31	375	4,098	10.93
335	3345	9.98	327	8,648	26.45
407	4841	11.89	311	7,440	23.92
327	2131	6.52	401	5,474	13.65
359	5302	14.77	436	6,156	14.12
354	2152	6.08	323	5,512	17.06
395	2424	6.21	353	6,372	18.05
380	4366	11.49	319	5,677	17.90
III					
417	10330	24.77			
443	4689	10.58			
355	9818	27.66			
352	4129	11.73			
355	5542	15.61			
389	7785	20.01			
340	6686	19.66			
366	9032	24.68			
313	10738	34.30			
411	5581	13.58			

NORMAL INSULIN AND NORMAL INSULIN WITH B CHAIN

(TABLE III)

Control rats received 2 ml each of a solution containing normal pancreas insulin and ^{14}C glucose.

Test rats received same mixture but with beef insulin B Chain*. added.

Insulin Assay	(1) Extract	(2) Dose Injected	^{14}C In Dose
Control	830 μ units	1180 μ units	1.365.290 CPM
Test	830 μ units	1200 μ units	1.389.300 CPM

S = 12.25

t = 0.601 - 15 degrees freedom

p = ⟨0.60⟩0.50

The concentration of B chain was 0.064 μg in the 2 ml dose of insulin/glucose mixture. This is equivalent to 1600 microunits of insulin/dose. Immunoassay of B chain solution at this concentration gave a zero value--the B chain did not react with anti-insulin serum.

*Gift of Dr. J. Kirtley, Lilly Laboratories.

TABLE III

	Diaphragm Weight	CPM ^{14}C/ Diaphragm	CPM ^{14}C/mg Diaphragm
Control	337	16558	49.13
n = 9	389	14035	36.08
$\bar{x}$ = 30.79	337	11345	33.66
S = 10.86	347	8911	25.68
	325	9231	28.40
	400	9676	24.19
	394	17011	43.17
	335	7623	22.75
	400	5618	14.04
Test	357	17513	49.05
n = 8	377	15842	42.02
$\bar{x}$ = 34.39	370	9988	26.99
S = 13.60	294	7739	26.32
	333	17100	51.35
	351	6999	19.94
	303	4849	16.00
	345	14994	43.46

BEEF INSULIN AND NORMAL HUMAN INSULIN

(TABLE IV)

Control rats received normal human insulin (#2 normal pancreas).

Test Rats received crystalline beef insulin.*

Insulin Assay	Insulin Injected	^{14}C Injected
Control	940 μ units/2 ml	1,753,480 CPM
Test	1000 μ units/2 ml	1,685,320 CPM

S = 21.43

t = 1.55 (17 degrees freedom)

p = ⟨0.20⟩0.10.

*Gift of Dr. J. Kirtley, Lilly Laboratories

TABLE IV

	Diaphragm Weight	CPM ^{14}C/ Diaphragm	CPM ^{14}C/mg Diaphragm
Control	450	29592	58.2
n = 10	545	25803	44.6
$\bar{x}$ = 66.2	519	29452	58.1
S = 19.0	378	23098	46.8
	523	43620	87.1
	705	45433	81.1
	496	20847	40.4
	571	37461	65.5
	461	35124	81.7
	449	52296	98.7
Test	518	20823	46.3
n = 9	578	20696	37.8
$\bar{x}$ = 50.9	507	31807	61.3
S = 21.6	493	33189	87.8
	501	26036	49.8
	560	26562	37.7
	516	14032	28.3
	572	14065	24.6
	530	38125	84.9

Pancreas Extract #1 (acid ethanol).

(TABLE V)

Patient was 66 years old, male with moderate--Kimmelstiel-Wilson syndrome. Moderate diabetic for years, controlled on diet.

Weight pancreas. 73 grams

Insulin content of final extract = 8500 μ units/ml in 75 ml.

Control pancreas Normal #2

	Mean Insulin/Dose	^{14}C CPM/Dose
Control	990 μ units/ml	1650640
Diabetic	920 μ units/ml	1810350

S = 20.18

t = 4.64 (13 degrees freedom)

p = <0.001

TABLE V

	Diaphragm Weight	CPM ^{14}C/ Diaphragm	CPM ^{14}C/mg Diaphragm
Control	648	32871	50.7
n = 8	561	16601	29.6
$\bar{x}$ = 58.4	518	15255	29.5
S = 25.1	591	24934	49.8
	528	21008	39.8
	455	40231	88.4
	548	52023	94.9
	605	51078	84.4
Test	479	10881	22.7
n = 7	441	4822	10.9
$\bar{x}$ = 10.5	490	2427	4.9
S = 6.0	535	7641	14.2
	475	3647	7.7
	367	6329	9.9
	617	2129	3.4

Pancreas extract #2 (acid ethanol).

