Use of Enzyme Proteolysis for the Immunochemical Measurement of Insulin

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SUMMARY

An immunochemical assay has been designed that utilizes the catalytic properties of glutathione-activated ficin to separate antibody-bound radioinsulin from unbound insulin I-131. Both the recovery of human insulin added to serum and the failure to detect chemically inactivated insulin provide evidence for the precision and specificity of the procedure. Measurements of endogenous insulin in serum (diluted 1:10) made after the administration of glucose or tolbutamide revealed rapid increases in circulating levels of hormone in nondiabetic subjects. The successful application of enzyme hydrolysis as the basis of a reliable and rapid immunoassay for insulin suggests that with the appropriate antibody and labeled antigen, the same principle could be employed to measure concentrations of other hormones or substances that are potentially antigenic. DIABETES *16*:656-63, September, 1967.

Most immunoreactive procedures currently used to measure the insulin content of serum differ from one another primarily in the means by which antibody-bound insulin I-131 is separated from the unbound radiohormone. Hydrodynamic flow chromatoelectrophoresis, one of the first practicable technics introduced,[1] was followed by a variety of others,[2-8] all of which have been applied successfully but require either time-consuming operations or specialized materials. For these reasons, an assay was conceived, based on the principle of enzyme hydrolysis, which eliminated the need for extended periods of equilibration, and utilized readily available materials.

Eventually a procedure was devised in which destruction of the free insulin I-131 by a proteolytic enzyme resulted in the distinct separation of the antibody-bound radiohormone.[9] Since then, modifications and refinements of the original method have resulted in an immunoassay system that can measure small amounts of insulin with precision, in a minimum of time and with standard laboratory equipment.

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PRINCIPLE

The concept of immunoassay by enzyme hydrolysis originated from the observation that insulin I-131 associated with antibody was protected against the destructive action of insulinase, whereas unbound or free radioinsulin was degraded.[10] Since the products of degradation of insulin I-131 are soluble in trichloroacetic acid solution, the antibody-bound hormone can be separated by precipitation and then measured.

Under appropriate conditions, the quantity of insulin I-131 bound by antibody will depend on the concentration of stable hormone; thus, by establishing a consistent relationship between small increments of stable insulin and the fraction of precipitated radioinsulin, it should be possible to measure unknown quantities of insulin.

Originally, homogenates of liver and kidney were used to degrade the insulin I-131, but because of their lack of stability search was made for an enzyme system with a predictable course of action. Subsequently, ficin, a mixture of proteolytic enzymes obtained from the latex extract of some fig trees, was found to be ideal. With more experience it became apparent, however, that the properties of the crude material changed during storage, resulting in an appreciable loss of potency. Measures taken to prevent or to correct the alteration were unsuccessful until cysteine or reduced glutathione was combined with the ficin. Although either chemical was capable of restoring full activity to the enzyme, the destructive effect of cysteine on insulin precluded its use.

Repeated trials at varying concentrations have shown that a 0.001 molar solution of reduced glutathione with 1 mg. ficin per milliliter provides optimal results. Although there is the possibility that some lots of ficin may require different amounts of reduced glutathione, this as yet has not been the case.

MATERIALS

1. Tris (hydroxymethyl) aminomethane-maleate (tris-maleate buffer), *p*H 7.8, prepared in the following fashion:

Stock Solution "A"—24.2 gm. of tris (hydroxymethyl) aminomethane plus 23.2 gm. of maleic acid (or 19.6 gm. of maleic anhydride) dissolved in distilled water to make 1 L.

Stock Solution "B"—8 gm. of sodium hydroxide dissolved in distilled water to make 1 L. (0.2 molar solution).

Fifty milliliters of "A" plus 63.5 ml. of "B" when diluted to 200 ml. with distilled water results in tris-maleate buffer, *p*H 7.8.

2. Tris-maleate-gelatin (TMG 2.5 per cent) (Gelatin U.S.P. [powder] obtained from J. T. Baker Chemical Co., Phillipsburg, New Jersey)—2.5 gm. were added to 100 ml. of tris-maleate buffer and heated gently over an open gas flame until the solution was clear and straw colored. This may be prepared several hours in advance of the assay and allowed to cool.

