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AN IMMUNOCHEMICAL ASSAY OF TOTAL EXTRACTABLE INSULIN IN MAN *

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For the precise assay of minute quantities of insulin in biological fluids, techniques using fibril formation (1) or paper chromatography (2), although specific for the insulin molecule, lack the required sensitivity. Methods based on the measurement *in vivo* of a fall in blood sugar in surgically modified rats (3) afford sensitivity, but lack both specificity and precision. *In vitro* biological assays, in which glucose metabolism is measured in rat diaphragm or epididymal fat, while perhaps more precise than *in vivo* methods, are subject to interference by other hormones such as somatotropin and epinephrine (4, 5), by unknown "insulin-like" substances whose extraction characteristics differ from those of insulin (6) and by numerous inhibitors of insulin activity (7–10).

In contrast, an immunological method should be both chemically specific and, especially after application of an isotopic method for the immunologic quantitation, reasonably sensitive and precise.

Berson and co-workers have originally reported (11) that when insulin-I^{131} is added to an excess of antibodies, it rapidly becomes bound. Furthermore, addition of unlabeled insulin in increasing quantities results in gradual saturation of the binding sites, reflected by an increase in the percentage of unbound insulin-I^{131}. We have subsequently confirmed these observations (12). The change in the percentage of unbound labeled insulin forms the basis of methods recently described for the measurement of injected insulin in rabbit plasma (13) and extracted insulin from mouse pancreas (14). Both of these methods utilized hydrodynamic flow techniques for the determination of the percentage of unbound insulin.

The modification of this approach for the assay of circulating human insulin is the subject of this report. Specific consideration has been given the following points: *1*) the preliminary extraction of the serum to remove endogenous antibodies that would interfere in the assay; *2*) the effective separation, by means of preferential salt precipitation, of free insulin-I^{131} from the bound labeled hormone produced after incubation with antibody and the elimination of nonspecific binding of insulin-I^{131} to extraneous proteins in the extracts; *3*) the cross reaction of human insulin with antibodies formed against beef insulin in the guinea pig; and *4*) the preparation of appropriate standard incubates with protein concentration and pH most nearly approximating those of the unknown extracts.

The described method was used to investigate the effect of carbohydrate ingestion, insulin injection and the presence of acromegaly on the levels of circulating insulin in man.

MATERIALS AND METHODS

Bovine crystalline insulin, lot no. T-2842, was obtained from Eli Lilly & Company. Insulin-I^{131} was prepared by a modification of the method of Burrows, Peters and Lowell (15), as described previously (12). *Insulin antisera* were obtained from guinea pigs treated as follows: 1 mg of insulin in 2 ml of Freund's adjuvant (16) was injected subcutaneously into adult guinea pigs. Eight days later the animals were re-injected with 0.5 mg of insulin in the adjuvant. When necessary, animals were given 10 per cent glucose intraperitoneally to overcome severe hypoglycemia. After 5 months the animals were injected with 0.25 mg of insulin in 2.5 ml of 10 per cent glucose. (The 5 month interval was arbitrarily chosen to conform with a concurrent study and does not represent an experimentally determined optimal period.) Anaphylaxis was general, but animals given adrenaline survived. Six days after the final injection blood was drawn by cardiac puncture; serum from the specimens was frozen and stored.

Expressing the insulin antibody titer as the dilution of serum, 1 ml of which would still bind 50 per cent of 0.1 μg of insulin-I^{131}, a typical antiserum had a value of 224 (12). Antiserum was diluted with 5 per cent human albumin until 10 μl bound about 75 per cent of 16 μU of

* Supported by a grant from Eli Lilly & Company. Cost of equipment was defrayed in part by a special grant from the Smith, Kline & French Laboratories.

TABLE I

*Inactivation of insulin-I^{131} in plasma measured by hydrodynamic flow (12)**

Experiment no	Before treatment			After 24 hours of treatment		
	Origin	Moving fraction	Insulin-degraded	Origin	Moving fraction	Insulin-degraded
			%			%
A. Alkali inactivation						
1	186	34	14	32	178	85
2	125	24	16	15	105	88
3	126	14	9	22	103	82
B. Cysteine inactivation						
1	125	22	18	87	53	38
2	108	14	11	57	30	34

* All values are given as activity in counts per second.

insulin-I^{131} (1 mg insulin considered equivalent to 27 units).

