Critical Variables in the Radioimmunoassay of Serum Insulin Using the Double Antibody Technic

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SUMMARY

An adaptation of the double antibody radioimmunoassay for insulin originally presented by Morgan and Lazarow is described. These studies have confirmed the presence of an inhibitor in serum and plasma (heparin) which delays the rate at which the insulin-insulin antibody complex is rendered insoluble by rabbit serum containing antibodies to guinea pig globulin. Serum dilution reduces the effect of the inhibitor but increases dilutional error. Heparin similarly reduces the effect of the inhibitor, but excess heparin produces falsely low values for immunoreactive insulin (IRI).

Data are presented for serum which show that if sufficient time (72 hrs.) is allowed to elapse after the addition of the precipitating antibody (rabbit, anti-guinea pig globulin, serum), the precipitating system reaches equilibrium. Employing this modification, additional data are presented, showing excellent recovery of human insulin added to serum, good duplication of respective serum IRI values in repeat assays, and constancy of fasting serum IRI in any one individual on repeated sampling. Normal adults exhibit fasting values of serum IRI from 1 to 20 μU./ml. with a mean of 8.4 μU./ml., and after rapid intravenous glucose, serum IRI usually reaches maximum levels in one to two minutes. DIABETES *14*:771-79, December 1965.

Systems for measuring immuno-reactive insulin (IRI) have found wide use in the exploration of disturbances in carbohydrate metabolism such as those seen in diabetes mellitus and in various other endocrine disorders. These systems have many advantages which include (a) specificity for insulin, (b) independence from biological variations inherent in bioassay procedures measuring insulin-like activity, and (c) the ability to assay simultaneously a large number of specimens.

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There are also possible disadvantages to these immunoassay procedures which should be kept in mind. The insulin value measured may not necessarily reflect the biological activity of endogenous serum insulin, as the ability to react with antibody may not be synonymous with biological effect. Furthermore, the extracted and purified pancreatic insulin used as a standard may not possess the same immunochemical properties as circulating endogenous insulin.

The basic concept of the immunoassay for insulin was set forth by Yalow and Berson[1,2] and consists in the competition between a constant amount of insulin I-131 and a variable amount of either serum insulin or insulin standards for available binding sites on a fixed amount of dilute guinea pig anti-insulin serum. However, efficient separation of the soluble antibody-bound insulin from nonantibody-bound, i.e., "free" insulin, may be difficult and can provide a source of inaccuracy unless optimal conditions are present.

Many technics have been devised to perform separations of the antibody-bound insulin and the "free" insulin moieties of the insulin assay system such as electrophoresis,[3] chromato-electrophoresis,[3] ion exchange resins,[4] salt precipitations,[5] acid precipitation after free insulin destruction by insulinase[6,7] and a double antibody technic.[8-10] Similar to other radioimmunoassays for insulin, the latter involves the reaction of diluted serum from guinea pigs immunized against pork insulin, and containing insulin antibodies, with pork insulin I-131 in the presence of either solutions of Crystalline (human) Insulin standards in buffer or human insulin in sera. The pork insulin I-131 and either the human insulin standard or the human insulin in serum combine with the guinea pig insulin antibodies (globulins) to form a soluble insulin-globulin complex. When serum from rabbits immunized against guinea pig globulin is added, this combines with the soluble insulin-globulin complex and forms an insoluble precipitate.

The precipitate can be collected by filtration[10] or isolated by centrifugation as proposed by Morgan and Lazarow.[8,9] The main advantage of the centrifugation technic is that a single tube can serve for the incubation of the reactants, isolation of the precipitate and the final measurement of radioactivity.

However, a special problem is introduced by the utilization of a second antibody, and the immunologic dynamics of this second antibody reaction in the presence of serum must be carefully assessed in order to validate the system. This study was designed to assess and quantify some of the variables of this technic of immunoassay for insulin and then to apply it to the measurement of immuno-reactive insulin in human serum.

MATERIALS AND METHODS

In general, these were adapted with some alterations from the procedure proposed by Morgan and Lazarow.[8-10]

Incubate tubes. All tubes employed were glass 13 x 100 mm. rimmed culture tubes.

Buffer. All dilutions of standard insulin, serum or plasma, pork insulin I-131, guinea pig anti-insulin serum and normal guinea pig serum were made in 5 per cent bovine serum albumin in borate buffer. This was prepared as follows: 8.25 gm. boric acid; 2.70 gm. soddium hydroxide and 12N HCl and H_2O to make one liter at *p*H 8.0. To this, 5 gm. of bovine serum albumin, fraction V (Armour Pharmaceuticals) was added to each 100 ml. (5 per cent BSA). Additional 0.1 N HCl was added if necessary to maintain a *p*H of 8.0.

