A SLOPING SCREEN METHOD FOR THE BIOASSAY OF INSULIN IN MICE

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A quantal response method for the bioassay of insulin is described employing a sloping screen for the detection of hypoglycaemic symptoms in insulin-treated mice. This procedure tends to eliminate the personal bias which may occur in assays using convulsive seizures or a state of collapse as the criterion of the response to insulin. In a comparison of this method with that described for insulin in the British Pharmacopoeia[1], it was found that although the slopes of the log dose-response lines did not differ significantly, the procedure using the sloping screen for detecting the response required a larger dose of insulin. Mice primed with 5 mU. of insulin before their routine use in assays were more uniform in their response than unprimed mice. The presence of a retarding agent such as gelatin or protamine added to insulin did not influence the slope of the log dose-response line, but may under special conditions delay the absorption of insulin from the injection site. Evidence has been obtained which suggests that the amount of daylight to which the mice are exposed may have a significant effect on the precision of the assay.

THE qualitative effect of the administration of insulin to laboratory animals may be observed either by measuring the fall in blood sugar or by the incidence of hypoglycaemic symptoms such as convulsions or muscle weakness. This response to insulin can be relieved by the administration of glucose.

Several procedures have been described for detecting hypoglycaemic reactions to insulin in mice[2]. An elevated temperature was employed in earlier studies to induce the characteristic convulsive seizures in insulin-treated mice. However in these assays trained personnel were required to recognise the symptoms attributable to insulin and to administer glucose quickly to prevent fatalities among the stricken mice. Thompson[3] found that mice showing a reaction to insulin would fall off a wire-mesh screen set at an angle of 60°. This type of equipment for the objective determination of the presence or absence of advanced insulin symptoms tended to eliminate personal bias and reduced the number of personnel required to perform an insulin assay. Young and Lewis[4] modified this apparatus by using revolving wire-mesh drums instead of the flat screens. The affected mice lost their foothold while the drum was turning and fell into trays containing food, the consumption of which was sufficient to alleviate the hypoglycaemia.

In the method of assay described in this communication, a modification of Thompson's[3] procedure for detecting the response to insulin has been employed, and various factors affecting this type of assay have been investigated.

EXPERIMENTAL

Female albino mice, raised in our own colony and weighing 18 to 24 g., are kept in cages in a room maintained at 27° ± 1° and are given Master Fox cubes* and water *ad libitum*. On the day of an assay, the food is withdrawn at 8.30 a.m., and the mice are weighed and distributed in containers according to their weight. All the mice in a single container have the same weight. Either 120 or 160 mice within a weight range of 4 to 5 g. are selected from the containers in such a way that the number from each weight group included in each of four dosage groups is the same. Thus the average weight of the mice is similar in each dose group.

The insulin diluent, unless otherwise specified, is an aqueous solution containing 0·90 per cent sodium chloride, 0·15 per cent phenol, and sufficient hydrochloric acid to adjust the acidity of the solution to pH 2·5 to 3·0. The stock insulin solution is prepared from the International Standard or the insulin preparation under test to contain 1·0 I.U./ml., and suitable dilutions are made from this stock solution. A volume of 0·20 ml., containing the desired dose of insulin, is injected subcutaneously into the mid dorsal area of each mouse. The log dose interval is either 0·2218 or 0·3010.