3. Tris-maleate-gelatin (TMG 1 per cent)—TMG 2.5 per cent was diluted with tris-maleate-buffer, *p*H 7.8.

4. Reduced glutathione (Nutritional Biochemicals Corporation, Cleveland, Ohio).

5. Ficin (Nutritional Biochemicals Corporation).

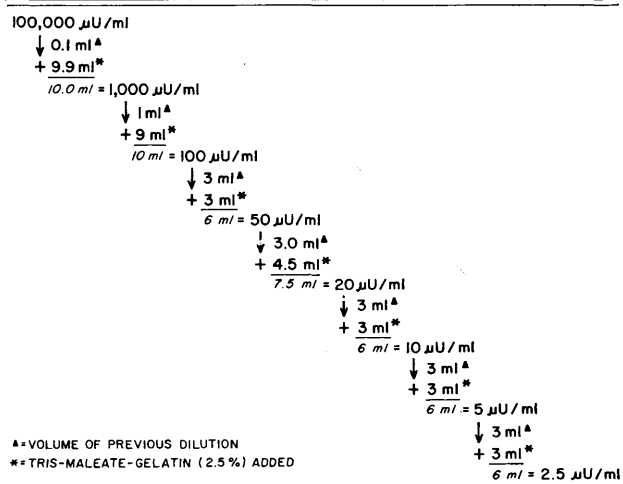
6. Ficin-glutathione solution—Reduced glutathione in a ratio of 3 mg. per 10 ml. of distilled water was stirred in a glass beaker with a magnetic bar for two to three minutes. Ficin, in a concentration of 1 mg. per milliliter of solution, was then added and the stirring continued for thirty minutes. At the end of this time, although some insoluble material can still be seen, the ficin-glutathione solution is ready to be used without filtration or centrifugation. Because of the possibility of change in the properties of the solution, it has been customary to allow no more than ninety minutes to elapse between the time of preparation of ficin-glutathione and its addition to the incubated reaction mixture.

7. Human insulin (Eli Lilly and Co., Indianapolis, Indiana)—Five ml. of 0.03 N hydrochloric acid were added to an ampoule of 0.5 U. of human insulin which resulted in a concentration of 100,000 μU. of insulin per milliliter. Individual 1 ml. aliquots were stored in stoppered tubes at 0° C. A single tube served as the source of the stock insulin solution from which further dilutions were made; the dilutions of stable insulin, as done in the assay, are shown in the flow sheet (table 1).

8. Pork insulin (Eli Lilly and Co.)—Crystalline pork insulin was treated in the same manner as the human insulin.

TABLE 1

Flow sheet for the dilution of human insulin standards



9. Radioinsulin-Pork insulin I-131, of intermediate specific activity (20 to 50 mc. per milligram), was purchased from Abbott Laboratories. One-tenth (0.1 ml.) was removed from the ampoule, diluted to 10 ml. with tris-maleate-gelatin (TMG-1 per cent) and stored at 0° C. Before each assay an aliquot was taken from the thawed solution and diluted with fresh tris-maleate-gelatin (TMG-1 per cent), producing a final concentration of 100 μU. of radioinsulin per milliliter.

10. Anti-pork insulin serum—Male guinea pigs (350 gm.) received weekly subcutaneous injections of 2.5 to 10 U. of Protamine Zinc pork insulin (80 U., Eli Lilly), for four to twelve weeks. Ten to fourteen days after the final injection, blood was collected by cardiac puncture and allowed to clot at 4° C. for several hours. The serum was removed and stored undiluted at 0° C. until its binding capacity and its ability to discriminate at various dilutions of insulin could be determined. Five per cent bovine serum albumin (Armour) in tris maleate buffer was used to dilute the antiserum. Guinea pig antiserum No. 1 was diluted 1:1,000 and 1 ml. samples were divided among a series of small test tubes which were stored at 0° C. Individual tubes were removed before each assay and the final dilution carried out with 5 per cent bovine serum albumin in tris-maleate buffer. In the experiments described here, the antiserum was found to be satisfactory at final dilutions ranging between 1:8,000 and 1:9,500.