Standards. Vials containing 0.5 unit of human insulin were generously supplied by Dr. Mary A. Root, Lilly Research Laboratories. To each vial was added 0.2 ml. of N/30 HCl. After the insulin was dissolved, the volume was made to 20 ml. by addition of 5 per cent BSA. Aliquots of 0.1 ml. containing 2,500 μU. were placed in separate tubes, sealed, frozen and stored at minus 20° C. On the day of assay, a single tube was allowed to thaw in an ice bath, and then diluted to a concentration of 400 μU./ml. with 5 per cent BSA. From this stock solution, dilutions of insulin were made with a final concentration of 2.5, 5, 10, 20, 40, and 80 μU./ml. and then employed without further dilution for the standard curve.

Pork insulin I-131. This was obtained from Abbott Laboratories with a specific activity greater than 20 millicuries per milligram and was diluted 100-fold on arrival with 5 per cent BSA and frozen. On day of assay, this was allowed to thaw in an ice bath, and a further dilution was made to a final concentration of 100 μU./ml. (10 μU./0.1 ml.).

Guinea pig, anti-pork insulin serum (AIS). Young animals (300 gm.), obtained from the Harvard Medical School animal farm, received weekly subcutaneous injections containing 5 to 15 units of Protamine Zinc Pork Insulin (U-40) (courtesy of the Lilly Research Laboratories) for three to four doses. The animals were bled by cardiac puncture ten days after the final injection, the blood clotted overnight at 4° C., and the serum was separated and frozen. Each serum was titrated at various dilutions. Serum from one guinea pig (BLK) at a dilution of 1:1600 demonstrated sensitivity over the range of standards employed and was utilized in all experiments.

Rabbit, anti-guinea pig globulin, serum (RAGS). RAGS was purchased from Arnel Products, New York, N.Y.

Normal guinea pig serum (NGPS). This was obtained from normal guinea pigs by cardiac puncture and diluted 1:400 in all experiments.

Heparin. The 1,000 or 10,000 U.S.P. U. per ml. preparations of Organon Laboratories were utilized at appropriate dilutions.

Table 1 shows in outline form the order in which reagents were added and the volumes used. All incubations were at 4° C. Serum and plasma samples were prepared for assay by a preliminary centrifugation at 2,000 RPM (1,000 × G.) for 20 min. at 4° C. in order to separate clear serum or plasma from high density particulate material and/or low density lipid, if present. The *p*H of this sample was then measured and adjusted to *p*H 7.6 to 8.2 if necessary with either 0.1 N NaOH or HCl, since samples with *p*H outside this range were found not to produce optimal precipitation. All standards and sera were assayed in dupli-

TABLE 1

The order and the volumes of the various reactants employed in the double antibody immunoassay for insulin*

Method	
Standard human insulin or prepared serum	1.0 ml.
Pork insulin I-131	0.1 ml.
Anti-insulin serum (diluted)	0.1 ml.
First incubation—48 hrs. at 4° C.	
Rabbit, anti-guinea pig globulin, serum	0.1 ml.
Normal guinea pig serum (diluted)	0.1 ml.
Second incubation—72 hrs. at 4° C.	
Centrifugation and decantation	

*Double antibody insulin radioimmunoassay adapted from Morgan and Lazarow.

cate. In all assays 10 microunits of pork insulin I-131 contained in 0.1 ml. were added to each tube. Similarly, AIS (BLK, 1:1600) was added in a volume of 0.1 ml. The time necessary for complete equilibration of the human insulin contained in standards of serum with the insulin I-131 and AIS was determined by hydrodynamic flow separation of antibody-bound and free insulin on Whatman 3 MM paper.[3] It was demonstrated that the diluted anti-insulin serum selected reached 98 per cent equilibration after 48 hrs. Consequently, all *first incubations* were of this length or longer. The term *second incubation* will be applied throughout as the phase after addition of rabbit, anti-guinea pig globulin, serum (RAGS).

The volume of RAGS (second antibody) that would insure complete precipitation of the antibody-bound insulin was determined by titration in the above-described system. However, to compensate for any crossreaction this material might have with human globulin, all titrations were performed in the presence of undiluted human serum as well as with buffer and insulin standards in buffer. Generally, a slightly larger volume of the second antibody was necessary to produce complete precipitation in the presence of serum, but to insure maximum precipitation, at least a 20 per cent excess was employed.

