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Received February 5, 1962. P.S.E.B.M., 1962, v110.

Immunoassay of Insulin Using a Two-Antibody System.* (27411)

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Immunoassay of insulin utilizing lysis of sensitized RBC(1), salt precipitation(2), and paper electrophoresis(3) has been reported.

It has been shown that I^{131}-insulin, which is bound *in vitro* by serum obtained from insulin resistant patients, can be quantitatively precipitated by an antibody to human gamma globulin(4). This suggested the possibility of using a similar system for insulin assay. The present paper describes a simple, rapid, and versatile assay method permitting large numbers of determinations to be carried out with high precision and great sensitivity.

The reaction consists of two steps. In the first, insulin forms a soluble complex with its specific antibody, which is obtained from immunized guinea pigs (AIS-GP). In the second, this soluble complex is precipitated by antibody to guinea pig serum, which is obtained from rabbits (AGPS-R).

The overall reaction is dependent upon the concentrations of the various reactants used, *i.e.*, AIS-GP, AGPS-R, and insulin. Using tracer amounts of I^{131}-insulin, the percent of radioactivity in the precipitate is dependent upon the total amount of insulin in the reaction mixture; as increasing amounts of unlabeled insulin are added, the percent of I^{131}-insulin in the precipitate is decreased correspondingly.

* This investigation was supported by research grants from Nat. Inst. of Arthritis and Metab. Dis., and from Division of General Medical Sciences, N.I.H., U.S P.H.S.

Materials. The AIS-GP used was obtained from guinea pigs immunized to insulin (Iletin, Lilly) using a Freund adjuvant(5,6). The AGPS-R was purchased from Sylvanna Chemical Co. Special preparations of I^{131}-insulin having specific activities greater than 200 mc/mg were supplied by Drs. J. L. Izzo and W. F. Bale.† A commercially available product of lower specific activity was also used.‡ All dilutions of serums and standards were made in 5% bovine serum albumin-borate buffer, at pH 8.5, containing 1:10,000 merthiolate (5% BSA).

Method. The following procedure was used to obtain the results shown in Fig. 1. The components were added to a 12 × 75 mm tube in the order indicated.

Step 1: (a) 0.5 ml of unlabeled human insulin solution containing 0.5 μU to 64 μU *i.e.*, 1-128 μU per ml. (b) 0.1 ml of tracer I^{131}-insulin solution containing 10 μU (25,000 cpm). (c) 0.1 ml of 1:10,000 dilution of AIS-GP. This reaction mixture was refrigerated (4°C) overnight (26 hours).

Step 2: The following were added: (d) 0.1 ml AGPS-R. (e) 0.1 ml 1:200 dilution of normal guinea pig serum (NGPS). The tubes were refrigerated for 2 hours and then centrifuged at 2000 rpm for 20 min. at room temperature. The supernatant was decanted. The precipitate was washed with 0.5 ml 1%

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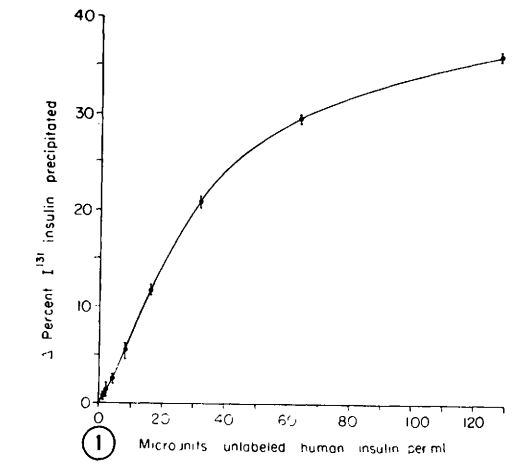
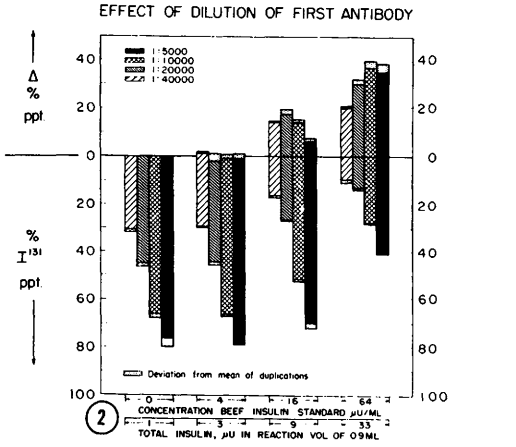
‡ I^{131}-insulin, sp. ac. 20-30 mc/mg, Abbott Pharmaceutical Co.