"Insulin-free" plasma, for use as carrier protein, was prepared by adjusting plasma to pH 13.0 with concentrated ammonium hydroxide and storing it at 4° C for 24 hours. Final pH was adjusted to 7.4 with concentrated hydrochloric acid. The effectiveness of alkaline inactivation of insulin was demonstrated by adding 0.1 μg of insulin-I^{131} to 1 ml of plasma, treating it as described above and subjecting the final mixture to hydrodynamic flow (insulin remains at the origin; degradation products move with serum proteins). Approximately 85 per cent of the radioactivity was found in the fraction representing degradation products (Table I). In similar samples to which 0.025 M cysteine (17) had been added, degradation during a 24 hour period at 4° C amounted to only 35 per cent.

Extraction of insulin from serum. To prevent the degradation of insulin that may occur during clot formation at room temperature, particularly when hemolysis has occurred, whole blood was allowed to clot in an ice bath.

A method for extracting insulin from pancreatic tissue (18) was modified for use with the resulting serum as follows. A mixture of 5 ml of serum and 20 ml of acidic alcohol (15 ml of 12 N hydrochloric acid diluted to 1 L with 75 per cent ethanol) was agitated for 1 hour at room temperature. After centrifugation, the supernatant was removed. The precipitate was re-extracted for 1 hour with an additional 20 ml of the acidic alcohol solution and centrifuged. The two supernatants were combined and adjusted to pH 8.5 to 9.0 with concentrated ammonium hydroxide. After 30 minutes at 4° C insoluble proteins were removed by centrifugation. To precipitate the crude insulin, 4 volumes of an alcohol-ether solution (1.5 : 2.5 vol/vol) were added and the mixture was chilled overnight at 4° C. After centrifugation the precipitate was exposed to a jet of air for 60 seconds to remove excess ether. The still moist precipitate was dissolved in 1.5 ml of 0.2 M glycine buffer (pH 8.5) by vigorous stirring with a glass rod. After a 1 or 2 minute interval to allow foam to settle, the solution was adjusted to 2.5 ml with additional glycine buffer. Protein that failed to go into solution was insulin-free and could be discarded. The final extract, representing a twofold concentration of the initial serum, was sufficient for 5 determinations and could be stored at 4° C for up to 2 weeks.

Assay of insulin. To obtain a standard curve, various amounts of nonradioactive insulin in 10 μl of 5 per cent albumin were incubated for 1 hour at 25° C with a mixture of 0.5 ml of 5 per cent albumin in the 0.2 M glycine buffer, 0.5 ml of 30 per cent urea in 5 per cent albumin solution, 10 μl (16 μU) of insulin-I^{131}, and 10 μl of the diluted guinea pig antiserum. After incubation, 0.4 ml of the "insulin-free" human plasma was added as carrier protein. The insulin bound to antibody was precipitated by adding 2 volumes of water containing sufficient sodium sulfite to make a final concentration of 17 per cent in the incubation mixture. The contents of the tubes were mixed and allowed to stand 1 hour at 4° C.

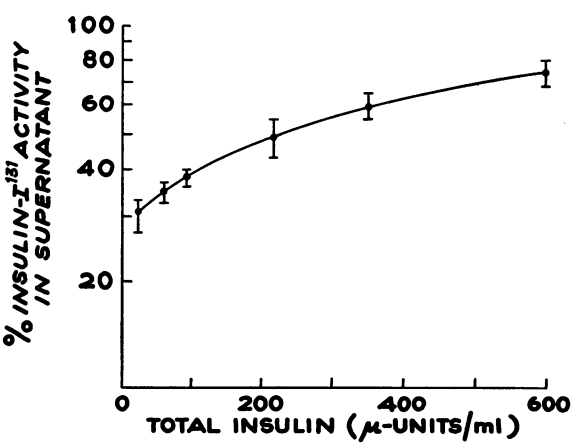

FIG. 1. EFFECT OF CONCENTRATION OF TOTAL INSULIN ON LEVEL OF FREE INSULIN-I^{131} REMAINING IN SUPERNATANT AFTER PRECIPITATION WITH SODIUM SULFITE. Mean of curves determined on 11 days during 2 month period ($\bar{x}$ = mean ± 2 × SD).

