Effect of Tolbutamide on "Free" and "Complexed" Serum Insulin

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SUMMARY

Serum insulin levels have been determined in non-diabetics and non-insulin requiring diabetics during the oral tolbutamide test. Using diluted sera, with the addition of heparin to measure "free" and "complexed" insulin by the rat diaphragm insulin bioassay, there was little evidence that tolbutamide has any appreciable effect on the dissociation of "complexed" insulin by this method. Several patients appeared to have no "complexed" insulin in their sera at any time. Obese subjects had higher serum insulin levels than those of normal weight. DIABETES *15*:663-67, September, 1966.

Hasselblatt and Schmieta[1] reported in 1960 that the addition of tolbutamide to anti-beef-insulin sera containing exogenous insulin brought about an increase in insulin activity, as measured by the rat epididymal fat pad assay, rather than the expected decrease from the inactivation by the insulin antibodies, and they suggested that the compound dissociated antibody-bound insulin. However, Otto and Korner[2] disagreed with this hypothesis, finding no increase under the same conditions, although they may have added too much insulin to draw an adequate conclusion.

On the other hand, they found that sulfonylurea in a concentration of 80 mg. per cent increased insulin activity in normal guinea pig sera. Kerp et al.,[3] using insulin antibody binding to I-131 insulin and subsequent ultracentrifugation, could find no change in binding with tolbutamide.

Since Antoniades et al.[5] have reported liberation of "complexed" insulin during the intravenous tolbutamide test in humans, we have investigated the relative amounts of "free" and "bound" insulin (determined by the dissociating effect of heparin) in our own laboratory in the blood of normal persons and noninsulin requiring diabetics, using the oral tolbutamide test,[4] which gives even higher serum drug levels than the intravenous test.

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MATERIALS AND METHOD

The oral tolbutamide test was given to a total of twenty-five subjects: fourteen untreated diabetics, three borderline cases, and eight normal individuals, as described by Vecchio et al.[4] Blood was drawn before, and thirty to forty minutes after the drug for blood sugar determinations. Sugar concentrations were determined by the AutoAnalyzer (Technicon) using the ferricyanide reaction. All samples were run in duplicate. Samples rarely differed by more than 2 mg. per cent. The blood was allowed to clot and the serum either used immediately or stored at 4° C. until assayed by the rat diaphragm insulin assay of Vallance-Owen.[6] The bicarbonate buffer contained 0.2 per cent Red Cross albumin. In almost every assay insulin in a concentration of 1,000, 500, 100 μU./cc.,* together with buffer alone,[7] was used for our standard curve. Glucose uptake (μg./10 mg. dry diaphragm) was plotted against the cube root of the standard insulin concentrations and the results of the unknown samples interpreted on this S shaped curve. The sera were diluted 1:4 or 1:8 in all except two cases, and incubation took place in open 10 cc. Erlenmeyer flasks on a Dubnoff shaker with continuous gassing with 95 per cent O_2-5 per cent CO_2 throughout the ninety-minute incubation time. Each serum was incubated both without and with 25 U. of heparin per cc., as we have found that heparin dissociates isolated insulin "complexes" and similarly increases insulin-like activity (ILA) of sera, as described.[8,9] Thus, insulin values obtained on serum without heparin added should represent "free" insulin in this dilution, while the addition of heparin should give values including both "free" and "complexed" insulin, the difference between the two values representing the latter portion, or, more properly, "heparin-liberated" ILA.

Diagnoses were made on the basis of prior oral glucose tolerance test. Borderline cases were so judged

*Recrystallized insulin with low glucagon content was kindly supplied by Dr. Alec Bowman of the Squibb Institute for Research, Rahway, New Jersey.

when the one or two-hour blood sugar was elevated, although in some cases the oral tolbutamide test indicated normal blood sugar response. It should also be noted that several patients were known diabetics of several years' duration (Nos. 13, 14, 15, 17 and 18), while two patients had received insulin several months prior to the test (Nos. 17, 20). No. 19 was discovered to have diabetes six months prior to the test. She was young and obese. No. 20 was an extremely obese female. No. 11 was moderately obese.

RESULTS

Table 1 shows the blood sugars obtained during the test. The patients in this series are numbered in accordance with tables 2 and 3.