The mice are starved for a period of 4 to 5 hours, injected with the diluted insulin, and then placed on a sloping screen set at an angle of 60°. The apparatus used in this work is a modification of that originally described by Thompson[3]. It consists of four compartments, 12 in. × 25 in. in size, of aluminium window-screening, and is set up in the laboratory in which the mice are housed. The framework holding the screens is also made of aluminium and is placed on a laboratory bench with the lower edge of the screens extending over the edge of the bench-top. The distance to the floor is 30 in. On rare occasions mice fall or jump from the screen at the beginning of the assay. To avoid false-positive responses, any mouse that falls off the screen during the first 20 minutes after the injection is replaced on the screen and watched carefully. Experience has shown that if the mouse falls again within a few minutes it is displaying a true hypoglycaemic response. After the initial 20 minutes' period of the assay, the mice appear to lose interest in exploring the lower part of the screen and usually remain clinging to one place for the remainder of the assay. The mice showing a positive response to the insulin fall into metal containers, partially filled with sawdust, which are placed immediately below each of the 4 screens. The affected mouse is usually given 0·5 to 1·0 ml. of a 5 per cent solution of glucose, and is permitted to recover from the hypoglycaemic reaction in a cage containing food and water. By using this technique very few of the mice die during an assay. The mice are left on the screen for a period of 90 minutes after the injection, and the number of mice remaining in each dose group is recorded at that time. The assay is carried out in the "mouse room" to avoid any change in the environmental temperature.

* Master Fox cubes are available from Toronto Elevators Ltd., Toronto, Canada.

Since there are 4 dosage groups, a 2 × 2 design is used, and the potency ratio and its confidence limits are calculated by the quantal response method using probits[5–7]. Each assay is checked for validity by suitable χ^2 tests. To increase the precision, 4 to 8 assays are usually combined by "method C" described by Perry[8]. This procedure permits, in addition to the calculation of the mean potency ratio and its confidence limits, the estimation of the mean slope as well as the average median effective dose and its standard error[7].

Results and Discussion

Comparison of the Sloping Screen Method with the Procedure Described in the British Pharmacopoeia[1]

A glass-fronted air incubator was used to determine the potency of the Third International Standard for Insulin by the method described in the British Pharmacopoeia[1] for the biological assay of insulin injection. The cabinet was maintained at 30 ± 1° during the assay period of 90 minutes. The mice were starved overnight, given a subcutaneous injection of the dilute insulin solution and placed in litre beakers in the cabinet. A total of 96 mice were used in a 2 × 2 assay design. The log dose interval was 0·3010. When a mouse displayed convulsive seizures or passed into a state of collapse, it was removed from the beaker in the cabinet and given an injection of a 5 per cent solution of glucose. The number of mice affected in this way by the insulin was recorded, and the potency ratio calculated by the probit method[7].

The sloping screen procedure was employed to assay the Fourth International Standard for Insulin, and the results are compared in Table I

TABLE I

COMPARISON OF THE SLOPING SCREEN METHOD WITH THAT DESCRIBED IN THE BRITISH PHARMACOPOEIA[1]

Method	Average slope of the log dose-response line b	Average median effective dose ED50±S.E.**	Potency of International standard for insulin	
		mU.	Found I.U./mg.	Adopted I.U./mg.
British Pharmacopoeia[1] air incubator at 30°C. (6 assays)	4·13±0·57*	13·4±0·5	25·6 (22·1-29·6)	24·5
Sloping screen at 27°C. (9 assays)	3·77±0·30	18·6±0·5	24·2 (21·7-26·9)	24·0

* $\sqrt{Vb} = \frac{1}{\sqrt{[nwx^2]}}$

** S.E. = $S_{\log ED50} \times 2{\cdot}303 \times ED50$ (7)

with those obtained with the method described in the British Pharmacopoeia[1]. Both assay procedures provided an estimate of the potency which did not differ significantly from that adopted after the completion of the collaborative assay to establish the biological activity of the International Standard Insulin preparations.

Although the values for the slopes (b) of the log dose-response lines estimated for the 2 assay methods are not the same, their standard errors suggest that this difference could occur by chance alone. However the median effective dose (ED50) calculated for the sloping screen procedure tends to be larger than that estimated for the method described in the British Pharmacopoeia[1]. Possibly the lower ED50 and therefore the increased sensitivity can be attributed at least in part to the higher temperature to which the mice are subjected in the latter procedure.