The procedure has been outlined in table 2; details are as follows:

With a self-adjusting micropipette, 0.1 ml. volumes

TABLE 2

Steps in the procedure for measurement of insulin by enzyme hydrolysis

Tube number	1 and 2	3 and 4	5 and 6	7 and 8	9 and 10	11 and 12	13 and 14	15 and 16	17 and 18
Insulin-131-I in TMG (1 per cent) 100 μU./ml.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
1 ml. TMG (2.5 per cent) containing human insulin	0	0	2.5 μU.	5 μU.	10 μU.	20 μU.	50 μU.	—	—
Serum diluted 1:10 in TMG (2.5 per cent)	—	—	—	—	—	—	—	1	1
AIS diluted in 5 per cent TMBSA	*	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
React at room temperature overnight; total radioactivity counted									
Ficin-GSH, in distilled water	1	1	1	1	1	1	1	1	1
Incubate one hour at 37° C.									
†Cold trichloroacetic acid (20 per cent)	2	2	2	2	2	2	2	2	2
Centrifuge at 900 × g for twenty minutes at room temperature; decant supernates									
Count precipitated radioactivity									

*0.2 ml. of BSA (5 per cent) are added to the first two tubes before the trichloroacetic acid.
†Cold sulfosalicylic acid (3 per cent) may be used instead.
Values in boxes represent milliliter per tube, unless otherwise indicated.
TMG = Tris-Maleate-Gelatin, AIS = Anti-Insulin Serum, TMBSA = Tris-Maleate Bovine Serum Albumin, GSH = Reduced Glutathione, BSA = Bovine Serum Albumin.

of diluted pork insulin I-131 (100 μU. per milliliter) are introduced into glass test tubes (13 mm. × 75 mm.). The number of tubes used in an assay should be sufficiently large to allow the standards and the serum samples to be analyzed in duplicate. For the standard curves, 1 ml. volumes of TMG (2.5 per cent) containing 0 (control), 2.5, 5, 10, 20 and 50 μU. of human insulin are added in consecutive order.*

For measurement of endogenous insulin, 1 ml. of 1:10 dilutions of serum (in TMG 2.5 per cent) is added to the tubes that contain radioinsulin but no stable insulin.

Following this, the tubes are shaken briefly by hand or by a mechanical mixer and 0.2 ml. of the diluted antiserum (optimal dilution depends on the antiserum) is transferred into all but the two control tubes (see table 2). Two-tenths (0.2) of a milliliter of 5 per cent bovine serum albumin (in tris-maleate buffer) is added to these two tubes in place of the antiserum to provide an adequate precipitate after the addition of the trichloroacetic acid. With stoppers in place, the tubes are shaken (the contents must be mixed thoroughly) and left overnight (fifteen to twenty hours) at room temperature (21 to 25° C.) to permit equilibration. The next morning, the total radioactivity in the tubes is measured in a well-type scintillation detector and the solution of ficin-glutathione is added.

One milliliter of the ficin-glutathione solution is measured into each test tube, after which great care should be taken to shake the tubes until the contents are thoroughly mixed. Then the tubes are placed in a metabolic incubator and shaken (approximately 100 cpm) for one hour at 37° C. At the completion of the incubation, 2 ml. of cold 20 per cent trichloroacetic acid are added to each tube to stop the hydrolytic action of the enzyme and to precipitate the proteins. The tubes are centrifuged at 2,000 rpm for twenty to thirty minutes at room temperature and the clear supernates are decanted carefully and discarded. The radioactivity in the precipitates is measured by "counting" the tubes in the detector and the fraction of precipitated radioactivity calculated in the following manner:

$$\text{precipitated radioactivity (per cent)} = \frac{\text{radioactivity in precipitate}}{\text{initial total radioactivity}} \times 100$$

The standard curve is obtained by plotting (on square-section graph paper) the percentage of precipitated radioactivity (vertical axis) against the concentration of exogenous insulin (horizontal axis).

*The TMG (2.5 per cent) used in the dilution of the crystalline insulin standard is adjusted with 5 per cent BSA (in tris-mal buffer) to approximate the protein concentration of a 1:10 dilution of serum. This is done by mixing TMG (2.5 per cent) and TMBSA (5 per cent) in a volume ratio of 9:1.

COMMENTS

1. *Ficin-glutathione solution*

The potency of the enzyme solution can be determined by noting the extent to which free insulin I-131 is degraded. It will be recalled that since the control test tubes (Nos. 1 and 2, table 2) contain no antiserum, less than 20 per cent of the radioactivity should be in the trichloroacetic acid precipitate. Failure to obtain this magnitude of hydrolysis can result from any one or all of the following conditions: (a) inadequate mixing of the ficin-glutathione solution with the contents of the tube before incubation; (b) unsatisfactory radioinsulin; (c) insufficient reduced glutathione.