(TABLE VI)

Patient was 43 years old, male, diabetes mellitus 8 years, controlled with low doses of insulin twice daily.

Weight of pancreas. 35 grams

Insulin content of final extract - 15.500 μ units/ml in 50 ml.

Control pancreas Normal #2

	Mean Insulin/Dose	^{14}C CPM/Dose
Control	890 μ u.	1908130
Diabetic	1065 μ u.	1847560

S = 10.02

t = 3.72 (17 degrees freedom)

p = <0.01

TABLE VI

	Diaphragm Weight	CPM ^{14}C/ Diaphragm	CPM ^{14}C/mg Diaphragm
Control	463	21198	45.8
n = 9	488	8069	16.5
$\bar{x}$ = 31.6	520	12888	24.8
S = 12.1	538	22237	41.3
	486	25935	53.4
	520	8458	16.2
	558	14771	26.4
	558	17832	32.0
	612	16993	27.5
Diabetic	507	3640	7.2
n = 10	543	12402	23.0
$\bar{x}$ = 14.5	590	6001	10.2
S = 6.2	538	14504	27.0
	483	4528	9.4
	605	9232	15.2
	544	9368	17.2
	604	6487	10.8
	526	4417	8.4
	604	10143	16.8

Diabetic Pancreas #3 (acid ethanol).

(TABLE VII)

Patient was 77 years old male. Diabetes Mellitus 20 years.

Weight of pancreas. 50 grams

Insulin concentration in final extract = 500.000 μ units/ml in 50 ml extract.

Control pancreas. Normal #2

	Mean Insulin/Dose	^{14}C CPM/Dose
Control	990 μ units	1.873.990
Diabetic	1025 μ units	1.931.980

S = 3.94

t = 3.40 (15 degrees freedom)

p =<0.01

TABLE VII

	Diaphragm Weight	CPM ^{14}C/ Diaphragm	CPM ^{14}C/mg Diaphragm
Control	429	9985	23.3
n = 8	563	6727	11.9
$\bar{x}$ = 12.04	445	6108	13.7
S = 4.7	486	4938	10.2
	516	6311	12.2
	619	4487	7.2
	559	5446	9.7
	543	4423	8.1
Diabetic	587	1332	2.3
n = 9	522	5654	10.9
$\bar{x}$ = 5.53	588	3757	6.4
S = 2.6	564	2773	4.9
	512	1029	2.0
	492	3357	6.8
	620	2139	3.5
	597	3215	5.4
	531	4098	7.7

Diabetic Pancreas #4 (acid ethanol)

(TABLE VIII)

Patient was 56 years old, female. Diabetes Mellitus 7 years on low doses of insulin.

Weight of Pancreas = 68 grams

Insulin concentration in final extract = 22.750 μ units/ml in 50 ml.

Control pancreas = Normal #2

	Mean Insulin/Dose	^{14}C CPM/Dose
Control	770 μ units	1381440
Diabetic	840 μ units	1413460

S = 8.54

t = 4.97

p = <0.001

TABLE VIII

	Diaphragm Weight	CPM ^{14}C/ Diaphragm	CPM ^{14}C/mg Diaphragm
Control	401	16098	40.14
n = 10	399	6807	17.06
$\bar{x}$ = 26.65	373	9198	24.65
S = 10.71	370	5374	14.52
	336	13008	38.71
	381	6073	15.93
	374	5205	13.91
	293	10049	34.29
	400	12923	34.30
	385	13488	35.03
Diabetic	317	788	2.49
n = 8	358	1877	5.24
$\bar{x}$ = 6.51	375	4345	11.58
S = 3.36	339	692	2.04
	351	3423	9.75
	342	2525	7.38
	340	1804	5.30
	378	3158	8.35

Diabetic Pancreas #5 (acid ethanol).

(TABLE IX)

Patient was 62 years old, male. Ten years history of Diabetes Mellitus, treated initially with insulin, subsequently with tolbutamide.

Weight of Pancreas = 94 grams

Insulin content of final extract = 69000 μ units/ml in 50 ml extract.