Effect of a Priming Dose of 5 mU. of Insulin on the Precision of the Assay

Mice employed in an assay for the first time frequently are more sensitive and behave more erratically than those which have been used in at least one test previously. Since it is necessary to include new mice from the stock colony from time to time to make up the quota of 120 to 160 mice for an assay, this apparent difference in sensitivity can lead to invalid assays or heterogeneity between the estimates of the potency ratios when the assays are combined.

Accordingly a study was made of the effect of giving the new mice a priming dose of 5 mU. of insulin before they were included in a regular assay. The individual estimates of the median effective dose for the unprimed mice were found to be heterogeneous when they were tested by χ^2, and semi-weights estimated by the method of Bliss[9] had to be used to calculate the average median effective dose for these mice. The results in Table II indicate that the ED50 for the unprimed mice was subject not

TABLE II

EFFECT OF A PRIMING DOSE OF 5mU OF INSULIN ON THE ED50 AND THE SLOPE OF THE LOG DOSE-RESPONSE LINE

Treatment of mice	No. of assays	Average ED50±S.E. mU.	Average slope
Unprimed	4	11·7±5·5	3·20±0·55
Primed with 5 mU of insulin	5	16·5±1·1	3·33±0·54

only to more variability as shown by the 5-fold larger standard error, but was also smaller than that for the primed mice. The values for b given in Table II are not significantly different, suggesting that priming of the mice has no real effect on the slope of the log dose-response line. As a result of this study, mice obtained from the stock colony are routinely given a dose of 5 mU. of insulin and placed on the sloping screen for a period of 90 minutes a few days before they are included in a regular insulin assay.

Effect of the Injection Medium on the Response of the Mice to Insulin

Various retarding agents were added to the acid saline injection medium in an attempt to improve the precision of the assay by increasing the slope of the log dose-response line. The materials employed are listed in

Table III together with the values for the slope of the log dose-response line and the median effective dose. The influence of the retarding agent on the relative potency is also shown in Table III. The acid saline injection medium was used as the reference standard in each of these assays.

TABLE III

EFFECT OF THE INJECTION MEDIUM ON THE RESPONSE TO INSULIN

Type of insulin preparation	No. of assays	Retardant, per cent	Slope of the log dose-response line		χ^2_b	Relative potency medium alone = 100 per cent P = 0·95
			Medium alone	Medium + retardant		
Cryst. insulin	4	carboxy-methylcellu-lose 1	4·22±0·67	3·31±0·56	1·08	142·9 (106·5–191·5)
Cryst. insulin	4	polyvinyl-pyr-rolidone 2	4·00±0·62	4·91±0·61	1·10	98·2 (86·0–112·0)
Cryst. insulin	1	pectin 3	5·23±1·25	2·30±1·10	3·10	77·6 (55·0–109·6)
Cryst. insulin	6	gelatin 16	3·87±0·50	2·69±0·46	3·01	96·1 (82·9–111·5)
Isophane insulin ..	5	gelatin* 16	2·45±0·51	3·57±0·60	2·03	56·2 (45·2–69·7)
Protamine zinc insulin	2	protamine* (1 mg./ml.)	4·02±0·85	3·06±0·81	0·67	83·8 (64·4–109·1)

* Injection medium at pH 7·2.

Although the values for b vary, the test for χ^2_b was not significant in any of the assays indicating that the presence of the retarding agent in the medium did not affect the slope to any extent. It is difficult to understand why the addition of 1 per cent carboxymethylcellulose to the medium enhanced the insulin effect. Perhaps the carboxymethylcellulose protected the insulin from tissue proteases at the injection site. Neither polyvinylpyrrolidone, pectin, nor gelatin, at the concentrations employed, had a significant effect on the relative potency. No delay in the onset of hypoglycemic symptoms was observed in the test animals when any one of these agents was added to the acid saline injection medium. Apparently at the dilution required in the mouse assay, insulin does not readily form a complex with any of the retarding agents employed in this study. The insulin probably occurred in the free state in the injection medium. In contrast, when isophane insulin was diluted with 16 per cent gelatin at pH 7·2, the crystalline protamine-zinc-insulin complex must have been partially dissociated only because little more than 50 per cent of the activity was available to the mice. This is probably not an effect of the hydrogen ions because the addition of excess protamine to protamine zinc insulin at pH 7·2 did not reduce the relative potency of the preparation.