The precipitated proteins were removed by centrifugation, and the radioactivity in an aliquot of the supernatant was counted in a well-type scintillation counter. When corrected for total volume, the value obtained represented the free insulin (and probably some degradation products) remaining after incubation. The standard curve was obtained by plotting the activity in the supernatant as per cent of the total radioactivity initially added against the concentration of total insulin (Figure 1).

Since the percentage of unbound insulin did not increase linearly with, or as a logarithmic function of, insulin concentration, at least 5 points along the curve were required for each day's determination. The curve was quite reproducible; over a period of 2 months during which several different preparations of insulin-I^{131} were used, the standard deviation for the low point and high point was 3.1 and 3.8 per cent, respectively ($n = 11$).

The unknown insulin was assayed by substituting 0.5 ml portions of the serum extract (equivalent to 1 ml of serum) for the glycine-albumin buffer used in the standards. Incubation and salt precipitation were carried out as described above. To determine the insulin content of the unknown sample the percentage of radioactivity in the resulting supernatant was read against the standard curve. Total insulin in serum was calculated by dividing this value by 0.8 (the mean per cent recovery of insulin by the extraction procedure).

Preparation and assay of human insulin. One g aliquots of biopsy specimens of a fresh human pancreas and of an islet cell tumor were extracted by an acid-alcohol procedure (14). The final precipitate from alcohol-ether was dissolved in 1 ml of dilute hydrochloric acid and stored as a stock source of crude human insulin. Prior to immunological assay, the stock extracts were diluted 500-fold with the glycine albumin buffer. The biological activity of these extracts was also determined by the mouse convulsion assay (19). Since only 10 animals were used for each standard or unknown sample, the results of this bioassay served as a crude approximation only. However, past experience in our laboratory indicates that variations in results rarely exceed 100 per cent. In addition, a sample of the extract from normal pancreas was determined more precisely by Dr. Otto Behrens in the laboratories of Eli Lilly & Company.

RESULTS

Extraction of insulin from serum. Table II shows the recoveries obtained when 1,250 μU of insulin-I^{131} was added to 5 ml of serum and extracted one hour later by the acid-alcohol procedure. Since degradation products produced during the iodination or the extraction procedures could have accounted for part of the radioactivity, aliquots of both the starting serum containing the labeled hormone and the final extract were subjected to hydrodynamic flow. The tabulated values have been corrected and represent the portion of the label that was chromatographically similar to insulin. When normal serum was extracted, approximately 80 per cent of the added insulin was recovered. As the donors had never received insulin, the serum did not contain significant amounts of endogenous antibodies (Table II). Recovery of insulin-I^{131} from sera (which had insulin antibody titers of 7 and 16, respectively) of two insulin-resistant diabetics was then evaluated. Despite the almost quantitative binding of the added insulin-I^{131}, recovery after extraction was essentially the same as that from normal serum. When extracts prepared from resistant sera were incubated with insulin-I^{131} and subjected to hydrodynamic flow, insulin-binding was no longer demonstrable. A final recovery of 80 per cent was assumed for all sera subsequently assayed for insulin content. No significant decomposition of the insulin could be demonstrated by hydrodynamic flow in extracts stored for as long as two

TABLE II

Acid-alcohol extraction of insulin-I^{131} from normal and insulin-resistant sera

Source of serum	Antibody titer* of serum	Insulin-I^{131} bound to serum protein	Recovery of insulin-I^{131} after extraction	Antibody titer of final extract
		%	%	
Normal	0	5	78	
Normal	0	11	80	
Normal	0	3	83	
Insulin-resistant diabetic	7	93	84	0
Insulin-resistant diabetic	16	96	80	0

* Titer = the dilution of serum, 1 ml of which will still bind 50 per cent of 0.1 μg of insulin-I^{131}.

weeks at 4° C. According to estimates by both biuret and Kjeldahl nitrogen methods, only 15 to 19 per cent of the total protein remained after the extraction procedure.

