IMMUNOASSAY OF ENDOGENOUS PLASMA INSULIN IN MAN

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For years investigators have sought an assay for insulin which would combine virtually absolute specificity with a high degree of sensitivity, sufficiently exquisite for measurement of the minute insulin concentrations usually present in the circulation. Methods in use recently depend on the ability of insulin to exert an effect on the metabolism of glucose *in vivo* or in excised muscle or adipose tissue. Thus, the insulin concentration in plasma has been estimated: *a*) from the degree of hypoglycemia produced in hypophysectomized, adrenalectomized, alloxan-diabetic rats (1); *b*) from the augmentation of glucose uptake by isolated rat hemidiaphragm (2); or *c*) from the increased oxidation of glucose-1-C^{14} by the rat epididymal fat pad (3). Since there have been reports indicating the presence, in plasma, of inhibitors of insulin action (4) and of non-insulin substances capable of inducing an insulin-like effect (5, 6), these procedures, while yielding interesting information regarding the effects of various plasmas on glucose metabolism in tissues, are of doubtful specificity for the measurement of insulin per se (5).

Recently it has been shown (7, 8) that insulins from various species (pork, beef, horse and sheep) show quantitative differences in reaction and cross reaction with antisera obtained from human subjects treated with commercial insulin preparations (beef, pork insulin mixtures). An immunoassay method for beef insulin has been reported in which the insulin content is determined from the degree of competitive inhibition which the insulin offers to the binding of beef insulin-I^{131} by human antisera (9–12). Although human insulin reacts with human antibeef, pork insulin antiserum and displaces beef insulin-I^{131} by competitive inhibition (7, 8, 10), the reaction is too weak to permit measurement of the low insulin concentrations present in human plasma (7, 8, 11–13). In preliminary communications we have reported that the competitive inhibition by human insulin of binding of crystalline beef insulin-I^{131} to guinea pig antibeef insulin antibodies is sufficiently marked to permit measurement of plasma insulin in man (11, 12, 14), and to be capable of detecting as little as a fraction of a microunit of human insulin (12, 14). Preliminary data on insulin concentrations in man before and after glucose loading have been reported (12, 14, 15). The present communication describes in detail the methods employed in the immunoassay of endogenous insulin in the plasma of man, and reports plasma insulin concentrations during glucose tolerance tests in nondiabetic and in early diabetic subjects and plasma insulin concentrations in subjects with functioning islet cell tumors or leucine-sensitive hypoglycemia.

METHODS

Immunization of guinea pigs. Guinea pigs were injected subcutaneously at 1 to 4 week intervals with 5 to 10 units of either protamine zinc beef insulin (Squibb) or commercial regular beef insulin (Squibb) emulsified with mannide mono-oleate. Insulin-binding antibodies were detected in all animals after 2 to 3 injections. The antiserum employed in the present study (GP 49, serum 6-25-59) was obtained from a guinea pig immunized with protamine zinc beef insulin without adjuvant and was selected for its relatively high antibody concentration and other suitable characteristics described below.

Preparation of insulin-I^{131}. Because of the desirability of keeping the concentration of added insulin-I^{131} as low as possible and yet assuring an adequate counting rate, it is necessary to prepare the insulin-I^{131} with a high specific activity. The lots of insulin-I^{131} employed in this study had specific activities of 75 to 300 mc per mg at the time of use. The preparation of such highly labeled preparations entails difficulties not encountered when the specific activity is very much lower. The Newerly modification (16) of the Pressman-Eisen method (17) was used for labeling with several further modifications designed to increase specific activity and to minimize damage to the insulin from irradiation and other causes. To approximately 0.3 ml chloroform in a 50 ml separatory funnel are added in turn, 0.2 ml of 2.5 N HCl, 20 μl of 10^{-3} M KI, 30 to 80 mc I^{131} (as iodide) and 1 drop of 1 M $NaNO_2$. Immediately after addition of the last reagent, the funnel is stoppered to prevent loss of I^{131} into the atmosphere and is shaken vigorously for 2 to 3 minutes. The chloroform layer (bottom) is then drawn

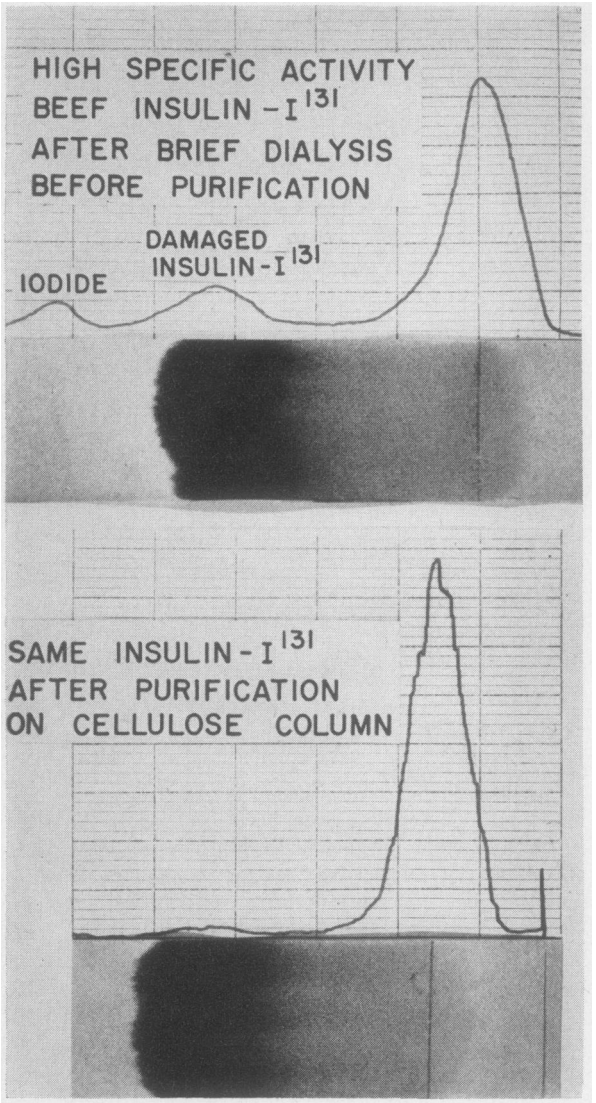

FIG. 1. PURIFICATION OF DAMAGED INSULIN-I^{131} BY CELLULOSE COLUMN ADSORPTION. *Top:* Chromato-electrophoretograms of beef insulin-I^{131}, with specific activity about 300 mc per mg, after 30 minutes' dialysis. *Bottom:* Same preparation after elution from cellulose column with control (nonimmune) plasma. At significantly lower specific activities the preparations appear as in the bottom figure *without* purification.

into a test tube beneath a layer of a few drops of water (to prevent loss of I^{131} into the air) and assayed for radioactivity in any low-sensitivity counting device. If much less than one-half of the starting radioactivity has been extracted, a second extraction with 0.2 to 0.3 ml chloroform is performed. The total amount of chloroform should be kept as small as possible to facilitate the subsequent extraction of iodine into the aqueous protein solution. The volume of the aqueous phase in the separatory funnel also should be kept small to favor the initial extraction of iodine into the chloroform. The chloroform-iodine mixture is added to 0.5 ml of 0.2 M borate buffer, pH 8, containing 20 μg of crystalline beef insulin in a 50 ml centrifuge tube, which provides for a broad interface between the two phases. The tube is shaken briskly but not violently for not more than 2 to 3 minutes following which an additional 1.0 ml borate buffer is mixed into the contents. A barely visible flocculate appears occasionally and should be allowed to settle, whereupon the top 0.5 ml (one-third of total) of the water phase is quickly removed and dialyzed against 2 L of distilled water.[1] Owing to the high concentration of radioactivity and low concentration of protein, the insulin is very susceptible to radiation damage (18, 19); therefore, exposure to I^{131} at this stage should be as brief as possible, not more than 5 to 10 minutes elapsing between addition of I^{131} to the insulin and the start of dialysis. Of the total radioactivity in the dialysis bag, approximately 65 to 80 per cent represents unbound I^{131} which is reduced to less than 1 per cent of the I^{131} bound to insulin after 2 hours of dialysis. Between 20 and 60 per cent of the insulin-I^{131} is adsorbed to the dialysis membrane during this time so that the procedure yields approximately 3 to 5 μg insulin labeled with about 1.0 to 2.0 mc I^{131}. Considerable sacrifices in total yield are made to expedite the procuring of a highly labeled preparation which usually contains no more than 4 to 6 per cent damaged components. We have the impression that the addition of 10^{-2} M KI or phenol (as radical scavengers) to the dialyzing solution may help to minimize radiation damage, but this has been difficult to establish since other factors are also responsible for damage to the protein during the procedure. Distilled water is used in the last dialysis following which 1 drop of human serum albumin (250 mg per ml) is added to the insulin-I^{131} solution to prevent losses of labeled insulin by adsorption to glassware (20, 21) and to minimize any further irradiation damage (18, 19). Solutions are kept frozen when not in use.

