A Method for Assay of Insulin Using Alloxan Diabetic Hypophysectomized Mouse. (22933)

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Interest in the development of methods for the assay of micro quantities of insulin stems from the desire to measure the amount of insulin in circulating blood. According to Waters and Best(1), if one estimates the concentration of insulin in blood on the basis of the daily dose of insulin required by patients who have had a total pancreatectomy, which is about 30 units, the amount of insulin would be no more than 200 microunits (μU) per ml of blood. By another method of estimation using insulin I^{131} Berson *et al.*(2) arrive at a value of 65 μU per ml body fluids. Two methods have been described for the micro assay of insulin, one using an *in vitro* technic and the other an *in vivo* technic. By the *in vitro* technic the isolated rat diaphragm is incubated in glucose buffer solution and the effects of added insulin upon the utilization of glucose and the deposition of glycogen in the muscle are measured. Willibrands and Groen(3) have prepared a critical review of this type of assay based on the reports of several workers (Stadie and Zapp(4); Krahl and Park(5); Perlmutter, Weisenfeld and Mufson(6); Groen, *et al.*(7); Vallance-Owen and Herlock(8); and Randle(9)). *In vivo* technics which measure the fall in blood sugar in response to insulin in animals which have been rendered exceedingly sensitive to insulin have been described by Hemmingsen, Nielsen and Nielsen(10), using the adrenalectomized rat; by Gellhorn, Feldman and Allen(11), using the adrenodemedulated hypophysectomized rat; by Anderson, Lindner and Sutton(12), using the adrenodemedulated alloxan diabetic hypophysectomized rat; and by Bornstein(13), using the adrenalectomized, hypophysectomized alloxan diabetic rat. Recently, Yenermen *et al.*(14) described a method using the alloxan diabetic hypophysectomized mouse, which purported to measure accurately amounts of insulin as low as three microunits. In an attempt to repeat the work described in the latter report the present authors were unable to reproduce the results. The method which will be described here uses, also, the alloxan diabetic hypophysectomized mouse. Amounts of insulin as low as 250 microunits can be measured if the insulin is administered intravenously or 1000 microunits if it is injected intraperitoneally.

Methods. Male mice (CAF_1) aged 3-4 months and weighing 25-30 g are used. The animals are made diabetic with alloxan. For this procedure the mice are placed in a well ventilated incubator set at 30°C for 2 hours without food or water. Alloxan monohydrate, 1.0% solution in physiological saline, is injected into the tail vein in a dose of 70 mg/kg body weight. In a group of 60 mice given this dose of alloxan 48 hours previously, the non-fasting blood sugar level of 56 mice was over 300 mg %, averaging 390 mg %. Forty-eight hours after alloxan administration the mice are put on daily subcutaneous injections of 2 units protamine zinc insulin (PZI) to restore them to a healthy state in preparation for hypophysectomy. A week is allowed to elapse between the alloxan administration and this operation. The day before operation regular insulin is substituted for PZI and no insulin is given on day of operation. Since hypophysectomy renders the mouse sensitive to insulin almost immediately, in order to avoid fatal hypoglycemia, it is well to give the animals food by stomach tube (egg-milk mixture) an hour before operation in case traces of insulin are still present in the tissues. The technic of hypophysectomy for the mouse is essentially the same as that originally described by Smith(15) for the rat. Veterinary nembutal (Abbott) is used as the anaesthetic, the intraperitoneal dose is 0.065 mg/g body weight. The best survival rates

