The State of Pancreatic and Serum Insulin

Results of Immunoassay

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SUMMARY

Insulin fractionation of human and canine pancreata and sera has been studied by immunoassay. Several procedures designed to separate bound and free insulin have been utilized. Immunoassay has demonstrated pancreatic insulin to have a very low solubility in saline. However, a cation-bound fraction could not be demonstrated in either the pancreas or serum. These results suggest that cation-bound insulin-like activity demonstrated by rat diaphragm or adipose tissue assay does not represent pancreatic insulin. DIABETES *14*:387-91, July, 1965.

Insulin bioassay studies have suggested that serum and the pancreas contain two forms of insulin-like activity (ILA), a "free" form which is assumed identical to crystalline insulin and an insulin-protein complex in which ILA is combined with a cation- or positively-charged protein.

Beigelman and co-workers[1] found that serum ILA, determined by bioassay in the hypophysectomized-alloxanized rat, was decreased if the serum was previously passed through a cation exchange resin. The ILA was recovered from the column by elution.[2] The eluate yielded ILA when incubated with the rat epididymal fat pad but not with the rat diaphragm. If the eluate was first incubated with either weak alkali or adipose tissue extract (ATE), it then possessed ILA to the rat diaphragm.[3,4] The hypothesis thus developed was that insulin is normally present in both a free and protein-complexed form. The rat diaphragm responds to only the free form. Incubation of the complexed insulin with ATE or alkali converts it to the "free" to which the rat diaphragm responds.[5,6] The epididymal fat pad, possibly because of its ability to break the insulin-protein complex, is sensitive to both forms.[7] Shaw and Shuey[8] were able to duplicate the above findings. They removed ILA from serum with the cation exchange resin and subsequently recovered ILA from the resin eluate. They also demonstrated a twelve-fold increase in ILA to the rat diaphragm after treatment of the eluate with ATE.

Antoniades and co-workers[9] also showed that the major portion of human or bovine pancreas insulin existed as the protein complex. This also combined with the cation resin and could be eluted and converted to "free" insulin the same as was shown for serum.[10] Crystalline insulin did not bind to the cation exchange resin. The work was expanded to compare the response of normals and diabetics to a glucose load. Antoniades[11,12] found that normal individuals respond with an increase in free serum insulin, whereas diabetic patients showed an increase mainly in the insulin complex. Tolbutamide was shown to accelerate the conversion of complex to free insulin.[13] Much of this work has been recently reviewed.[14] The finding that the complex is physiologically inactive and that conversion to free insulin is delayed or absent in diabetes could explain the poor insulin response observed in adult onset diabetes.

Samaan and co-workers[15,16] and Froesch, et al.[17] have suggested that insulin is present in peripheral vein blood largely as antibody "non-suppressible" or "atypical" insulin. In the pancreatic vein it is mostly "suppressible" or "typical" insulin and only this fraction increases in response to a glucose load.

Using their immunoassay technic,[18] Berson and Yalow were unable to increase the insulin activity of plasma by incubation with adipose tissue extracts.[19] Kipnis and Stein[20] found, by immunoassay, that plasma insulin was unchanged after treatment with adipose tissue extract, alkalinization, heparin or acid alcohol extraction.

Using a modified immunoassay procedure, several studies were carried out in an attempt to demonstrate two forms of circulating insulin. The results of these studies are presented.

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METHODS

Immunoassay of insulin. All assays were done by a modified procedure which separates the antibody-bound from the free insulin with an anion exchange resin.[21] Both human and canine insulins were compared to a pork insulin standard* using I-125 labeled insulin.† All assays were performed in quadruplicate and the average of results are reported. The variation was usually less than 10 per cent of the mean.

Solubility of pancreatic insulin. Both human pancreata obtained at autopsy and dog pancreata obtained at surgery were utilized in this study. The tissue was extracted with either acid alcohol (15 ml. of 12 N HCl in one liter of 75 per cent ethanol) or 0.15 M. sodium chloride in a blender at 4° C. From 50 to 2,000 mg. of pancreatic tissue was extracted in 20 ml. of either solvent. The insulin concentration of both the supernatant solution and the whole homogenate was determined. Aliquots of these solutions were necessarily diluted from 1,000 to 60,000 times for assay.

