PLASMA INSULIN ACTIVITY FOLLOWING THE ADMINISTRATION OF BZ 55 (N-SULFANYLYL-N'-n-BUTYL-CARBAMIDE) IN DOGS

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Previously Takeuchi *et al.* (1957) reported on the necessity controlling the experimental conditions in the rat-diaphragm method for applying it to micro-determination of insulin. In the same report was especially remarked the presence of a factor in the blood plasma, which inhibited glucose uptake by the isolated rats diaphragm, and which was removed by diluting plasma more than 20-fold. Further Ohashi *et al.* (1960) demonstrated by this method that the insulin activity increased in peripherally circulating blood when 1.6% solution of mesoxalate was continuously infused into the pancreatic circulation of normal dogs. In the present experiments, change in plasma insulin activity in fasting dogs was observed after administration of insulin, glucose and BZ 55 (N-sulfanylyl-N'-n-butyl-carbamide). At the same time, action of BZ 55 *in vitro* was investigated. Below is given the description about a fact of interest concerning the action mode of BZ 55.

MATERIALS AND METHODS

1. *Diaphragm donors and preparations* Wistar strain female rats, weighing 80~120 g, were bred for more than 1 week in an animal stock room, kept at constant temperature of 28~30°C, feeding the standard solid food (Oriental MC 5) and fresh vegetables. From 3 hrs. after the last feeding the animals were fasted for 16~18 hrs., and then used for experiments. Materials were prepared by pooled method following the hemidiaphragm technique and quarterdiaphragm technique. The standard insulin was prepared as follows: 23.52 U/mg of crystalline insulin (Eli Lilly & Co.) was dissolved in distilled water and adjusted to pH 2.8 with glacial acetic acid, making the final concentration 20 U/ml; when used, it was diluted with Krebs-Ringer phosphate buffer solution containing 0.2% glucose, to desired concentration.

2. *Experimental animals* For blood sampling, normal dogs, weighing 10~15 kg, were used. They were bred for a certain period, fasted from the day previous to the experiment, and blood was taken from the cubital or jugular vein. Heparinized plasma was first diluted to 2 folds with glucose saline solution to make the final concentration of glucose 0.2%, and then desired dilution was prepared with Krebs-Ringer solution containing 0.2% glucose.

Solution of BZ 55 was prepared as follows: For experiments *in vivo*, 1 g of crystalline powder BZ 55 was dissolved in 5 ml of 1N NaOH solution and then physiological saline solution was added to make the whole 35 ml. For *in vitro* use, the same powder was dissolved in 1N NaOH solution at the same rate as the above, and the balanced saline solution was added to make the concentration of each component of incubation medium exactly Krebs-Ringer glucose solution's one.

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3. *Computation* The measure of glucose in mg, taken up per 100 mg wet weight of diaphragm in 90 mins, was designated as basal uptake by diaphragm, and the remainders of subtraction of it from glucose uptake in insulin solution and test plasma dilution were designated as extraglucose uptake (Δ -uptake) due to insulin and test plasma dilution, respectively. Δ -Uptake due to insulin is also called insulin effect. For the computation of insulin activity, the standard regression coefficient assay was used. That is, the Δ -uptake for test plasma dilution was converted into insulin equivalent by means of the regression line, the location of which was determined by the preliminarily measured slope of regression line (b) and Δ -uptake for 1 mU/ml of the standard insulin, obtained in each measurement. Insulin activity was calculated by multiplying the insulin equivalent by the dilution rate of plasma.

RESULTS

I. Dose-Response Curve

Average basal uptake for 24 cases was 0.461 ± 0.071 mg/100 mg of wet weight/90 mins.

Figure 1 records the Δ -uptakes of the rats hemidiaphragms in the presence of different concentrations of insulin added *in vitro*. The graph and the table of variance analysis show that there is a linear regression of response with log-concentration from an insulin concentration of 10.0 to 0.01 mU/ml. This regression line has $b=0.6610$, standard deviation from regression line $s=0.1674$, and index of precision (by Bliss) $\lambda=0.253$ (interval between consecutive dose-levels, 10-fold).

