Failure to Demonstrate "Bound" Insulin in Human Serum

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SUMMARY

The reported insulin-like activity, ILA, eluted from a cation resin column, following passage of serum, has been confirmed using the rat diaphragm bioassay. Four methods reported to convert "bound" to "free" insulin did not increase assayable ILA.

A quantitative estimate of ILA in the untreated preparation using the rat diaphragm assay was greater than the estimate obtained using the mouse in vivo assay. It is concluded that rat diaphragm measures total ILA without prior activation. These studies fail to support the concept of "free" and "bound" insulin in human serum. DIABETES *17*:369-73, June, 1968.

Beigelman et al.[1] in developing an in vivo bioassay for insulin found that serum insulin-like activity, ILA, was decreased if the serum was first passed through a cation exchange resin. It was subsequently shown that the ILA could be recovered from the resin.[2] Elution of the cation resin with either acid or alkali yielded material which exhibited ILA when determined using the rat epididymal fat pad,[3] but showed no activity using the rat diaphragm.[4,5] Activity was increased following acid alcohol extraction of the resin eluate,[6] or incubation with alkali,[4,5] adipose tissue extract,[5,7,8] or heparin.[9]

From these studies, Antoniades and co-workers proposed that serum of normal and diabetic individuals contains two materials having ILA. The "free" insulin was assumed to be the physiologically active form of crystalline insulin. The second, an insulin-protein complex termed "bound" insulin, was postulated to be physiologically inactive. It was further thought that extraction or incubation of the complex liberates "free" insulin to which the diaphragm responds. These investigators also assumed that the fat pad was able to dissociate the complex and therefore measured both the "free" and "bound" form.[10-12]

Antoniades et. al.[6] subsequently suggested that the fat pad assayable ILA of either "bound" insulin or its acid-alcohol extract is inhibited after two-hours incubation with guinea pig anti-insulin sera. Crystalline insulin, however, was inhibited within fifteen minutes. "Bound" insulin injected intravenously or intraperitoneally in intact as well as hypophysectomized or adrenalectomized rats has been shown to stimulate fat synthesis in adipose tissue and glucose-C-14 incorporation into muscle glycogen similar to that of crystalline insulin.[13-15] Antoniades and Gershoff[16] obtained similar hypoglycemic effects with crystalline insulin and "bound" insulin injected intravenously in hypophysectomized and adrenalectomized rats but no effects, from the amounts used, in intact animals. They suggested that when studied in vivo, "bound" insulin represents total insulin activity.

As previously reported,[17] insulin immunoassay studies in this laboratory have not confirmed the above findings. The immunoassayable insulin concentration in serum or pancreatic extracts is not changed by passage through a cation exchange column. Also, the cation resin eluate did not yield any immunoassayable insulin either before or after attempts to convert "bound" to "free" insulin. Because of the possibility that the immunoassay technic does not measure either the "bound" or "free" ILA, the rat diaphragm assay method was employed in an attempt to demonstrate the existence of "bound" insulin and the reported activation phenomenon.

MATERIALS AND METHODS

A. *Rat diaphragm insulin assay procedure*

The procedure for insulin bioassay using the rat hemidiaphragm was essentially that of Vallance-Owens and Hurlock.[18] The material to be assayed was alter-

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nately added to the left or right hemidiaphragm. By this technic, no difference from the opposite control hemidiaphragm was obtained without added insulin or cation resin eluate. Glucose was determined by the Somogyi-Nelson procedure. The amount of glucose incorporated into glycogen was determined by the method of Rafaelsen et al.[19] Values were calculated as micrograms glucose uptake per 10 mg. dry tissue or glycogen production per 10 mg. wet tissue. Results are expressed as difference from control hemidiaphragm.