The hypoglycemic activity of the insulin extracts was studied in fasting mice using the 20 ml. extracts from 2 gm. of pancreatic tissue. The acid alcohol extracts were lyophilized and made to original volume with 0.15 M. sodium chloride. Each animal received an intraperitoneal injection equal to the extract from 125 mg. of pancreas per 100 gm. mouse weight. This was one-half milliliter for a 40 gm. mouse. Five mice were included in each group. Thirty minutes following injection, the mice were decapitated and bled. Whole blood glucose was determined by a modified Somogyi-Nelson technic.

Resin extraction of pancreatic insulin. Pancreatic tissue was ground in 0.15 M. sodium chloride in a Potter homogenizer. Concentrations were adjusted to range from 5 mg. to 1 gm. of pancreas per milliliter of saline. The saline-pancreas homogenate was mixed with one-fifth volume of cation exchange resin, Dowex 50-8X, 20-50 mesh Na^+ for fifteen minutes as a batch procedure. The mixture was centrifuged and the supernatant pancreas-saline homogenate was removed. The resin was rewashed with saline, centrifuged and supernatant removed. The procedure was repeated until all pancreatic particulate matter had been removed from the resin. Insulin was then extracted from the saline-pancreas homogenate by acid alcohol extraction. The amount of insulin extracted from the resin treated pancreas was compared to the acid alcohol extract of comparable control pancreas.

Resin extraction of serum. Extraction of serum with cation exchange resin was accomplished on a 11 mm. diameter chromatography column. Three cation exchange resins Dowex 50-8X, Rexyn CG-50, and Amberlite CG-120 were all used in the sodium form. The results were the same with each resin. The resin was added to a height of 10 cm. and washed with cold 0.15 M. sodium chloride. As the saline solution progressed below the top of the column, 10 to 15 ml. of serum was carefully added. The first 5 ml. of effluent was rejected. The remainder of material was collected and assay was carried out as for control serum.

Elution of insulin activity from resin. Following the above treatment of pancreatic tissue, the resin was transferred to a chromatography column and washed with two volumes of cold normal saline. Resin columns used for serum extraction were also washed with two volumes of cold saline. The insulin-like activity was extracted from the resin by one volume of 0.1 N-ammonium hydroxide and dripped into 0.1 N HCl or H_2SO_4. In later studies, elution was carried out with one volume of 0.02 N ammonium hydroxide dripped into phosphate buffer *p*H 7.0. Some eluates were studied directly after neutralization. Others were first treated at alkaline *p*H, see below, to convert to "free" insulin.

Conversion of complexed to free insulin. Two methods suggested by Antoniades were used to dissociate the insulin-protein complex; incubation with adipose tissue extract, ATE, and treatment at alkaline *p*H.

The ATE was prepared from fresh human or canine mesenteric fat, obtained at surgery, which was frozen at —20° C. The tissue was homogenized in a blender in cold 0.15 M. sodium chloride at a weight:volume ratio of approximately 1.5:1. The homogenate was centrifuged at 2,300 rpm for ten minutes and the fat free supernatant collected. Two ml. of the extract was added to each 10 ml. of media and incubation was carried out at 37° C. for thirty minutes and immunoassay followed immediately after cooling.

Alkaline dissociation was carried out at *p*H 9.8. One part Gey and Gey buffer was added to two parts of serum and adjusted slowly to *p*H 9.8. No samples exceeded *p*H of 10.0. After centrifugation for thirty minutes at 4° C., the supernatant was removed and adjusted at *p*H 7.2. Dialysis against 0.15 M NaCl was carried out for twenty-four hours prior to immunoassay.

*Kindly supplied by Dr. Mary A. Root, Eli Lilly and Company, Indianapolis, Indiana.

† Abbott Laboratories, Oak Ridge, Tennessee.

