Studies of an Inhibitor of the Two Antibody Immunoassay System

Carl Robert Morgan, Ph.D., Robert L. Sorenson, B.A., and Arnold Lazarow, M.D., Ph.D., Minneapolis

SUMMARY

A heat labile plasma inhibitor of the two antibody insulin immunoassay system has been investigated by means of paper electrophoresis, ultracentrifugation, and DEAE cellulose anion exchange resin. These studies indicate that the inhibitor does not interfere with the binding of insulin to its specific antibody (first antibody reaction); but that the inhibitor interferes with the reaction of anti-guinea pig serum (rabbit source) with the insulin-anti-insulin soluble complex (second antibody reaction). When plasma samples are appropriately diluted the effect of the inhibitor is minimized.

It has been demonstrated that insulin forms a soluble complex with its specific antibody obtained from immunized guinea pigs (AIS-GP) and that this soluble complex is precipitated by antibody to guinea pig serum obtained from rabbits (AGPS-R).[1-3] The over-all 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 per cent 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 per cent of I-131 insulin in the precipitate is decreased correspondingly. In studies using a two antibody system for immunoassay of insulin in rat plasma, a decrease in the per cent of I-131 insulin precipitated was observed when undiluted plasma was used. This decrease was not attributable to endogenous insulin.[4] "Inhibition" was not observed when the plasma was diluted 1:8 or more. Further studies on the nature of this inhibition are reported here.

MATERIAL AND METHODS

Immunoprecipitation of I-131 insulin added to plasma

Normal male rats of the Holtzman subline of the Sprague-Dawley strain were anesthetized with Nembutal* and bled by cardiac puncture. The blood was mixed with heparin (100 USP units per milliliter of blood). The heparinized blood samples were kept chilled in an ice bath until centrifugation (2,000 rpm for ten minutes at room temperature). The plasma was withdrawn and merthiolate was added to a final concentration of 1:10,000; the plasma was stored at −20° C. Thawed plasma samples were filtered through glass wool before use.

The two antibody immunoprecipitation technic was used to recover I-131 insulin added to plasma or dilutions of plasma.[4] Bovine serum albumin in borate buffer (5 per cent BSA) was used as the diluent. Table 1 is a schema of the method used in these studies.

TABLE 1

The two antibody immunoassay procedures used in these studies

First antibody reaction:
(1) 1.0 ml. 5 per cent BSA or rat plasma (various dilutions).
(2) 0.1 ml. anti-insulin guinea pig plasma (1:100).
(3) 0.1 ml. I-131 insulin (35 μU) in 5 per cent BSA.
(4) Twenty-four hours at 4° C.

Second antibody reaction:
(5) 0.2 ml. anti-guinea pig rabbit plasma.
(6) One hour at 4° C.

I-131 insulin recovery:
(7) Centrifuged at 2,000 rpm. (15 min.).
(8) Supernatant decanted.
(9) Supernatant and precipitate counted to determine distribution of I-131 insulin.

Paper electrophoresis

It has been demonstrated that insulin remains at the origin of electrophoretic paper strips, whereas insulin bound to antibody migrates with serum proteins during hydrodynamic flow paper electrophoresis.[5]

Various mixtures containing I-131 insulin (First Antibody Reaction, table 1) were added to equal volumes of normal guinea pig serum (NGPS), and immediately ten microliters were placed on the paper strips (Whatman #3) that had previously been saturated with buffer.* The ten microliters of material were

From the Department of Anatomy, University of Minnesota, Minneapolis 14, Minnesota.

*Pentobarbital Sodium, Abbott Laboratories, Chicago, Illinois.

*Veronal buffer, pH 8.5.

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placed 4 cm. from the cathode end of the paper. A constant current of 5 milliamperes per cell was used. The top of the chamber was left open. Serum proteins were well separated from the origin (9 cm.) in sixteen hours. The paper strips were dried at 120° C. for thirty minutes, dyed with bromophenol blue to identify the location of the protein, and then cut into 1-cm. pieces. These pieces were counted in a Packard Autogamma Spectrometer 410A. The distribution of the I-131 insulin in relation to the serum protein was recorded.