Control pancreas = Normal #2

	Mean Insulin/Dose	^{14}C CPM/Dose
Control	890 μ units	1647800
Diabetic	1150 μ units	1623100

S = 8.36

t = 2.944 (17 degrees freedom)

p = <0.01

TABLE IX

	Diaphragm Weight	CPM ^{14}C/ Diaphragm	CPM ^{14}C/mg Diaphragm
Control	545	12484	22.9
n = 10	523	11635	22.2
$\bar{x}$ = 21.93	540	3127	5.8
S = 8.8	466	13113	28.1
	550	5002	9.1
	531	10998	20.7
	444	14336	32.3
	444	14662	33.0
	508	11860	23.3
Test	423	6911	16.3
n = 9	423	1255	3.0
$\bar{x}$ = 9.96	470	5647	12.1
S = 6.22	526	2164	4.1
	518	8885	17.15
	570	7096	12.4
	494	8869	18.0
	541	2960	5.5
	450	476	1.1

Diabetic Pancreas extract #1a (antibody complexed--gel filtered)

(TABLE X a+b)

Patient was 55 years old, female, diabetes 7 years, initially on insulin subsequently on orinase.

Weight of Pancreas = 82 grams

Insulin content of final extract = 950 μ units/ml in 50 ml.

Control pancreas = #1 Normal

Four groups of rats were inoculated on four different days. The CPM ^{14}C/mg diaphragm were corrected to a dose of 1.700.000/2 ml to avoid bias.

Insulin Level/Dose (μ units)		^{14}C CPM/Dose	
Diabetic	Normal	Diabetic	Normal
990	1076	1767040	1747600
1080	1180	1847010	1789750
1050	1140	1725680	1667250
1152	1086	1623810	1529170

S = 18.92

t = 3.33 (48 degrees freedom)

p = <0.01

TABLE X a

	Diaphragm Weight	CPM ^{14}C/ Diaphragm	Corrected CPM ^{14}C/mg Diaphragm
Control	485	16190	32.48
n = 25	454	40227	86.22
$\bar{x}$ = 42.17	489	39051	73.82
S = 21.9	452	21325	45.76
	473	12905	25.91
	466	8590	17.48
	429	9032	19.99
	470	20281	44.01
	420	26926	65.39
	417	20325	49.72
	416	31132	76.33
	433	29199	68.78
	403	33854	85.68
	453	20325	45.76
	384	16815	44.66
	443	8553	21.43
	467	13339	31.70
	421	9880	26.05
	463	11489	27.54
	456	14254	26.98
	434	9462	24.20
	449	14809	36.61
	403	15448	42.55
	471	8724	20.56
	409	5794	15.72

TABLE X b

	Diaphragm Weight	CPM ^{14}C/ Diaphragm	Corrected CPM ^{14}C/mg Diaphragm
Diabetic	497	10319	20.02
n = 25	589	20064	32.85
$\bar{x}$ = 24.35	498	10787	20.89
S = 15.3	414	20965	48.83
	487	14205	28.13
	458	2336	4.68
	450	6366	12.98
	514	2687	4.80
	492	6957	13.93
	516	24824	47.40
	479	17181	35.34
	412	9692	23.18
	440	25185	56.39
	497	25813	57.80
	462	14303	30.50
	489	4453	9.53
	418	5273	13.21
	402	5891	15.34
	436	6578	15.80
	512	2041	4.17
	326	5944	19.21
	423	13383	33.12
	389	7372	19.84
	403	8033	20.87
	519	9984	20.14

DISCUSSION

(a) Validity of experimental procedures

Glycogen extraction

Essentially this is the precipitation of glycogen with 66% ethanol. Incubation of rat diaphragms with ^{14}C labelled glucose yields ^{14}C activity in lactate, glucose, alanine, maltose, carbon dioxide and glucose oligo saccharides (37). Only glycogen and the higher oligo saccharides are insoluble in 66% ethanol.

The hydrolysis of labelled "glycogen" derived by this technique, with subsequent formation of glucose phenylhydrazone yields specific activities comparable to the theoretical (36).

The control experiments confirm that the glycogen ^{14}C activity is not due to glucose ^{14}C either from contamination by the injected material or from glucose ^{14}C in the muscle itself. The experiments on quenching show that the ^{14}C activity measured is a true representation of the ^{14}C activity present.

It is assumed, therefore, that this is a valid technique for the measurement of ^{14}C glucose incorporation into glycogen.

The test of biological activity

Stadie has shown that rat diaphragms will take up labelled insulin in vitro and that this activity cannot be removed by washing. When exposed to glucose 90 minutes after exposure to insulin and subsequent washing such diaphragms will incorporate glucose at an increased rate (38).