Solubility of insulin-I^{131} and insulin-I^{131} antibody in sodium sulfite. Attempts to precipitate preferentially the insulin antibody complex with increasing amounts of trichloroacetic acid proved unsuccessful, since both the free and bound insulins precipitated simultaneously. Preliminary experiments substituting sodium sulfite as a precipitant indicated that this approach was feasible, provided that carrier protein was added. "Insulin-free" plasma or gelatin was utilized as carrier

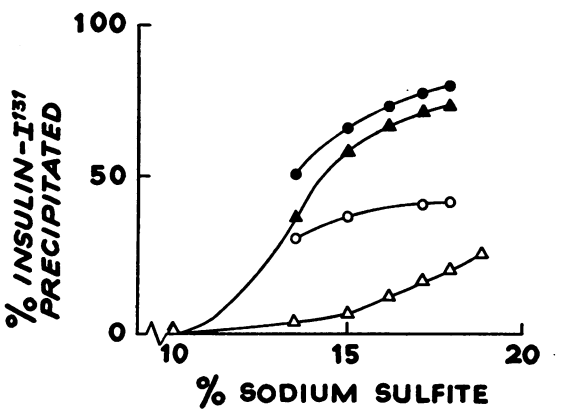

FIG. 2. PREFERENTIAL PRECIPITATION OF FREE AND BOUND INSULIN-I^{131} IN ALBUMIN-GLYCINE BUFFER AND SERUM EXTRACTS BY SODIUM SULFITE. ▲——▲ Bound insulin-I^{131} (antibody added) in buffer; △——△ free insulin-I^{131} (no antibody) in buffer; ●——● bound insulin-I^{131} (antibody added) in serum extract; ○——○ free insulin-I^{131} (no antibody) in serum extract.

protein. The latter was eventually discarded when it proved too soluble in sodium sulfite at the pH of 8.5 used for the assay.

Since the standards contained less precipitable protein than did the unknown extracts, it was necessary to add sufficient carrier protein to both solutions to minimize the difference. Too little carrier caused incomplete precipitation in the standards, resulting in erroneously low values for insulin in the extracts. The concentration of carrier protein necessary to overcome this situation, but not great enough to increase coprecipitation of the free insulin, was determined by adding increasing amounts of carrier to incubates of insulin-I^{131} plus antibody both in the standard glycine-

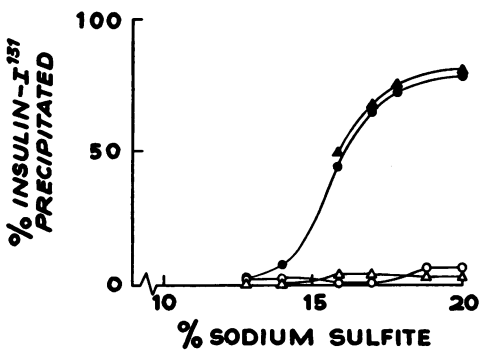

FIG. 3. EFFECT OF 15 PER CENT UREA ON PREFERENTIAL PRECIPITATION OF FREE AND BOUND INSULIN-I^{131} IN ALBUMIN-GLYCINE BUFFER OR SERUM EXTRACTS BY SODIUM SULFITE. ▲——▲ Bound insulin-I^{131} (antibody added) in buffer; △——△ free insulin-I^{131} (no antibody) in buffer; ●——● bound insulin-I^{131} (antibody added) in serum extract; ○——○ free insulin-I^{131} (no antibody) in serum extract.

albumin buffer and in an extract of "insulin-free" plasma until the resulting supernatant counts in each were identical.

Figure 2 shows the effect of the concentration of sodium sulfite on the solubility of insulin-I^{131} suspended in glycine-albumin buffer or serum extract, with and without the addition of sufficient antibody to bind approximately 80 per cent of the labeled hormone. In the glycine-albumin solutions a concentration of 15 per cent sodium sulfite resulted in quantitative precipitation of insulin-I^{131} bound to antibody, whereas free insulin-I^{131} remained in solution. In contrast, when serum extracts were incubated without antibody, 40 per cent of the total insulin-I^{131} was bound and precipitated by extraneous proteins contained in the extract.