DEAE cellulose

Four glass columns (1.6-cm. inside diameter by 45-cm. length) were packed with DEAE cellulose* to a height of 10 cm. Glass wool was used at the top and bottom of the cellulose. The columns were washed with distilled water until the effluents were clear. Then the columns were washed with borate buffer until the effluents were *p*H 8.5.

Four separate test solutions were prepared (table 2). These mixtures (*p*H 8.5) were allowed to react for twenty-four hours at 4° C. A 4.0-ml. aliquot was withdrawn from each reaction mixture and applied to its respective column.

Each column was washed with five successive 5.0-ml. aliquots of buffer† at *p*H 8.0, 7.0, 6.5, 6.0, 5.5, 4.5, 3.5, 3.0, and then with acid alcohol (*p*H < 1). Each aliquot of buffer was passed through the column at a gravitational flow rate of 5 ml. per 2-3 min. The collected fractions were counted to determine the distribution of I-131 insulin in the effluents.

Ultracentrifugation

Four solutions were prepared (table 2). These mixtures were reacted for twenty-four hours at 4° C.; then they were centrifuged in the Spinco Ultracentrifuge (rotor #40) at 30,000 rpm for twenty-one hrs. A series of 1-ml. aliquots were withdrawn successively from each centrifuge tube from the top to the bottom.[5] These were counted to determine the distribution of I-131 insulin in each tube.

RESULTS

Immunoprecipitation of I-131 insulin added to plasma

When I-131 insulin (35 microunits) was reacted with AIS-GP in 5 per cent BSA and immunoprecipitated

*Selectacel Ion Exchange Cellulose, DEAE Standard, Carl Schleicher and Schuell Co., Keene, N.H.

†Buffers: (a) *p*H 5.5-8.0 $Na_2PO_4 + KH_2PO_4$ (b) 3.0-5.5 acetic acid + sodium acetate (c) acid alcohol: 15 ml. 12 *N* HCl in 1 liter 75 per cent ethanol.

TABLE 2

The reaction mixtures used in DEAE cellulose and ultracentrifugal studies. These mixtures were kept at 4° C. for twenty-four hours prior to application to cellulose columns or initiation of centrifugation.

	5 per cent BSA ml.	I-131 insulin 1,350 μU/ml.	Rat plasma ml.	AIS-GP 1:100	NGPS 1:100
DEAE cellulose					
Column 1	4.0	0.1 ml.	—	—	0.4 ml.
Column 2	—	0.1 ml.	4.0	—	0.4 ml.
Column 3	4.0	0.1 ml.	—	0.4 ml.	—
Column 4	—	0.1 ml.	4.0	0.4 ml.	—
Ultracentrifuge					
Tube 1	10.0	1.0 ml.	—	—	1.0 ml.
Tube 2	4.0	1.0 ml.	6.0	—	1.0 ml.
Tube 3	10.0	1.0 ml.	—	1.0 ml.	—
Tube 4	4.0	1.0 ml.	6.0	1.0 ml.	—

(table 1), 88 per cent of the I-131 insulin was precipitated (figure 1). However, when 1 ml. of undiluted rat plasma was used in place of the 5 per cent BSA, only 8 per cent of the I-131 insulin was precipitated. With increasing dilutions of rat plasma, the per cent of I-131 recovered increased; 90 per cent of the immunoprecipitable insulin was recovered from mixtures containing rat plasma dilutions of 1:8 or more.

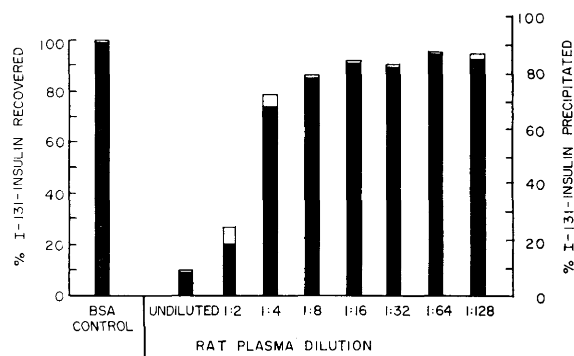

FIG. 1. Effect of dilution of rat plasma "inhibitor" on the per cent of I-131 insulin precipitated (right scale) and on the per cent of immunoprecipitable I-131 insulin recovered (left scale). The amount of antibody used was capable of binding 3,000 microunits of insulin.
□ = Minus (−) one standard deviation (σ) where

$$\sigma = \sqrt{\frac{\Sigma(x-x)^2}{N-1}} \text{ and } N = 3.$$

Effect of heating

Some samples of rat plasma were preheated in a water bath at 56° C. for 5, 10, 20, 40, 80, and 160