Gamatt, in work confirming this, shows that there is a continuous uptake of ^{131}I insulin by isolated rat diaphragms; the rate of glucose uptake increasing with rise in insulin absorption.

This uptake of glucose is not linear, and at a diaphragm insulin level of about 120 u units/100 mg tissue there is no further increase in glucose uptake (39).

When insulin is injected intraperitoneally into rats, it is without effect on tissues save those in direct contact with the peritoneal cavity (36). This is shown by the differing patterns of labelled glucose uptake following intravenous or intraperitoneal injection together with a tracer dose of ^{14}C glucose. The insulin levels in the bio-assay fell between 800 and 1200 units per rat. If about 50% of this was absorbed by the diaphragm there would be about 80-120 u units insulin/100 mg tissue for most of the animals, the level at which diaphragm is saturated in vitro. The control experiment using insulin at three different levels tends to confirm this by showing no increase in glucose uptake with an insulin dose of 1100 u units.

The test of biological activity is thus shown to be a sensitive indicator of insulin activity.

The pancreas extractions

Gel filtration, antibody complexed. With this treatment, insulin is freed from any combination with protein by acetic acid and gel filtration. It is then complexed with anti-insulin antibody. This complex will contain antibody, insulin and substances cross reacting with the antibody. The possibility that isolated B chains could be carried through with insulin was rejected by the demonstration that B chains are unreactive with the antiserum used.

This final extract contains only immunologically pure insulin.

Acid-ethanol extraction

The initial extraction removes insulin from attachment to protein. Neutralisation of the acid ethanol extract precipitates protein, including gamma globulins. The final extract was crude, so it was necessary to determine if substances which would interfere in the test of biological activity or the immunoassay were present.

As shown by the control experiments, catecholamines and glucocorticoids, if present, are not precipitated by the addition of ether to the neutral acid-ethanol extract and so are not present in the final extract.

Acid alcohol extraction of pancreas results in quantitative yield of glucagon (40). As the B chain of insulin is also a polypeptide of approximately the same molecular weight it will also be recovered. As was shown in table II, B chain did not compete with insulin in the test of biological activity.

Glucagon per se does not directly stimulate or inhibit the incorporation of glucose into muscle glycogen. Glucagon absorbed into the blood stream from the peritoneal cavity will stimulate pancreatic insulin release, thereby increasing the rate of muscle glycogen formation.

Immunoassay for glucagon performed on the acid-ethanol pancreatic extracts showed that the glucagon content of the normal pancreas extract was no greater than that of the diabetic pancreas extracts.

The increased rate of glycogen formation with the normal extract cannot, therefore, be attributed to glucagon.

Trypsin and chymotrypsin have been shown to have an insulin--like effect on the rat diaphragm in stimulating glycogen formation (41). Furthermore, chymotrypsin shows an insulin--like effect in the immunoassay, presumably by causing partial hydrolysis of the labelled insulin (42). Both of these enzymes are precipitated when the acid-ethanol extract is brought to neutrality, and so are not present in the final product.

The biological activity of insulin extracted from a non-diabetic pancreas did not differ significantly in its biological activity of crystalline beef insulin. This was accepted as proof that the insulin itself had not been altered by the process of extraction.

A possible explanation for the difference in biological activity observed between normal and diabetic pancreases is that it is due to an artifact in the immunoassay. This would imply that something in the diabetic extract influences the immunoassay to a greater degree than does a normal pancreas extract. While this cannot be excluded, it is difficult to conceive the mechanism for such an effect, which has not been reported elsewhere.

All the diabetic pancreas extracts tested showed a highly significant difference in biological activity compared with a normal pancreas extract. This difference is assumed to reflect a change in the insulin molecule per se, perhaps leading to an altered state of aggregation. Some possible changes in the molecule which could account for the decreased biological activity will be briefly discussed.

(b) The Abnormal Insulin Molecule

It is well established that human insulin is a protein comprising two polypeptide chains joined by disulphide bonds.

An abnormal insulin must therefore differ in its' amino acid composition by substitution or transposition of one or more amino acids.

Primary amino acid sequence

(1) Carboxy-terminal asparagine. Although 29 of the 51 amino acids of the insulins so far described have at least two alternatives; with one possible exception all insulins so far reported have asparagine as the A chain carboxy-terminal amino acid (43). In the Lidley-Rollett model of the insulin molecule, an important role in the tertiary structure of insulin is attributed to this amino acid,--coupling to the arginine in position 22 of the B chain (44).

Several investigators have shown that insulin may exist as more than one isomeric form.