Table 2 shows the diaphragm bioassay data of forty assays, together with the standard error of the mean. Most of the diluted sera caused a glucose uptake similar to the 100 μU./cc. standard. Our assay also seems to be most sensitive in this region, although data below 10-15 μU./cc. do not differ significantly from buffer alone in some assays. The distinction between 500 and 1000 μU./cc. also is poor.

In table 3 the bioassay data have been expressed as microunits of insulin per cc. of whole serum, with appropriate adjustment made for dilution, and the net effect of the addition of heparin ("heparin-liberated" insulin-like activity) represented in the third column. With the sera of two subjects (Nos. 21 and 22) heparin appeared to be consistently inhibitory on the glucose uptake of the rat diaphragm which corresponds with some cases already reported,[8] while in three cases (Nos. 4, 6 and 15) heparin caused inhibition before tolbutamide was given. In one case (No. 10), inhibition was found after tolbutamide administration. The high serum "free" ILA values in some individuals in the normal group may be due to previous carbohydrate abnormalities in Nos. 1 and 6 (hypoglycemic episodes). No. 3 had a strong family history of diabetes. Our normal series also shows no significant difference in "free" insulin-like activity values before and after tolbutamide, a finding which may be due to the time of sampling, as normal individuals usually have a peak value at an earlier time. Twenty cases show no change, or even an increase in "heparin-liberated" ILA after the administration of tolbutamide by mouth. All patients with unusually high serum insulin levels were obese, and were either suspected or overt diabetics.

TABLE 1

Tolbutamide test. Blood sugars (mg. per 100 ml.)

	Patient	Before tolbutamide	Thirty minutes after tolbutamide
	Normal		
1.	J.R.	80	31
	J.R.*	82	47
2.	F.P.	89	50
3.	I.D.	86	44
4.	E.A.	107	83
5.	J.L.	97	46
6.	Mc.P.	77	50
7.	L.C.	80	66
8.	H.S.	100	58
	Borderline		
9.	J.Mc.G.	80	55
10.	F.S.	77	64
11.	J.C.	84	44
	Diabetics		
12.	St.W.	96	72
13.	A.S.	222	213
14.	Mc.H.	97	65
15.	M.B.	195	205
16.	M.Br.	107	75
17.	J.S.	183	185
18.	M.Bro.	91	86
19.	L.M.	269	254
20.	F.E.	81	72
21.	B.D.	111	91
22.	A.M.	90	59
23.	M.M.	118	110
24.	L.A.	110	90
25.	H.F.	90	74

* = one year later

DISCUSSION

Hasselblatt, in a communication three years after the first publication,[10] repeated his previous statement concerning the prevention of antibody binding by tolbutamide and also verified this phenomenon in an insulin-resistant patient, adding that the carbonic acid of tolbutamide, which is inert, did not prevent insulin-antibody binding. He also stated that a concentration of 20 mg. per cent tolbutamide in normal rat serum liberated endogenous insulin, and that this "bound" insulin-like activity was only present six to ten hours after food intake, with none present in the fasting state or after an oral glucose load. Since the concentration of tolbutamide in vitro corresponded well with concentrations reached in vivo during the intravenous tolbutamide test, he saw no reason to doubt a similar liberation in vivo. He considered the possibility that tolbutamide could liberate "bound" insulin present in the pancreas, too, but he felt that a portion of the effect of tolbutamide represented liberated insulin from peripheral binding sites, thus assuming a multiple action of the drug, perhaps with a minor role being played by pancreatic release.

Lacy[11] studied the effect of 100 mg. per cent tolbutamide in vitro on insulin-like activity in sera from twelve diabetic patients on insulin and thirteen non-

TABLE 2

Diaphragm bioassay data. Glucose uptake (μg. glucose/10 mg. dry diaphragm ± S.E.M.) above basal level (buffer alone). Serum diluted one-eighth unless otherwise marked.