Preliminary studies indicated that the addition of diluted normal guinea pig serum as a carrier increased the mass of the precipitate and the ease in which it could be separated. However, increasing the amount of this necessitated an increase in the volume of the RAGS which was necessary for complete precipitation. It was found that the addition of 0.1 ml. of a 1:400 dilution produced a satisfactory mass of precipitate, and also could be fully precipitated by small volumes of potent RAGS.[8,9]

After all incubations at 4° C. were complete, the tubes were centrifuged at 2,000 RPM (1,000 × G.) for twenty minutes at 4° C. Prolongation of centrifuging time or increased speed did not produce precipitates of higher radioactivity. After centrifugation, the supernatant fluid from each tube was gently decanted into a clean 13 × 100 mm. rimmed test tube and both tubes were then stoppered and counted successively in a Nuclear-Chicago gamma spectrometer equipped with an automatic tube changer set for 1,000 counts or more. If a pair of tubes containing a respective precipitate and supernatant fluid did not exhibit a total count rate within 5 per cent of 10 μU. of pork insulin I-131, the results were discarded. The radioactivity of the precipitate was calculated as a per cent of the total radioactivity in both precipitate and supernatant fluid for each pair. All studies were done in duplicate and if the calculated per cent activity of both precipitates did not agree within 2 per cent after a recount, the results were discarded. Approximately one pair of duplicate tubes in each 200 were discarded due to loss of counts or poor agreement of the per cent precipitated.

When normal guinea pig serum diluted 1:1600 was substituted for the AIS diluted 1:1600, less than 3 per cent of the radioactivity appeared in the precipitate.

RESULTS

Illustrating the above described system, figure 1 shows the results obtained when series of tubes containing various concentrations of human insulin were prepared at the same time and allowed a first incubation of 48 hrs. To each was added 0.1 ml. of RAGS and 0.1 ml. of NGPS diluted 1:400. At the end of a second incubation period of 1, 4, 24, 48, 72 and 96 hrs., separate series of standard curve tubes were centrifuged and decanted, the precipitates and supernatant fluids were counted, and the per cent of total activity precipitated was plotted versus human insulin concentration. There was no significant difference among any of the standard curves.

Figure 2 depicts the results of seventeen standard human insulin curves employing twelve separate lots of pork insulin I-131 over a period of nine months. All other reactants were identical, and aliquots of the same lot of human insulin were used throughout. The first incubation periods were of 48-hr. duration, the second were of 72-hr. duration.

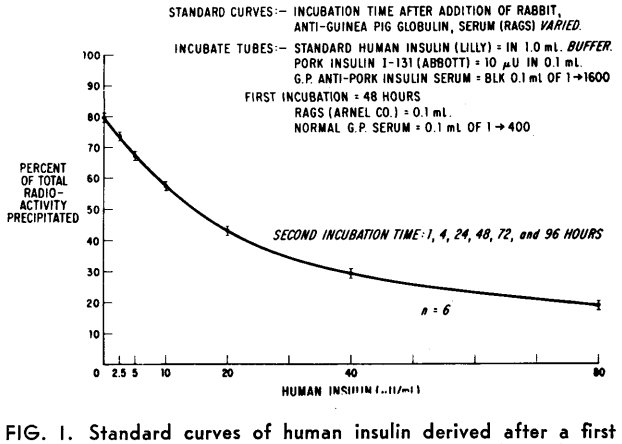

FIG. 1. Standard curves of human insulin derived after a first incubation period of 48 hr., and second incubation times of 1, 4, 24, 48, 72 and 96 hr.

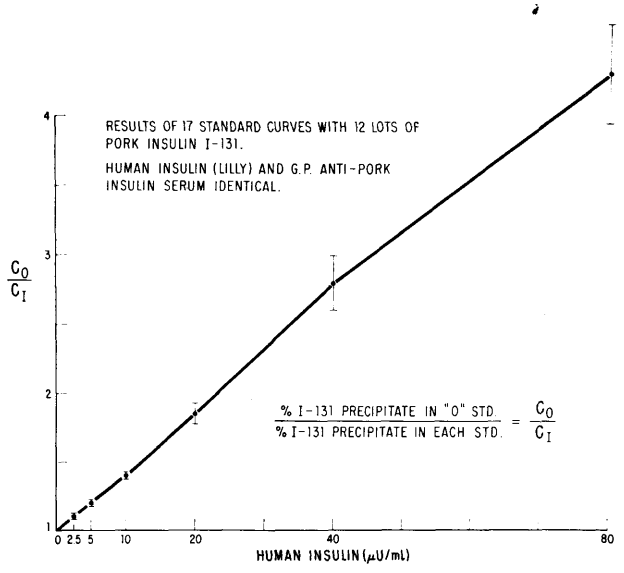

FIG. 2. Mean performance of seventeen standard human insulin curves. Results expressed as the per cent precipitated in "O" standard insulin tubes divided by the per cent precipitated in each respective insulin containing tube.