after hypophysectomy are obtained if the animals are given 6% glucose in a solution of 0.5% NaCl in place of tap water to drink and fresh whole wheat bread and crisp kale twice a day. Overcrowding of animals in the cages is avoided. The cages are kept in a ventilated incubator at 30°C. If the animals are used for assay not oftener than once a week, they may survive for as many as 6 assays. The animals remain in the incubator during the test, except for the few minutes when they are removed for injection or the taking of blood samples. Blood glucose is determined on 0.01 ml of blood taken from the tip of the tail. The method of Park and Johnson(16) is used. The starting blood sugar values in a group of 15 to 30 mice, which is the number used in one day's assay, may vary from 100 to 350 mg %. The practice is to use 1.0 ml of the supernatant fluid after protein precipitation for all the samples and to bring the color of the most concentrated solutions into a satisfactory range for the spectrophotometer reading by diluting with blank solution. Crystalline glucagon-free insulin† is used for the standard. One stock solution has served as the insulin standard during the entire period in which the data of this report have been collected. The stock solution of insulin is prepared by dissolving 2 mg of insulin in 5 ml of 0.2% phenol solution containing a drop of N acetic acid. This solution contains 10 U/ml. Aliquots are diluted with sterile saline to the desired concentration for injection upon the day of the experiment. The standard solution is stored in the refrigerator at 4°C. *Intravenous method.* It has been found useful to have at least 2 trained people carry out this procedure. since the exact timing of each step is critical for this method. An example of the routine procedure in our laboratory is given here. Two groups each of 12 to 15 test mice are used for one assay; the first group is started at 9:00 a. m. and the second group at 9:45 a. m., while the first group is being processed. *9:00 a. m.* 25 mg glucose in 1.0 ml saline are injected subcutaneously into mice in Group I. The animals are placed in clean cages without food or water and returned at once to the incubator. *9:45 a. m.* The animals are placed in individual mouse holders, given Nos. #1 to #15, respectively, and returned to the incubator. *10:00 a. m.* Test dose of insulin in 0.1 ml saline is injected intravenously into mouse #1. The rest of the mice are injected one minute apart, in numerical order. *10:05 a. m.*‡ Initial blood specimen from mouse #1 and from a mouse every minute thereafter in numerical order. Blood proteins are precipitated immediately according to the method for the blood sugar determination. *10:25 a. m.* Final blood specimen from mouse #1, etc. The second group is carried through in the same way.

The injections consist of saline and 3 or 4 dose levels of insulin with 6 test animals for each. In most experiments the data from one or 2 animals have to be discarded for such reasons as: failure to deliver the dose intravenously, failure to obtain a blood sample, or because the blood glucose levels are too low for quantitative determination.

Intraperitoneal method. The number of test animals used in this procedure is 24 to 30. For identification purposes the mice are separated into groups of 6 to a cage and each is given a distinguishing mark, such as: pink head, pink back, etc. *9:00 a. m.* 25 mg glucose in 1.0 ml saline are injected subcutaneously. The order in which the animals are injected is maintained for subsequent procedures. The animals are without food or water and are returned to the incubator as soon as injected. *10:00 a. m.* Initial blood specimen from a mouse every minute in numerical order. *10:01 a. m.* Test dose of insulin in 0.1 ml saline injected intraperitoneally into mouse #1, etc. *10:31 a. m.* Final blood specimen from mouse #1, etc.

Results. A summary of the blood sugar response to different doses of intravenously administered insulin is presented in Fig. 1, and for intraperitoneally administered in-

† We wish to thank Dr. Otto K. Behrens, Eli Lilly and Co., for the glucagon-free insulin.

‡ The reason for taking the initial blood specimen 5 minutes after insulin injection is to avoid the necessity of correcting for dilution caused by intravenous injection.

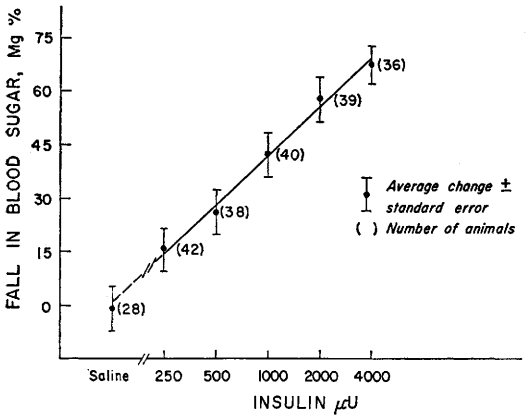

FIG. 1. Change in blood sugar for different doses of insulin, intravenous method.