A satisfactory solution of ficin-glutathione, under the conditions outlined here, degrades approximately 85 per cent of unbound insulin I-131 in less than thirty minutes and has an unchanged rate of reaction at stable insulin concentrations up to 80,000 μU. per milliliter.

2. *Guinea pig antiserum*

Anti-insulin serum can be frozen in 5 per cent bovine serum albumin for many months without any appreciable loss of binding potency but should such loss occur, the dilution may be decreased until binding and discrimination are once again satisfactory. For any particular lot of antiserum, the optimal titer is determined by a series of standard curves with varying dilutions of antiserum. In general, the most satisfactory results were obtained with a dilution of antiserum that protected approximately 60 to 70 per cent of the radioinsulin (10 μU.) from enzymatic hydrolysis (tubes 3 and 4, table 2) but allowed adequate discrimination with small amounts of added stable insulin.

Any unused antiserum may be returned to the freezer and pooled with a lot made up for a subsequent assay.

Unsatisfactory standard curve

One of the major sources of difficulty has been traced to the nonhomogenous distribution of the constituents within the test tubes which results in insufficient equilibration or incomplete hydrolysis. Because of the consistency of the gelatin it is essential that the tubes be shaken both before the overnight equilibration and after the addition of the ficin-glutathione.

A sample of serum from a pooled source should be included in each set of determinations in order to recognize any interassay deviation in values and to assess the reproducibility of the procedure.

RESULTS

1. *Replicate variability and reproducibility*

Calculations carried out on the results of 300 determinations (unknowns and standards) made in duplicate during twelve assay views, extending over a period of six months, revealed that the duplicate variability (or coefficient of variation) was 1.35 per cent (S.E.), thus, 95 per cent of the time the true value will be within 2.7 per cent of the sample reading.

The mean value for the concentration of endogenous insulin obtained on replicate samples of pooled serum from five separate assays over a period of three months was 59 ± 5 μU. (S.D.).

2. *Standard curve*

The standard curve characteristically seen with human insulin has been illustrated in figure 1 together with a representative pattern obtained with crystalline pork insulin. Despite the fact that the same antiserum was used (guinea pig No. 1, dilution 1:9,500) for both standard curves, the configurations differ considerably. Regardless of the similarity in chemical structure between human and pork insulin, the two species cannot be used interchangeably unless it can be shown that a particular lot of antiserum reacts identically with both, a point that has been emphasized by others in previous publications.[11,12]

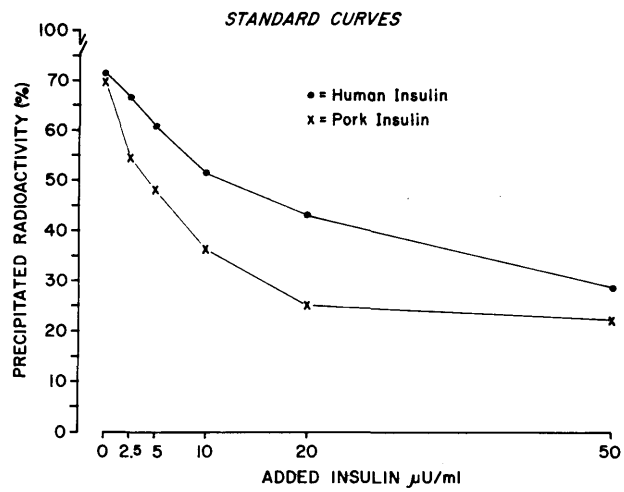


FIG. 1. Representative standard curves obtained with human and with pork insulin. Guinea pig antiserum No. 1, diluted 1:9,500.

It should be understood that the patterns of the standard curves can differ among assays and that the shape of the curve may vary with a particular antiserum.

3. *Effect of pH*

Standard curves obtained with buffers of varying *p*H (figure 2) disclosed that the most satisfactory patterns were obtained at an alkaline *p*H.

