Effect of Heparin on Insulin-like Activity in Rat Bioassay

Comparison between Rat Diaphragm and Epididymal Fat Pad Assay in Normal and Untreated Diabetic Subjects

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SUMMARY

A comparison between the rat diaphragm and the rat epididymal fat pad insulin bioassay has been undertaken, making use of serial dilution on sera from untreated diabetic and normal individuals and preincubation with heparin. Heparin, in our hands, has been found to split isolated basic insulin protein complexes and we believe that it also can do this in diluted sera. Our results indicate that by the use of dilution and preincubation with heparin, the two assays correspond surprisingly well in their results. Untreated diabetics, both in the fasting and postprandial state, have more insulin in their serum than normals. There appears to be an antagonistic factor present in postprandial diabetic serum, which is revealed by dilution. Diabetic sera appear to contain more "complexed insulin" than normal ones. No dissociation of "complexed insulin" could be found when glucose was given orally. DIABETES *14*:805-10, December 1965.

Since the inception of the rat diaphragm and rat epididymal fat pad assays for insulin, it has been noted that the fat pad assay measures considerably higher levels of "insulin-like activity" than the diaphragm method when undiluted sera are used. It has also been noted that untreated diabetic adult subjects show higher insulin levels than normals particularly in the postprandial state by the former method, although somewhat elevated values have also been reported by the diaphragm assay postprandially in mildly diabetic subjects.[1]

It is of interest that several workers have reported very high insulin levels in diabetic subjects with the diaphragm assay when sera were diluted 1/8 to 1/16,[2,3] and it was assumed that this dilution had caused the disappearance of a substance which inhibited the glucose uptake in the rat diaphragm.

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Another "form" of insulin in blood has also been reported, which could be absorbed on a cationic exchange resin and recovered by elution with dilute alkali or acid. This material had little or no activity when tested with the diaphragm, while considerable activity was reported with the fat pad assay. It was furthermore found that exposure to *p*H 10 or an aqueous adipose tissue extract (ATE) enabled the diaphragm to respond to this substance, the assumption being that these technics dissociated a type of insulin from a basic (protein) element.[4,5] Further studies have shown that considerable amounts of these insulin complexes may exist in the fasting state in normal individuals, with a rapid disappearance after intravenous glucose.[6] Furthermore, a number of untreated adult diabetics have this variety in their serum, and the disappearance noted in normals did not take place to an appreciable extent after intravenous glucose, thus giving another possible explanation for the discrepancies in insulin levels reported by the two assays. Other studies[7,8] done with the fat pad assay and the use of insulin antibodies have indicated a fairly constant level of "atypical" insulin in blood, with no relationship to blood sugar. The latter variety is not inactivated by insulin antibodies, and thus appears to be similar to the previously mentioned "complexed" insulin except for the apparent stable blood levels. Considerable doubt has been raised as to whether these substances are insulin, as reported by Berson, who could get no binding to insulin antibodies.[9] However, Shaw et al. have reported at least partial inactivation of "complexed" insulin by antibodies after dissociation with adipose tissue extracts,[10] and have also noticed an effect of these complexes on glycogen synthesis, a function which only insulin seems to be able to perform.

An extensive comparison of the two bioassays has not been reported so far, and we have attempted to study the insulin levels in sera with both the diaphragm and the rat epididymal fat pad, making use of serial

dilution. Also, in our hands, addition of heparin prior to tissue incubation has resulted in recovery of insulin-like activity from basic "insulin complexes," similar to that seen with ATE.[11] This probably is effected by a binding of heparin to the basic moiety of the complex, as is seen in the combination of protamine with heparin. Therefore, we have also added heparin to the most dilute sample of serum in an effort to find out if further insulin-like activity could be found. Since in general, insulin levels reported by the diaphragm bioassay (undiluted sera) and the immunoassay seem to correspond fairly well in contrast to the much higher levels of insulin (or "insulin-like activity") found with the epididymal fat pad assay, a further study of the discrepancies seemed to be necessary.