(a) Crystalline pork, beef and sheep insulins of full biological potency were subjected to countercurrent distribution (45). All showed a major component and several minor components. In each insulin two materials of equal biological activity were obtained--termed A and B--with the B component having one less amide group.

(b) Crystalline beef insulin when chromatographed on DEAE cellulose with elution at varying pH also showed two components (46).

Fraction I crystallised with zinc salts, and had full biological activity, while fraction II did not crystatallise with

zinc salts and had about 50% of biological activity. These two fractions had different optical rotation, migrated differently in electrophoretic field but recombined at pH 8.5.

Fraction I possessed a higher positive charge.

(c) Two fractions, I and II were obtained when crystalline insulin (beef and pork) was subjected to either countercurrent distribution or partition chromatography (47). Fraction I had six free amino groups, while fraction II had five. The authors suggest that these components are the same as the A and B fractions of Craig and Harfenist (45).

As Craig and Harfenist did not give details of their bio-assay procedure it is difficult to account for the discrepancy between their findings and those of Volini and Mitz (46).

Loss of asparagine from the A carboxy-terminal position results in loss of biological activity.

(a) Beef insulin from which the carboxy-terminal amino acids have been removed has forty percent of the biological activity of unaltered insulin by the mouse convulsion assay (48). Loss of alanine only was without effect.

(b) Similar treatment yielded a product which had less than ten percent of biological activity by rat diaphragm and rabbit blood sugar techniques (49). Loss of alanine alone was without effect, but loss of asparagine alone gave the above findings.

Coupling of most of the free amino groups of insulin resulted in varying losses of activity (50). However, there is no indication as to which amino groups were blocked.

Although insulin from human diabetic pancreas was found to have the same quantitative amino acid composition as normal human

pancreas insulin, it must be remembered that acid hydrolysis followed by column chromatography will not distinguish between asparagine and aspartic acid (51).

More refined analysis of the abnormal insulin molecule may therefore show that asparagine is missing from the carboxy-terminal of the A chain, possibly substituted by aspartic acid.

(2) Substitution in the B chain

The greatly increased requirement for insulin in diabetic subjects with insulin resistance due to antibody formation may be reduced by the substitution of insulin from a different species. Thus, in clinical experience, the substitution of pork for beef insulin has this desired effect (52). As the difference between these insulins lies in their A chains, it may be assumed that the A chain is involved in antigenic activity.

This has been confirmed by the hybridisation of cod A chain with beef B chain and beef A chain with cod B chain. The former reacted only weakly with guinea pig anti-beef serum, but the latter reacted as strongly as pure beef insulin (53).

As the abnormal human insulin reacts with antisera, this suggests the possibility of an altered B chain with intact A chain.

(a) Incubation of insulin with trypsin results firstly in the loss of the carboxy-terminal amino acid of the B chain and subsequently loss of the adjacent seven amino acids as a group by hydrolysis of the B22-B23 peptide bond (49). The larger product, desheptapeptide insulin (DHA) was found to have about 15% of the biological activity of the original material, while the heptapeptide fragment was inactive.

(b) Other workers have been unable to confirm these findings with regard to biological activity (54,55). They found DHA to be biologically inactive using (as did the previous author) the rabbit-blood sugar assay. In addition, it was observed that the rate of release of heptapeptide followed the same kinetics as the loss of biological activity.

(c) DHA reacts fully with antisera prepared against the appropriate intact insulin (56).

From this evidence, the abnormality may be associated with the B23-B30 portion of the insulin molecule.

(3) Amino terminal glycine

Glycine is present as the amino terminal amino acid of the A chain in all insulins examined. Its' positive charge appears to be neutralised by the free carboxyl group on A4 (aspartic or glutamic acid), thus maintaining the structure of the molecule. Substitution of glutamine or asparagine at the A4 position presumably would distort the molecule.

While no evidence of such alteration exists, the effect of removal of the initial amino-terminal sequences of the A and B chains has been studied (57).

Insulin containing zinc resists the action of leucine amino-peptidase, while zinc free insulin is hydrolysed, with the probable loss of B1-B7 and A1-A6. Insulin treated in this manner lost 56% of its' activity as judged by mouse convulsion assay.

(4) Interchain leucine bonds

In the Lindley-Rollett structure, attachment between the side chains of the leucine molecules at A16 and B15 and A13 and B11 by

hydrophobic bonds is a major contribution to the tertiary structure.

Alteration of the structure could occur by substitution of these molecules by other amino acids with more polar side chains. Determination of the amino acid sequence of abnormal insulin will reveal such an abnormality.