This work supports the observation made earlier by Young, Reid, and Romans[10] that the mouse convulsion method of assay is a satisfactory procedure for determining the insulin content of commercial preparations such as protamine zinc insulin and globin insulin with zinc. Excellent results have been obtained in this laboratory by using the sloping screen method for determining the insulin content of protamine zinc insulin, isophane insulin, globin insulin with zinc, and insulin zinc suspension.

Effect of Daylight on the Response of the Mice to Insulin

A survey of the average slopes of the log dose-response lines obtained in routine insulin assays over a period of several years revealed that the values tended to be higher during the winter months than they were during the summer. Since the mice were kept in air-conditioned quarters, this variation could not be attributed to changes in environmental temperature.

TABLE IV

INFLUENCE OF LIGHT ON THE RESPONSE OF MICE TO INSULIN

Time of year	No. of assays	Slope of log dose-response line b_S	Median effective dose of standard ED50mU + S.E.	Remarks
June 1956 ..	5	2·83 ± 0·46	42·6 ± 2·8	Bright light, no blinds on windows
August 1956 ..	12	4·57 ± 0·36	18·7 ± 0·5	Dark, covers over windows, room lights on during assays
December 1957	4	5·10 ± 0·78	16·4 ± 0·6	Diffuse light, venetian blinds over windows
June 1958 ..	6	3·57 ± 0·63	21·8 ± 1·1	Diffuse light, venetian blinds over windows
December 1958	7	6·16 ± 0·69	16·2 ± 0·4	Diffuse light, venetian blinds over windows
May 1959 ..	4	4·13 ± 0·92	22·1 ± 1·5	Covers over windows, lights on from 8 a.m. until 5 p.m.
June 1959 ..	4	4·20 ± 0·80	20·2 ± 1·0	Covers over windows, lights on from 8 a.m. until 5 p.m.

According to Table IV, in June, 1956, the slope of the line for the International Insulin Standard was only 2·83 ± 0·46, while in December, 1957 and 1958, it was 5·10 ± 0·78 and 6·16 ± 0·69 respectively. Also the highest value for the median effective dose was obtained in June, 1956, while the lowest values were observed in December, 1957 and 1958. During June, 1956, the mouse room was exposed to daylight all day, but in July, 1956, the windows were covered with cardboard and the lights were turned on in the mouse room during the period of the assay only. The data in Table IV indicate that the slope of the line estimated during August, 1956, was significantly steeper than that calculated in June, 1956, and the median effective dose was closer to that found during the winter months. Eventually venetian blinds replaced the cardboard covers and this change permitted a diffused light to enter the mouse room during the daylight hours. These conditions prevailed in June, 1958, and it will be seen that the average slope tended to be lower than it was in August, 1956. In May, 1959, the cardboard covers were replaced on the windows and the lights were turned on at 8.0 a.m. and off at 5.0 p.m. by a time switch. The slopes found under these conditions were slightly higher than that obtained in June, 1958, but the difference was not actually significant.

Although these data are by no means complete, there is enough evidence to suggest that prolonged exposure to daylight reduces the precision of the assay. Mice appear to be more resistant to the insulin and do not differentiate between the dose levels as well as they do when kept in the dark for longer periods. Apparently the seasonal variation in the steepness of the log dose-response line which was observed with our mice can

be explained on the basis of the amount of daylight which entered the mouse room at the various times of the year. Perhaps the activity of the mice, which is greater in subdued light or darkness than it is in daylight, is one of the factors responsible for this effect of light on the precision of the assay.

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