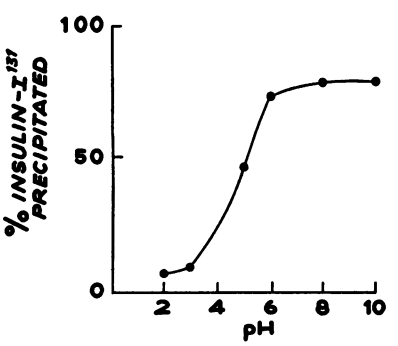

FIG. 4. EFFECT OF pH ON BINDING OF INSULIN-I^{131} TO ANTIBODY.

Figure 3 shows that the addition of 15 per cent urea to the incubation mixtures effectively eliminated almost all of the nonspecific binding in typical serum extracts. Additional salt was required to achieve optimal preferential precipitation. The amount of insulin-I^{131} precipitated by antibody in serum extract was no longer notably different from that in the standard which contained no unlabeled insulin, indicating that the level of circulating human insulin was too low to be detected.

The effect of pH on insulin-antibody complex formation (*Figure 4*). Binding was not detectable below pH 3.0 and reached a maximum at about pH 6. Since insulin and many of the proteins in the serum extracts were insoluble at slightly acid and neutral pH's, pH 8.5 was used for all incubations.

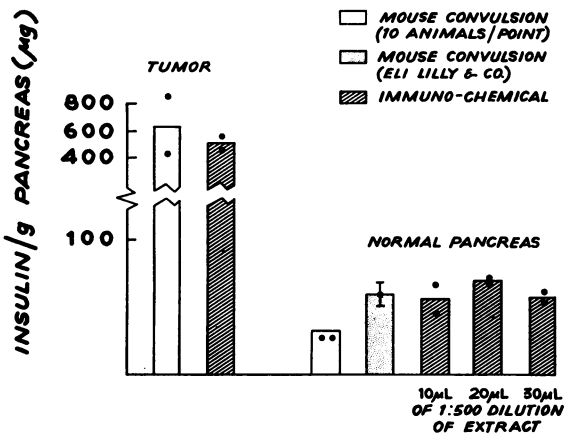

FIG. 5. COMPARISON OF BIOLOGICAL AND IMMUNOLOGICAL ASSAY OF HUMAN INSULIN. Each point represents the mean of duplicate values.

Measurement of human insulin from pancreatic tissue (*Figure 5*). Measurements of human insulin levels in the pancreatic tumor and normal pancreas by biological and immunochemical methods were in good agreement. The extremely good duplication of the assays on normal pancreas by the mouse convulsion method in the Eli Lilly laboratory and by the immunochemical approach was probably coincidental (58.7 μg per g tissue, SD = 4.2 per cent; and 61.5 μg per g tissue, SD = 7.6 per cent, respectively). Results obtained by assaying three different dilutions of the human extract and correcting for dilution differed only within the limits of experimental error. Assay by both methods showed ten times more insulin in the tumor tissue than in the sample of normal pancreas.

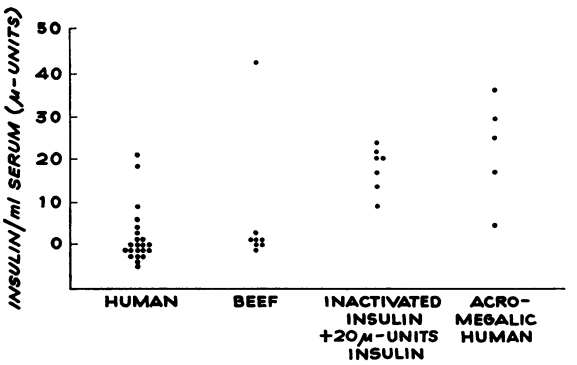

FIG. 6. CIRCULATING LEVELS OF INSULIN IN HUMAN AND BOVINE SERUM. Each point represents the mean of duplicate values.

Circulating level of insulin in human and bovine serum (*Figure 6*). Determination of the circulating insulin in extracts of sera from 22 fasting subjects was attempted. Except on two occasions insulin could not be detected. As seen from the recovery studies on extracts with added insulin, a level of 20 μU per ml of serum is within the minimum sensitivity of this method. Serum insulin levels were notably elevated in four of five subjects with active acromegaly.

The circulating insulin levels in extracts of bovine serum were also determined. This insulin is species identical with the insulin used originally to form the antibodies and to make the standards. In seven of the eight samples tested, as in the human sera, insulin levels were undetectable.