Each of the seventeen standard curves was plotted according to the method utilized by Hales and Randle,[10] as the absolute concentration of insulin in each lot of pork insulin I-131 varied slightly from the stated concentrations. The ratio of the per cent precipitated radioactivity in the "O" standard of each standard curve (Co) to the per cent precipitated radioactivity in each standard in the curve (Ci) has been plotted versus the concentration of human insulin. The solid line represents the mean values and the horizontal bars indicate one standard error of the mean. The plot is essentially linear with some tendency to show a decreased slope between 40 and 80 μU./ml. There was no tendency for the slope of the line to rise or fall throughout the period and any slope variations were random.

After reliable results were achieved with standard human insulin, further studies were designed to explore the ability of the assay system to measure endogenous human insulin in serum and plasma. Two hundred milliliters of venous blood was collected from a normal fasting subject, allowed to clot at room temperature for four hours, centrifuged, and the serum separated and frozen at minus 20° C. until use.

The serum was thawed and prepared as previously described in Materials and Methods. Six series of incubate tubes were prepared with human serum diluted at 1:10, 1:5, 2:5, 3:5, 4:5, and 1:1. A series of standards were made at the same time, and identical reactants were added to all tubes. The first incubation period was 48 hrs., following which equal amounts of RAGS and NGPS (1:400) were added to all tubes. The tubes containing one series of standards and one series of human serum dilutions were centrifuged, decanted and counted for radioactivity after the second incubation phase had proceeded to 1, 4, 12, 24, 48 and 72 hrs. All levels of apparent immunoreactive insulin in serum or plasma were corrected for the respective dilutions to μU./ml. serum or plasma.

The results are shown in figure 3. The apparent IRI values at any one dilution declined until at least 72 hrs. of second incubation had elapsed. No further changes were noted on prolonging this phase. At 72 hrs., equal values of IRI were obtained at all serum dilutions. Those specimens which had been centrifuged before 72 hrs. showed higher apparent levels of IRI, but on the other hand, the greater dilutions showed lower values of IRI. The standard curves for all the incubation times employed were identical to those shown in figure 1.

Figure 4 shows the results obtained when identical studies were performed on heparinized (100 U. per ml.) blood plasma from the same subject collected at the same time as the serum used in the preceding experiment. Similar results were seen after 72 hrs. of the second incubation; however, the plasma IRI values appeared lower than those seen in similar dilutions of serum. At second incubation times of less than 72 hrs., lower apparent IRI values were seen at the lower dilutions when compared to the respective serum values. The highest apparent values were noted at dilutions of 1:5. However, lower values than these were seen at 1:10 dilutions.

The low levels of IRI in fasting serum or plasma diluted more than 3:5 were near the lower limit of sensitivity for this assay procedure. To insure that there was no significant effect of dilution upon the levels of IRI, the dilution experiment was repeated employing serum obtained from normal subjects one to two hours after a meal when levels of circulating insulin would be high. Table 2 shows these studies in three sera employing a 72-hr. second incubation period. There was no consistent significant degree of inhibition apparent.

Since the values obtained for apparent IRI in serum after 72 hrs. of second incubation were close to those obtained in heparinized plasma at one hour of incubation, the following experiment was designed to test the effect of heparin on the final value for IRI. Pre-