Fig. 1. Standard regression line for insulin determination

Table 1. Effect of intravenous injection of insulin (1 U/kg) on dog plasma insulin activity

Dog	Before injection			Time after injection in mins.					
	B.s.	Δ -uptake	InA	10			30		
				B.s.	Δ -uptake	InA	B.s.	Δ -uptake	InA
8	89	0.038	0.150	70	0.137	5.283	75	0.072	0.510
9	99	0.036	0.140	86	0.138	5.476	55	0.059	0.319
18	103	0.062	0.357	125	0.137	5.284	57	0.088	0.906
19	102	0.045	0.267	126	0.108	2.574	65	0.077	0.843
Mean		0.045	0.229		0.130**	4.655		0.074**	0.645
Standard deviation			± 0.103			± 1.390			± 0.278

B.s.: Blood sugar level (mg/100 ml)

Δ -uptake: Extraglucose uptake in 20-fold dilution of plasma (mg/100 mg of wet weight/90 mins.)

InA: Plasma insulin activity (mU/ml)

These abbreviations stand throughout the following tables.

** : Significant increase over pre-injection level ($P < 0.01$)

Fig. 2. Plasma insulin activity and blood sugar level after intravenous injection of 1 U/kg of insulin in dogs

The minimum detective concentration is 0.003 mU/ml. From the above results, this regression line is considered to be usable as the standard line for the determination of insulin. Plasma insulin activity estimated by this method is 0.347 ± 0.184 mU/ml in 14 normal fasting dogs.

II. Effect of Pre-Treatment with Drugs

1. *Intravenous administration of insulin* Regular insulin in a dose of 1 U/kg was given intravenously to normal fasting dogs, and change in blood sugar level and in plasma insulin activity was pursued. The results are shown in Table 1, and that for a typical case is represented in Figure 2. Blood sugar level shows decrease of

Table 2. Effect of subcutaneous injection of insulin on plasma insulin activity in dogs

No. of dog	Dose of insulin (U/kg)	Before injection			Time after injection in hrs.					
		B.s.	Δ -uptake	InA	1			2		
					B.s.	Δ -uptake	InA	B.s.	Δ -uptake	InA
14	0.2	94	0.033	0.267	47	0.037	0.308	55	0.039	0.331
16		83	0.047	0.441	50	0.044	0.396	66	0.040	0.343
Mean			0.040	0.354		0.041	0.352		0.040	0.337
10	0.4	100	0.006	0.101	72	0.047	0.441	48	0.021	0.173
20		88	0.035	0.267	49	0.042	0.343	56	0.044	0.369
Mean			0.021	0.184		0.045	0.392		0.033	0.271
11	0.6	75	0.027	0.215	47	0.079	1.395	41	0.066	0.874
15		70	0.038	0.319	44	0.049	0.474	43	0.047	0.441
Mean			0.033	0.267		0.064	0.935		0.057	0.658
17	0.8	83	0.043	0.382	61	0.078	1.346	65	0.049	0.474
21		89	0.040	0.308	49	0.084	2.666	49	0.058	0.589
Mean			0.042	0.345		0.081	2.006		0.054	0.532

Fig. 3. Plasma insulin activity and blood sugar level after subcutaneous injection of insulin in a dose of 0.2 U/kg (left) and 0.6 U/kg (right) in dogs

20~50%, and plasma insulin activity remarkably rose from the pre-injection value 0.229 mU/ml to 4.655 mU/ml at 10 mins., but lowered to 0.645 mU/ml at 30 mins. (all are averages for 4 cases). Increases in Δ -uptake at 10 and 30 mins. following the injection are significant ($P < 0.01$).

2. *Subcutaneous administration of insulin* Various doses of regular insulin, ranging 0.2~0.8 U/kg, were subcutaneously given, and changes in blood sugar level and plasma insulin activity were pursued for 2 hrs. following the administration. The results are shown in Table 2, and that for representative cases are illustrated in Figure 3. The maximum decrease in blood sugar level ranged 40~50% after

Table 3. Effect of intravenous injection of 1 g/kg of glucose on plasma insulin activity in dogs

No. of dog	Before injection			30 mins. after injection		
	B.s.	Δ -uptake	lnA	B.s.	Δ -uptake	lnA
12	30	0.049	0.474	124	0.065	0.843
13		0.052	0.528	133	0.080	1.447
22	92	0.043	0.223	231	0.073	0.656
23	105	0.034	0.161	239	0.078	0.785
Mean		0.045	0.347		0.074	0.933
Standard deviation			± 0.182			± 0.352

Significant increase over pre-injection level ($P < 0.05$)