B. *Preparation of cation resin eluate*

Serum obtained from both nonfasted and fasted normal individuals was treated according to the method of Antoniades and Gundersen.[11] One liter was passed, at a flow rate of 10 ml. per minute, through a Dowex 50-×8 (Na^+ cycled) 4.7 × 60 cm. resin column which was then washed with two volumes of 0.15 M NaCl at the same rate. The serum and saline wash were not utilized. The resin was eluted at 4° C. with 1 L. of 0.02 M NH_4OH over a 1 to 2-min. period. The eluate was neutralized with 0.2 N H_2SO_4. When lyophilized, the eluate yielded about 4 gm. of powder, consisting mainly of sodium and ammonium salts. This material will hereafter be referred to as cation resin eluate. Prior to assay, the powder was dissolved in water and dialyzed for 24 hrs. at 4° C. against two changes of Gey and Gey bicarbonate buffer.[20] Before being used, the dialysis tubing was boiled for approximately eight hours with several changes of water to free it of volatile or soluble sulfur compounds.

All water referred to in these studies was distilled water deionized by passing through an Amberlite MB-1 column.

C. *Preparation of adipose tissue extract (ATE)*

Both crude and partially purified ATE were prepared according to the method of Antoniades and Gundersen.[7] The lyophilized powder was redissolved in water, protein nitrogen determined on an aliquot,[21] and the material stored at —20° C. until used.

D. *Methods of treatment of the cation resin eluate*

1. *Incubation with ATE.* After dialysis, the cation resin eluate was diluted with Gey and Gey buffer. The partially purified ATE was added to yield a protein nitrogen concentration of 15 μg. per ml. This was equivalent to 60 μg. per ml. of crude ATE. The crude ATE was not used as it consistently showed an inhibitory effect.

2. *Alkali treatment.* The cation resin eluate was treated according to Gundersen and Antoniades.[4] After centrifugation at 1,100 g for 30 min., the supernatant was removed from the precipitate of calcium phosphate and the *p*H adjusted to 7.2. The treated and nontreated solutions were dialyzed overnight against Gey and Gey buffer.

3. *Acid alcohol extractions.* Four-hundred milligrams of the lyophilized cation resin eluate, obtained from approximately 100 ml. of serum, was dissolved in 5 ml. of water and dialyzed against 100 volumes of deionized water at 4° C. with three changes. The *p*H was adjusted to 7 with 1 N NaOH and the residue removed by centrifugation. Control aliquots were lyophilized and stored at —20° C. until used. The remaining cation resin eluate was extracted with acid alcohol according to Antoniades et. al.[6] For assay, the acid alcohol treated and control samples were diluted to the equivalent of 7 mg. of the lyophilized cation resin eluate per ml. The diluent was deionized water containing 0.5 mg. of human serum albumin per ml. The samples were dialyzed overnight against 100 volumes of Gey and Gey buffer.

4. *Heparin incubation.* Heparin (Liquaemin) solution was extracted three times with diethyl ether to remove the benzyl alcohol preservative. Residual ether was removed under vacuum and last traces of benzyl alcohol removed by treatment with a small amount of acid-washed activated charcoal. This purified heparin solution was frozen at —20° C. until used. It was added to the incubation mixture at a concentration of 145 μg. per ml. just prior to rat diaphragm assay.

E. *Comparison of in vivo and in vitro ILA of cation resin eluate*

Based on the ILA determined by rat diaphragm assay, the cation resin eluate was compared to the same activity of insulin. Both cation resin eluate supplied to us by Dr. Antoniades and materials prepared in this laboratory were studied. Following an overnight fast, 20 gm. mice were injected intraperitoneally with the study material or saline, each containing one microcurie of uniformly labeled C-14-glucose per injection. One hour after injection, the mice were decapitated, blood collected in a dry heparinized tube and diaphragms removed. Blood glucose and diaphragm C-14-glycogen were determined.