The State of Association of Insulin

While insulin exists as the monomer (6000 M.W.) in strongly acid solution and at alkaline pH, the dimer is found at weakly acid and neutral pH (58,59). The dimer is the smallest form which contains zinc. It seems probable that insulin, in the physiological state exists as the dimer or possibly tetramer; but the techniques available for extraction of insulin do not allow confirmation of its' state of polymerisation.

The attachments between the insulin molecules in the dimer are most logically through the B10, B10 histidine side chains and the B13 B13 glutamic acid side chains with a zinc atom centrally coordinated.

Two further points of attachment are B17 B16 leucine hydrophobic bonds and a B21 B1 salt link between the B21 glutamic free carboxyl and the B1 terminal amino group.

Obviously there are several points where an alteration insufficient to radically distort the structure of a monomeric insulin molecule could prevent its' association into a dimer.

In this model of a dimer, the only significant possibility of interaction between the A chains is by Van der Waals forces acting between tyrosine residues A14 A14 (44,60).

Coupling of more than half the hydroxyphenyl groups results in marked biological inactivation, so interaction between the A chains may be an important factor in maintaining the insulin dimer as the biologically active form (50).

(c) The Heredity of An Abnormal Insulin

The data which have been presented lend themselves to two interpretations.

(1) That all the insulin extracted from the diabetic pancreases is abnormal insulin, or,

(2) That the abnormal insulin, is, in fact, a mixture of normal insulin and abnormal insulin with little or no biological activity.

If (1) is correct, it indicates that the diabetes of adult onset represents a homozygous condition. This is in line with the hypothesis advocated by Steinberg that diabetes results from the homozygous inheritance of a recessive gene of variable expression (61). While this hypothesis is the most in accord with family studies, it is difficult to reconcile with the differing clinical pictures of juvenile and adult onset diabetes.

If (2) is correct, then this indicates that diabetes is inherited in a recessive manner, but that the adult diabetic is the heterozygous state, the juvenile diabetic being homozygous. (32,33) Assuming that plasma insulin level is a true indication of the pancreatic insulin production, the values which have been obtained on immunoassay of plasma from diabetics tends to support this hypothesis. Immunoassayable insulin is present at normal or elevated levels in asymptomatic juvenile diabetics and in diabetics of adult onset; but is present in low values only in the plasma of clinical juvenile diabetics. This is in contrast to the insulin levels obtained by bio-assay reporting low values in adult diabetics and complete absence of insulin in juvenile diabetics. The difference between the assays is a reflection of the reduced biological activity of abnormal insulin.

It is then logical to postulate that the juvenile diabetic in the pre-clinical stage is producing sufficient abnormal insulin to maintain metabolic homeostasis, and becomes diabetic when insulin production falls behind metabolic requirements.

Indirect evidence to support this was obtained when two pancreases from juvenile diabetics were processed for insulin. One was extracted by the acid-ethanol technique, the other by the antibody complex--gel filtration. In each case the insulin content of the entire final extract was less than 5000 microunits.

CONCLUSION

While the evidence presented here for the existance of an abnormal insulin molecule in diabetes mellitus must be regarded as presumptive only, the considerable practical and theoretical importance of this concept will fully justify an investigation for the abnormality in the molecular structure of diabetic insulin.

REFERENCES

1. Dale, H.H., The physiology of insulin, Lancet, I, 989 (1922).

2. Hemmingsen, A.M., Nielsen, A., and Nielsen, A.L., The effect of insulin on adrenalectomised rats and mice, Acta. Med. Scand. 90, 105 (1938).

3. Gelhorn, E., Feldman, J., and Allen, A., Assay of insulin on hypophysectomised, adrenodemedullated and hypophysectomised-adrenodemedullated rats, Endocrinology, 29, 137 (1941).

4. Groen, J., Kamminga, C.E., Willebrands, A.F., and Buckman, J.R., Evidence for the presence of insulin in blood serum. A method for an approximate determination of the insulin content of blood, J. Clin. Invest., 31, 97 (1952).

5. Willebrands, A.F., Van der Geld, H., and Groen, J., Determination of insulin using the isolated rat diaphragm, the effect of serum dilution, Diabetes, 7, 119 (1958).

6. Martin, D.B., Renold, A.E., and Dagenais, Y.M., An assay for insulin-like activity using rat adipose tissue, Lancet, 2, 76 (1958).

7. Berson, S.A., and Yalow, R.S., Insulin in blood and insulin antibodies, Am. J. Med., 40, 676 (1966).

8. Yalow, R.S., and Berson, S.A., The assay of plasma insulin in human subjects by immunoassay, Nature, 184, 1648 (1959).