Effects of oral glucose on circulating insulin levels (*Figure 7*). Fifty g of glucose was ad-

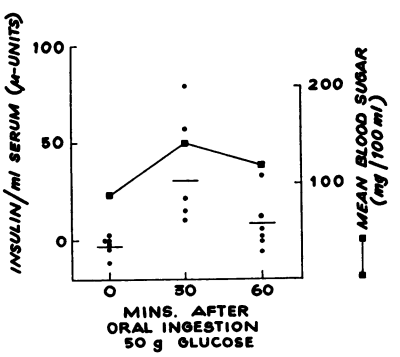

FIG. 7. EFFECT OF ORAL GLUCOSE ON CIRCULATING INSULIN. Each point represents the mean of duplicate values.

ministered orally to six normal fasting subjects. After 30 minutes the serum insulin levels were increased in five of the six subjects (mean, 31 μU). By 60 minutes the insulin levels had commenced to fall and in most instances were no longer detectable. The blood sugar level, elevated at 30 minutes, had also begun to decrease.

Disappearance of crystalline bovine insulin (Figure 8). To demonstrate the efficiency of the immunoassay in measuring changing levels of serum insulin, periodic determinations were made after intravenous administration of 10 U of crystalline hormone to a normal subject. Insulin

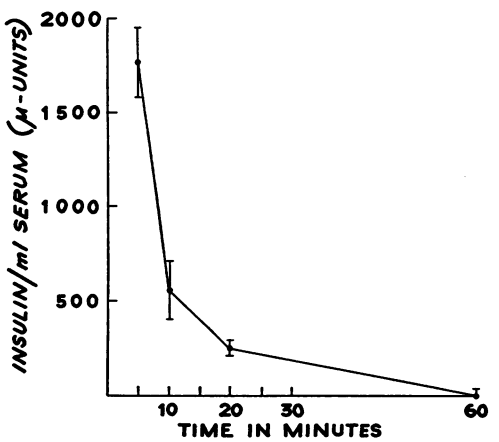

FIG. 8. INSULIN SERUM LEVELS AFTER INJECTION OF CRYSTALLINE BEEF INSULIN (10 UNITS INTRAVENOUSLY) IN NORMAL SUBJECT.

levels were extremely high 5 minutes after the injection; within 20 minutes, however, 90 per cent of the insulin had disappeared. After 60 minutes circulating insulin was no longer detectable.

An insulin-resistant diabetic was given 500 U of insulin (U-500, Eli Lilly & Company) intramuscularly. Twenty-four hours later the serum was collected and extracted to remove the endogenous antibody. Assay of the extract showed a high concentration of insulin (3,100 μU per ml), despite a concomitantly high blood sugar level (301 mg per 100 ml) and severe glycosuria and ketonuria.

DISCUSSION

An immunological method for the measurement of circulating insulin offers the distinct advantage of being chemically specific for the insulin molecule. Growth hormone, epinephrine, steroids, glucagon or tolbutamide do not interfere with the binding of insulin to antibody (12). In the method described in this report the inherent specificity of such techniques is further augmented by the removal of gross impurities from the serum by acid-alcohol extraction. This preliminary step not only removes endogenous insulin antibodies, thereby allowing application of the procedure to serum from individuals previously receiving insulin (11, 12), but also eliminates about 85 per cent of the extraneous serum proteins. Baird and Bornstein (20) have shown that a similar extraction procedure effectively removes many of the globulin inhibitors that plague investigators attempting biological assays. Indeed, preliminary extraction of the serum is of such obvious advantage in increasing specificity that it conceivably could be universally adopted in all insulin assay methods.

The efficiency of extraction with acid alcohol may result in part from the dissociation of insulin antibody complexes noted at acid pH (21). Singer and Campbell (22) have observed that albumin fails to form a precipitating antibody at low pH values. Sri Ram and Maurer (23) suggested that the spontaneous precipitation, rather than affinity of the antigen to the antibody, was the pH-sensitive factor. The insulin-antibody complex, however, is soluble and is quantitated not by spontaneous precipitate formation but by chemical means. The lack of insulin-binding at low pH in this soluble system, as shown by both sodium sulfite and hydrodynamic flow methods (12), indicates the existence of a pH sensitivity at the level of antigen-antibody combination.