Fig. 4. Plasma insulin activity and blood sugar level after intravenous injection of 1 g/kg of glucose in dogs

administrations of 4 doses from 0.2 to 0.8 U/kg. It is observed that low blood sugar level persists longer, the greater the dose of insulin administered. After the administration of 0.2 U/kg of insulin, plasma insulin activity, which was 0.354 mU/ml before the administration, became 0.352 mU/ml at 1 hr., and 0.337 mU/ml at 2 hrs. (average for 2 cases), thus remaining within the range of fluctuation around the normal value. After the administration of 0.4 U/kg, 0.184 mU/ml before the administration became 0.392 mU/ml at 1 hr. (average for 2 cases), showing tendency of slight increase but still remaining within the normal pattern. After the administration of 0.6 U/kg, one of 2 cases showed increase to 1.395 mU/ml, which is above the normal range. In the case of 0.8 U/kg, 0.345 mU/ml before the administration became 2.006 mU/ml (average for 2 cases) at 1 hr. after it showing evident increase. From these results it is estimated that the minimum dose of

Table 4 (a). Effect of administration of BZ55 on plasma insulin activity in dogs

No. of dog	Before injection			Time after injection in hrs.								
				1			2			4		
	B.s.	Δ -uptake	lnA	B.s.	Δ -uptake	lnA	B.s.	Δ -uptake	lnA	B.s.	Δ -uptake	lnA
Intraperitoneal injection (50 mg/kg)												
1	92	0.035	0.027				66	0.040	0.343			
2	85	0.043	0.382	66	0.022	0.180						
Intravenous injection (100 mg/kg)												
3	97	0.042	0.369				74	0.040	0.343	78	0.046	0.426
4	90	0.062	0.757	71	0.051	0.510						
5				64	0.029	0.231	65	0.033	0.267			
6	73						55	0.030	0.239	36	0.022	0.180
7	81	0.035	0.287	77	0.033	0.267				53	0.036	0.267
Mean		0.046	0.471		0.038	0.336		0.034	0.283		0.035	0.301
Standard deviation		± 0.251				± 0.152			± 0.054			± 0.123

Fig. 5. Plasma insulin activity and blood sugar level after intraperitoneal injection of 50 mg/kg of BZ 55 in dogs

insulin which can raise plasma insulin activity in subcutaneous injection would be found between 0.4 and 0.6 U/kg.

3. *Intravenous injection of glucose in a dose of 1 g/kg* The results are recorded in Table 3 and that for a representative case is plotted in Figure 4. At 1 hr. after the injection of glucose, plasma insulin activity rose from 0.347 mU/ml before the injection to 0.933 mU/ml (average for 4 cases), showing significant increase. In this way, endogenous insulin in blood seemed to increase following administration of glucose.

Table 4 (b). Analysis of variance calculated from Δ -uptake shown in Table 4 (a)

Factor	SS	DF	MS	Sig.
Administration of BZ 55	2.8	1	2.8	
Residual	0.2	2	0.1	
Between groups	3.0	3	1.0	
Within groups	10.7	8	1.3	
Total	13.7	11		

Dog No. 7

Fig. 6. Plasma insulin activity and blood sugar level after intravenous injection of 100 mg/kg of BZ 55

Table 5. Effect of BZ 55 upon the basal uptake and insulin effect by the isolated rats diaphragm *in vitro*

Incubation medium	No. of cases	Glucose uptake	Difference		t	P
		mg/100 mg of wet weight/90 mins.				
By the hemidiaphragm technique						
KRPG	4	0.462±0.053				
KRPG+BZ 55 (20 mg%)	4	0.461±0.049	-0.001	0.19	0.8	P<0.9
KRPG	4	0.481±0.046				
KRPG+BZ 55 (100 mg%)	4	0.469±0.043	-0.012	1.27	0.2	P<0.3
By the quarterdiaphragm technique						
KRPG (a)	3	0.461±0.037				
KRPG+BZ 55 (20 mg%)(b)	3	0.468±0.032	(b)-(a)	0.006	1.46	0.2<P<0.3
KRPG+Insulin (c)	3	0.591±0.041	(c)-(a)=(e)	0.130		
KRPG+Insulin			(d)-(b)=(f)	0.127		
+BZ 55 (20 mg%)(d)	3	0.595±0.039	(f)-(e)	-0.003	0.40	0.7<P<0.8

Krebs-Ringer solution containing 0.2% glucose (pH 7.2)

Insulin concentration, 1 mU/ml

4. *Intraperitoneal injection of BZ 55* Table 4 shows result of observation as late as 2 hrs. after the administration in a dose of 50 mg/kg, and Figure 5 represents one typical case. The maximum decrease in blood sugar level was about 25%, but plasma insulin activity was 0.287~0.382 mU/ml before the administration, 0.180 mU/ml at 1 hr. and 0.343 mU/ml at 2 hrs., scarcely showing any change.