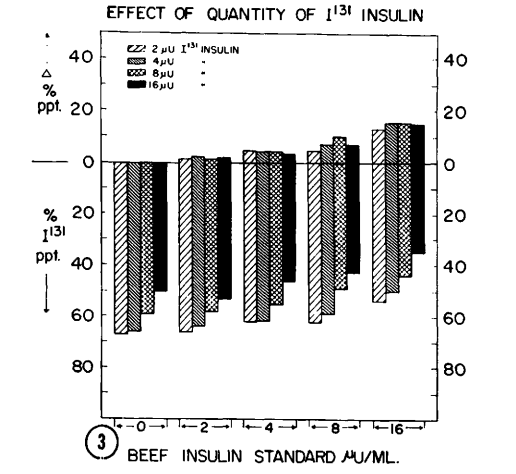


FIG. 1. Assay curve for insulin (Human, Lilly). Mean values of 5 determinations are shown by dots; stand. dev. indicated by vertical bars. $\sigma = \sqrt{\frac{\Sigma (x - \bar{x})^2}{N - 1}}$. Similar *(but not identical)* assay curves were used to obtain values in Table I.

BSA and recentrifuged. The second supernatant was added to the first; the precipitate and the combined supernatants were counted in a gamma spectrometer, and the percent of I^{131}-insulin precipitated was calculated. The decrease in percent precipitated (Δ% ppt.) was calculated for each increment of unlabeled insulin added; *i.e.*, the percent of I^{131}-insulin precipitated was subtracted from that obtained when only the tracer I^{131}-insulin was used.

When the amount of unlabeled insulin added is plotted against the Δ% ppt., the curve passes through the origin and it approximates a straight line at the lower insulin concentrations (Fig. 1). Note that in the range 8 μU/ml to 64 μU/ml of insulin the Δ% precipitated for a 2-fold increase of insulin is greater than 3 times the standard deviation of replicate determinations.

This two-antibody system is highly versatile; it may be used for insulin assay over a wide range of concentrations by varying the amounts of the reactants, or it may be used for selective precipitation of insulin(7).

Parameters. Experiments were carried out to check various parameters of the two-antibody system. Percent of I^{131}-insulin precipitated varied with concentration of AIS-GP used (Fig. 2). A dilution of AIS-GP was selected which gave sizable differences in percent of I^{131}-insulin precipitated when small quantities of unlabeled insulin were added.

Although with dilute AIS-GP the first antibody reaction reaches equilibrium within one to several days, adequate assays can be obtained when shorter periods of incubation are used. Under the conditions used here, *i.e.*, a large concentration of AGPS-R, the second antibody reaction reaches equilibrium rapidly; equivalent results were obtained with incubation times of 1, 2, 4, and 24 hours.

With appropriate concentrations of AIS-GP, the tracer dose of I^{131}-insulin added can be increased from 1 to 16 microunits without

FIG. 2. Effect of dilution of the first antibody (AIS-GP) on % of I^{131}-insulin precipitated and on Δ % of I^{131}-insulin precipitated.

FIG. 3. Effect of quantity of I^{131}-insulin tracer on % of I^{131}-insulin precipitated and on Δ % of I^{131}-insulin precipitated.

TABLE I. Immunoassay of Insulin.

	Hr	Glucose, mg/100 ml	△ % ppt	Estimated insulin content, micro-units/ml of plasma
Human plasma during a glucose tolerance test (human insulin standard)§				
J. L.	0	81	11.9*	14
	1	76	13.0‡	19
	2	78	18.4‡	27
R. S.	0	83	7.9†	10
	1	138	30.4‡	50
	2	45	11.5†	14

	Dry wt of tissue, μg	△ % ppt	μU/sample	Estimated beef insulin equiv-alent, units/g dry wt
Rat pancreas tissue (beef insulin standard)§				
NGPS pretreated				
Islet	.070	32.5	25	357
"	.080	32.3	25	313
Acinar	.096	4.6	3	32
AIS-GP pretreated				
Islet	.080	27.8	20	250
"	.076	20.3	14	182
Acinar	.036	3.6	2	56

* .50 ml of plasma.
† .45 " " " .
‡ .40 " " " .

§ 1 μU of I^{131}-insulin used as a tracer.

decreasing the sensitivity of the assay system (Fig. 3). Therefore, this method of assay is not limited to the use of I^{131}-insulin of extremely high specific activity and commercially available I^{131}-insulin of lower specific activity can be used.

A small amount of NGPS was added to increase the mass of protein precipitated; this increased the effectiveness of the assay. In control studies, where either the first or second antibody was omitted, insignificant amounts (1-2%) of I^{131}-insulin were recovered with the precipitate.