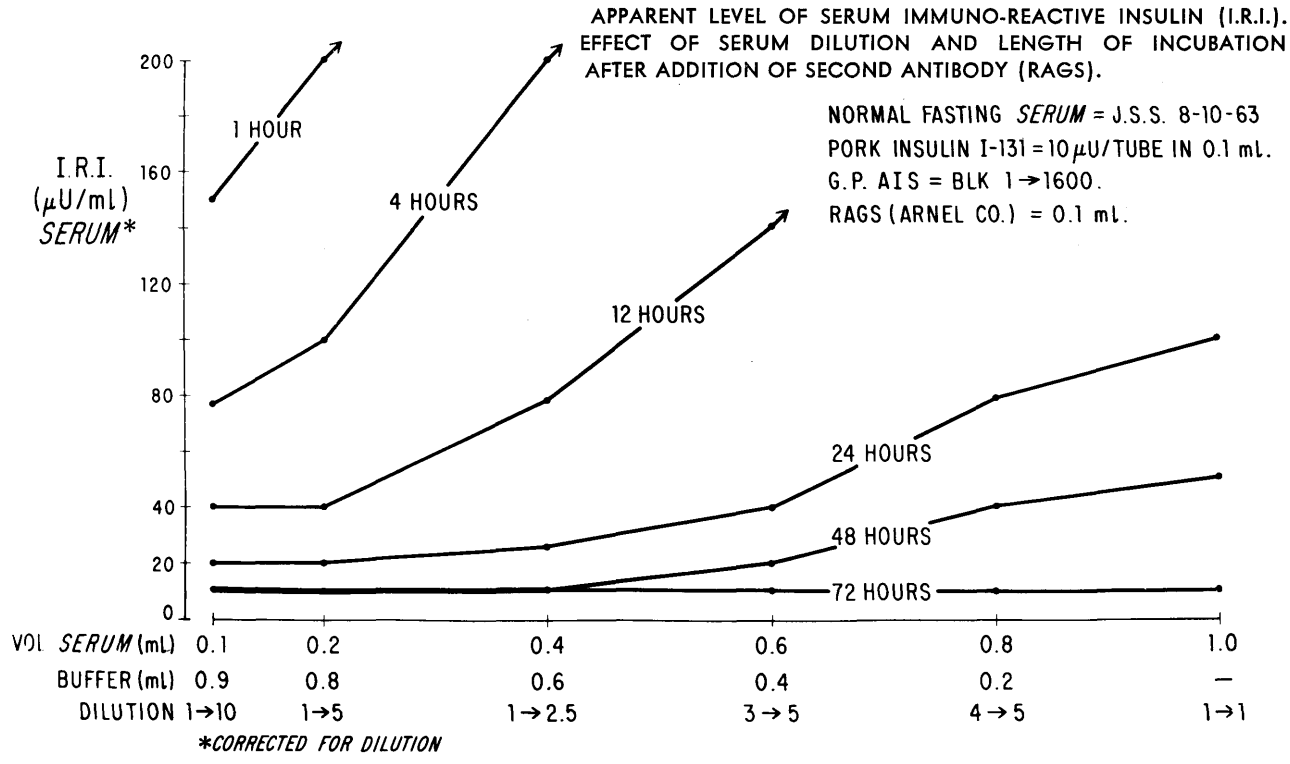

FIG. 3. Apparent level of serum IRI (μU./ml.) at dilutions of 1:1 to 1:10 when the second incubation period was varied from 1 to 72 hr. All values corrected for dilution.

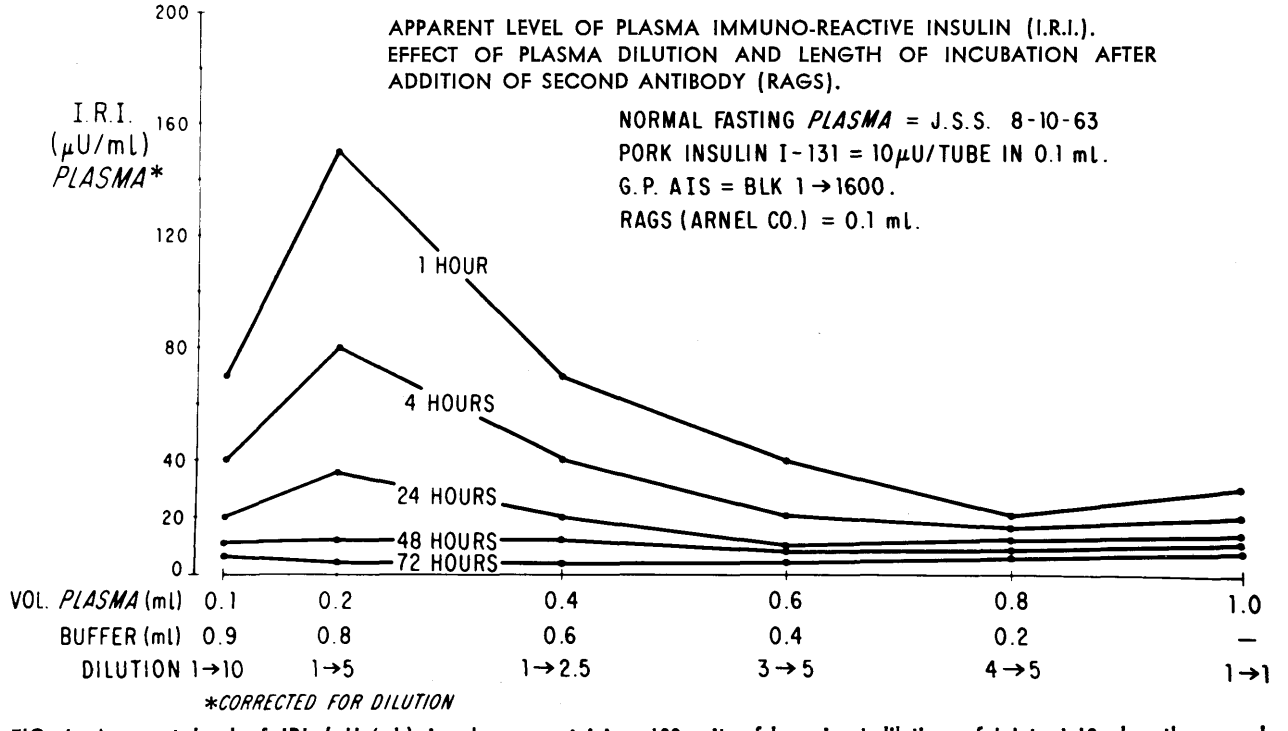

FIG. 4. Apparent level of IRI (μU./ml.) in plasma containing 100 units of heparin at dilutions of 1:1 to 1:10 when the second incubation period was varied from 1 hr. to 72 hr. All values corrected for dilution.