5. *Intravenous injection of 100 mg/kg of BZ 55* Change in blood sugar level and plasma insulin activity was pursued as late as 4 hrs. after the administration. As seen in Table 4(a), blood sugar level decreased about 30~50%. And in many cases, low blood sugar level persisted as late as 4 hrs. after the administration. However, plasma insulin activity (average for 3 cases) was 0.471 mU/ml before, 0.336 mU/ml at 1 hr., 0.283 mU/ml at 2 hrs., and 0.301 mU/ml at 4 hrs. after the administration, without showing significant differences. Figure 6 represents one of these cases, and Table 4(b) is the table of variance analysis calculated from the date of Δ -uptake shown in Table 4(a).

III. Effect of BZ 55 added *in vitro*:

BZ 55 in the incubation medium was at concentration of 20 and 100 mg%, and effect on basal uptake was investigated by hemidiaphragm technique. Further, by quarterdiaphragm technique, the direct action of BZ 55 at concentration of 20 mg% upon the basal uptake and the insulin effect (insulin concentration, 1 mU/ml) were investigated simultaneously. The results are shown in Table 5. There could not be found any significant change of glucose uptake by the isolated rats diaphragm even at high concentration (100 mg%) of BZ 55. Also in 3 cases, on which the direct action (b) and the influence on the insulin effect (f) were simultaneously investigated by the quarterdiaphragm technique, no significant differences were observed between the basal uptake (a) and the insulin effect (e), respectively.

DISCUSSION

In 1955 Francke and Fuchs reported on hypoglycemic action of BZ 55. Since then many reports have been published on the clinical observations of antidiabetic effect of sulfonylurea derivatives. There is, however, no agreement as yet concerning the mode of their hypoglycemic action, although many pharmacological studies have been performed on it. The contradictory views on it at present can be grossly grouped into the following three. The first includes the view as entertained by Holt *et al.* (1956) and Gept *et al.* (1957) on the basis of histological findings that BZ 55 stimulates β -cell in Langerhans' islet. It also includes views of those who attribute hypoglycemia, induced by BZ 55, to increase of insulin secretion from β -cell in Langerhans' islet, because alloxan diabetic animals (Holt *et al.*, 1956), pancreatectomized animals (Houssay and Penhos, 1956) and hepato-pancreatectomized dogs (Ohashi, 1959) did not respond to sulfonylurea derivatives. Under the second is grouped the views of those who place emphasis on the action of sulfonylurea to increase glycogen synthesis in the liver (Fry and Wright, 1957), on blocking the path-way to glucose from fructose in the liver (Renold *et al.*, 1956), who consequently attribute hypoglycemia to hepatotropic action of sulfonylurea. The third includes various other views such as those of Mirsky *et al.* (1956),

of Bertram *et al.* (1956) and Achelis and Hardebeck (1956), and of Goetz *et al.* (1956), who consider, as mechanism of the hypoglycemic action, inhibitory effect on insulinase in tissue, decrease in glucagon discharge from α -cell in Langerhans' islet, and direct stimulation to glucose uptake of peripheral tissue, respectively. In all these views, the point in dispute seems to concern the relation between insulin and sulfonylurea derivatives. Is there any insulin-like stimulation for glucose uptake by tissue? Does it potentiate the action of insulin? or does it increase the secretion of insulin? or does it exert its action independently of insulin in all senses? It is considered that clear cut answers can be obtained for all these, if insulin content in blood can be determined exactly and if the effect of these compounds on the glucose uptake by the isolated tissue can be elucidated perfectly. In view of this, the authors attempted the application of the rat-diaphragm method.

Previously, Takeuchi (1958), one of the authors, gave detailed description of procedures to determine the plasma insulin activity by means of the rat-diaphragm method. In the present experiments, plasma insulin activity in dogs under the various conditions was determined to investigate the degree of precision of this method, and the following results were obtained: When 1 g/kg of glucose was intravenously injected, increase in plasma insulin activity which occurred 30 mins. after was confirmed, and when 1 U/kg of insulin was intravenously injected, increase in plasma insulin activity could be traced as late as 40 mins., and the minimum dose of insulin in subcutaneous injection which could produce increase in plasma insulin activity was 0.4 U/kg.