If the insulin-I^{131} solution is surveyed for radioactivity at completion of dialysis, the specific activity of the insulin-I^{131} may be estimated approximately. If the yield of labeled insulin has been sufficient to produce a specific activity in excess of 150 mc per mg, it can be anticipated that damage will be significantly in excess of 4 to 6 per cent, and at 300 mc per mg may be as great as 15 to 18 per cent. It is then necessary to effect partial purification of the insulin-I^{131}. Since the damaged components do not adsorb to paper but are observed to migrate with serum proteins on paper strip chromatography or electrophoresis (22), it is possible to use a cellulose column for the purification procedure as follows: The dialyzed insulin-I^{131} solution is added to 0.1 ml control (nonimmune) serum and the mixture is then passed through a column packed

[1] Removal of unbound iodide^{131} by anionic exchange resins is usually unsatisfactory because much of the insulin-I^{131} at this low concentration is lost by adsorption to the resin.

with a cellulose powder [2] about 1 ml in volume following which the column is washed 3 or 4 times with 1 ml of veronal buffer, 0.1 ionic strength. Most of the damaged components pass through the column with the serum while the undamaged insulin remains adsorbed to the cellulose in the column and can now be eluted slowly with undiluted control serum or plasma. Usually 3 to 4 eluates (each 0.5 ml of plasma) are collected and diluted immediately 1:20 to 1:100 with veronal buffer containing 0.025 per cent serum albumin to prevent further damage to the insulin by the concentrated plasma. Although the elution of insulin-I^{131} from the column is far from complete, adequate amounts are obtained for almost any number of insulin assays. Most of the damaged fraction is removed by this procedure (Figure 1).

Principles of immunoassay. The basis of the technique resides in the ability of human insulin to react strongly with the insulin-binding antibodies present in guinea pig antibeef insulin serum (11, 12, 14), and by so doing, to inhibit competitively the binding of crystalline beef insulin-I^{131} to antibody. The assay of human insulin in unknown solutions is accomplished by comparison with known concentrations of human insulin. The use of I^{131}-labeled animal insulin as a tracer is necessitated by the lack of a crystalline preparation of human insulin.

The determination of antibody-bound insulin-I^{131} and free insulin-I^{131} by paper chromato-electrophoresis has been described previously (22). Briefly, the separation of antibody-bound insulin from unbound insulin in plasma results from the adsorption of all free insulin (when present in amounts less than 1 to 5 μg) to the paper at the site of application ("origin"), while the antibody-bound insulin migrates toward the anode with the inter-β-γ-globulins. Thus, in the presence of insulin-I^{131} there appear two separate peaks of radioactivity; measurement of the areas beneath the two peaks (by planimetry) yields the relative proportion of bound insulin-I^{131} (migrating with serum globulins) and free insulin-I^{131} (remaining at origin). The ratio of bound insulin-I^{131} to free insulin-I^{131} (B/F) is a function of the concentration of insulin-binding antibodies, of both insulin concentrations, and of the characteristic kinetic and thermodynamic constants for the reactions between the insulins and the particular antiserum (23). Selection of an antiserum for purposes of this assay is determined primarily by the desirability of obtaining a relatively marked decrease in B/F ratio with small increments in the concentration of human insulin. Although the antibody concentration is of only secondary importance, it should be high enough to permit at least 1:100 dilution of the antiserum (preferably 1:1,000 dilution or greater). On the basis of preliminary tests the antiserum is diluted appropriately to yield an initial B/F ratio between 2 and 4 for tracer beef insulin-I^{131} alone, in the absence of added human insulin. Provided that the amount of the beef insulin-I^{131} used is truly a tracer quantity, the initial B/F ratio is inversely proportional to the dilution factor (23). In the presence of human insulin, the B/F ratio decreases progressively with increase in insulin concentration; with sensitive antisera the B/F ratio is reduced by about 50 per cent in the presence of 15 μU per ml human insulin.

Standard curves. Two preparations of human insulin were employed as standards. The first ("Tietze human insulin")[3] is reported (24) to have a potency of 1.8 U per mg crude preparation; the second ("Fisher human insulin"),[3] was assayed at 6.8 U per mg in 1956 (25), but it was believed that the activity of the latter preparation might have decreased slightly since its initial preparation (25). A tentative value of 6 U per mg for the Fisher insulin was assigned. However, since a value as low as 22 U per mg could be placed on a crystalline sample of the latter preparation (25), whereas the Tietze insulin was assayed relative to a standard of 27 to 29 U per mg, we have regarded the Tietze crude insulin preparation as $1.8/28 \times 100 = 6.45$ per cent pure insulin by weight, and the Fisher insulin powder preparation as $6/22 \times 100 = 28.2$ per cent pure insulin by weight. When compared on this basis, no consistent differences in potencies of the two preparations were observed in the immunoassay procedure and the value of 6 U per mg for the Fisher preparation was accepted as the correct value. Since the Fisher preparation is the more highly purified, it was employed as standard in most of the studies.

All dilutions of insulin and antiserum are prepared in 0.1 ionic strength veronal buffer containing 0.25 per cent human serum albumin to prevent adsorption of reactants to glassware. (There is no detectable insulin in commercial supplies of human serum albumin.) Standard solutions each contain identical concentrations of tracer beef insulin-I^{131} (about 0.05 to 0.15 mμg per ml but differing in different runs) and antiserum, but varying concentrations of human insulin ranging from 0.05 to 5.0 mμg per ml (calculated as "pure" human insulin). The antiserum is added last in all cases. Mixtures are refrigerated at 4° C for 4 days. These conditions provide sufficient time to reach equilibrium between bound and free insulin. The mixtures are then subjected to chromato-electrophoresis (22) in a cold room at 4° C (Whatman 3 MM paper, veronal buffer, 0.1 ionic strength, pH 8.6, constant voltage 20 to 25 v per cm, cover of apparatus open), which produces a satisfactory separation of the peaks of bound and free insulin-I^{131} in about 1 to 1.5 hours. Earlier immunoassays (10) were performed after prolonged incubation at 37° C. However, it has since been shown (23) that the standard free energy change of the reaction in the direction of antigen-antibody complex formation is increased considerably at 4° C, which results in an approximately twofold greater slope in the B/F versus insulin concentration curves at low insulin concentrations. Just prior to chromato-

[2] Genuine Whatman Cellulose Powder, W & R Balston Ltd., England.

[3] We are greatly indebted to Dr. F. Tietze of the National Institutes of Health and Dr. A. M. Fisher of the Connaught Laboratories, Toronto, Canada, for these preparations.

electrophoresis, control (nonimmune) guinea pig plasma is added to the mixtures to prevent trailing of antibody-bound insulin on the paper strips, since the very low concentrations of serum proteins in these mixtures are insufficient in themselves to prevent adsorption of the serum proteins (including antibody) to the paper.

The chromato-electrophoretograms are developed until the albumin band has moved about 2.5 to 3 inches from the origin, which, under the conditions employed here, usually takes about an hour. The peak of antibody-bound insulin-I^{131} moves about 2.25 inches under these conditions. The use of several large boxes, each with a capacity for 16 strips, makes it possible to run 250 to 300 strips a day. After drying, the strips are assayed for radioactivity in an automatic strip counter (Figure 2A). A "standard curve" is obtained by plotting the B/F ratio as a function of the concentration of added human insulin (Figure 2B) after correction for damaged components of insulin-I^{131}. From 3 to 6 per cent of the insulin-I^{131} was damaged after final preparation of the lots employed in this study. These damaged components migrate nonspecifically with the serum proteins, primarily with the α-globulins (22), and are demonstrably not available for binding by antibody. The short run chromato-electrophoresis does not resolve the serum proteins well enough to distinguish between antibody-bound insulin-I^{131} and damaged insulin-I^{131} so that the damaged fraction is determined by using either control (nonimmune) plasma, or antiserum whose binding capacity for undamaged insulin-I^{131} is completely saturated with beef insulin. Since the antiserum used here has a maximal beef insulin-binding capacity of about 1 mμg per ml at the dilutions employed, it has been general practice to include one or more samples made up with 1 to 4 μg per ml beef insulin for the purpose of determining the damaged fraction. Damaged insulin-I^{131} migrating with serum proteins is corrected for by subtracting the fraction damaged from the total area in the chromato-electrophoretogram. The area under the free insulin peak is then divided by the corrected total area to yield the fraction of free insulin. The fraction "bound insulin" is then 1.00 minus the fraction "free insulin."

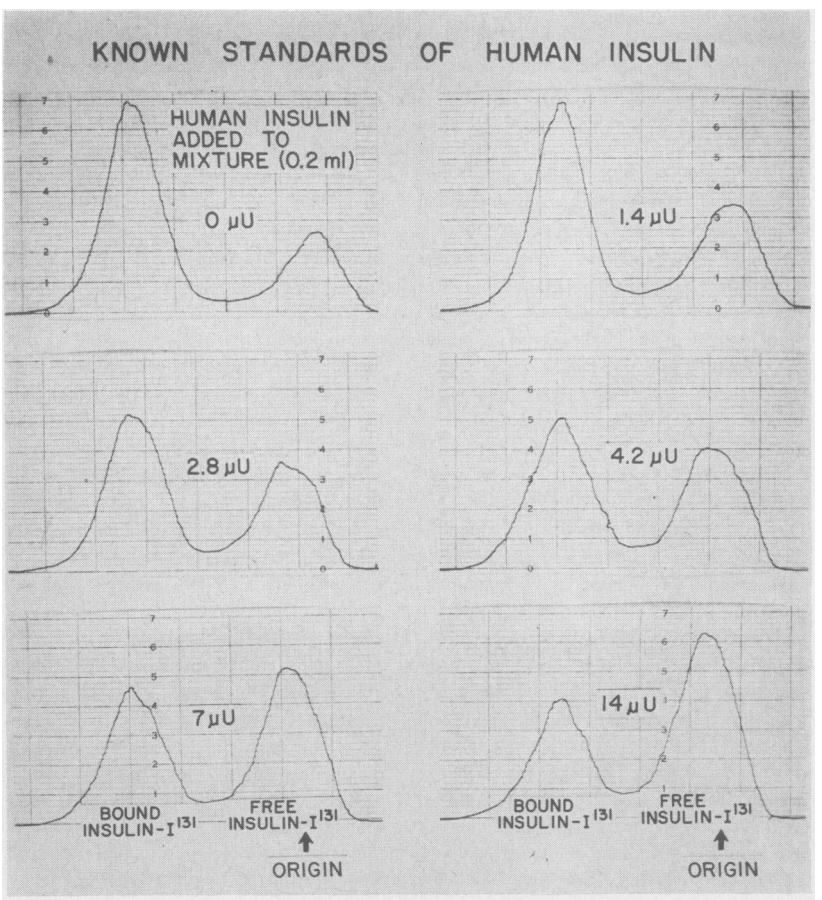

FIG. 2. A: RADIOCHROMATO-ELECTROPHORETOGRAMS OF ANTISERUM, INSULIN MIXTURES. Mixtures contained the same concentrations of guinea pig antibeef insulin serum and beef insulin-I^{131} but varying concentrations of human insulin as indicated.