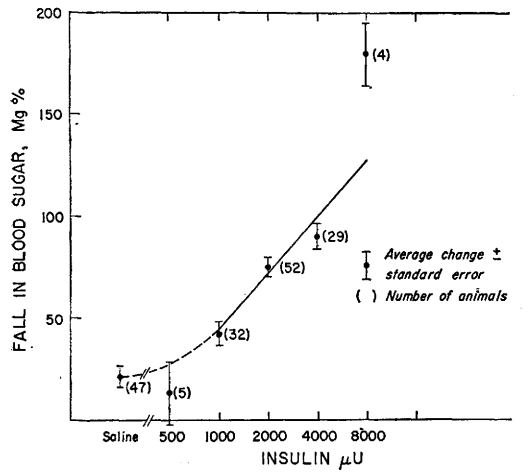

FIG. 2. Change in blood sugar for different doses of insulin, intraperitoneal method.

sulin in Fig. 2. Each observed point is an average of the falls in blood sugar for the number of mice indicated in parentheses. The standard error of each mean is indicated by the vertical bar. For the intravenous route the biological response is clearly a linear function of log dose in the range of 250 to 4000 μU. The least amount that can be measured is in the neighborhood of 250 μU, since response to any smaller amount will not differ appreciably from response to saline. For the intraperitoneal assay administration of saline is accompanied by a significant fall in blood sugar. In consequence the least detectable amount of insulin is higher than for the intravenous assay, something between 500 and 1000 μU.

These results are obtained by combining experiments done at a number of different times and results for animals used once or several times. In order to distinguish between these two kinds of experiments as well as to indicate the nature of the variation from experiment to experiment we show the separate results for each experiment for the intravenous assay in Table I. The number of animals used in any one experiment is small and the results in consequence somewhat variable. When appraised against the variation in response from animal to animal treated alike within an experiment, however, the variation between experiments is no greater than might be expected by chance. We have therefore pooled all experiments separately for intravenous and intraperitoneal assays, but have maintained the distinction between results obtained with first and subsequent use. (There is some difficulty with survival of animals during the summer months. Assays carried out at that time have been discarded.)

A summary of these results is shown in Table II. It will be noted that for both the intravenous and the intraperitoneal methods steeper slopes are obtained on second and subsequent use than on first use. Whether this is because of some effect of use, such as accustoming the animal to handling, or related to the time elapsing after hypophysectomy, we are unable to say. The steeper slopes after repeated use are counter balanced to some extent by higher standard deviations, so that indices of inherent precision, λ, are much the same on first as on subsequent use.

The value of λ for the intravenous assay, 0.7, is somewhat higher than that reported for other animal assays of insulin (Bliss and Cattell, 17). This simply means that to attain any required level of precision more observations are required. Thus, if one wishes to estimate the potency of an unknown preparation the number of animals required to attain any desired precision is given by the formula $n = (2\ \lambda/s_M)^2$, where s_M is the selected standard error of log potency. If s_M is chosen as 0.301, corresponding to a standard

TABLE I. Intravenous Method, Average Fall during 20 Min. in Blood Sugar (mg %) by Dose Level and Number of Previous Times Assay Animal Was Used. A negative value stands for a rise in blood sugar.

Date of exp.	Saline	Insulin dose (μU) 250	500	1000	2000	4000
		First use				
11/14/55	−25 (1)*		−19 (1)	2 (2)	64 (2)	
16	8 (3)		26 (4)	31 (6)	38 (5)	
18		14 (4)			45 (3)	77 (3)
25	26 (2)	10 (2)	41 (1)	45 (1)		
28	7 (4)	5 (5)	35 (3)	36 (4)		
12/22	− 4 (1)	24 (1)	17 (3)	42 (4)		42 (2)
1/ 4/56	8 (3)	43 (3)	14 (3)	24 (2)	60 (2)	68 (2)
11	− 8 (3)	33 (5)	30 (3)	29 (4)	35 (3)	47 (6)
All exp.	4.4(17)	21.0(20)	23.0(18)	32.3(22)	45.2(15)	56.4(13)
		Second or later use				
11/14/55	−15 (1)			59 (1)	32 (1)	
18		−30 (1)			68 (4)	65 (3)
21		20 (4)			52 (4)	63 (9)
23			45 (7)	59 (5)	93 (3)	125 (2)
25	−36 (2)	0 (4)	24 (4)	8 (1)		
1/ 6/56	3 (2)	4 (4)	26 (4)	73 (4)	67 (4)	51 (3)
13	2 (3)	14 (4)	16 (2)	46 (4)	78 (4)	80 (2)
17	−10 (3)	18 (5)	13 (3)	46 (3)	53 (4)	103 (4)
All exp.	−9.5(11)	10.9(22)	29.3(20)	54.2(18)	66.0(24)	75.5(23)

* No. of assay animals on which avg is based shown in parentheses.

error of twofold in the estimation of potency. 22 animals would be required, half for the standard and half for the unknown. The value of λ for the intraperitoneal assay is less than for the intravenous, but the greater sensitivity of the latter makes it the method of choice.