9. Morgan, C.R., and Lazarow, A., Immunoassay of insulin using a two antibody system, Proc. Soc. Exp. Med. (N.Y.), 110, 29 (1962).

10. Morgan, C.R., and Lazarow, A., Further studies on an inhibitor of the two antibody immunoassay system, Diabetes, 13, 579 (1964).

11. Kuzuya, T., and Samols, E., The plasma insulin "inhibitor" and immunoprecipitation assay, Metabolism, 13, 493 (1964).

12. Berson, S.A., and Yalow, R.S., Plasma insulin in health and disease, Am. J. Med., 31, 874 (1961).

13. Vallance-Owen, J., Hurlock, B., and Please, N.W., Plasma insulin activity in diabetes mellitus, measured by the rat diaphragm technique, Lancet, 2, 583 (1955).

14. Yalow, R.S., and Berson, S.A., Dynamics of insulin secretion in hypoglycemia, Diabetes, 14, 341 (1965).

15. Yalow, R.S. and Berson, S.A., Immunoassay of endogenous plasma insulin in man, J. Clin. Invest., 39, 1157 (1960).

16. Vallance-Owen, J.E., Dennes, E., and Campbell, P.N., Insulin antagonism in the plasma of diabetic patients and normal subjects, Lancet, 2, 336 (1958).

17. Vallance-Owen, J., Insulin antagonists, in "On the nature and treatment of diabetes", Leibel, B.B., and Wrenshall, G.A., (eds.) Exerpta Medica Foundation, New York, (1965).

18. Meade, R.C., Stiglitz, R.A., and Kleist, T.J., The state of pancreatic and serum insulin, Diabetes, 14, 387, (1965).

19. Antoniades, H.N., Renold, A.E., Dagenais, Y.M., and Steinke, J., Preliminary observations on the state of insulin in human and bovine pancreas, Proc. Soc. Exp. Biol. Med., (N.Y.), 103, 677 (1960).

20. Antoniades, H.N., and Gunderson, K., Studies on the state of insulin in blood, Endocrinology, 68, 36 (1961).

21. Antoniades, H.N., Huber, A.M., Boshell, B.R., Saravis, C.A., and Gershoff, S.N., Studies on the state of insulin in blood: properties of circulating "free" and "bound" insulin, Endocrinology, 76, 709 (1965).

22. Levine, R., Goldstein, M.S., Klein, S.P., and Huddleston, B., The action of insulin on the distribution of galactose in eviscerated, nephrectomised dogs, J. Biol. Chem., 179, 985 (1949).

23. Levine, R., The action of insulin at the cell membrane, Am. J. Med, 40, 691, (1966).

24. Manchester, K.L., and Young, F.G., The effect of insulin on the incorporation of amino acids into the protein of normal rat diaphragm, Biochem. J., 70, 373 (1958).

25. Adachi, K., Inoue, H., Tanioka, H., and Takeda, Y., The biochemistry of isolated animal cells, III, Effect of insulin on lipid synthesis in dispersed rat liver cells, J. Biochem., (Tokyo), 58, 68 (1965).

26. Krahl, M.E., The action of insulin on cells, Academic Press, New York, (1961).

27. Rodbell, M., The similar effects of phospholipase C (Cl. perfringens toxin) and of insulin on glucose and amino acid metabolism, J. Biol. Chem., 241, 130 (1966).

28. Blecher, M., On the mechanism of action of phospholipase A and insulin on glucose entry into free adipose cells, Biochem. and Biophys, Res. Comm., 23, 68 (1966).

29. Weber, G., Singhal, R.L., and Srivastava, S.K., Insulin: suppressor of biosynthesis of hepatic gluconeogenic enzymes, Proc. Nat. Acad. Sci., 53, 96 (1965).

30. Weber, G., Stamm, N.B., and Fisher, E.A., Insulin: inducer of pyruvate kinase, Science, 149, 67 (July 1965).

31. Wool, I.G., Insulin and the regulation of protein biosynthesis in-"Mechanism of hormone action," P. Karlson ed., Academic Press, New York, (1964).

32. Stimmler, L., and Elliot, R.B., Inheritance of a diabetic serum factor inhibiting normal utilisation of insulin, Lancet, I, 956 (1964).

33. Elliot, R.B., O'Brien, D., and Roy, C.C., An abnormal insulin in juvenile diabetes mellitus, Diabetes, 14, 780 (1965).