It is evident that variation in the volumes of solution applied to the paper strips is of no consequence. Generally 100 to 200 μl is applied, the larger volume permitting use of a smaller quantity of tracer beef insulin-I^{131} for the same counting rate.

Assay of insulin in plasma. Mixtures containing unknown samples are prepared at the same time and in the same way as are standard solutions except that the unknown sample is substituted for the human insulin. Plasma insulin is best determined in a 1:10 final dilution unless the insulin concentration is unusually high; then a 1:20 or 1:40 dilution may be used. Mixtures may be made up to any desired volume. However, since only 100 to 200 μl is applied to the paper strips, it is convenient to prepare all mixtures in 0.2 or 0.5 ml volumes containing 20 or 50 μl of plasma, respectively.

Since insulin may be damaged by plasma during incubation (22), an effect which is more marked in concentrated plasma than in diluted plasma, and at 37° rather than at 4° C,[4] it is advisable to run a control mixture with unknown plasma but without antiserum to correct for "incubation damage." However, at 1:10 dilution of plasma after 4 days at 4° C, incubation damage amounts only to 0 to 3 per cent, an observation which contributed to the selection of these conditions. Therefore, only a negligible additional correction for damage is required in the plasma samples.

The insulin concentration in each plasma sample is determined from the standard curve by referring to the insulin concentration which corresponds to the corrected B/F ratio observed in the plasma sample (10–12, 14, 15).

Subjects for glucose tolerance tests. Subjects were chosen at random from patients sent to the general laboratory for glucose tolerance tests and from known diabetic and apparently nondiabetic patients on the wards of the Veterans Administration Hospital, Bronx, N. Y. Patients who had *ever* been treated with insulin were excluded from this study in order to obviate effects of antibodies in their own serum (22). Other than the exception noted below, subjects were classified as diabetic or nondiabetic on the basis of the following criteria applied to the 2-hour blood sugar curve following oral ingestion of 100 g of glucose: *diabetic*—a peak blood sugar concentration of 180 mg per 100 ml or greater, and a 2-hour blood sugar concentration of 120 mg per 100 ml or greater; *nondiabetic*—a peak blood sugar concentration not exceeding 160 mg per 100 ml, and 2-hour level no more than 120 mg per 100 ml. One subject with marginal ulcer and a dumping syndrome, with a blood sugar concentration of 286 mg per 100 ml at 0.5 hour falling to 134 mg per 100 ml at 1 hour and 44 mg per 100 ml at 2 hours, is included in this group. Because of the exclusion of insulin-treated patients, only mild or early maturity-onset diabetes is represented in the diabetic group. Subjects who did not qualify by these criteria for either group are considered in an *"undetermined status."* The criteria employed are modified from those suggested by Fajans and Conn (26) and are designed to eliminate questionable cases from diabetic and nondiabetic categories.

[4] For this reason plasma is separated in a refrigerated centrifuge immediately after withdrawal of blood and is used immediately or kept frozen until used in order to minimize loss of the endogenous insulin present.

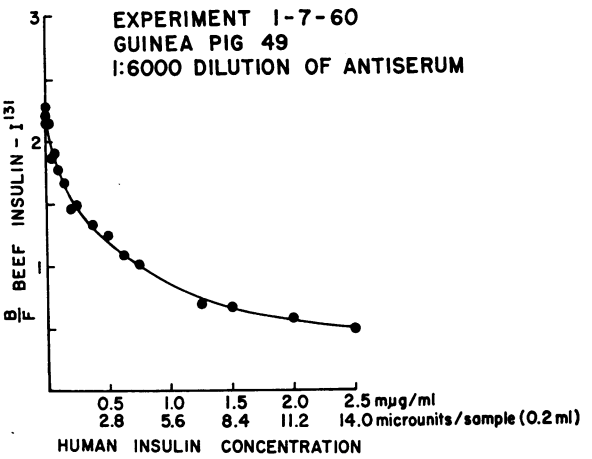

FIG. 2. B: RATIO, $\frac{\text{BOUND INSULIN-I}^{131}}{\text{FREE INSULIN-I}^{131}}$, AS A FUNCTION OF THE CONCENTRATION OF ADDED HUMAN INSULIN. The ratios were obtained from the complete series of radiochromato-electrophoretograms, a few of which are shown in Figure 2A.

All subjects were to have fasted for 14 hours prior to the glucose tolerance test, but from the fasting blood sugar concentration in one subject (Ri) it is suspected that this restriction was not observed in his case. All subjects were to have consumed a diet containing at least 300 g carbohydrate per day for 3 days preceding the glucose tolerance test, but there is no assurance that this regimen was followed in all cases. Blood samples were obtained in the fasting state immediately before, and 0.5 hour, 1 hour and 2 hours following glucose feeding. In a small group of cases an additional 50 g glucose was administered at 1.5, 2 and 2.5 hours, and blood collections were continued to 3 hours.

Blood sugar determinations were determined according to the method of Somogyi (27).

RESULTS

Standard curves. Several representative standard curves are shown in Figure 3. The amount of insulin-I^{131} employed as tracer varied somewhat from experiment to experiment. In the experiments shown in Figure 4, the effects of Tietze and Fisher insulins are compared with each other and with the effect of crystalline beef insulin. As in other experiments no significant differences between the two human insulin preparations were observed. Since 100 to 200 μl of solution was as-

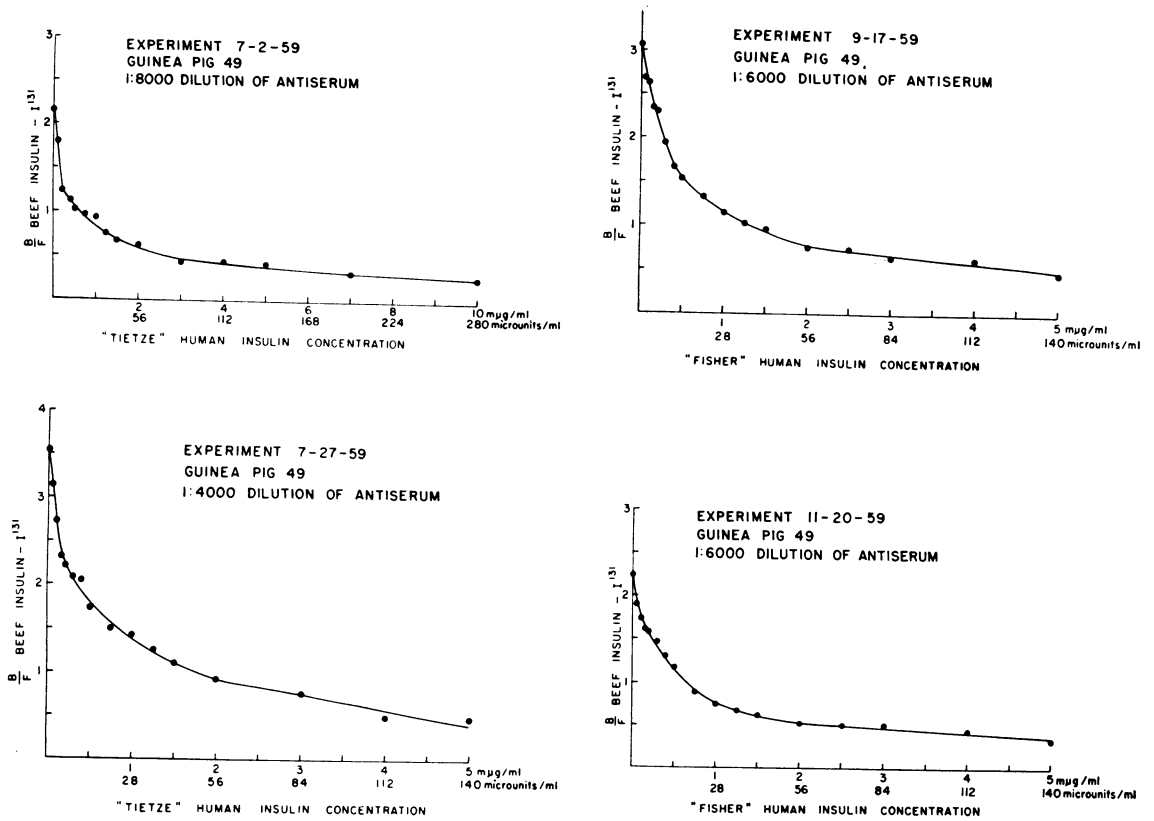

FIG. 3. STANDARD CURVES: B/F (BEEF INSULIN-I^{131}) RATIO AS A FUNCTION OF THE CONCENTRATION OF TIETZE OR FISHER HUMAN INSULIN.

sayed, less than 1 μU of human insulin was readily detectable with this antiserum. At low insulin concentrations, random variations in B/F produce only small errors in the absolute quantity of insulin but the percentage error is high; conversely, at high insulin concentrations the absolute error is likely to be higher but the percentage error lower. By increasing the dilution of the antiserum, the entire concentration range is easily scaled down by a factor of 2 or 3 and the limit of sensitivity increased to about 0.1 to 0.2 μU of insulin. However, the conditions employed are suitable for determination over the 50- to 100-fold range of insulin concentrations ordinarily encountered in man.