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min. I-131 insulin was added, and immunoprecipitation was carried out. Approximately 80 per cent of the I-131 insulin that was immunologically reactive was recovered from plasma samples that had been preheated for 40 min. or more (figure 2).

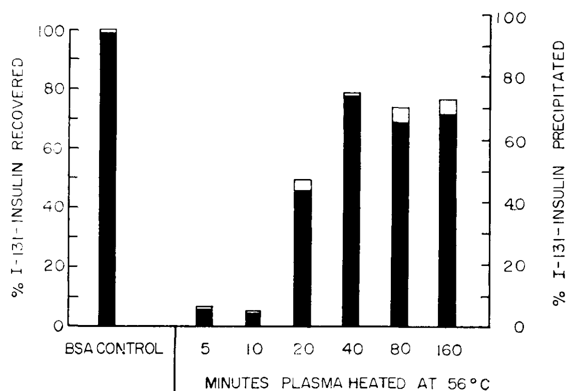

FIG. 2. Effect of heat on the plasma "inhibitor" of the two antibody immunoassay method.
□ = Minus (−) one standard deviation (σ) where $\sigma = \sqrt{\frac{\Sigma(x-x)^2}{N-1}}$ **and N = 3.**

Thirty-five microunits of I-131 insulin in 5 per cent BSA were dispensed to each of several tubes and heated in a water bath at 56° for 5, 10, 20, 40, 80, and 160 min. Heating of I-131 insulin at 56° C. for 40 min. altered its immunological reactivity so that over 50 per cent was not immunoprecipitable.

Paper electrophoresis

I-131 insulin was mixed with AIS-GP or NGPS in solutions of 5 per cent BSA or with various dilutions of rat plasma (First Antibody Reaction, table 1). After twenty-four hours, these reaction mixtures were placed on paper strips and hydrodynamic flow electrophoretic separation of the plasma protein was carried out. In those samples containing AIS-GP, more than 70 per cent of the I-131 insulin migrated with the plasma protein, whereas in those containing NGPS nearly all of the I-131 insulin remained at the origin.

Over 70 per cent of the I-131 insulin migrated with the plasma protein when the first antibody reaction (table 1) was carried out in 5 per cent BSA, undiluted rat plasma, or various dilutions of rat plasma (table 3). Most of the I-131 insulin remained at the origin when NGPS was used instead of AIS-GP. When I-131 insulin and undiluted rat plasma were mixed without either AIS-GP or NGPS and allowed to stand at 4° C. for twenty-four hours prior to electrophoresis, over 90 per cent of the I-131 insulin remained at the origin.

In experiments where AIS-GP and I-131 insulin were mixed together and electrophoresis was carried out immediately, only 30 per cent of the I-131 insulin migrated with the plasma protein.

DEAE cellulose

When I-131 insulin was mixed with NGPS in 5 per cent BSA or rat plasma (table 2), the I-131 insulin was subsequently adsorbed by this anion exchange resin at *p*H 8.5. The insulin remained on the column during a series of forty washes (5 ml. each) with buffer solutions of decreasing *p*H values (*p*H 8 to *p*H 3). This I-131 insulin was readily eluted from the columns with acid alcohol (figure 3).