METHODS

The rat diaphragm insulin bioassay was used as described by Vallance-Owen,[12] except that the medium was incubated in open 10 ml. Erlenmeyer flasks in the Dubnoff shaker[13] under constant gassing with 95 per cent O_2 and 5 per cent CO_2. The gas was passed through a water trap. We have noted no change in sugar content by this method when media were incubated without diaphragms. Standard insulin* concentrations of 100, 500, and 1,000 μU./cc. were run in each of the diaphragm assays, together with buffer alone. Unknowns were interpreted on the S-curve previously described by the use of these insulin concentrations.[13] The values of unknowns between 0 and 100 μU. also conformed well with data obtained by Cunningham, using a straight linear relationship in this region.[14]

The fat pad assay of Martin and Renold[15] was employed, using 1-C-14 glucose and absorbing C-14-O_2 with hyamine for counting in the scintillation counter. Incubation period was two hours.

Sera were separated from blood after proper clotting and clot retraction at room temperature. Most sera were kept in the cold room (4° C.) until testing time, although some were frozen (—10° C.). Hemolysed sera were not used.

Blood was drawn from normal individuals and untreated, newly discovered diabetics while fasting and after oral glucose (100 grams). Most diabetics were moderately to grossly obese. The normal subjects were within normal weight range.

Sera were diluted with our usual bicarbonate buffer (Gey and Gey),[23] to which 1 cc. of 25 per cent normal human albumin/500 cc. was added in the diaphragm method and 0.2 per cent gelatin in the fat pad assay.

Heparin (Liquaemin Sodium, 1,000 μ/cc.) was added to the most dilute sample, usually 0.1 or 0.2 cc./8 cc. of diluted serum, although we have lately used powdered heparin* dissolved in Gey and Gey buffer in a concentration as high as 10,000 U./cc. with similar results; .1 cc. aliquots were used per 8 cc. of sample, with a preincubation of one-half hour at room temperature before addition of tissues. Repeated freezing of sera appeared to decrease the effect of heparin, although final values remained the same.

"Insulin complexes" were obtained by the methods described by Antoniades and Gundersen.[13] The lyophilized powder was dissolved in Gey and Gey buffer and dialysed against the same buffer for twenty-four hours, with one change. Heparin was then added as described above.

RESULTS

Preliminary studies

Figure 1 shows the effect of heparin on the amounts of insulin activity recovered from five different samples of complexed insulin. It can be seen that considerably higher values are found with the diaphragm assay after heparin is added, while high values are seen with the fat pad assay even when the latter substance is not used. In a couple of assays the amounts of eluate used precluded the full measurement of activity in the fat pad assay, since this method uses 500 μU./cc. as maximal standard while the diaphragm can give fairly accurate measurements up to 1,000 μU./cc.

Figure 2 shows experiments performed to rule out

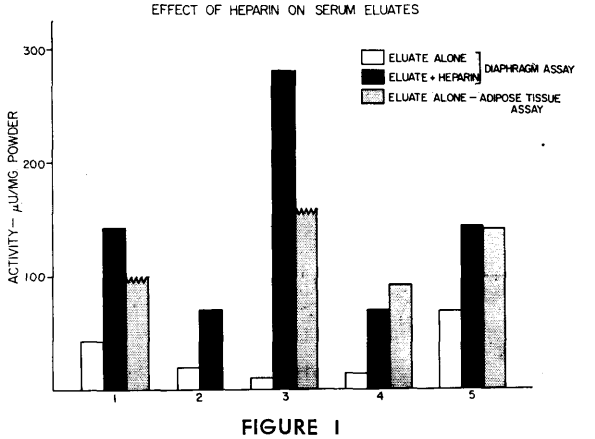

FIGURE 1

*Supplied by E. R. Squibb and Sons, Dr. A. Borman, Rahway, New Jersey.

*Supplied by The Upjohn Company, Kalamazoo, Michigan.