Patient	Insulin standard (μU./cc.) 1,000	500	100	Before tolbutamide: Serum alone	Serum + heparin	30-40 minutes after tolbutamide: Serum alone	Serum + heparin
1. J.R.	320±23	274±20	116±10	82±22‡	136±10‡		
J.R.	338±6	336±6	120±17			96±11‡	146±12‡
J.R.*	314±50	310±60	132±7	176±20‡	176±20‡		
J.R.	266±5	226±6	106±10			110±17‡	154±20‡
2. F.P.	312±26	302±27	178±24	30±25	102±6	54±9	104±24
3. I.D.	—	184±8	116±20	54±29	126±12	54±9	92±9
I.D.†	312±10	300±7	172±19	124±3	188±18	128±20	192±19
4. E.A.	338±18	308±6	176±27	60±23	38±17		
E.A.	414±27	360±20	188±43			144±17‡	154±24‡
5. J.L.	254±18	234±17	172±36	112±18	122±3		
J.L.	256±26	212±16	172±19			90±12‡	128±6‡
6. Mc.P.	228±20	224±24	138±38	108±20	74±18	110±20	114±18
7. L.C.	252±25	216±15	134±18	48±13‡	108±11‡	62±11‡	128±19‡
8. H.S.	240±20	200±12	136±42	38±22	40±9		
H.S.	222±32	206±38	90±36			70±20	86±16
9. J.Mc.G.	320±38	226±32	88±27	52±19‡	170±13‡		
J.Mc.G.	338±6	336±6	120±17			258±9‡	306±23‡
10. F.S.	310±20	278±14	152±24	62±16	72±22		
F.S.	236±2	200±26	100±20			134±24‡	114±22‡
11. J.C.	226±28	164±18	94±24	104±3	130±20	110±15	182±9
12. St.W.	306±24	272±19	—	0±6	76±9		
St.W.	278±18	204±8	158±20			50±18	158±19
13. A.S.	304±2	276±40	142±10	108±16‡	152±7‡		
A.S.	256±17	244±18	100±18			92±13	114±16
14. Mc.H.	290±18	296±18	144±24	150±26	158±38	116±13	150±24
15. M.B.	338±28	336±14	188±14	112±9‡	106±20‡	82±4‡	128±25‡
16. M.Br.	306±10	296±4	—	36±7‡	38±22‡		
M.Br.	316±28	296±24	142±16			136±8‡	146±45‡
17. J.S.	414±27	360±21	188±43	146±13‡	220±18‡		
J.S.	250±7	240±9	140±18			128±6‡	158±16‡
18. M.Bro.	290±19	—	—	16±6‡	90±19‡	26±12‡	94±14‡
19. L.M.	292±50	—	—	50±15§	126±28§		
L.M.	210±20	—	—			116±16	158±10
20. F.E.	206±46	190±46	70±20	30±9§	62±11§		
F.E.	240±28	206±32	92±22			32±34	168±64
21. B.D.	274±14	250±15	166±25	134±11	112±12	170±12	154±22
22. A.M.	280±10	274±8	124±38	156±10	90±20	184±14	154±22
23. M.M.	280±7	300±38	158±38	104±24	220±26	158±17	242±24
24. L.A.	246±18	230±26	108±30	24±2	54±4		
L.A.	294±1	226±15	152±6			0±9	54±5
25. H.F.	390±43	356±55	134±20	78±23	104±10	118±20	156±2
Mean±S.E.M.	289±8	262±9	136±5				

* = 1 year later
† = repeat
‡ = 1/4 dilution
§ = 1/16 dilution

diabetic subjects, and found increased activity, using the fat pad assay, in eight diabetic and six normal sera after addition of the drug. This concentration is four times as high as serum levels during the oral tolbutamide test. Antoniades et al.,[5] using the intravenous tolbutamide test in fifteen nondiabetic and nine patients with noninsulin dependent diabetes, isolated "complexed" ("bound") insulin and measured amounts of both "free" and "bound" insulin on the rat diaphragm. They found a distinct dissociation of "complexed" insulin in nine nondiabetic subjects, a "mild" response in four, and none in two subjects. In noninsulin dependent diabetics six patients showed a "good" response. Their conclusions are essentially the same as Hasselblatt's as far as pancreatic release is concerned, with the addition of a hypothesis of a deficiency in a trigger mechanism which activates tissue factors which presumably furthers the utilization of "complexed" insulin.