The use of salt precipitation to separate free from bound insulin, which is similar to the method described by Farr for albumin (24), requires only a single count of a sharply defined supernatant. In contrast, the hydrodynamic flow method (11, 14) requires additional apparatus for multiple simultaneous determination and, since it distributes the insulin-I^{131} over 10 cm of paper, calls for a higher initial level of radioactivity. Furthermore, at least ten samples must be counted to determine the bound and free peaks, and it is often difficult to differentiate the peaks, particularly in areas of very low or very high binding. Other methods for the preferential isolation of bound insulin have

been reported. Mitchell, Whitehead and O'Rourke (25) used an Amberlite resin for separating the insulin-antibody complex, but noted serious interference by extraneous serum proteins. Skom and Talmage (26) separated the insulin complex from free insulin by precipitating the latter with anti-gamma-globulin sera, a method requiring a second antibody system. Arquilla and Stavitsky (27) utilized a hemolytic technique for the quantitative measurement of insulin-antibody formation, but reported some lack of specificity due to unknown factors affecting red cell lysis.

Nonspecific binding of insulin to serum protein was effectively inhibited by the addition of urea, implying that this phenomenon is caused by hydrogen bonding. On the other hand the comparative ineffectiveness of urea in dissociating the insulin-antibody complex was not surprising, since the affinity of this antigen to its antibody may be primarily due to interatomic van der Waals' forces (12).

The most serious drawback to an insulin assay based on an immunological reaction is the necessity for assuming that the human insulin from extracts and the beef insulin used in the standards have the same capacity for binding to antisera that is produced against beef insulin in guinea pigs. Thus our inability to detect circulating insulin in human sera could be ascribed to its failure to cross react with these antibodies. Although Burrows and associates (15) reported that human insulin did not bind, Moloney and Aprile (21), Arquilla and Stavitsky (28), Berson and Yalow (29) and Wright (30) have independently demonstrated the cross reactivity of human insulin with antibodies produced from beef insulin. The close agreement between our measurements by the immunological and the mouse convulsion assay of human insulin extracted from pancreas indicates that effective cross reactivity does occur. (Exact evaluation of the quantitative relationship between human and beef insulin, however, must await the availability of a crystalline human insulin of sufficient purity to allow unequivocal determination of its insulin content by biological assay.) In addition, a very low level of circulating insulin is not necessarily the result of poor cross reaction, since insulin was not detected in beef blood in which no species difference exists between standards and circulating hormone.

The circulating form of insulin may be different from that extracted from the pancreas. This hypothesis has been suggested by the observation that insulin and the "insulin-like" activity in serum are found in different fractions after chromatographic separation on a Dowex-50 column (31). In the hope of minimizing this problem, we have adopted an acid alcohol procedure for extracting the serum similar to that used for commercial extraction of insulin from pancreas.

Circulating insulin was not detectable by immunochemical assay, although this method is sufficiently sensitive to measure as little as 20 μU of insulin per ml of serum. This level is lower than those estimated from biological techniques, which vary from 65 μU to 13.5 mU per ml (32–35). In most of these techniques, values obtained with *unextracted* serum were compared with results from standards dissolved in various arbitrary media including saline, different buffers or serum albumin. Recently, however, Anderson, Wherry and Bates (36), using extracts of plasma, reported normal levels as low as 25 μU per ml, and Field, Weinberg, Johnson and Spoont (37) have suggested that the circulating level may be less than 10 μU per ml.

The stimulating effect of glucose on insulin secretion was demonstrated by immunological assay. An increase in circulating insulin (mean, 31 μU) was found 30 minutes after oral administration of glucose. The decrease in circulating insulin after 60 minutes was paralleled by a fall in the blood sugar level.

An increase in the serum insulin concentration of patients with acromegaly has been reported by Wright (38) and also by Randle (34), who suggested that the apparent rise may be a reflection of the interference of large amounts of growth hormone in the assay. In contrast, Bornstein and Lawrence (10) not only reported no detectable insulin but described a hyperglycemic factor in blood from acromegalic patients which they believed might be due to glucagon. Since the immunochemical assay is not affected by either growth hormone or glucagon, our results indicate that the level of circulating insulin is truly elevated in acromegalic patients.