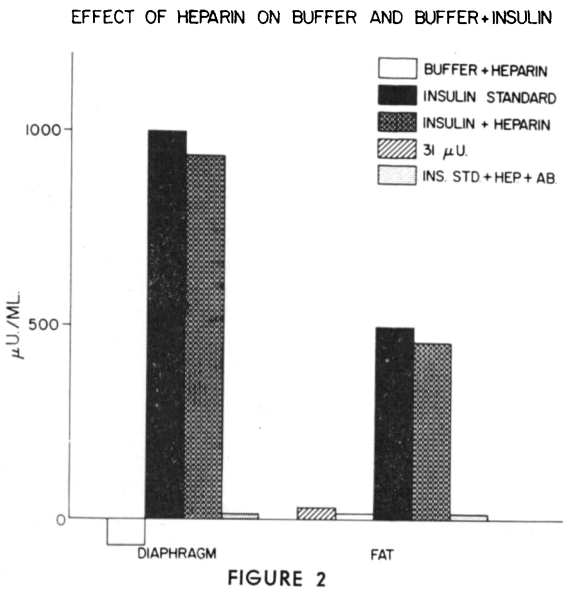

FIGURE 2

any specific effects of heparin on glucose uptake by the diaphragm or CO_2 production in the fat pad. The same amount of heparin was added to buffer alone, insulin standards in buffer, and insulin standard to which specific antibodies from guinea pigs were added. Each category represents nine hemidiaphragms or fat pads. It can be seen that heparin does not enhance the activity of the already present insulin, nor does it enhance the glucose uptake or CO_2 production in buffer alone. The inhibitory action of insulin antibodies is unimpeded.

Table 1 shows the effect of insulin antibodies on "complexed insulin" with the addition of heparin. Although the effect seems to be incomplete, it is consistent.

Effects of dilution and heparin on sera

Table 2 shows comparative studies done on a series of sera in a group of nondiabetic fasting subjects. Various dilutions have been made, and heparin was added to the last dilution. In a number of cases the serum was only diluted 50 per cent (½), and tested without and with heparin. All figures have been corrected for dilution. The averages quoted thus do not give complete information, but are rather given to compare the results of the two assays, neither one of which has a high index of precision. The increment in insulin-like activity when heparin is added to the diaphragm assay can be seen in most cases, and these values correspond in general well with those obtained by the fat pad assay. We have noted that sera from a few patients consistently show no heparin effect, and often less insulin activity is found when heparin is added.

Table 3 shows a series of insulin values obtained in normal individuals after oral glucose. Again the data correspond well as far as the two assays are concerned when heparin is added. It appears that "complexed insulin" is not dissociated, or incompletely so, when glucose is given orally, which may be due to the failure of the blood sugar to reach the levels obtained with intravenous glucose.[6]

In table 4 we give the results in a series of fasting sera from recently discovered, untreated diabetic subjects. No appreciable increment in values is seen with dilution in the diaphragm assay, while some is seen with the epididymal fat pad assay, similar to that shown by Lyngsoe.[22] Considerably higher insulin values are obtained from the addition of heparin in the diaphragm assay, with little effect of this substance on the fat pad. The comparative values in the last columns are again quite similar, and considerably higher than in the normal subjects.

A number of postprandial sera from the same type of patients as in table 4 is reported in table 5. Glucose was given orally in this series, too. In some instances dilution gave a striking increase in insulin values in the diaphragm assay, with some increase on the fat pad, too. With heparin added, higher values than in any other category were found, and the two assays were again generally in agreement, although wider discrepancies are seen than in the other tables, probably because of inherent variation in the assays and the higher values found. Thus, our data should not be considered as

TABLE 1

Effect of insulin antibody on "complexed" insulin (μU./cc.)
Heparin added

	Diaphragm			Fat Pad		
	without	with AB	1,000 μU. +AB	without	with AB	1,000 μU. +AB
Eluate II	1,000	225	85	150	100	<31
Eluate II	166	90	15	103	42	35
Eluate II	132	100	15	65	49	
Eluate II	200	10		260	51	
Eluate II	250	120	6	50	35	<31