RESULTS

Stimulant effect of cation resin eluate on glucose metabolism (ILA)

As shown in table 1, as little as 3 mg. per ml. of the cation resin eluate has significant ILA, 7.2 μg. glycogen per 10 mg. wet tissue. This confirms Antoniades reported ILA in cation resin eluate. However, these results

TABLE 1

Effects of untreated and treated cation resin eluates upon glucose uptake and glycogen production in isolated rat hemidiaphragms

Cation resin eluates				Stimulant effect on glucose metabolism (ILA)			
				Glycogen production (μg./10 mg. wet tissue)		Glucose uptake (μg./10 mg.dry tissue)	
Source	Concentration (mg./ml.)	"n"	Treatment*	Untreated	Treated	Untreated	Treated
Antoniades	6	5	ATE	5.2±1.6†	3.9±1.3		
Authors	3	5	ATE	7.2±1.5	6.0±2.9		
Authors	15	20	ATE	19.2±1.8	15.4±0.9‡		
Antoniades	18	5	Alkali	10.4±0.8	11.4±1.8		
Authors	12	5	Alkali	13.9±1.5	10.8±1.9		
Authors	7	9	Acid-alcohol	6.3±1.7	3.7±1.2		
Authors	16	6	Heparin			154±39	179±41
Insulin	1,000 (μU./ml.)	15		26.6±2.3		224±32	
Standard	100 (μU./ml.)	15		5.7±1.0		94±30	

*ATE = Adipose tissue extract. Heparin concentration = 145 μg./ml.
†Mean ± S.E.M. Difference from control hemidiaphragm.
‡A significant decrease $p < 0.001$, see text.

differ in that rat diaphragm response was obtained without prior treatment of the eluate.

Effect of treatment of cation resin eluate.

The results of the four methods of treatment proposed for the activation of cation resin eluate are shown in table 1. The ATE treatment of both material supplied by Dr. Antoniades and that produced in our laboratory did not increase ILA. Quite the contrary, a significant decrease in activity was obtained in the largest study. A similar failure to show activation was obtained following acid alcohol treatment of the same materials. Likewise, alkali or heparin incubation of the cation resin eluate produced in our laboratory did not change the ILA.

Comparison of in vivo and in vitro ILA of cation resin eluate

Changes in blood glucose and diaphragm muscle C-14-glycogen following the intraperitoneal injection of cation resin eluate or crystalline insulin are shown in table 2. Neither the eluate from Antoniades nor that produced in our laboratory had any significant hypoglycemic effect at the concentrations used. Both materials stimulated incorporation of C-14-glucose into glycogen in vivo. However, both of the eluates had less

TABLE 2

In vivo comparison of crystalline insulin and cation resin eluate ILA

Material	Source	Dose μU.	No. of animals	Blood glucose mg. per 100 ml.	P†	Diaphragm C-14-glycogen cpm/10 mg. wet wt.	P†
Control—saline		0	5	110.9±12.5		2± 0.5	
Crystalline insulin		1,500	5	74.7± 4.6	<.01	468±278	<.01
Cation resin eluate	Authors	1,500	5	121.8±16.2	NS	148± 69	<.05
Control—saline		0	4	84.6±14.0*		28±14*	
Cation resin eluate	Antoniades	1,500	4	59.1±18.3	NS	415± 92	<.01
Control—saline		0	5	100.8± 8.6		12± 10	
Crystalline insulin		500	5	77.8± 6.8	<.05	396±141	<.05
Cation resin eluate	Authors	500	5	99.3± 5.4	NS	111± 19	<.01
Control—saline		0	4	88.0± 5.8		22± 11	
Cation resin eluate	Antoniades	500	4	92.7± 9.0	NS	159± 79	NS
Control—saline		0	5	93.3± 6.4		6± 3	
Crystalline insulin		100	5	101.5± 6.8	NS	120± 35	<.05
Cation resin eluate	Authors	100	5	87.7± 8.5	NS	17± 12	NS

*Mean ± S.E.M.
†Probability of no difference from control.

activity than the equivalent dose of insulin predicted from the in vitro assay. There was no evidence to suggest that the in vivo activity of the eluate exceeded that obtained using the rat diaphragm.

DISCUSSION

In this study, we have been unable to confirm the presence of "bound" insulin in human serum. Contrary to Antoniades and Gundersen,[4,5] we find that the untreated cation resin eluate possesses ILA in the rat diaphragm assay. The ILA remained unchanged following all proposed methods of activation.[4-9] In addition, the in vivo ILA of cation resin eluate did not exceed the rat diaphragm bioassay.