TABLE 2

Levels of IRI μU./ml. in postprandial sera diluted from 1:1 to 1:10 with a second incubation period of 72 hrs. All levels corrected for dilution

Dilution	J.S. serum	J.W. serum	G.S. serum
1:1	98	25	42
4:5	98	28	40
3:5	101	34	38
1:2.5	94	30	36
1:5	85	25	40
1:10	88	25	40

liminary studies ascertained that plasma prepared from blood collected into measured amounts of heparin behaved identically to serum to which had been added identical amounts of heparin. A large volume of serum from a fasting normal subject was obtained, and various amounts of heparin were added to aliquots of this serum. Table 3 shows the levels of apparent IRI after second incubations of one hour and 72 hrs. in undiluted serum containing various concentrations of heparin. It can be seen that after a second incubation period of one hour, the apparent IRI decreased as heparin concentration increased. However, after 72 hrs. of a second incubation, all apparent IRI values were much lower, but the value still decreased as heparin concentration increased.

Since these data suggested that the concentration of heparin affected the value of IRI, further studies were confined to serum, and only a 72-hr. second incubation period was employed.

Table 4 shows the results of recovery experiments in which various amounts of human insulin dissolved in 5 per cent BSA were added to serum from normal fasting subjects, then assayed at a 1:2 dilution. From 10 to 50 μU./ml. were added and the total mean recovery ± S.E.M. was 94.5 ± 5.6 per cent ($n = 10$). Furthermore, pre-incubation of human serum alone or with

TABLE 3

The effect of various concentrations of heparin upon the apparent levels of IRI in undiluted fasting serum from a normal subject when second incubation times were 1 and 72 hrs.

Heparin concentration (U. per ml. serum)	Apparent IRI (μU./ml.) incubation time after adding second antibody 1 hr.	72 hr.
0	>80	14
10	>80	13
20	75	12
50	50	11
100	35	11
200	22	10
500	20	9
1,000	17	8

TABLE 4

Levels of IRI (μU./ml.) in serum before and after the addition of various quantities of Crystalline (human) Insulin

Serum alone	Added insulin (μU./ml.)	After added insulin	Per cent total recovery
3	20	23	100
19	20	39	100
11	20	29	94
9	20	30	103
12	10	20	91
3	10	12	92
8	10	12	67
8	10	12	67
16	50	70	106
6	50	70	125
			Mean 94.5
			S.D. ± 17.6
			S.E. ± 5.6

added insulin at 4° C. for five days prior to assay did not significantly alter the level of IRI. When the first incubation period was increased from 48 to 96 hrs., allowing a longer period for damage to occur to the labeled insulin, little difference was noted in the final level of serum IRI.

To determine the reproducibility of repeated assays on aliquots of the same serum, blood was taken from four normal subjects, the serum was prepared as described and assayed on four separate occasions undiluted and diluted 1:2 over a six-month period. The values for IRI are shown in table 5. No serum showed a deviation of more than 2 μU./ml. from the mean value.

TABLE 5

Levels of IRI (μU./ml.) in aliquots of the same fasting serum obtained from four normal subjects when assayed repeatedly over a six-month period

Subject:	G.H.	G.C.	P.C.	G.S.
Serum IRI (μU./ml.)	3	5	8	12
	2	3	10	9
	3	5	6	9
	4	7	9	13
Mean:	3	5	8	11

To assess the uniformity of fasting serum IRI in the same subject, serum from two normal fasting subjects was collected on five occasions over a one-year period and assayed at a 1:2 dilution. As seen in table 6, there was excellent constancy of IRI values for each individual, since none deviated more than 2 μU./ml. from the mean value.

In order to assess the range and distribution of fasting serum IRI, seventy-five normal subjects were selected who had no clinical evidence of diabetes mellitus,

TABLE 6

Levels of IRI (μU./ml.) in multiple fasting serum samples collected from the same normal subjects over a period of one year

Subject:	G.C.	G.S.
Serum	5	11
IRI	7	11
(μU./mi.)	7	14
	4	12
	6	13
Mean:	6	12

carbohydrate intolerance, obesity, malnutrition, endocrinopathy, and who were not on any drug or medication.