TABLE 2

Effect of dilution and heparin on serum insulin-like activity (μU./cc.) in normal fasting subjects

Patient	Age	BS	Diaphragm ½	Diaphragm ¼	Diaphragm ⅛	Diaphragm + hep.	Fat Pad ½	Fat Pad ¼	Fat Pad ⅛	Fat Pad + hep.
G.O.	21	72	40	120	0	560	170	380	480	380
B.D.	31	81		700	780	880		240	340	1,280
L.P.	50	75	60	100	120	320	180	260	420	540
M.L.	45	88	30	150	0	280	70	110	200	500
M.D.	34	93	110	40	80	400	90	120	⌣80	⌣80
B.L.	34	85	50	0	0		80	120	⌣80	
M.J.	20	80	20			80	230			270
P.M.	22	82	10			170	200			240
L.H.	40	85	100			50	380			260
T.A.	25	87	0			140	90			100
B.S.	27	72	120			40	160			270
J.M.	37	80	40			90	180			130
L.M.	42	76			70	640			390	400
C.P.	40	85		360		440		500		460
J.M.	17	78		40		600		420		330
J.M.	17	78			0	640			600	680
I.D.	27	86			440	880			620	390
	Average		53	192	165	388	166	270	365	423

TABLE 3

Effect of dilution and heparin on serum insulin-like activity (μU./cc.) in normal postprandial subjects

Patient	Age	Time pc.	BS	Diaphragm ½	Diaphragm ¼	Diaphragm ⅛	Diaphragm + hep.	Fat Pad ½	Fat Pad ¼	Fat Pad ⅛	Fat Pad + hep.
H.H.	60	2½	90		260	220	230		760	1,040	610
J.H.	40	3	70		<10	<10	90		40	⌣80	⌣80
G.O.	21	1	89	320	660	90	100	130	1,300	260	200
G.O.	21	3	75	250	460	0	240	420	310	320	460
B.S.	55	2½	75	0	0	0	740	440	640	760	750
L.P.	45	2½	75	250	320	0	520	260	420	450	500
M.D.	34	½	100	200	130	90	640	330	340	500	650
M.D.	34	2	91	60	140	0	400	250	150	420	360
L.M.*	42	1	160			440	730			720	820
J.R.†	50	1	196			550	640			660	610
		Average		180	247	140	433	310	495	521	504

*Except for borderline BS, GTT was normal.
†Glucose-cortisone test.

TABLE 4

Effect of dilution and heparin on serum insulin-like activity (μU./cc.) in untreated diabetic fasting subjects

Patient	Age	BS	½	¼	⅛	+ hep.	½	¼	⅛	+ hep.
W.S.	53	100	70	280	130	2,400	250	720	730	1,160
L.C.	45	135	20	180	130	370	620	720	1,080	520
L.C.	45	125	120	260	110	400	640	720	1,120	1,320
L.C.	45	135	80	90	220	730	470	970	1,360	840
W.C.	70	75	90	90	60	1,000	270	680	1,020	2,100
J.S.	56	170	40	110	0	400	390	420	560	540
E.L.	53	90	140	520	160	400	280	400	520	650
B.Y.	65	100	70	160	0	480	100	180	270	240
M.C.	60	105	40	110	160	360	300	400	430	540
St.W.	58	96	40	100	0	240	200	160	220	180
	Average		72	190	97	778	352	534	731	809

TABLE 5

Effect of dilution and heparin on insulin-like activity (μU./cc.) in untreated postprandial subjects