After injecting insulin-I^{131} and measuring the trichloroacetic acid-precipitable activity in serum, Elgee, Williams and Lee (39) found that 90 per

cent of the starting level of insulin was cleared from the blood within 15 to 20 minutes. This technique could be subject to error, since part of the circulating radioactivity might have represented partially degraded insulin that was still insoluble in trichloroacetic acid. Others (11, 17), using hydrodynamic flow-electrophoresis to differentiate degraded from unmodified insulin-I^{131}, also noted this rapid clearance. A parallelism between the rate of disappearance of insulin-I^{131} and immunochemically measurable insulin in the rabbit has also been reported (13). These observations, as well as the validity of using insulin-I^{131} as a metabolic tracer, have been confirmed in man by the finding that 90 per cent of the immunologically active insulin was cleared from the circulation in the first 15 to 20 minutes.

The demonstration that resistant diabetics may have high concentrations of insulin in their blood 24 hours after administration of the hormone despite concurrent hyperglycemia, glycosuria and ketonuria agrees with results obtained by biological assays of serum extracts (20, 40). It is apparent that in investigations measuring antibody titers or insulin-binding capacity in resistant serum, the results can be grossly affected by the partial saturation of the serum antibodies by variable unmeasured amounts of endogenous insulin. To minimize this effect, serum should be collected from the donor subjects at the greatest interval possible after insulin administration. A concomitant assay of total insulin would also be useful in this type of study.

Allowing the assumption that insulin-I^{131} and endogenous circulating insulin are extracted from serum in a similar manner, the immunological method for insulin assay described in this report is not affected by antibodies, antagonists or "insulin-like" substances. The method, at present, is applicable to quantities of total insulin above 20 μU per ml of serum. It is expected that increased sensitivity can be achieved by the synthesis of insulin-I^{131} of higher specific activity.

That a portion of the insulin measured by immunological techniques may include biologically inactive precursors or degradation products retaining antigenic activity cannot at present be excluded. Furthermore, as seen in the insulin-resistant diabetic, the total circulating insulin is not always biologically available to the subject. The measurement of "total insulin" in conjunction with the determination of "net active insulin" by biological assays, however, should lead to a greater understanding of the interplay between circulating insulin and its inhibitors, antagonists and "insulin-like" factors in normal and disease states.

SUMMARY

The new method for the immunochemical assay of circulating human insulin described in this report is based on the per cent change in binding of insulin-I^{131} to insulin antibodies in the presence of varying quantities of unlabeled insulin. The degree of binding is measured quantitatively after preferential precipitation of bound insulin with sodium sulfite.

Preliminary extraction of serum with acid alcohol resulted in an 80 per cent recovery of insulin-I^{131} regardless of the presence of endogenous antibodies. The method is therefore applicable to serum from subjects who have previously had insulin therapy.

Nonspecific binding caused by extraneous proteins in the extracts was effectively removed with 15 per cent urea. Affinity of insulin to its antibodies was pH-sensitive, being negligible at acid pH and reaching a maximum at neutral or alkaline pH.

Measurements of insulin extracted from human pancreas by the described chemical method and by mouse convulsion assay were in close agreement, indicating that effective cross reaction occurs between human insulin and antibodies made against beef insulin in the guinea pig.

Circulating levels of human insulin were lower than the minimum sensitivity of the assay (20 μU per ml of serum). Elevated insulin levels were detected in normal subjects after oral administration of glucose and in patients with active acromegaly.

Studies with the immunochemical assay demonstrated a rapid disappearance from serum of injected crystalline bovine insulin, confirming the results of others who employed the clearance of an insulin-I^{131} tracer. Insulin-resistant diabetics still retain large amounts of circulating insulin 24 hours after intramuscular injection of the hormone, despite severe hyperglycemia, glycosuria and ketonuria.

ACKNOWLEDGMENTS

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ADDENDUM

In a recent report published during the final preparation of this manuscript, Yalow, R. S., and Berson, S. A. [Nature (Lond.) 1959, **184**, 1648] have described an immunochemical assay for insulin in unextracted human plasma. These authors reported a fasting circulating level for insulin of 64 to 98 μU per ml, which could be enhanced notably after glucose administration.

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