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RESULTS

Pancreatic insulin solubility. The amount of insulin recovered from pancreas by saline and acid alcohol extraction is shown in figure 1. Insulin determinations were by immunoassay only. The study clearly demonstrates the very low solubility of pancreatic insulin in saline. The quantity of insulin extracted by acid alcohol increases directly as the quantity of pancreas. The saline supernatants contain approximately 10 milliunits per milliliter regardless of the amount of pancreas extracted. This value would appear to represent the solubility constant for pancreatic insulin in saline. As the quantity of pancreas is decreased, a point is reached at which the saline and acid alcohol extracts contain the same amount of insulin. Since it is assumed that acid alcohol extracts the total insulin, it would follow that at low concentrations the total insulin is also soluble in saline. However, the insulin insoluble in saline is assumed different than crystalline insulin because the low solubility coefficient is not characteristic of crystalline insulin. If the saline-insoluble fraction was re-extracted with acid alcohol, the total insulin was recovered, demonstrating that the insulin was not destroyed during this saline extraction procedure. The fact that immunoassay gives the same value for saline as for acid alcohol extractions at these low concentrations and the fact that these values represent nearly four units of insulin per gram of pancreas, which is in agreement with the standard mouse convulsion assay,[22] indicates that the immunoassay procedure detects total pancreatic insulin regardless of its free or combined state.

Hypoglycemic activity of pancreatic extracts. A comparison of the hypoglycemic activity of acid alcohol and saline extracts of pancreas is shown in table 1. The acid alcohol extracts contained ten to twenty times the quantity of insulin needed for a hypoglycemic response

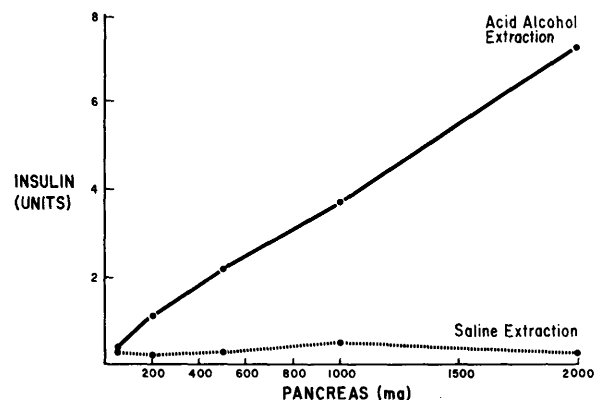

FIG. 1. Solubility of pancreatic insulin.

TABLE 1

Hypoglycemic effect in mice of acid alcohol and saline extracts of pancreas

Group	Whole blood glucose forty-five minutes after injection
Control	76
Acid alcohol	12
Saline	80

Extracts diluted on basis of 2 U. per gram of pancreas to inject 250 mU./100 gram mouse.

and accounted for the marked hypoglycemia observed. The saline extracts showed no insulin activity and are in agreement with the immunoassay which showed the saline to contain less than 0.02 per cent of the insulin of the acid alcohol extract. The hypoglycemic activity is thus shown to correlate with the insulin immunoassay.

Resin extraction of pancreatic insulin. The results of resin treatment of pancreatic tissue homogenates are shown in table 2. Insulin concentration is essentially unchanged after resin treatment. One study showed a persistant decrease (4.3 to 1.6 mU. per ml.). This, however, was the exception. Elution of the resin columns did not recover any immunoassayable insulin in any case even though the eluate had been dissociated at alkaline *p*H. This seems conclusive evidence that the resin is not removing any immunoassayable insulin.

Resin extraction of serum. Treatment of human or dog serum with any one of three cation exchange resins yielded results similar to pancreatic tissue studies (table 3). There was no change in serum insulin after cation treatment regardless of the source or the clinical state of the individual. Elution of the resin columns did not yield any detectable insulin in any case regardless of whether the eluate was treated to convert to free

TABLE 2

Treatment of saline-pancreas homogenates with cation resin (Dowex 50-8X Na+)

Insulin concentration mU. per ml.		
Control	Resin-treated	Alkaline dissociated resin eluate
1,673	1,595	
146	405	
103	119	
130	248	
4.0	5.3	0.005
4.5	5.2	0.005
3.2	3.2	0.005
4.5	4.5	0.005
*4.3	*1.6	0.005

*Average of five samples.