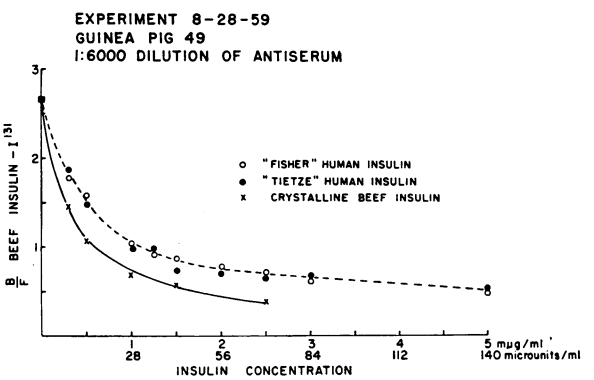

FIG. 4. COMPARISON OF THE EFFECTIVENESS OF VARIOUS CONCENTRATIONS OF TIETZE AND FISHER HUMAN INSULINS AND CRYSTALLINE BEEF INSULIN IN REDUCING THE B/F RATIO FOR BEEF INSULIN-I^{131}.

It is evident that beef insulin reacts about two to four times more strongly (depending on the insulin concentration employed) with the guinea pig antibeef insulin serum than does human insulin (Figure 4). Other guinea pig antisera to beef insulin have shown even greater differences in reaction of beef insulin and human insulin. On this account *beef insulin cannot be used as a standard for the assay of human insulin in the guinea pig antibeef insulin system.* Because of the differences in reactivity of human and beef insulin, differences in the specific activity of the beef insulin-I^{131} preparations result in different

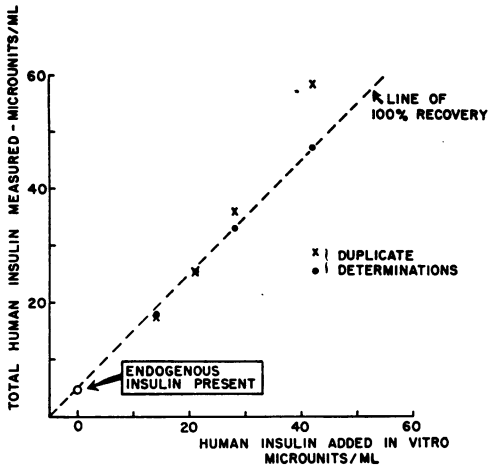

FIG. 5. A: RECOVERY OF HUMAN INSULIN ADDED *IN VITRO* TO A FASTING PLASMA SAMPLE. Endogenous insulin concentration in undiluted plasma was 48 μU per ml. All assays were performed in 1:10 dilution of plasma.

initial B/F values and somewhat differently shaped curves even at the same dilution of antiserum if approximately the same radioactivity (and therefore different amounts of beef insulin) is used. These differences could be abolished if each lot of beef insulin-I^{131} were assayed for its beef insulin concentration and if the same amount of beef insulin were employed, independent of its content of radioactivity. However, it is more expedient to include a standard curve with human insulin for each run of unknowns. When 250 or more unknown samples have been run in a single

TABLE I

*Effect of cysteine on endogenous plasma insulin**

Subj.	Plasma sample	Insulin concentration: Original plasma before incubation and dialysis	Control sample incubated and dialyzed without cysteine	Sample incubated with cysteine and dialyzed
		μU/ml	*μU/ml*	*μU/ml*
Yo.	1 hr	324	238	14
Un.	1 hr	337	216	0

* See text for conditions of experiments.

experiment, an added set of 15 to 16 standard solutions is a negligible addition.

Recovery of added human insulin and effect of plasma dilution. The virtually quantitative recovery of human insulin added to plasma *in vitro* (Figure 5A) indicates that the plasma has neither an inhibitory nor an augmentative effect and this conclusion is confirmed by the proportionate decrease in measured insulin concentration when the plasma is diluted over a large range (Figure 5B).

Effect of cysteine and cellulose on endogenous plasma insulin. Since insulin is destroyed by incubation with cysteine at alkaline pH and is adsorbed by powdered cellulose, the effects of these agents on endogenous insulin were tested. Plasmas of relatively high insulin concentration were incubated at 37° C with 0.02 M cysteine at pH 8 for 1.5 hours and then dialyzed against normal saline for 3 hours to remove the cysteine. Aliquots of the same serum samples were treated

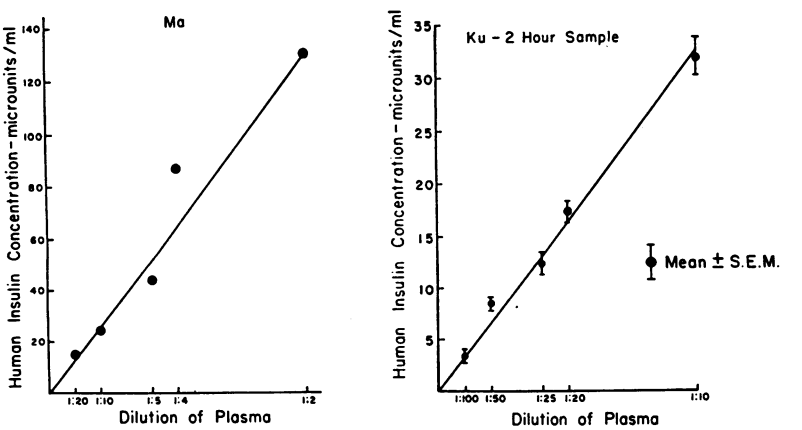

FIG. 5. B: EFFECT OF DILUTION OF PLASMA ON MEASURED CONCENTRATION OF ENDOGENOUS PLASMA INSULIN. Four replicate determinations were made for each point in the experiment on the right.

TABLE II

Cellulose adsorption of beef insulin-I^{131} and endogenous human plasma insulin

Subj.	Plasma	Per cent adsorbed by cellulose column: Beef insulin-I^{131}	Per cent adsorbed by cellulose column: Endogenous human insulin
	ml		
Y.	0.1	69	64
	0.2	49	44
U.	0.1	71	88
E.	0.1	81	84

similarly except that cysteine was omitted. Although incubation and dialysis alone led to a 26 to 36 per cent loss in endogenous insulin concentration in the control samples, cysteine was almost completely effective in destroying the endogenous insulin (Table I). In simultaneous experiments insulin-I^{131} was found to be virtually completely destroyed under these conditions as determined by paper chromato-electrophoresis.

To evaluate cellulose adsorption of endogenous insulin, a minute amount of tracer beef insulin-I^{131}, negligible compared to the amounts used in the immunoassay, was added to 0.1 or 0.2 ml plasma which was then passed through a packed powdered cellulose column (about 0.5 ml in volume) and eluted, by suction, with veronal buffer to recover all the plasma. Assay of the radioactivity remaining on the column and that eluted from the column, and immunoassay of endogenous insulin eluted from the column revealed that approximately the same fractions of endogenous insulin and added beef insulin-I^{131} were adsorbed by the cellulose (Table II). The larger the amount of plasma per unit volume of cellulose the less the fraction of insulin that was adsorbed. With significantly smaller amounts of plasma virtually all insulin-I^{131} and endogenous insulin are adsorbed, but the insulin concentrations then become unmeasurable. Only negligible fractions of albumin-I^{131} and γ-globulin-I^{131} are adsorbed by cellulose under these conditions.

Insulin concentrations in early maturity-onset diabetic and control subjects. The average fasting insulin concentrations tended to be only slightly

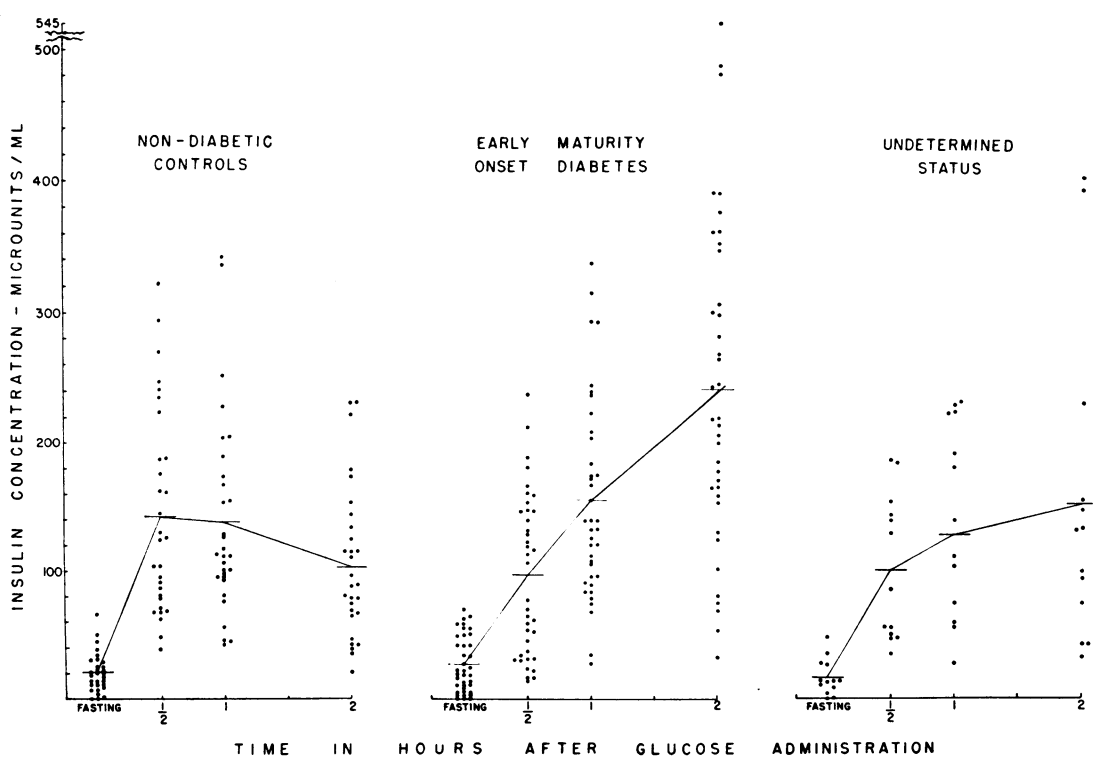

FIG. 6. PLASMA INSULIN CONCENTRATIONS DURING STANDARD 100 G (P.O.) GLUCOSE TOLERANCE TEST IN VARIOUS GROUPS OF SUBJECTS.