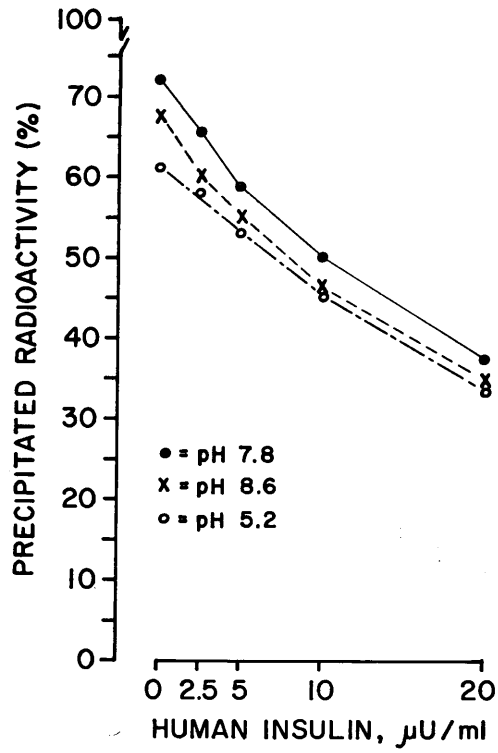


FIG. 2. Effect of pH on the standard curve.

4. *Temperature and time of reaction*

Because of the danger of bacterial contamination and potential damage at room temperature, prolonged periods of reaction were carried out at 4° C. It was apparent from the reduction in binding and the flat standard curve (figure 3) that a time of reaction of eighteen hours at 4° C. under the conditions described is too short. However, extending the period of reaction to forty hours resulted in augmented binding and an enhanced sensitivity. As a matter of practicality and convenience, this implies that the time of reaction can be carried out over a weekend or longer (at 4° C.) with assurance that the assay can be completed satisfactorily.

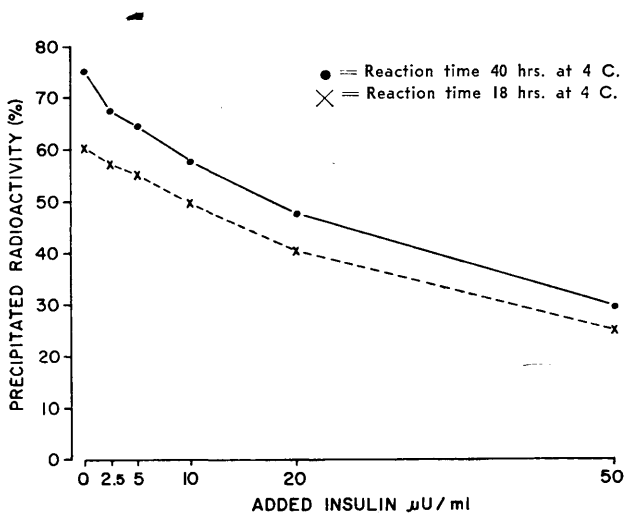


FIG. 3. Effect of temperature and time of reaction on the standard curve.

5. *Measurement of circulating insulin*

The concentration of endogenous insulin was determined in whole and diluted samples of serum from normal subjects and untreated diabetic patients. In each sample, larger amounts of insulin were detected in the 1:10 dilutions than in either whole serum or in serum diluted 1:5 (figures 4 and 5). In the latter instance the values seemed to bear a constant relationship, which was approximately two thirds of the con-

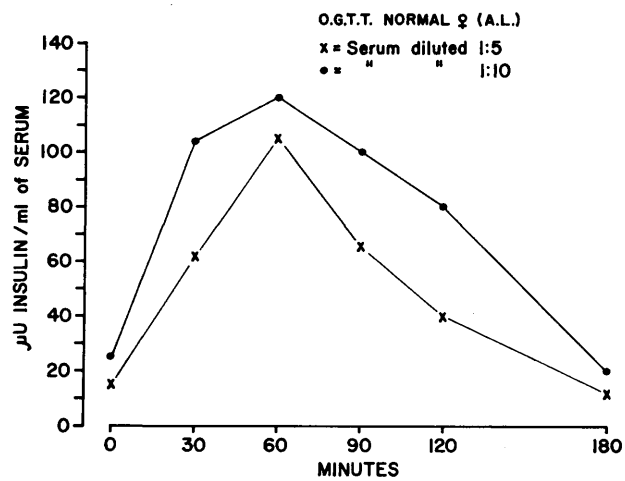


FIG. 4. Differences in levels of endogenous insulin seen at 1:5 and 1:10 dilutions of serum after oral glucose in a normal subject.