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TABLE 3

Treatment of serum with cation exchange resin (Dowex 50-Na)

Serum source		Insulin concentration μU./ml. Control	Resin treated
Dog splenic vein		16	14
Post tolbutamide		67	64
Dog hepatic vein		20	22
Post tolbutamide		51	51
Human—normal		34	34
		33	40
Glucose tolerance test			
Diabetic, E.S.	3-hr.	80	71
Diabetic, L.L.	30-min.	82	77
	3-hr.	99	102
Diabetic, C.D.	30-min.	72	86
Normal, R.G.	30-min.	111	122

insulin prior to assay.

Conversion of insulin complex to "free" insulin. Incubation with ATE or precipitation at *p*H 9.8 in an effort to convert all insulin to the free form did not increase the insulin detectable by immunoassay (table 4). Incubation with ATE caused a consistent decrease in the remaining insulin. To study further the possibility of insulin destruction by ATE, known amounts of crystalline insulin were added to 6 per cent albumin and the effect of ATE and alkali incubation is shown in table 4. There was a loss of assayable insulin following ATE incubation in all cases. The same result has been noted with several extracts from separate adipose tissues. Centrifugation at *p*H 9.8 produced no effect.

TABLE 4

Effect of insulin "dissociation" with ATE or alkali

Insulin source	Insulin recovery μU./ml. Control	ATE incubation*	Alkaline precipitation*
Pancreas—canine	2,165	1,072	1,380
canine	104	67	
canine	60	33	
canine	47	39	
Serum—human—			
normal	153	66	
diabetic	112	99	
diabetic	233	74	
Pancreas—human—			
normal	23		19
Serum—human—			
normal	192		170
normal	118		92
normal	93		60
normal	153		138
Crystalline beef	180	110	173
	276	187	308

*See text.

DISCUSSION

The inability of immunoassay to demonstrate a cation-bound or inactive form of insulin even after attempts to convert it to an active or free form raises some doubt as to whether the protein-bound fraction of ILA actually represents insulin. If it can be converted to a "free" form of crystalline insulin, it should then be detectable by immunoassay.

In the pancreas, Antoniades found a major portion of insulin bound and not detectable by immunoassay. The studies here reported, however, indicate that although pancreatic insulin is only slightly soluble in saline, immunoassay can detect the total pancreatic insulin which equaled nearly four units per gram of pancreas. Pancreatic insulin could not be bound to any of three cation exchange resins studied. If, in spite of the above findings, one still reasons that immunoassay does not detect the "bound" fraction and therefore removal of the fraction cannot be detected, the inability of alkaline precipitation or ATE incubation to produce any increase in the pancreatic homogenates or resin column eluates suggests that the "free" form, demonstrated by Antoniades to be liberated by either of the above procedures, is not crystalline insulin.

The complete lack of two demonstrable fractions in serum studies also suggests that the bound fraction is not insulin. Likewise, the clinical studies reported by Antoniades are in sharp disagreement with results of immunoassay. He states that in noninsulin dependent adult-onset type diabetes, the response to glucose results in an increase in the biologically inactive bound fraction with no increase or drop in free insulin. Insulin immunoassay studies during a standard oral glucose tolerance test in this laboratory revealed a rise from an average fasting value of 11 microunits per ml. to a one-hour peak of 78 microunits per ml. in ten normals and in eighteen diabetics an average fasting level of 22 microunits per ml. reached a peak average of 121 microunits per ml. at one and one-half hours. If one assumes the immunoassay values represent only free insulin, they are in sharp contrast to the decreasing values reported by Antoniades but agree with the "typical" and "suppressible" insulin reported by Samaan and Froesch. If this is total insulin, the inability to reremove a portion on a cation resin is unexplained. The inability of insulin-antisera to neutralize the ILA of insulin complexes or to detect insulin after several attempts to dissociate these complexes strongly suggests that these fractions do not represent pancreatic insulin.

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ACKNOWLEDGMENT

This investigation was supported in part by PHS Grant AM-08536-01 from the National Institutes of Health, Public Health Service, U.S. Dept. of Health, Education and Welfare.

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