TABLE III

Blood sugar and plasma insulin concentrations during a standard 100 g oral glucose tolerance test

Subj.	Blood sugars						Plasma insulin concentrations					
	F	0.5 hr	1 hr	2 hrs	3 hrs	4 hrs	F	0.5 hr	1 hr	2 hrs	3 hrs	4 hrs
	mg/100 ml						*μU/ml*					
A. *Nondiabetic controls*												
La.	87	122	78	91			31	162	112	89		
Pa.	73	109	68	77			25	270	154	155		
Hu. J.	85	120	113	113			7	103	95	116		
Wh.	78	110	85	88			66	294	168	174		
Don.	88	108	135	115			45	67	98	39		
St.	89	120	104	93			13	95	46	43		
Kan.	83	113	103	83			28	62	229	117		
Ra.	91	131	135	90			20	176	128	112		
Jo.	92	113	113	80			25	124	118	21		
Wa.	98	128	147	118			20	68	93	75		
Dor.	90	140	148	118			31	81	126	90		
Ei.	83	100	70	95			50	235	101	79		
Cor.	110	133	133	115			21	130	155	145		
Cal.	91	117	147	119			7	39	76	47		
Te.	83	143	155	73			17	78	190	126		
Ru.	83	103	113	98			3	145	204	222		
Dam.	90	118	120	110			18	187	205	180		
Kas.	98	150	135	108			11	48	112	65		
Kr.	98	115	125	120			2	71	95	117		
Him.	93	140	120	108			22	322	252	232		
Hu. J. J.	95	128	110	105			9	126	56			
Dan.	83	115	73	100			0	224	18	98		
Sc.	83	158	140	113			22	91	114	135		
Un.	88	133	153	115			0	247	337	233		
Ke.	103	138	128	112			34	163	174	67		
Hig.	100	148	88	120			14	241	42	70		
Con.	90	133	135	115			11	104	45	36		
Pop.	93	148	143	95			39	67	101	79		
Ry.	90	143	150	110			11	84	107	81		
Al.	96	296	134	44			14	188	342	42		
						Mean	21	143	139	106		
B. *Early maturity-onset diabetes*												
Ri.	245	346	436	472			51	123	173	179		
Wa. D.	96	178	218	150			6	32	97	166		
Mor.	93	110	180	218			3	77	158	300		
Moh.	95	145	193	135			59	162	339	355		
Ko.	93	240	360	120			3	14	294	364		
Fl.	138	173	248	266			56	70	190	270		
Fel.	100	152	190	141			19	107	121	102		
Fr.	113	238	310	195			35	113	316	378		
Sh.	118	232	300	190			13	38	175	76		
Go.	100	146	218	173			22	54	156	216		
Bl.	114	168	236	223			19	59	108	221		
Ma.	163	240	256	320			51	46	35	54		
We.	96	178	218	150			6	32	97	166		
Qu.	93	155	180	178			56	120	121	283		
Ok.	143	202	244	204			11	24	79	160		
Ha.	110	155	193	200			50	154	185	482		
Cr.	92	200	245	225			0	31	75	70		
Mi.	105	180	211	170			25	117	112	187		
Ro.	113	177	233	240			0	182	224	266		
Wo.	100	163	170	180			42	160	168	207		
Poi.	103	188	243	190			5	17	83	173		
Fo. F.	152	244	266	380			11	22	28	23		
No.	105	195	215	185	91	83	59	190	238	392	159	59
Ny.	93	158	193	138	60		11	53	90	154	17	
Moo.	113	153	205	268			63	148	91	131		
Fo. J.	90	169	193	185	83		34	238	249	490	173	
Br.	90	167	198	163	86	67	20	62	133	201	79	29
Pl.	100	183	193	160	63		42	168	241	308	191	
Mu.	130	233	326	374			3	17	68	126		
Fla.	93	140	170	178			22	140	126	220		

TABLE III—*Continued*

Subj.	Blood sugars F	0.5 hr	1 hr	2 hrs	3 hrs	4 hrs	Plasma insulin concentrations F	0.5 hr	1 hr	2 hrs	3 hrs	4 hrs
	mg/100 ml						*μU/ml*					
B. *Early maturity-onset diabetes*—Continued												
Car.	108	165	238	183	90		42	34	106	247	106	
How.	125	198	223	250			14	132	140	245		
Coo.	95	145	163	178	128		28	213	205	350	233	
Fele.	100	193	266	235			0	31	84	81		
Le.	90	173	250	193			65	65	294	392		
Ga.	105	148	178	135			70	148	210	302		
Hor.	95	165	147	195	211		8	148	140	364	386	
Eh.	91	156	211	309	246		17	129	176	545	531	
						Mean	27	97	156	243		
C. *Undetermined status*												
Wa. D.	85	100	150	148			0	35	56	42		
Wr.	98	123	158	138			27	86	75	75		
Ric.	100	138	158	143			13	140	226	100		
Ba.	88	140	150	129			14	144	230	405		
Har.	88	148	178	140			36	187	232	134		
Hew.	83	145	163	105			4	56	64	94		
Jos.	95	147	171	136			49	130	192	157		
Leh.	100	152	162	145			14	186	224	395		
Wis.	103	150	158	128			0	155	182	148		
Doh.	70	110	155	128			8	48	28	33		
Lut.	100	118	160	130			28	51	112	132		
Sa.	100	170	143	110			11	48	140	42		
Wi.	90	123	164	118			14	56	104	233		
						Mean	17	101	128	153		
D. *Decompensated cirrhosis*												
Man.	96	164	146	136			54	240	356	486		
Mar.	92	168	150	100			8	140	226	143		
Cara.	95	143	140	93			14	40	57	30		
Cro.	85	143	118	85			2	16	14	8		
Di.	78	100	82	65			25	33	19	5		
Fo.	80	135	100	80			5	36	22	8		
E. *Pituitary tumors*												
Sil.*	92	122	147	134			32	350	570	175		
Sin.†	90	135	140	117			5	177	192	180		
Led. (Acromegaly)	93	163	174	103			8	156	203	109		
F. *Thyrotoxicosis*												
El.	103	215	240	210			38	275	230	240		
Yo.	93	170	184	103			65	247	324	81		
G. *Others*												
Ku. (Hemochromatosis)	73	135	175	160			48	121	321	330		
Cra. (Acute pancreatitis)	100	120	138	125			5	20	100	135		
Bl.‡	48	108	148	135			28	90	56	67		
Coh.§	36	52	78	76			118	190	199	98		

* Chromophobe adenoma.
† Eosinophilic and chromophobe adenoma with acromegaly.
‡ Hypoglycemia, cause undetermined, after partial pancreatectomy.
§ Proven islet cell adenoma (courtesy of Dr. H. Epstein).

higher in the diabetic (mean, 27 μU per ml) than in the nondiabetic (mean, 21 μU per ml) subjects, although 34 per cent of the diabetics exceeded 40 μU per ml in contrast to only 10 per cent of the nondiabetics. In none of the 68 patients in both of these groups did the fasting level exceed 70 μU per ml (Table III, A and B, Figure 6). These values are in good agreement with those reported

earlier in a smaller series of subjects (15). The responses to orally administered glucose in diabetic and nondiabetic patients differed more markedly than did the fasting insulin concentrations. Nondiabetic subjects were about equally divided in showing the peak insulin concentration at 0.5 hour or 1 hour (Table IIIA), whereas with few definite exceptions diabetic patients showed the maximal insulin concentration at 2 hours (Table IIIB). The average insulin concentration at 0.5 hour was lower in diabetic (mean, 97 μU per ml) than in nondiabetic (mean, 143 μU per ml) subjects, but the diabetics appeared to form two groups at this point (Figure 6). A delayed insulin response is suggestive in the lower of these two groups.

Although there is a large scatter of individual values, the mean curves for the two groups illustrate these differences clearly (Figure 6). The average integrated insulin concentration during the 2 hour glucose tolerance test was 26 per cent higher for the diabetic (147 μU per ml) than for the nondiabetic (117 μU per ml) group. The "undetermined" group (Table IIIC) probably represents a mixture of early diabetic and nondiabetic subjects and nothing can be concluded definitely about the variable insulin response to glucose loading.

Insulin concentrations in four diabetic and five nondiabetic subjects given an additional 50 g of glucose at half hour intervals from 1.5 to 2.5 hours are shown in Figure 7 and Table IV. Insulin concentrations rose to higher levels in both groups but more marked increases were observed in the diabetic subjects.