Our results fail to confirm those of others who have claimed that tolbutamide dissociates "complexed" or "bound" insulin. The oral tolbutamide test gives highest drug levels at thirty minutes, while the intravenous test

TABLE 3

Effect of tolbutamide on "heparin-liberated" insulin
(figures are serum insulin — μU./cc.)

Patient	Before tolbutamide			Thirty minutes after tolbutamide		
	Serum only	Serum + heparin	Heparin effect	Serum only	Serum + heparin	Heparin effect
NORMAL						
1. J.R.	260	460	200	320	500	180
J.R.*	660	660	0	420	780	360
2. F.P.	120	480	360	360	680	320
3. I.D.	400	880	480	400	640	240
I.D.†	560	880	320	560	880	320
4. E.A.	280	160	—120	300	320	20
5. J.L.	260	300	40	220	310	90
6. Mc.P.	640	440	—200	640	680	40
7. L.C.	140	280	140	280	480	200
8. H.S.	230	240	10	460	650	190
Mean±S.E.M	355±60	478±77	123±71	396±39	592±56	196±36
BORDERLINE						
9. J.McG.	200	1,100	900	1,320	1,720	400
10. F.S.	320	360	40	540	460	—80
11. J.C.	880	1,320	440	1,000	2,720	1,720
DIABETICS						
12. St.W.	0	400	400	240	800	560
13. A.S.	300	480	180	340	460	120
14. Mc.H.	420	480	60	280	420	140
15. M.B.	480	440	—40	180	270	90
16. M.Br.	~90	100	10	380	420	40
17. J.S.	300	560	260	200	440	240
18. M.Bro.	10	200	190	120	300	180
19. L.M.	320	1,280	960	940	1,730	790
20. F.E.	720	1,460	740	160	2,640	2,480
21. B.D.	640	520	—120	840	760	—80
22. A.M.	1,120	600	—520	1,480	1,080	—400
23. M.M.	240	1,540	1,300	400	1,900	1,500
24. L.A.	200	400	200	0	320	320
25. H.F.	340	510	170	640	1,000	360
Mean±S.E.M.	370±76	640±116	271±119	436±106	896±186	453±188

* = 1 year later
† = repeat

produces the highest levels initially. This difference, however, should not be important so far as our results are concerned, as Antoniades et al.[5] report one diabetic showing a fall in "complexed" insulin substantially later than this initial peak, with no significant change at twenty minutes, while others show a rapid fall.

We feel at present that the primary effect of tolbutamide in the treatment of diabetes is directly on the pancreas, especially since the drug blood levels on maintenance therapy are lower than the ones reported either with the intravenous or oral tests. Furthermore, islet cell degranulation, as seen by electron microscopy by Williamson et al.,[12] strongly suggests a direct effect on insulin secretion.

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Galactose Utilization in Young and Adult Rats

(*Continued from page 662*)

Liver galactokinase activity in adult animals was not changed by fasting or by insulin administration in vivo or in vitro. Fasting of two-day old animals also did not affect liver enzyme activity.

When young rats were fed the diet containing a galactose, the level of liver galactokinase remained significantly higher for a longer period of time than when they were fed the nongalactose diet. However, in order to show this effect, it was necessary to feed the galactose diet before the animals were twenty-four days of age. Feeding the 40 per cent galactose diet for eight days to sixteen-day old rats resulted in a higher level of activity than that found in nursing sixteen-day old rats. This increase did not appear when eighteen-day old rats were fed the galactose diet for eight days, although this diet prevented the fall in activity which would have occurred as the animals matured. These results suggest that the predominant form of the enzyme in the young animal can adapt quite rapidly in response to dietary galactose, but that the principal enzyme form in the adult animal is less responsive to dietary galactose.

The greater galactokinase activity and the increase in response to feeding galactose would appear physiologically useful in a young rat because of the much larger intake of galactose. In addition, enzyme activity in the fetal and newborn liver preparations was more readily inhibited by galactose and galactose-1-phosphate than in preparations from the adult animals. These characteristics of the enzyme—rapid increase when galactose was fed, and greater inhibition by galactose-1-phosphate—could be advantageous, since they would permit initial utilization of large amounts of galactose, but prevent accumulation of galactose-1-phosphate. Glactose-1-phosphate is considered the cause of galactose toxicity, perhaps because of its inhibitory effects on a variety of enzymes involved in glucose metabolism.

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