Preliminary assays. We have estimated the insulin concentration of human plasma samples obtained from individuals during a glucose tolerance test.§ The plasma samples (0.5 ml and 0.45 or 0.40 brought to 0.5 ml with 5% BSA) were run simultaneously with 0.5 ml standard solutions containing from 0.5 μU to 64 μU, *i.e.*, 1-128 μU/ml, of insulin (Human, Lilly). An assay curve similar to Fig. 1 was constructed; the equivalent units of insulin in the plasma samples were read from the curve, and are expressed as μU per ml of plasma (Table I).

An estimate of the insulin content of rat islet tissue microdissected from frozen-dried sections of pancreas was also made‖ The rats had been pretreated with i.p. injections of NGPS or AIS-GP 3 hours before sacrifice. The frozen-dried samples of tissue weighed less than 0.1 μg; they were dissolved in 0.5 ml 5% BSA and run simultaneously with 0.5 ml standard solutions containing 0.5 μU to 64 μU of insulin (Beef, Lilly). An assay curve similar to Fig. 1 was graphed; the equivalent units of insulin in the tissue samples were read from the graph and are expressed as units per gram (dry weight) of tissue (Table I).

The results of these preliminary assays indicate that this two-antibody method is sufficiently sensitive to determine the insulin in the plasma of fasting humans, to demonstrate changes in insulin levels following glucose treatment, and to detect submicrogram quan-

§ Plasma samples and blood sugar values supplied by Dr. F. Goetz, Dept. of Medicine, Univ. of Minnesota.

‖ Microdissected islets prepared by Dr. I. P. Lowe, Dept. of Anatomy, Univ. of Minnesota.

tities of insulin in microdissected islet tissue. The relative simplicity of this method suggests that it will have wide application.

Summary. Tracer amounts of I^{131}-insulin are precipitated by specific antibodies. The change in percent precipitated, as increasing amounts of unlabeled insulin are added, forms the basis of this assay. The method is sensitive to less than one microunit.

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Received February 12, 1962. P.S.E.B.M., 1962, v110.

Comparison of Protein and Zinc Electrophoretic Patterns of Lobes of Rat Prostate.* (27412)

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The presence of large amounts of zinc in prostate has intrigued many investigators since Bertrand and Vladesco's(1) report of its occurrence in the human gland. Others have since established that zinc occurs in high concentrations in rabbit prostate(2) and in dorsolateral prostate of rat(3). The rat lends itself well for study of the prostate since its lobes, ventral and dorsolateral, are distinctly separated. The dorsolateral lobe can, in turn, be separated by gross dissection into its dorsal tip, an area which contains only acini of holocrine type, and its lateral tip, an area which is comprised exclusively of acini of apocrine type(4,5). Histochemical(4,6,7,8), autoradiographic(9,10) and Zn^{65} distribution (4,5) studies in rats have established that the large amounts of zinc are localized predominantly in lateral acini of the dorsolateral prostate. This report presents results of studies on electrophoretic separation of various zinc components found in prostatic lobe secretions and in blood serum. Parallel studies were made of protein migratory patterns in prostatic secretions and serum.

Materials and methods. 1. *Experimental animals.* Previous reports indicate that administered Zn^{65} concentrates in various lobes of rat prostate(4,5,11) parallelling the distribution of natural zinc in various parts of the gland(3,6). To facilitate identification of zinc in prostatic secretions and in serum, Zn^{65}† was administered to rats used in this study; and the presence of zinc on electrophoretic paper strips was thereby determined by radioactive measurement. Mature male Wistar rats, 16-20 weeks of age (350-400 g), were used in these experiments. For studies on lateral prostatic lobe secretion, rats were injected with 0.04 μc/g of Zn^{65} intracardially under ether anesthesia. Twenty-four hours later, when Zn^{65} concentration in lateral prostatic lobe was maximal(4,11), rats were sacrificed by chloroform and exsanguination. Lateral tips of dorsolateral prostate were dissected, as described previously(5).

Since Zn^{65} concentration in dorsal and ven-

* Research supported in part by grants from U. S. Atomic Energy Commission and U. S. Public Health Service, Nat. Cancer Inst.

† Zn^{65} was purchased from Union Carbide Nuclear Co. as $Zn^{65}Cl_2$ in HCl solution with a specific activity of approximately 1000 mc/g. The solution was diluted with physiological saline containing sufficient NaOH to neutralize some of the acid present compatible with solubility. The dilution was made so that the administered dose was contained in less than 0.25 ml.