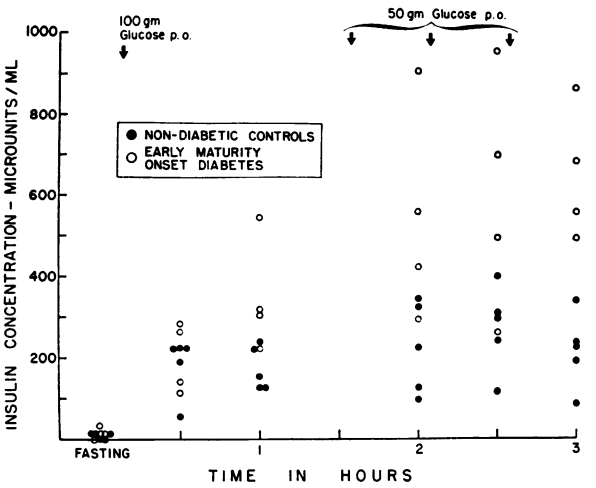

FIG. 7. PLASMA INSULIN CONCENTRATIONS DURING HEAVY GLUCOSE LOADING EXPERIMENTS IN DIABETIC AND NONDIABETIC SUBJECTS.

It should be emphasized that insulin-I^{131} when administered intravenously exhibits a rapid fall in concentration due to a marked and continuous increase in its apparent volume of distribution for a period of about 30 to 60 minutes and to a metabolic turnover rate with a half-time of about 35 minutes (22). It may be reasonably expected that endogenously secreted insulin behaves similarly,[5] and therefore that any particular peak concentra-

[5] Endogenously secreted insulin is, in addition, subject to removal by the liver before it reaches the peripheral circulation (28, 29).

TABLE IV

Effect of heavy glucose loading on blood sugars and plasma insulin levels*

Subj.	Blood sugars						Plasma insulin concentrations					
	F	0.5 hr	1 hr	2 hrs	2.5 hrs	3 hrs	F	0.5 hr	1 hr	2 hrs	2.5 hrs	3 hrs
	mg/100 ml						*μU/ml*					
Nondiabetic controls												
Hea.	84	136	106	130	108	110	3	190	129	345	308	190
Gas.	78	118	86	108	104	98	14	224	129	322	300	341
Rei.	90	127	129	105	103	101	15	224	224	125	400	225
Keh.	94	158	142	98	90	86	0	224	238	224	241	235
McC.	100	144	150	104	102	90	17	56	151	98	118	84
Maturity-onset diabetes												
Cri.	100	188	214	208	188	162	17	265	548	910	960	685
All.	86	140	152	166	126	122	12	284	223	560	496	496
Ab.	90	143	170	167	177	155	31	112	309	420	700	870
Ti.	86	170	181	155	149	145	3	140	313	294	255	578

* Glucose 100 g p.o., immediately after fasting specimen; glucose, 50 g p. o., at 1.5, 2 and 2.5 hours.

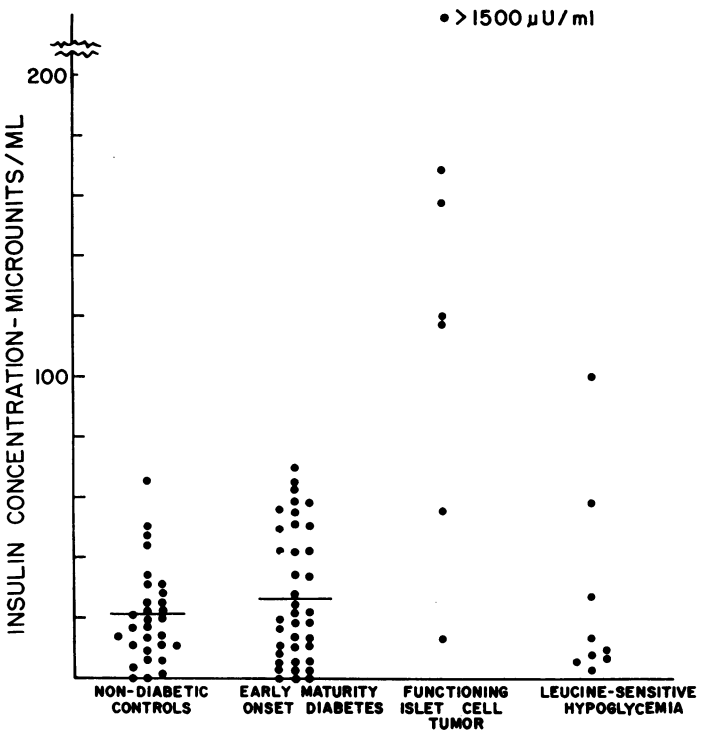

FIG. 8. FASTING PLASMA INSULIN CONCENTRATIONS IN VARIOUS GROUPS OF SUBJECTS. The subject with plasma insulin concentration greater than 1,500 μU per ml had an islet cell adenocarcinoma with widespread metastases (patient of Dr. J. Field).

tion depends on the precise moment of sampling. A very rapid and pronounced fall from the peak concentration would be anticipated in the case of a single secretory spurt. Conversely, a sustained elevation or continued rise in insulin concentration implies a continued secretion during the time interval under observation.

Insulin concentrations in patients with islet cell tumors or leucine-induced hypoglycemia. Insulin concentrations in fasting plasmas from five of seven patients [6] with proven islet cell tumors were elevated above normal levels (Figure 8), but the response to glucose was normal in the one patient studied during a glucose tolerance test (Coh., Table IIIG).

Four of six subjects [7] with leucine-induced hypoglycemia showed increased insulin concentrations following administration of L-leucine (75 to 150 mg per kg) in six of nine experiments (Figure 9), although fasting insulin concentrations were elevated in only a single patient (Figure 8), the only adult in the series and the one patient suspected on clinical grounds to have an islet cell tumor.[8] The peaks of insulin concentration, when observed, were in good time correspondence with the induced hypoglycemia.

Plasma insulin in cirrhosis, acromegaly and hyperthyroidism. Six patients with decompensated cirrhosis were studied (Table IIID). In two cases the glucose curves were high, but not within the diabetic range, and were associated with relatively high insulin concentrations. In three cases insulin concentrations were very low throughout the 2 hour glucose tolerance test, and in two of these the glucose concentration curves were quite flat. Intravenous glucose tolerance tests are necessary before it can be decided whether the observed association in the latter cases is to be at-

[6] We are indebted to Doctors H. Epstein, J. Field, E. D. Furth, E. Gordon, A. Renold and J. Steinke for these sera.

[7] We are indebted to Doctors A. DiGeorge, M. Goldner, M. Grumbach, I. Rosenthal and S. Weisenfeld for these sera.

[8] Courtesy of Doctors S. Weisenfeld and M. Goldner.

tributed to poor glucose absorption or to heightened insulin sensitivity.

In three patients with pituitary tumors, two of whom had clinical acromegaly, and in two thyrotoxic subjects, insulin concentrations during the glucose tolerance test were in the high normal range (Table III, E and F).

Results in a few individual cases that do not fall into the other categories are also included in Table IIIG.

In the absence of glucose loading, plasma insulin concentrations did not change significantly in two control subjects (Fra. and Gre., Table V).

In seven cases, sera were refrozen and repeat determinations were performed one or more months later with a different lot of insulin-I^{131}. The reproducibility of determinations performed under these conditions is shown in Figure 10.

TABLE V

*Blood sugar and plasma insulin concentrations in the absence of glucose loading**

Nondiabetic controls	Time	Blood sugar	Plasma insulin concentrations
	min	*mg/100 ml*	*μU/ml*
Gre.	0	93	17
	20	90	19
	40	90	18
	60	88	18
	120	78	25
Fra.	0	85	3
	20	85	5
	40	80	5
	60	85	3
	120	88	3

* Subjects were fasted overnight and throughout the period of blood sampling.

DISCUSSION

The demonstration that unlabeled insulin could displace insulin-I^{131} from complexes with insulin-binding antibody (22) and that the fraction of insulin-I^{131} bound to antibody decreases progressively with increase in insulin concentration (22) laid the foundation for the immunoassay of insulin employing isotopically labeled insulin. In initial reports describing results with the present method for immunoassay of beef insulin (9, 10) it was emphasized that species differences in the reaction of insulin with insulin antisera exist and that

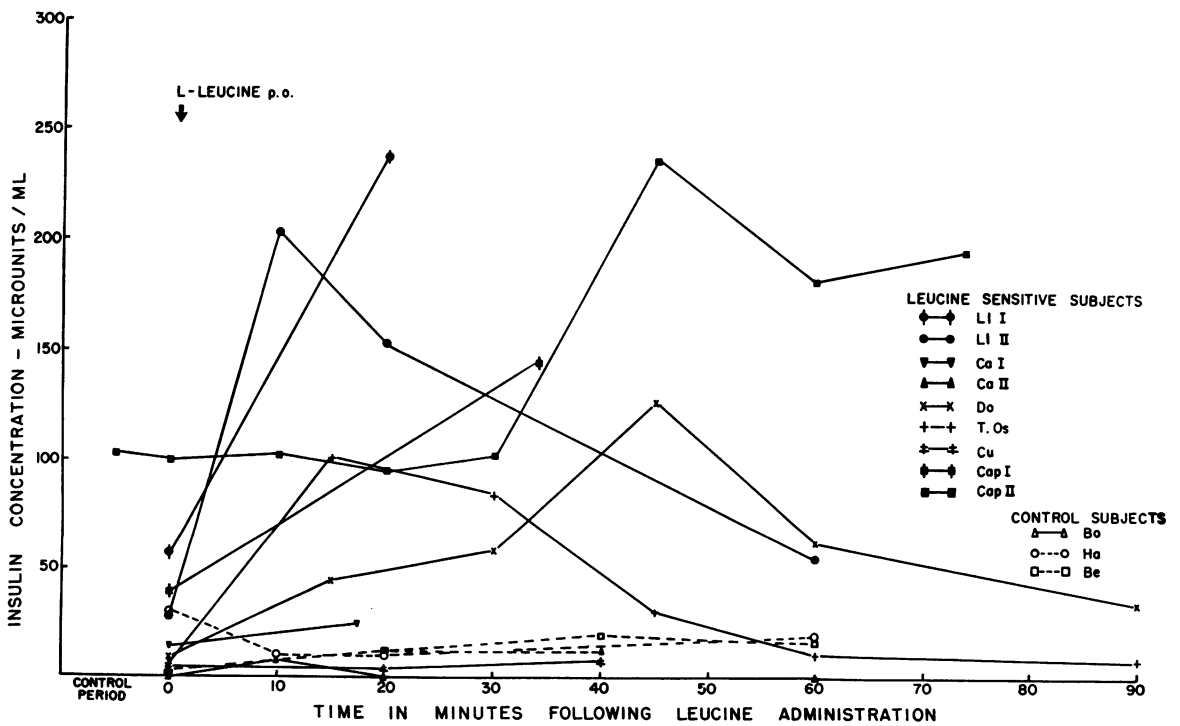

FIG. 9. PLASMA INSULIN CONCENTRATIONS FOLLOWING ADMINISTRATION OF L-LEUCINE TO CONTROL AND LEUCINE-SENSITIVE HYPOGLYCEMIC SUBJECTS.