Patient	Age	Time	BS	Diaphragm ½	Diaphragm ¼	Diaphragm ⅛	Diaphragm + hep.	Fat Pad ½	Fat Pad ¼	Fat Pad ⅛	Fat Pad + hep.
J.M.	42	2½	130		0	240	750		740	1,120	960
K.M.	58	3	300	180		690	1,570	480		960	1,680
L.V.	40	2½	250		340	310	580		600	500	1,070
L.L.	60	3	110	40		320	2,000	380		490	560
J.H.	65	2½	235	220		50	580	590		880	740
F.E.	47	3	572		30	740	2,240		370	390	690
O.F.	64	2½	130		560	2,100	4,610		840	960	2,240
L.C.	60	½	145	390	20	260	1,060	390	640	1,260	1,400
L.C.	60	2½	230	150	390	310	260	560	840	1,040	1,280
I.M.	65	5	197		80	1,060	1,550		390	540	1,360
G.C.	45	2½	180	20	80	470	780	600	700	900	1,480
I.P.	61	2½	135	140	280	280	320	700	800	900	680
T.W.	70	3	221	40	120	80	640	310	640	780	820
R.A.	58	1½	201	180	200	190	730	190	290	370	1,020
R.A.	58	2½	200	120	240	0	320	630	430	660	870
M.M.	68	5	208	100	160	320	640	350	400	700	580
R.P.	55	2½	165	160	380	320	960	620	650	1,320	1,010
F.E.	46	3	561	250	500	1,400	1,800	480	560	840	1,080
		Average		153	279	508	1,177	483	459	756	1,084

being quantitatively accurate, but rather as indicative of relative values. However, in some cases much higher values are seen consistently with the fat pad assay.

The dilution effect seen in some instances may be due to the antagonist reported by Vallance-Owen and others.[16,17] This factor does not seem to be dependent on previous treatment with insulin or oral hypoglycemic drugs, as shown in table 6, nor is the blood sugar always grossly elevated.

DISCUSSION

Our comparative studies with the two bioassays indicate that serum dilution and the addition of heparin "unmasks" a considerable amount of insulin-like activity, especially when the rat diaphragm assay is employed. Furthermore, in a significant number of instances the values reported by both assays coincide quite closely. The finding of higher insulin levels in recently discovered, untreated diabetics corresponds well with previous reports.[18,19] It is tempting to associate recovery of large amounts of insulin-like activity after dilution with the disappearance of antagonistic substances, such as have been described by Vallance-Owen and others.[16,17] Recovery of large amounts of insulin-like activity after electrophoresis and chromatography[17,20,21] also seem to indicate the removal of such factors, and the finding of several peaks of activity would indicate that insulin exists in several forms in the blood. Since the addition of heparin frequently results in higher insulin values than with dilution alone, it appears that this substance releases more insulin, perhaps bound to basic proteins and that the latter moiety does not represent an antagonistic factor per se, but is of a different nature. It is also interesting that heparin seems to work better in high serum dilution than low ones, as seen in our results. The apparent persistence of "complexed insulin"

TABLE 6

Effect of dilution on insulin content of diabetic sera (μU./cc.)

	Post-prandial blood sugar	Diaphragm 1/2	1/4	1/8	1/16
1. Insulin resistant	560	10	24	3,750	4,000
2. 500 mg. chlorpropamide	198	480	254	2,520	5,250
3. Postprandial untreated diabetic	200		44	1,720	1,460
4. Postprandial untreated diabetic	572		30	740	2,130
5. Postprandial untreated diabetic	130		560	2,100	2,530
6. Postprandial untreated diabetic	197		80	1,060	1,890
7. Postprandial untreated diabetic	70	94	320	1,100	

in the postprandial state may indicate that this moiety is not important for maintenance of serum glucose levels in normal individuals where adequate amounts of "free" insulin are present. Although there is also an apparent abundance of the latter in the diabetics, when diluted sera are assayed, earlier experiments have shown that levels are found to be lower in the undiluted sera.[17] Thus, antagonists may prevent the use of "free" insulin in muscle tissue, indicating that these may be equally important. They also seem to be important in uncontrolled diabetics who take insulin and appear to be present when oral drugs are used, too.

On the basis of our findings, we believe that the so-called "insulin antagonists" and "complexed insulin" are two different entities, the origin and role of which are not known at the present time. We also believe that the rat epididymal fat pad insulin bioassay does measure predominantly insulin, although further work is necessary to determine why the immunoassay fails to measure a great proportion of serum insulin.

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