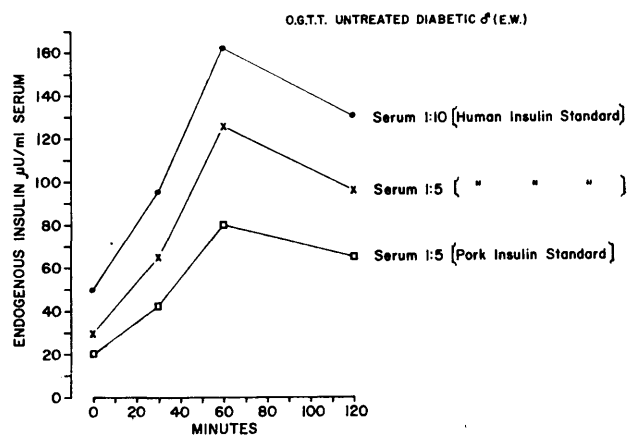


FIG. 5. Comparison of insulin levels in serum diluted 1:5 and 1:10 after ingestion of glucose in an untreated subject with maturity-onset diabetes. Insulin values using a pork insulin standard (bottom plot of chart) are significantly lower than the values for the identical serum samples using a human insulin standard (middle plot).

centrations found at the 1:10 dilutions. Dilutions of serum greater than 1:10 yielded essentially the same results as obtained with 1:10, indicating that further dilution was unnecessary. Attention is directed to figure 5, where the values for endogenous insulin in serum at a 1:5 dilution have been derived from a pork insulin standard. Identical samples of serum, also diluted 1:5, gave significantly higher values for circulating insulin with a human insulin standard, emphasizing the profound influence that a particular species of insulin can have on the reactivity of the antibody.

6. *Recovery of added insulin*

The recovery experiments substantiated the impression that complete recovery of insulin was possible only in a 1:10 dilution of serum. In whole or in weakly diluted serum (1:5) the maximum amount of insulin that could be detected, regardless of the level, seldom exceeded two thirds of the added quantity. However, with serum diluted 1:10, the recovery of insulin at all concentrations of enrichment was almost perfect (figure 6), indicating that a 1:10 dilution of serum is an absolute requisite for the valid measurement of exogenous or endogenous insulin by this method.

7. *Comparison with double-antibody technic*

Determinations of insulin in serum were carried out "blindly" and the results compared with the values obtained with the double-antibody procedure.[13] As shown in figure 7, the agreement between the two technics was close, although the low concentrations of insulin resulted in higher values when measured by enzyme hydrolysis. Whether this difference represents the fraction of insulin uncovered by the dilution of the serum[14] or reflects variations in the sensitivity of the technics is unclear.

8. *Specificity of the assay*

In view of the recognized effect that tolbutamide has on the release of insulin from the pancreas, blood was collected at regular intervals from several individuals who were given 1 gm. of the drug intravenously. Measurements of insulin revealed that within thirty minutes (figure 8) the endogenous level had risen significantly and was accompanied by a corresponding decline in the blood glucose.

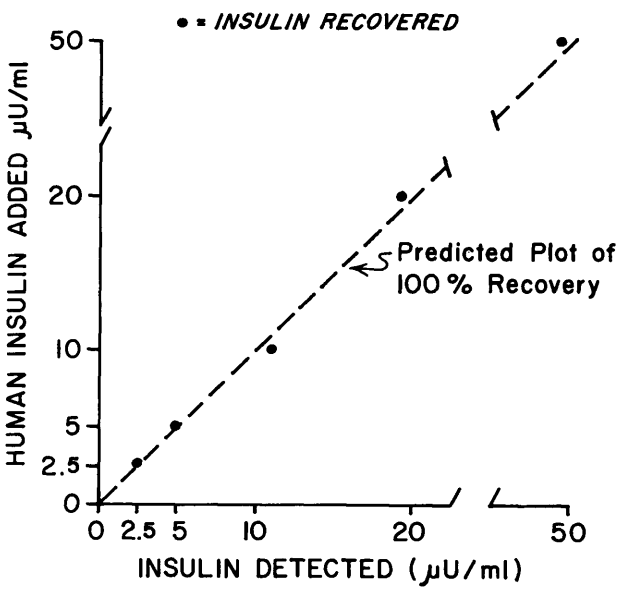


FIG. 6. Measurement of human insulin added to 1:10 dilution of serum. The endogenous concentration of hormone was subtracted from the amounts recovered before the values were plotted. When the assay was carried out with the same serum diluted 1:5, less than two thirds of the added insulin was recovered, regardless of level of enrichment.