After subjects had fasted overnight, venous blood was collected and allowed to clot for three hours at room temperature or overnight at 4° C. and the serum was separated by centrifugation. All serum samples were frozen until the day of assay and prepared as previously described. All were assayed diluted 1:2, those with levels below 4 μU./ml. were re-assayed undiluted. As shown schematically in figure 5, the values ranged between one and 20 μU./ml. with a mean ± S.E.M. of 8.44 ± 0.35 μU./ml.

Finally, the assay system was applied to the measurement of IRI in similarly selected normal adult subjects before and throughout intravenous glucose tolerance testing. After subjects had fasted overnight, blood samples were drawn, then glucose (0.5 gm. per kg. of body weight) was administered intravenously in a 2- to 3-min. period. After the end of the infusion, blood samples were withdrawn at 1, 2, 3, 5, 10, 20, 30, 40, 50, 60, 120, and 180 min. The serum was prepared, diluted 1:2, and the serum IRI determined in the same assay. Table 7 illustrates the results. Five of the six subjects showed the highest value for IRI in the 1- or 2-min. sample.

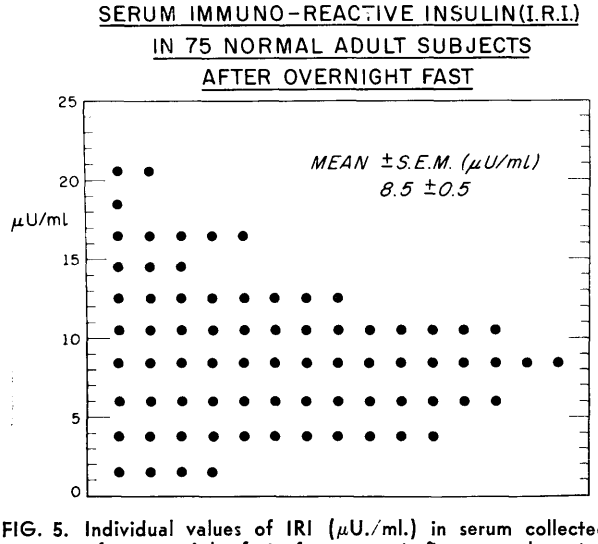

FIG. 5. Individual values of IRI (μU./ml.) in serum collected after overnight fasts from seventy-five normal control subjects with mean and standard error of mean.

TABLE 7

Values of IRI (μU./ml.) before and after rapid intravenous glucose infusion in normal control subjects (0.5 gm./kg. body weight)

		Time (mins.)										
Normals	F	1	2	3	5	10	20	30	40	50	60	120	180
D.C.	6	41	54	51	36	38	30	32	—	—	25	—	—
O.T.	6	30	29	29	22	22	22	21	24	21	15	6	3
P.C.	6	106	94	75	70	64	39	13	18	11	10	8	13
R.C.	12	90	77	71	83	88	104	78	75	68	58	—	—
T.J.	3	60	56	42	37	30	26	30	36	31	30	—	—
J.S.	7	106	108	88	66	44	33	24	27	20	14	—	—
Mean	7	72	70	59	52	48	42	33	36	30	25	7	8
S.E.M.	1	14	12	9	10	10	13	9	10	10	7	—	—

DISCUSSION

Although many types of radioimmunoassays for insulin have been proposed, the double antibody technic may achieve acceptance. However, the recognition of an inhibitor to the system in plasma led to studies by Morgan and Lazarow,[11] who documented that the inhibitor affects the second incubation phase, i.e., the reaction between the soluble insulin, anti-insulin-globulin complex and the precipitating anti-guinea pig globulin serum produced in rabbits. They proposed either diluting the plasma or adding 0.01 M EDTA to neutralize the inhibitor. Samols and Bilkus[12] and Kuzuya and Samols[13] similarly have examined this problem and suggest that suboptimal precipitation is due to cross-reaction between the rabbit serum containing antibodies to guinea pig globulin and human globulin. Investigators using the double antibody system as described by Hales and Randle[7] have not reported inhibitors to the measurement of plasma insulin. However, these investigators reversed the order of antibody reactions and employed different buffers from those used here in addition to employing filtration to isolate the precipitate.

Studies reported here using a system adapted from that proposed by Morgan and Lazarow have shown that this achieves excellent performance when applied to solutions of human insulin standards (figure 2). However, an effect similar to the inhibitor described by Morgan and Lazarow could be seen in human serum and, to a lesser extent, in human plasma. Since the volumes of the second antibody were in excess, having been titrated in the presence of serum, crossreaction

with human globulins does not account for the inhibition.