However, when I-131 insulin was reacted with AIS-GP in the presence of 5 per cent BSA or undiluted rat

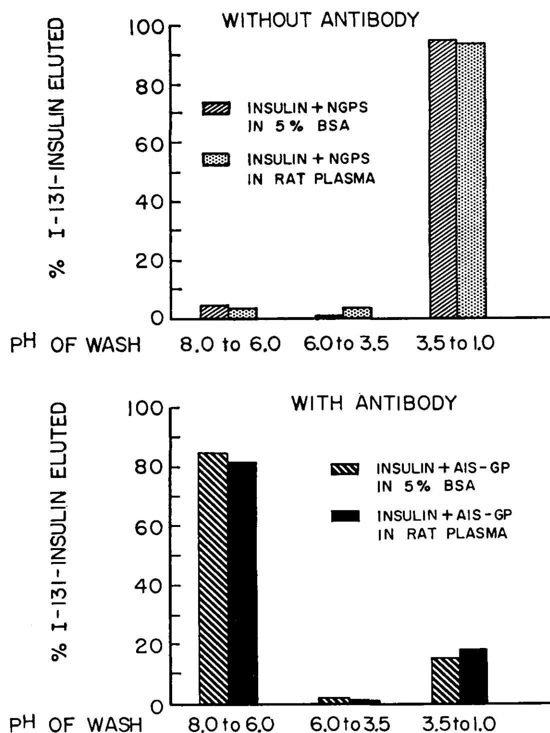

FIG. 3. The "inhibitor" does not interfere with the binding of insulin to this anion exchange (upper graph). Likewise, the "inhibitor" does not prevent the binding of insulin to its specific antibody (lower graph).

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TABLE 3

The distribution of I-131 insulin after hydrodynamic flow paper electrophoresis of various reaction mixtures. The mixtures were refrigerated at 4° C. for twenty-four hours prior to the electrophoresis.

	AIS-GP									NGPS	NONE
	5 per cent BSA	Rat plasma								5 per cent BSA	Undiluted rat plasma
		Undiluted	1:2	1:4	1:8	1:16	1:32	1:64	1:128		
Per cent I-131* insulin at origin	3	9	10	7	7	12	10	10	12	90	96
Per cent I-131 insulin with plasma protein	76	78	71	71	82	78	82	84	76	6	2

*A small amount of I-131 insulin was distributed on the paper between the origin and the serum protein.

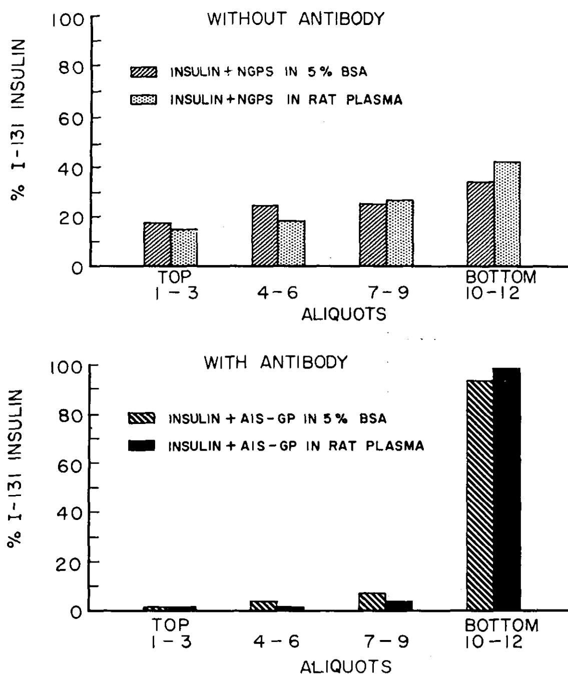

FIG. 4. The sedimentation rate of insulin in rat plasma is only slightly altered from that in 5 per cent BSA (upper graph). However, when antibody was present, the insulin sedimented with the globulins (lower graph).

plasma (table 2), the radioactive insulin was not subsequently adsorbed during passage through DEAE cellulose. The I-131 insulin was recovered in the first 30 ml. of buffer washed through the column (figure 3).

Ultracentrifugation

I-131 insulin was reacted with AIS-GP in 5 per cent BSA or rat plasma and the reaction mixtures were ultracentrifuged as described above. Eleven 1-ml. aliquots were removed from each centrifuge tube (top to bottom) and counted. The centrifuge tube plus the remaining residue was also counted. Ninety per cent of the I-131 insulin was recovered in the bottom of the tubes when AIS-GP was used, whereas the radioactive insulin was more evenly distributed throughout the tubes when NGPS was used (figure 4).