Summary. Two methods for the assay of small amounts of insulin are presented. Both involve using the alloxan diabetic hypophysectomized mouse. One involves measuring the fall in blood sugar in test animals over a 20 minute period after the intravenous administration of insulin, the insulin being given one hour after the subcutaneous injection of 2.5 mg glucose. The other method involves measuring the fall in blood sugar 30 minutes after the intraperitoneal administration of the insulin, which, as in the intravenous method, is given one hour after a 2.5 mg glucose meal. The intravenous method, which is the more sensitive of the two, is capable of detecting as little as 250 μU of insulin.

TABLE II. Comparison of Precision of Intravenous and Intraperitoneal Method, First and Subsequent Use of Animals.

	Intrav. method	Intraper. method
	1st use	
Stand. dev. (mg %) (σ)	23.3 ± 1.9*	33.6 ± 3.1
Slope (mg %/10-fold dilution) (b)	30.9 ± 4.6	70.2 ±12.5
Index of precision (σ/b) (λ)	.76± .13	.48± .10
	2nd and subsequent uses	
Stand. dev.	38.9 ± 3.0	35.9 ± 2.9
Slope/10-fold dilution	57.4 ± 7.3	97.5 ± 9.1
Index of precision	.68± .10	.37± .05

* Stand. error.

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Received November 13, 1956. P.S.E.B.M., 1957, v94.

Lessened Insulin Hypophosphatemia Following N-Sulfanilyl-N′-Butylurea (BZ-55 or Carbutamid).* (22934)

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N-sulfanilyl-N′-butylurea (BZ-55 or Carbutamid) was administered to juvenile diabetics prior to insulin tolerance tests in an attempt to determine whether the amelioration of clinical diabetes reported, at a conference, with this compound can be attributed to potentiation or prolongation of insulin action.

Materials and methods. Twenty-seven juvenile diabetics, previously maintained for 3 to 5 days on short-acting insulin received *per os* 100 mg of BZ-55/kg body weight 45 minutes before start of intravenous insulin tolerance test, 0.1 u of crystalline insulin/kg given at zero time. Samples of venous blood obtained without stasis at –45, –20, 0, 30, 60, 90, 120 minutes, and in some patients at 3 and 4 hours, were analyzed for concentrations of sugar by Hagedorn-Jensen procedure. Levels of inorganic phosphorus, potassium, total carbon dioxide, chloride, and sodium in sera of venous bloods drawn between zero and 2 hour points were measured by standard procedures(1). The patients were fasted and at bed rest. Control insulin tolerance tests were made in 14 juvenile diabetics under the same circumstances save that BZ-55 was not given and blood sampling at –45 and –20 minutes was omitted.

Results. From Fig. 1 it is evident that BZ-55 neither potentiates nor prolongs the hypoglycemic action of exogenous insulin. The mean decrements in blood sugar concentrations at 30 minutes (–52 and –36 mg % without and with BZ-55) and at 60 minutes (–77 and –69 mg % without or with BZ-55, respectively) are of the same order of magnitude in the 2 groups with p values of 0.11 and 0.56. On the other hand the decrease in serum inorganic phosphorus which accompanies the hypoglycemia is distinctly less in patients receiving BZ-55, p value of 0.02 (Fig. 1), with mean values of –0.53 and –0.80 mg % at 30 minutes. Changes in serum carbon dioxide content, potassium, chloride and sodium were not influenced by BZ-55.

Discussion. A number of possible explanations have been advanced for amelioration of diabetes mellitus in certain patients by N-sulfanilyl-N′-butylurea, including suppression of pancreatic alpha cell activity(2), decreased absorption from the gastro-intestinal tract (3), decreased gluconeogenesis, increased hepatic glycogenation(4), diminished glucose-6-phosphatase activity(5), inhibition of cytochrome oxidase(6), increased production of insulin(7), and inhibition of insulin destruction(8,9). Insofar as the last of these is concerned there is no evidence in our studies that BZ-55 intensifies or prolongs hypoglycemia which follows administration of insulin in these dosages to juvenile diabetics. As a matter of fact the lesser degree of hypophosphatemia is in keeping with a diminution of

* Aided by grant from Department of Health.
† Established Investigator of Am. Heart Assn.