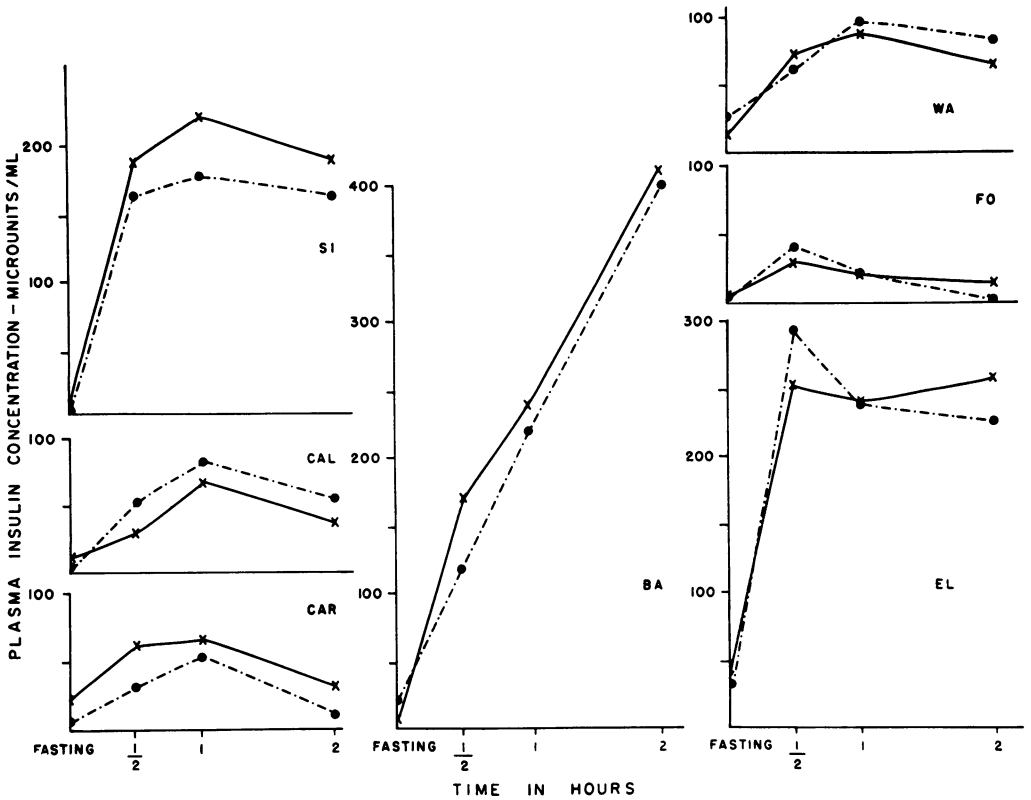

FIG. 10. REPRODUCIBILITY OF PLASMA INSULIN DETERMINATIONS ON THE SAME PLASMA SAMPLES PERFORMED ONE OR MORE MONTHS APART WITH DIFFERENT LOTS OF INSULIN-I^{131}. Plasmas were stored frozen between determinations. In these cases, the average values of the two determinations are presented in Table III.

human antibeef, pork insulin serum is useful for microassay of animal insulins (10). However, the human antisera react too weakly with human insulin (7, 8, 11) to serve as a basis for assay of the latter hormone in plasma. Fortunately, however, the serum of guinea pigs immunized with beef insulin was reported to react sufficiently strongly with human insulin for purposes of assay (11, 14) and this finding has recently been confirmed by Grodsky and Forsham (30). The latter workers have employed a salt fractionation technique that produces a partial separation of antibody-bound insulin-I^{131} from free insulin-I^{131} and have measured insulin concentrations in extracts of plasma. However, since, in the assays of Grodsky and Forsham, human insulin was assumed to react quantitatively like beef insulin, which is not valid for guinea pig antibeef insulin serum, their absolute values for human plasma insulin concentrations are questionable. Furthermore, since the *relative* degree to which human insulin and beef insulin react at different insulin concentrations varies several-fold, even relative values obtained with beef insulin as a standard are subject to large errors. Grodsky and Forsham (30) were unable to detect insulin in most fasting plasmas and reported mean values of 31 μU per ml after glucose loading in five patients. These values are very much smaller than those reported earlier by us (14) or those of the much larger series presented here. However, a significant underestimate of human insulin concentration is precisely the anticipated consequence of the invalid assumption of equal reactivity of beef and human insulins in the guinea pig antibeef insulin system.

To our knowledge there have been only two other immunologic methods employed for the assay of insulin. Arquilla and Stavitsky (31) developed an assay for insulin based on the inhibi-

tion of hemolysis of insulin-sensitized red blood cells; however, the lower limit of detectability by this technique was approximately 0.1 μg (2.8 mU) making it unsuitable for determination of plasma insulin. Loveless (32) has used certain normal human subjects, in whom the skin can be locally sensitized to insulin (by the intracutaneous injection of human anti-insulin serum) to assay insulin by the whealing response obtained. Aside from the inconvenience associated with this method, the lower limit of detectability was 200 μU beef insulin per ml and human plasma insulin was not detectable, a result attributed in part to the lesser reactivity of human insulin (32).

Reported estimates of plasma insulin concentrations, derived from the various biological assay procedures, have varied widely. Thus, the *in vitro* diaphragm assay has yielded values ranging from 40 to 80 μU per ml (33) to as high as 4,600 μU per ml (34) in fasting plasmas and from about 130 to 800 μU per ml (33) to 9,000 to 22,000 μU per ml (35) after glucose in normal subjects. Measuring the increase in oxidation of glucose-1-C^{14} to $C^{14}O_2$ by rat epididymal adipose tissue *in vitro,* Martin, Renold and Dagenais (3) found that the insulin-like activity of fasting normal plasma in this preparation corresponded to 50 to 350 μU of insulin per ml. Pfeiffer, Pfeiffer, Ditschuneit and Ahn (36), using the same assay, found that plasma diluted 1:2 gave higher and more consistent insulin concentrations and reported normal fasting levels of 135 to 680 μU per ml in 15 normal human subjects, with concentrations frequently exceeding 2,000 to 4,000 μU per ml after tolbutamide and metahexamide. Employing the immunoassay method we have observed generally much smaller increases in peripheral insulin concentration after large doses of sodium tolbutamide, administered intravenously or by mouth, than after glucose given by the same routes to normal or diabetic men (37).

It is generally agreed (5, 34) that dilution of plasma or serum increases markedly the estimated insulin concentration in the diaphragm assay and similar observations have been made in the rat epididymal fat pad assay (36). This phenomenon has been either attributed to the presence of inhibitory substances in the plasma (5, 34) or interpreted as indicating that insulin-like activity of serum as measured by the isolated rat diaphragm is not specific for insulin per se. Randle has found that albumin and other proteins may exert a nonspecific stimulation of glucose uptake by rat diaphragm (5) and that 1 ml of plasma exhibiting an insulin concentration of 13,000 μU per ml in the diaphragm assay had no effect on blood sugar when injected into alloxan-diabetic hypophysectomized rats, whereas 2,000 μU insulin produced a marked hypoglycemia (35).

The recent report by Leonards (6), that insulin-like activity in plasma, when tested on the rat epididymal fat pad, persists after total pancreatectomy and that insulin-neutralizing antiserum from guinea pigs has no inhibitory effect on the insulin-like activity of human serum in this system, has raised a serious question as to what part of the insulin-like effect on fat tissue is due to insulin itself.

In vivo insulin assays have also yielded variable estimates of plasma insulin concentration. Measuring the fall in blood sugar induced in adrenodemedullated alloxan-diabetic hypophysectomized rats, Anderson, Lindner and Sutton (38) were unable to detect circulating insulin in fasting plasma although the method was sensitive to 125 μU insulin. Bornstein and Lawrence (1) using adrenalectomized hypophysectomized diabetic rats reported plasma insulin concentrations 2 hours after glucose to average about 340 μU per ml in normal subjects and 100 to 320 μU per ml in diabetic patients not subject to ketosis, but Randle (5) was unable to confirm the suitability of these animals for insulin assay. More recently, Baird and Bornstein (39), employing adrenalectomized alloxan-diabetic mice, have found that normal fasting plasma extracted with acid ethanol-*n*-butanol-toluene (which is thought to separate insulin from insulin antagonists) contains about 1,000 μU per ml. Values about three times as high were observed after glucose feeding. Values as high or higher were found in four of six diabetic subjects.

At the present time it does not appear possible to resolve all the apparently divergent findings summarized here. It is necessary, however, to point out that plasma insulin concentrations determined by the immunoassay technique are in agreement with the lowest estimates derived from other methods of assay, notably the *in vivo* bioassays of Bornstein and Lawrence (1), and of Anderson

and co-workers (38), and the diaphragm assay of Vallance-Owen and Hurlock (33). By comparison with the biological effects of exogenous insulin the lower concentrations appear most reasonable.