DISCUSSION

The low recovery of I-131 insulin by immunoprecipitation when the reaction was carried out in undiluted rat plasma (figure 1) confirms our previously reported work.[4] It should be noted that the 1:100 dilution of the AIS-GP pool used in these experiments is capable of binding more than 3,000 microunits of insulin (in 5 per cent BSA).[3] The fact that over 80 per cent of the added I-131 insulin was recovered when rat plasma was diluted 1:4 and that over 90 per cent of the added I-131 insulin was recovered from rat plasma dilutions of 1:8 or more indicates that this "inhibitor" does not seriously interfere with immunoassay by the two antibody method when plasma samples are appropriately diluted.

This "inhibitor" is heat labile (figure 2), but the heat lability of insulin precludes the use of preheated plasma samples for immunoassay.

Electrophoretic migration of I-131 insulin with the plasma protein occurred only with those reaction mixtures containing insulin antibody. When the first antibody reaction (table 1) was carried out in undiluted rat plasma, subsequent electrophoresis demonstrated that I-131 insulin migrated with insulin antibody (table 3); therefore, the "inhibition" noted in figure 1 does not affect the first antibody reaction. This is corroborated by the observations made with DEAE cellulose columns and ultracentrifugation studies.

The fact that only 30 per cent of the I-131 insulin migrated with the plasma proteins during electrophoresis, when the antibody and insulin were mixed and immediately applied to the paper, indicates that there

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was not sufficient time for the insulin-antibody reaction to occur after samples are applied to the paper strips, i.e., the insulin binds to the paper and the antibody migrates with the plasma protein before a substantial amount of the insulin is bound to the antibody. Therefore, the migration of over 70 per cent of the I-131 insulin with the plasma protein from reaction mixtures containing undiluted rat plasma indicates that the insulin was bound to the AIS-GP before the sample was applied to the paper.

Heparin was excluded as the plasma "inhibitor" during immunoprecipitations by finding that equal amounts of I-131 insulin were precipitated from reaction mixtures containing 1,000 USP units of heparin or no heparin.

Also, some plasma samples were mixed with equal amounts of ether, frozen overnight, thawed and then centrifuged. The plasma was withdrawn from beneath the ether layer. When compared with non-ether-extracted plasma from the same normal rat plasma pool immunoprecipitation of I-131 insulin from ether-extracted plasma samples was equally inhibited.

The visible precipitate of the second antibody reaction does not appear to be less when the reaction is carried out in undiluted rat plasma as compared to when it is carried out in 5 per cent BSA. This indicates that the inhibition is not of the second antibody reaction per se, but that the "inhibitor" is interfering with the reaction of the insulin-anti-insulin complex with AGPS-R.

The same type of "inhibition" has been noted with undiluted human plasma samples.

Inhibition similar to that reported here has been noted by workers attempting to use two antibody methods for immunoassay of growth hormone[6] and adrenocorticotropin.[7] This information together with the data presented here indicates that the "inhibitor" of the two antibody immunoassay method is not specific for insulin, but that it is a nonspecific inhibitor of the second antibody reaction (table 1). By contrast, a heat labile inhibitor specific for insulin has been reported by workers using a hemagglutination technic.[8]

The data presented here and in our earlier report demonstrate the necessity of using diluted plasma samples for immunoassay by the two antibody method. When plasma samples are appropriately diluted, this method offers a convenient, reliable immunoassay procedure.

SUMMARIO IN INTERLINGUA

Studios de un Inhibitor del Systema de Immunoessayage a Duo Anticorpores

Un thermolabile inhibitor plasmal del systema de immunoessayage de insulina a duo anticorpores esseva investigate per medio de electrophorese a papiro, ultracentrifugation, e resina de excambio anionic a cellulosa DEAE. Iste studios indica que le inhibitor non interfere in le ligation de insulina a su anticorpore specific (prime reaction anticorporee) sed que le inhibitor interfere in le reaction de conilian sero anti porco de India con le solubile complexo insulina-anti-insulina (secunde reaction anticorporee). Quando le specimens de plasma es diluite appropriatemente, le effecto del inhibitor es reducite marcatemente.

ADDENDUM

Since these studies were completed, we have observed that EDTA (final concentration 0.01 *M*) prevents "inhibition." This addition makes possible the use of this method for assay of insulin in undiluted plasma samples.

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