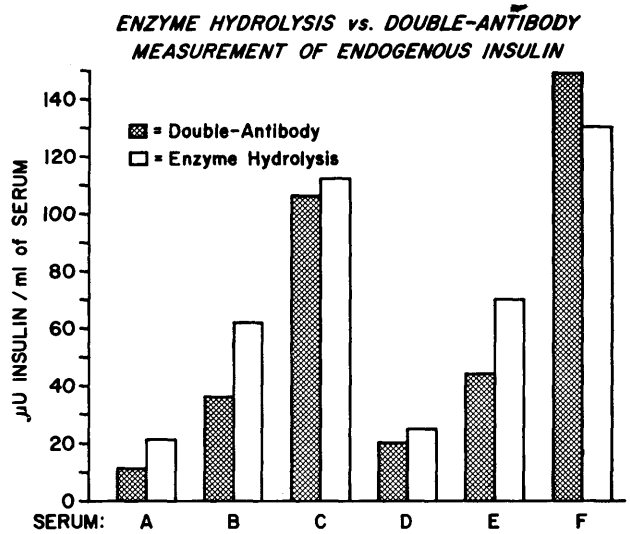


FIG. 7. Endogenous insulin concentrations in several different samples of serum determined by both enzyme hydrolysis and double antibody technics.

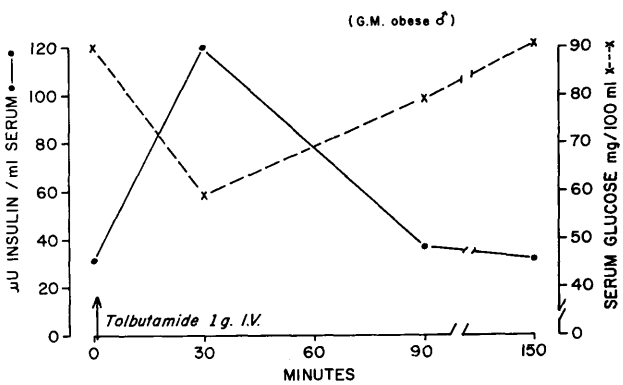


FIG. 8. Changes in levels of endogenous insulin and glucose in an obese individual given 1 gm. of tolbutamide intravenously.

The specificity of this method was further demonstrated by an assay on serum in which insulin was inactivated by a modification of the method of Grodsky.[4]* No insulin could be detected in the serum that had formerly contained 80 μU. per milliliter.

DISCUSSION

The development of a satisfactory immunoassay for insulin, based on the enzymatic proteolysis of insulin I-131, depended largely on the selection of an appropriate enzyme. Insulinase was the first enzyme system to be used,[9] but in our hands proved to be both inconvenient and unreliable. Others, however, have reported the successful application of an insulinase system,[15] but it is difficult to evaluate their results because of lack of data on reproducibility, replicate variability or recovery of added stable insulin.

A variety of proteolytic enzymes was surveyed (including papain, pancreatin, pepsin, trypsin), but none achieved the desired results until ficin was tried. The unique properties of ficin could be exploited only by deviating from the initial design of the assay and by introducing several modifications. Originally, 5 per cent bovine serum albumin had been used for all dilutions, but because of the erratic and unsatisfactory behavior of ficin in that medium, 2.5 per cent gelatin in tris-maleate buffer was tested and found to be suitable. Shortly thereafter it became evident that ficin suffered considerable loss in potency during storage, albeit in the dry state and protected from light and heat. The problem was eventually resolved when it was noted that catalytic activity could be fully restored by incubation with reduced glutathione. Presumably, most of the activity of ficin is dependent on the availability of sulfhydryl groups, since the activating effect is also seen with cysteine.