Because of the complexity of the various procedures used to demonstrate "bound" insulin, one might speculate that there are many factors to account for the difference between our results and Antoniades. However, in reviewing all possible differences, we are unable to find any which seems significant.

Antoniades found "bound" insulin concentration was greater in fasting than nonfasting serum.[2] In our studies which extended over several months, cation resin eluate from both fasting and nonfasting sera showed no increased ILA with four proposed activation technics.

The rat diaphragm bioassay procedure used did not differ appreciably from that of Antoniades. He usually reported glucose uptake; whereas, we report C-14-glycogen production which in our hands was less variable and more reliable than glucose uptake. However, glucose uptake results were reported for the heparin activation study. Although "bound" insulin should have no ILA on the rat diaphragm Antoniades and Gundersen[7] obtained an average net increased glucose uptake due to untreated "bound" insulin of about 75 μg. per 10 mg. dry tissue which increased to 192 μg. upon incubation with ATE. Our untreated cation resin eluate value of 154 μg. per 10 mg. dry tissue (table 1) is an intermediate value and does not support the statement that the rat diaphragm is nonreactive to the resin eluate. Similar results were obtained with cation resin eluate donated by Antoniades. He stated[22] that up to 50 mg. per ml. of the powder would have no rat diaphragm ILA until activated; however, significantly increased glycogen synthesis was obtained with as little as 2 mg. per ml. of the crude powder. These results suggest that the authors' rat diaphragm bioassay procedure is more sensitive than that of Antoniades.

The method for preparation of partially purified ATE did not differ from that of Antoniades and Gundersen.[7] The equivalent of 60 μg. protein nitrogen per ml. used for cation resin eluate incubation is well above the 18 μg. per ml. which Antoniades found gave optimum results.[7] The other methods for treatment of cation resin eluate were unchanged from those suggested by Antoniades and associates, with the exception that the benzyl alcohol preservative was removed from heparin solution.

Another possible explanation for the lack of activation response could be a difference in the type of resin used for preparation of the cation resin eluate. Beigelman and co-workers[1] first noted a loss of ILA from serum passed through IRC-50 resin. Subsequently, Antoniades[5] used Dowex 50-×2 or Dowex 50-×8 and reported no difference although the ILA values were higher in eluates from Dowex 50-×8. Horwitz, Alp and Recant[23] showed binding of ILA from pancreas homogenates to Dowex 50-×2 but not to Dowex 50-×8. Using Dowex 50W-×8 resin, Shaw and Shuey[8] demonstrated a twelvefold increase in ILA of resin eluate with ATE incubation. However, Dr. Shaw[24] stated: "In later studies, there was considerable difficulty in consistently duplicating the published results. Nevertheless, many subsequent experiments confirmed that incubation of ATE with undiluted serum resulted in a significant increase in ILA measured by the rat diaphragm technique." In their first study, a single batch of resin had been used for all of the published data and new batches of resin did not always yield "bound" insulin. In the present study, several different batches of Dowex 50-×8 or Dowex 50W-×8 were used, none of which yielded "bound" insulin. Furthermore, it appears that the type of resin used is not the cause of the discrepancy between our studies and Antoniades', since cation resin eluate prepared in his laboratory gave results similar to material prepared by the authors.

Visking casing, used for dialysis in several steps, has been reported to contribute to ILA activity.[25] Antoniades does not mention prior treatment of dialysis tubing. All casings used in this study were treated as stated in methods.

Stimulation of C-14-glycogen deposition has been demonstrated using intact[13-15] and adrenalectomized or hypophysectomized[16] rats. In our in vivo study, the intact mouse was used. The rat diaphragm, which measures only "free" insulin,[4,5] yielded equal or greater ILA than the in vivo study which measures total "bound" and "free" insulin.[16] Our results suggest that the rat diaphragm measures total ILA of the cation resin eluate. Furthermore, the value of 10 μU. per mg. powder

which we obtained by rat diaphragm assay without treatment compares to Antoniades' stated value of 10-15 μU. per mg. after activation.[22] If, in the authors' hands, the rat diaphragm measures total ILA, no increase in activity would be expected.

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