34. O'Brien, D., and Ibbott, F.A., Laboratory manual of pediatric micro and ultra micro biochemical techniques, p. 134, 3rd. ed., Harper and Row, New York, (1962).

35. Best, C.H., Haist, R.E., and Ridout, J.H., Diet and the insulin content of pancreas, J. Physiology, 97, 107 (1940).

36. Rafelson, O., Lauris, V., and Renold, E., Localised intraperitoneal action of insulin on rat diaphragm and epididymal adipose tissue in vivo, Diabetes, 14, 19 (1965).

37. Chain, E.B., Recent studies on carbohydrate metabolism, Brit. Med. J. 5154 (1959).

38. Stadie, W.E., Haugaard, N., and Vaughn, M., The quantitative relation between insulin and it's biological activity, J. Biol. Chem., 200, 745 (1953).

39. Garratt, C.J., Cameron, J.S., and Menziger, G., The association of 131-insulin with rat diaphragm muscle and it's effect on glucose uptake, Bioch. Biophys. Acta., 115, 179 (1966).

40. Unger, R.H., personal communication.

41. Rieser, P., and Rieser, C.H., Anabolic response of diaphragm muscle to insulin and other pancreatic proteins, Proc. Soc. Exp. Biol. Med., (N.Y.), 116, 669 (1964).

42. Pruitt, K.M., Boshell, B.R., and Kreisberg, R.A., Effect of pancreatic proteins on insulin assay systems. Diabetes, 15, 342 (1966).

43. Smith, L.F., Species variation in the amino acid sequence of insulin, Am. J. Med., 40, 662 (1966).

44. Lindley, H., and Rollett, J.S., An investigation of insulin structure by model building techniques, Bioch. and Biophys. Acta., 18, 183 (1955).

45. Harfenist, E.J. and Craig, L.C., Countercurrent distribution studies with insulin, J. Am. Chem. Soc., 74, 3083 (1952).

46. Volini, M. and Mitz, M.A., The fractionation of insulin on diethylaminoethyl cellulose, J. Am. Chem. Soc., 82, 5572 (1960).

47. Carpenter, F.H. and Chrambach, A., On the amide content of insulin fractions isolated by partition column chromatography and countercurrent distribution, J. Biol. Chem., 237, 404 (1962).

48. Harris, J.E. and Choh, H.L., The biological activity of enzymatic digests of insulin, J. Am. Chem. Soc., 74, 2945 (1952).

49. Nicol, D.H.S.W., The biological activity of pure peptides obtained by the enzymic hydrolysis of insulin, Bioch. J., 75, 395 (1960).

50. Fraenkel-Conrat, J. and Fraenkel-Conrat, H., The essential groups of insulin, Biochem. and Biophys. Acta, 5, 89 (1950).

51. Mirsky, I.A., Personal communication.

52. Berson, S.A. and Yalow, R.S., Species specificity of human anti-beef, pork insulin serum, J. Clin. Invest., 38, 2017 (1959).

53. Wilson, S., Dixon G.H., and Wardlaw, A.C., Resynthesis of cod insulin from it's polypeptide chains and preparation of cod-ox "hybrid" insulins, Biochem. et Biophys. Acta, 62, 382 (1962).

54. Carpenter, F.H. and Baum, W.E., Rates of production of alanine and heptapeptide and loss of biological activity during the digestion of insulin with trypsin, J. Biol. Chem., 237, 409 (1962).

55. Young, J.D. and Carpenter, F.H., Isolation and Characterization of products formed by the action of trypsin on insulin, J. Biol. Chem., 236, 743 (1961).

56. Yalow, R.S. and Berson, S.A., Immunologic aspects of insulin, Am. J. Med., 31, 882 (1961).

57. Smith, E.L., Hill, R.L. and Borman, A., Activity of insulin degraded by leucine aminopeptidase, Biochem. et Biophys. Acta, 29, 207 (1958).

58. Harfenist, E.J. and Craig, L.C., The molecular weight of insulin, J. Am. Chem. Soc., 74, 3087 (1952).

59. Marcker, K., Association of zinc free insulin, Acta Chem. Scand., 14, 194 (1960).

60. Laskowski, M. Jr., Leach, S.J., and Scheraga, H.A., Tyrosyl hydrogen bonds in insulin, J. Am. Chem. Soc., 82, 571 (1960).

61. Steinberg, A.G., "Genetics and Diabetes" in On the nature and treatment of diabetes, Leibel, E.S. and Wrenshall, G.A. eds., Exerpta Medica Foundation, (1965).