In contrast to these intraperitoneal administration of 50 mg/kg or intravenous administration of 100 mg/kg of BZ 55 could not elicit alteration in plasma insulin activity. In other words, increase in insulin content in blood sufficient to cause the observed hypoglycemia could not be demonstrated after the administration of BZ 55 in the above mentioned doses and the ways. This seems to be in agreement with the report by Fry and Wright (1957) that glucose uptake by the diaphragm, which was isolated from the rats treated with D 860 (N-Toluyyl-sulfonyl-N'-butylurea) did not differ from that for the control.

It is, however, impossible to deny pancreatotrophic action of BZ 55, that is, action to increase the secretion of insulin, on account of the above described results; for it can be suspected in the 1st place that the administration of BZ 55 may produce in blood an inhibitory factor to Δ -uptake by rats diaphragm. Of course, BZ 55 itself has not such an inhibitory action, because it was confirmed in the present experiments that application of BZ 55 *in vitro* produces scarcely any alteration in the basal uptake and insulin effect. However, no conclusion can hurriedly be made from the present results as to the presence or absence of inhibitory substance other than BZ 55. In the 2nd place, a question is raised from the results of subcutaneous injection of 0.2 U/kg of insulin in the present experiments. In this case, the degree of hypoglycemia induced by it was almost equal to that in cases which were intraperitoneally given 50 mg/kg of BZ 55. On the other hand, the minimum dose of insulin in subcutaneous injection for plasma insulin activity increase as demonstrated by the authors' method was 0.4 U/kg, and no increase was observed in cases given 0.2 U/kg of insulin. Consequently when increase of plasma insulin activity caused by BZ 55 administration is below that which is produced

with 0.4 U/kg of regular insulin injected subcutaneously, it may not be demonstrated by the authors' method.

Holt *et al.* (1957), Plestschner and Gey (1957), Aiman and Kulkarni (1957), Mohnicke *et al.* (1957) and Wrenshall (1957) published various results on the direct or indirect action of sulfonylurea derivatives on glucose uptake by the isolated tissue. But it seems difficult to say that their experimental conditions are suitable. It is naturally concluded that it is difficult to make clear the action mode of these derivatives only by determining plasma insulin activity by means of the response of the isolated tissue like in the rat-diaphragm or the rat-adipose tissue methods.

SUMMARY

(I) Determination of insulin was examined by the rat-diaphragm method, and the following results were obtained: A linear regression was observed between extragluco uptake and log-concentration of insulin in the range of 10 to 0.01 mU/kg. The minimum detectable concentration was 0.003 mU/ml, slope of regression line (regression coefficient) $b=0.6610$, standard deviation of regression line $S=0.1674$, index of precision $\lambda=0.253$ (intervals between consecutive doses, 10-fold).

(II) Plasma insulin activity in 14 normal fasting dogs, estimated by this method was 0.347 ± 0.184 mU/ml. Change in plasma insulin activity was observed after administration of insulin, glucose and BZ 55 (N-sulfanylyl-N'-n-butyl-carbamide) with the following results:

1. After intravenous administration of 1 U/kg of insulin, plasma insulin activity increased from 0.229 mU/ml before to 4.655 mU/ml at 10 mins. became 0.645 mU/ml at 30 mins. after.

2. After subcutaneous administration of 0.2~0.8 U/kg of insulin the results were as follows: In case of 0.2 U/kg, plasma insulin activity always remained in the normal range; but in the case of 0.6 U/kg, it increased significantly after the injection. The minimum dose of insulin in subcutaneous injection which produced increase in plasma insulin activity was 0.4 U/kg.

3. After the intravenous injection of 1 g/kg of glucose, 0.347 mU/ml before the injection became 0.933 mU/ml at 30 mins., indicating increase.

4. After intraperitoneal administration of 50 mg/kg of BZ 55, 0.287~0.382 mU/ml before the injection became 0.180 mU/ml at 1 hr. and 2 hrs. After the intravenous administration of 100 mg/kg, 0.471 mU/ml before the injection became 0.336 mU/ml at 1 hr., 0.283 mU/ml at 2 hrs. and 0.301 mU/ml at 4 hrs. In all the cases, there was no significant difference in plasma insulin activity between before and after the administration.

(III) No significant effect of BZ 55 was observed *in vitro* experiments on the basal uptake and insulin effect by the isolated rats diaphragm.

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