The importance of the enzyme system has received considerable emphasis because the ultimate success of the assay depends on a predictable degree of proteolysis. Without the assurance of uniform action, the possibility exists that insufficient or excessive hydrolysis could invalidate the procedure. The problem of excessive hydrolysis may be more readily appreciated when it is recalled that the measurement of endogenous and exogenous insulin is accomplished despite a short period of reaction between antibody and insulin, a period considered too short for the antigen-antibody reaction to have gone to completion.[11] In this system, however, while completion of reaction seems to be unnecessary, a certain minimal degree of association between antibody and antigen is essential, for it seems that ficin is capable of degrading both free and antibody-bound insulin I-131. Support for this statement can be found in observations made during the time when the optimal concentration of reduced glutathione was being sought. It was noted then that increased concentrations of glutathione (0.1 to 0.01 molar) in combination with ficin resulted in appreciably more trichloroacetic acid-soluble radioactivity after incubation with antibody-bound insulin I-131 than did the weaker concentrations of glutathione with ficin. Since glutathione alone, in the amounts used above, does not fragment radioinsulin, it is reasonable to assume that a larger fraction of the antibody-insulin I-131 complex was degraded by the more potent enzyme system. Faced with this fact it is difficult to escape the conclusion that an extreme degree of enzyme activity might destroy more than the optimal amount of bound insulin, or alter the integrity of the complex, thereby precluding a satisfactory assay. On the other hand, the opposite condition of inadequate proteolysis can be just as damaging, for failure to degrade bound insulin I-131 sufficiently would be reflected in a flat and consequently useless standard curve.

The most intriguing problems which arose, with respect to the detection of insulin in serum were, (a) the inability to recover all of the stable insulin added to undiluted serum, and (b) the augmentation of measurable levels of endogenous insulin produced by dilution of serum. It is possible that both of these phenomena are related to the inhibitory effect of serum proteins on ficin, although studies done by us indicate that the degradation of free insulin I-131 by ficin is virtually the same in serum diluted 1:5 or 1:10.

That the peculiar effect of serum on the recovery of insulin can explain the differences in values observed between the enzyme hydrolysis and double-antibody technics seems unlikely. It is worth noting, that in the Yalow-Berson procedure,[11] where the serum is also diluted 1:10, their published values for fasting normal subjects are similar to those reported here, and also substantially higher than those obtained by the double antibody technic. Since the augmentation of insulin-like activity has been observed with dilution of serum in a bioassay system,[14] one might postulate that the state of

*Sodium hydroxide (2 N) is added drop by drop to serum until the *p*H is between 11.5 and 11.6. The serum is set aside for three days at 4° C. and then centrifuged at 900 × g to remove the insoluble material. The supernate is neutralized with 2 N hydrochloric acid and is then ready to be assayed.

the hormone (types or aggregates of insulin molecules) undergoes change with dilution of serum.

One should not lose sight of the possibility that some of the discrepancy in insulin values might be related to the greater sensitivity of the double-antibody assay with concentrations of insulin below 10 μU. per milliliter. Since the serum is diluted 1:10 in the enzyme method, the chance of detecting 5 μU. per milliliter of circulating insulin may not be great as in the double-antibody procedure, where a 1:2 or 1:4 dilution of serum is used. Moreover, there is no way of knowing whether or not some dissociation of the antigen-antibody complex during incubation with the ficin might contribute to loss of sensitivity at the lower levels of insulin. One final comment regarding sensitivity might be made and that is, sensitivity of any immunoassay is related to the shape of the standard curve which in turn depends on the characteristics of a particular antiserum.

Although there is no guarantee that any immunoassay measures a biologically active form of insulin, since antigenically reactive constituents may exist in a metabolically inert molecule, the specificity of this procedure seems evident. Changes in the response of endogenous insulin to tolbutamide or glucose, the inability to detect insulin after chemical inactivation of the hormone and the excellent recovery of exogenous insulin added to serum provide ample proof of the specificity of the assay. Furthermore, various peptide hormones such as human growth hormone (1 μgm.), prolactin (1 μgm.) and thyrotropin (50 μU.) had no demonstrable effect in this system. Crystalline glucagon (Eli Lilly, lot 258-234B-167-1) at a concentration of 1 μgm. produced a change that was equivalent to 7μU. of insulin. This was not, however, unexpected for information kindly provided by the Lilly Laboratories indicated that the glucagon contained some insulin (less than 0.05 U. per milligram).

The significance of this system is not necessarily to be found in the advantages of daily determinations, simplicity or economy, but rather in the principle that underlies the procedure. The successful introduction of enzyme hydrolysis as a distinctive technic in the measurement of insulin offers promise that the basic design can be applied to the measurement of other hormones, or antigenic substances.

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