A rough estimate of the amount of insulin secreted can be derived from the insulin concentrations reported here according to the following considerations. From the area under the mean insulin curve in nondiabetic subjects it is found that the average insulin concentration during the 2 hour period following glucose administration was 117 μU per ml. It has been shown previously (22) that I^{131}-labeled insulin in man is metabolized at a rate of about 2 per cent per minute and is distributed into an apparent volume of distribution of about 37 per cent of body weight in about 45 minutes, distribution being about half completed at 15 minutes. If we now assume that the distribution and metabolism of endogenous insulin that reaches the peripheral circulation is similar quantitatively to that of exogenous insulin [9] and make the conservative estimate that, on the average, the endogenous insulin was distributed in a volume corresponding to 30 per cent of body weight (21 L) over the 2 hour period, we can calculate that 0.117 U per L $\times$ 21 L was being degraded at the rate of 2 per cent per minute during these 120 minutes. This computation leads to the estimate that approximately 6 U of insulin reached the peripheral circulation during the 2 hour glucose tolerance test. Madison, Combes, Unger and Kaplan (28) have found that approximately 50 per cent of insulin given into the portal vein is removed from the circulation during its first passage through the liver, and this value is in good agreement with the figure of 40 per cent given by Mortimore and Tietze in the rat (29). If we accept the 50 per cent value for the liver of man, it can be concluded that an average of about 12 U of insulin was secreted during the glucose tolerance test in nondiabetic subjects. This is what might be expected in each of three feedings per day. If, also, there is added into the calculation (on the same basis) the amount of insulin necessary to maintain a fasting level of 0.021 U per L for the other 18 hours, we arrive at the estimate of 36 U (postprandial secretion) plus 19 U (fasting secretion) = 55 U for the average total insulin secretion per day in nondiabetic subjects.[10] Since, even at the end of the 2 hour glucose tolerance test the insulin concentration was still above fasting levels, calculations over a more extended time period would yield even slightly higher estimates. These figures are certainly consistent with the insulin requirement of 30 to 40 U daily in totally depancreatized human subjects (40), since exogenously administered insulin does not experience the initial hepatic removal to which endogenously secreted insulin is subjected.

In order to resolve the much higher estimates of plasma insulin concentration given by Willebrands, van der Geld and Groen (34), Randle (35), and Pfeiffer and associates (36) with these considerations, we must assume either that the turnover rate of endogenous insulin is very much slower than that of exogenously administered insulin (in which event it is difficult to understand why human subjects do not remain in prolonged jeopardy of hypoglycemia from the high insulin concentrations that follow glucose administration) or that endogenous insulin is confined almost exclusively to the plasma. Even if the latter alternative (which also is in strong conflict with the results on distribution of exogenous insulin) were true, a fasting level as high as 4,600 μU per ml (34) would mean that there is almost 14 U of insulin in the circulation of fasting human subjects, a conclusion which is still difficult to accept. Randle's (35) values of 9,000 to 22,000 μU per ml in normal plasma 2.5 hours following glucose would mean a total of 27 to 66 U in plasma alone, neglecting insulin in extravascular space, at a time when the blood sugar is usually at a normal level. However, as already noted, Randle has indicated his conviction that this "insulin-like" activity is not due entirely to insulin alone (5).

To return now to the results of the present study, it will be noted that the high insulin con-

[9] It has been established that exogenous unlabeled crystalline beef insulin and I^{131}-labeled crystalline beef insulin show virtually identical plasma disappearance curves in the rabbit (10). Furthermore, the precipitous fall in insulin concentration from peak levels, observed in many patients of the present study (Table IIIA), even when insulin secretion may be presumed to be continuing, is evidence that endogenous insulin also is rapidly removed from the circulation.

[10] These calculations ignore any increase in insulin secretion that would result from small feedings between meals.

centrations observed in diabetic subjects during the glucose tolerance test are not inconsistent with the less extensive data of Bornstein and Lawrence (1) and Baird and Bornstein (39). Very recently Seltzer and Smith (41), employing the rat diaphragm assay of Vallance-Owen and Hurlock (33), have reported insulin concentrations one hour after glucose, in tolbutamide-sensitive adult diabetics, almost in the normal range, but significantly lower values were observed in juvenile diabetics and adult tolbutamide-insensitive diabetics. To resolve the present finding of a higher than normal integrated insulin output in diabetics during the glucose tolerance test with sustained hyperglycemia in these patients, it must be concluded that the tissues of the maturity-onset diabetic do not respond to his insulin as well as the tissues of the nondiabetic subject respond to his insulin. However, from these observations it cannot be concluded that the early diabetic has the same maximal potential insulin output as the nondiabetic, since in the latter the return of blood sugar to normal levels does not allow for the continued stimulus of prolonged hyperglycemia as in the diabetic. The attempt to produce a sustained stimulus to insulin secretion by repeated administration of glucose to a total of 250 g did result in a more marked insulin secretion in nondiabetic subjects. However, the response of diabetics was still greater indicating that their insulin reserve is not depleted during the 100 g glucose tolerance test. The experiments failed, however, to test maximal insulin secretory capacity of the nondiabetic subjects since a sustained hyperglycemia was not achieved in these patients.

Appreciation of the lack of responsiveness of blood sugar, in the face of apparently adequate amounts of insulin secreted by early maturity-onset diabetic subjects, is obviously of importance in the interpretation of the pathogenesis of this type of diabetes. However, the data at hand can only indicate that absolute insulin deficiency per se is not the cause of the hyperglycemia and suggest other possibilities that merit investigation, namely, *1*) abnormal tissues with a high threshold for the action of insulin; *2*) an abnormal insulin that acts poorly with respect to hormonal activity *in vivo* but reacts well immunologically *in vitro; 3*) an abnormally rapid inactivation of hormonally active sites [a suggestion in accord with the ideas expressed by Mirsky (42)] but not of immunologically active sites on the insulin molecules; and *4*) the presence of insulin antagonists. The last suggestion has been made many times by previous workers. A joint attack on the problem, utilizing both the specific immunoassay for plasma insulin and an assay method that measures the net biological effect of insulin and its inhibitors would seem to be indicated.

The high fasting insulin concentrations observed in hypoglycemia associated with functioning islet cell tumors are not unexpected. However, the normal response to glucose in the one patient studied suggests that the insulin-producing tumor may be secreting insulin continuously or sporadically but that it is not stimulated specifically by hyperglycemia. The failure to detect high plasma concentrations of insulin in two cases can possibly be explained by the normally rapid turnover of insulin and the sampling at a time when insulin production by the tumor had been quiescent for an hour or two previously.

Leucine-induced hypoglycemia in children with idiopathic hypoglycemia was first reported by Cochrane, Payne, Simpkiss and Woolf (43) but a satisfactory interpretation of the disturbance has not been given. From the results of the present study it appears that leucine serves as an abnormal stimulus to insulin secretion in these subjects but may also have other effects. Most of the patients whose sera were assayed here have been studied in detail in other respects as well by the various investigators who supplied the sera and are to be reported by them individually.

SUMMARY AND CONCLUSION

1. An immunoassay for plasma insulin in man is presented, based on the reaction of human insulin, competing with beef insulin-I^{131}, with insulin-binding antibodies in the sera of guinea pigs immunized with beef insulin. The method is sensitive to less than 1 μU of insulin, permitting measurement of insulin concentrations in 10 to 20 μl of plasma.

2. Human insulin added *in vitro* to plasma is recovered quantitatively, and measured endogenous insulin concentrations decrease proportionately on dilution of plasma over the range 1:2 to 1:100.

3. Endogenous plasma insulin is destroyed by incubation with cysteine and endogenous insulin adsorption by cellulose columns is quantitatively similar to the adsorption of added beef insulin-I^{131}.

4. Repeat determinations of insulin concentrations on the same plasma samples (stored frozen in the interim) one or more months apart, with different lots of insulin-I^{131}, were generally in good agreement.

5. Fasting plasma insulin concentrations in early maturity-onset diabetic patients who had never been treated with insulin, (mean, 27 μU per ml) and in nondiabetic subjects (mean, 21 μU per ml) did not differ markedly. Following 100 g of glucose by mouth, nondiabetic subjects usually showed peak insulin concentrations at 0.5 hour (mean, 143 μU per ml) or 1 hour (mean, 139 μU per ml) and a decline by 2 hours (mean, 106 μU per ml). In contrast, insulin concentrations in diabetic subjects showed a lesser increase at 0.5 hour (mean, 97 μU per ml) but continued to rise to a peak at 2 hours (mean, 243 μU per ml). The integrated average insulin concentration during the 2 hour glucose tolerance test was 26 per cent higher in diabetics (mean, 147 μU per ml) than in nondiabetics (mean, 117 μU per ml).

6. In a small series of patients subjected to additional glucose loading at 1.5, 2 and 2.5 hours, very high insulin concentrations were observed in both groups, but levels in diabetic patients far exceeded those in nondiabetic subjects.

7. Fasting insulin concentrations were elevated in five of seven subjects with functioning islet cell adenomas but insulin secretory response to glucose was normal in the one patient studied.

8. Four of six subjects with leucine-sensitive hypoglycemia showed increased insulin concentrations following administration of leucine in six of nine experiments.

9. Insulin responses were generally in the high normal range in three patients with pituitary tumors (two associated with acromegaly) and in two patients with thyrotoxicosis.

10. Plasma insulin concentrations measured by imunoassay are compared with values obtained by other assay methods and found to yield the lowest estimates.

11. Calculation of the average normal daily insulin secretion rate, on the basis of the data presented, yields an estimate of about 55 U of insulin per day.

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