Dilution of human serum neutralized part of the effect of the inhibitor. Although high values were achieved for the IRI of fasting human serum even when diluted, prolongation of the second incubation period to 72 hrs. or more allowed the measurement of IRI to be accomplished even in undiluted serum. Furthermore, as seen in table 2, when 72 hrs. are allowed for the second incubation time, similar values are measured in serum whether undiluted or diluted 1:10. The effect of the inhibitor in human serum is to delay the rate of reaction between the first and the second antibody. Dilution of serum reduces the effectiveness of the inhibitor and decreases the time necessary for the antibodies to reach full equilibration. Collection of blood in heparin solution or addition of heparin to serum produces the same effect. When heparin-containing plasma is undiluted, near-complete equilibration between antibodies is achieved rapidly; however, dilution delays the rate of reaction and gives falsely high values of IRI, and at greater dilutions (1:10), rapid equilibration is again noted. It is speculated that as the heparin concentration is reduced to a critical level in a respective plasma, the inhibitor effect is partially unmasked and delays antibody precipitation. However, as further dilution is made, the inhibitor itself is reduced in concentration and ceases to play an effective role in delaying precipitation. This present study does not elucidate the character of the inhibitor nor the effect of heparin, but it suggests that the physical-chemical mechanisms responsible for the rate of precipitation reaction between these two globulin antibodies is affected by heparin. Increasing the heparin concentration in serum allows more rapid precipitation. However, as seen in table 3, even after prolonged second incubation periods, smaller amounts of IRI are observed as heparin concentration increases. It is uncertain whether heparin in serum alters the insulin-insulin antibody reaction to favor binding or causes nonspecific binding of free insulin I-131 to the precipitate mass. Similar levels of heparin added to the standard human insulin solutions cause no discernible change in the standard curve values.

Crystalline (human) Insulin added to serum was recovered, as seen in table 4. Pre-incubation of serum alone or with added human insulin, and prolonged first incubation periods did not influence the level of serum IRI. This suggests that the amount of insulin damage which occurs during the prescribed periods of incubation is not sufficient to affect the measurable serum IRI to any significant degree.

Little deviation is seen when the same serum is repeatedly assayed (table 5). This demonstrates the constancy of the system on repeated assays and indicates that endogenous IRI is stable in frozen serum. Also, little deviation is seen when repeated samples of fasting sera are obtained from normal subjects over a one-year period. Similar findings noted by Steinke et al.[14] who employed the rat adipose tissue bioassay indicate a constancy of fasting insulin levels in any one normal subject.

The range and mean of normal fasting values are slightly lower than those reported by Samols and Bilkus,[12] Yalow and Berson,[3] Hales and Randle,[10] moderately lower than those reported by Cerasi and Luft,[15] and Goetz et al.,[16] and similar to those reported by Grodsky and Forsham.[5]

Serum IRI responds rapidly to intravenous glucose, as shown by table 7. The highest values are usually seen between one and two minutes after the end of the glucose infusion, which demonstrates that acute hyperglycemia provokes rapid release of IRI. The subsequent fall in IRI shows that the magnitude of the insulin release is not sustained but falls off soon after the glucose load is administered and parallels the decline of blood glucose.

Employing an immunoassay, Seltzer and Allen[17] have described similar changes in plasma insulin after intravenous glucose administration. The earliest sample measured (5 min.) showed the highest level of insulin, and subsequent samples showed a rapid fall parallel to blood sugar decline.

Using glucose uptake by rat hemidiaphragm as an index of insulin activity, Antoniades et al.[18] described similar changes in "free" insulin after intravenous glucose in normal subjects. The maximum rise was seen 5 to 8 min. following the glucose infusion and was approximately ten times the fasting level. Subsequently, the insulin levels declined until near fasting levels were demonstrated at 20 to 40 min. after the infusion. Although the pattern of serum "free" insulin is similar to the pattern of serum IRI levels after similar glucose infusions, the absolute levels measured by this diaphragm method are approximately five to eight times those of the immunoassay.

Similar studies were done by Steinke et al.[14] employing the rat adipose tissue technic. These workers demonstrated that in normal subjects, the maximum rise of serum insulin-like activity occurred twenty minutes following the glucose infusion.

The explanation for the discrepancies of these serum insulin patterns is unknown and will await further characterization of these three insulin assay systems.

Addendum: Since this paper was submitted, Morgan et al. have published further studies of the inhibitor in the two antibody immunoassay system.[19] Their immunoelectrophoretic studies demonstrated no crossreactivity of rat or human gamma globulin with rabbit anti-guinea pig plasma. The inhibitor was identified as complement on the basis of inactivation by heat, antigen-antibody complexes, EDTA or ammonia.

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