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Owing to the almost complete lack of knowledge regarding the chemical composition of the active principle in insulin preparations no means have so far been found of determining the potency of a sample of insulin in terms of a chemical reaction. It is necessary, therefore, to resort to biological methods using as indicators the most marked symptoms following the administration of insulin. The observation that insulin causes a fall in the blood-sugar in normal animals and that collapse or convulsions appear when the blood-sugar has fallen to a certain low level, has led to the development of blood-sugar tests and convulsive dose tests. These criterions also suggest a means of establishing a unit of potency in terms of an animal reaction, but the considerable fluctuations of sensitivity in the animals has made this impossible, and the international unit has therefore been defined in terms of an exact weight of a standard preparation. The blood-sugar tests have been preferred by many workers because of the parallelism existing between the degree of hypoglycæmia in normal animals and the therapeutic effect in diabetic organisms, while the obvious simplicity of using convulsions as an indicator has led others to adopt the convulsive dose tests. We have made use of both kinds of tests and paid special attention to a comparison of their results. Like other workers engaged in insulin standardisation we have been much embarrassed by the wide range of variation in sensitivity shown by different individuals of the same species

and the sudden fluctuations in the average sensitivity which may affect the whole stock of animals at a time. We have spent much time in investigating the cause of the difficulties, but with slight success. Various systems of procedure have been tried in order to overcome the difficulties and at the present time the methods described in the following are considered most adapted to the purpose.

1. Rabbit blood-sugar test.

For the estimation of insulin on rabbits we have adopted with some minor modifications the method described by Marks (1). It depends on the fact, that the hypoglycæmic action of insulin within certain limits is proportional to the dose, when measured as the average fall of the blood-sugar over a period of five hours following the injections. Our essential modification is the use of the average of the first and final values of the blood-sugar as the base-line, instead of the initial value only, as used by Marks. The other modifications are only slight as it will be evident from the following.

We used rabbits weighing about 2 kilos. They were fed on oats and beet-roots and starved for 24 hours before each experiment. The insulin solutions used for the injections contained, what was assumed to be 2,5 unit per cc, and the dose given subcutaneously was 0,2 cc per kilo body weight ($^1/_2$ unit per kilo).

For the purpose of comparing the potency of two insulin preparations each preparation was injected on a group of five rabbits in doses assumed to be equivalent. After an interval of two or three days the experiment was repeated on the same rabbits with reversal of the groups. The number of animals used generally seemed sufficient to se-

cure about the same average sensitivity of the group during the period of assay, and by way of reversal we endeavoured to eliminate the difference of sensitivity in the two groups. If the average sensitivity of the groups shows fluctuations during the period of assay, these generally affect the whole stock of animals in the same way, and consequently they will be eliminated by the reversal. On the other hand in case the sensitivity varies differently in the two groups, this may give rise to errors, which can only be reduced by repetition of the estimation.

For the estimation of the blood-sugar the method of HAGEDORN and NORMAN-JENSEN (2) was used.

A sample of blood for the estimation of the normal fasting blood-sugar was taken half an hour before the injection and further samples every hour subsequently, until six samples had been obtained. At the time of the last sample the blood-sugar has returned to the normal level, and the average of the first and the last sample was considered as the normal blood-sugar. The difference between this average and the average of the other four samples was considered as the effect of the dose injected. The effect of each preparation is evaluated by averaging the effects on each of the five rabbits. The ratio of the effects of the two preparations directly gives the ratio of their potency. In table I. such an experiment and the way of calculating the result is described in detail. As the numerical value of the ratio is the only thing we wish to know, we need not calculate the averages but only sum up the figures as described in the table.

As a trial of the method we instituted a series of comparisons between various amounts of the same preparation and found the results summarized in table II.

Table

Designation of Rabbits	Weight of Rabbits in grs.	cc injected	Date of Experiment	Pre- $10^3 \times$ percentage ½ hour before injection	4½ hours after injection	½ hour after injection
No. 1 white; long ears	1930	0,385	12/4-26	96	94	92
- 2 white	2100	0,42	—	91	92	89
- 3 black	1900	0,38	—	89	92	49
- 4 white	2320	0,465	—	96	96	71
- 5 grey	2150	0,43	—	100	98	89
- 6 white; long ears	2100	0,42	15/4-26	94	92	82
- 7 black	2160	0,43	—	91	92	60
- 8 greyish brown	2225	0,445	—	100	96	60
- 9 white	2370	0,475	—	100	96	85
- 10 black	1930	0,385	—	100	98	60
				957	946	737
				Sum $\times 2 = 3806$		

Differ-

$$\frac{\text{potency of Preparation 1}}{\text{potency of Preparation 2}} =$$

Table II.

True ratio of doses	1,33	1,50	1,59	1,33	1,00
Ratio found by test	1,35	1,59	1,59	1,30	1,01

It is obvious from the table, that reliable results can be obtained, even when the doses of the two preparations under comparison differ considerably. In other stocks of rabbits however the proportionality of effect to dosage might perhaps be less exact, and it is recommended, therefore, to adjust the doses so as to be as nearly as possible equivalent, and neither of them must exceed $^3/_4$ unit per kilo, because with larger doses the effects are no longer proportional to the dose. When the experiment is repeated on another batch of rabbits, generally the same result is obtained, as will

I.

paration 1.				Preparation 2.					
of blood-sugar			Date of Experiment	$10^3 \times$ percentage of blood-sugar					
$1^1/_2$ hours after injection	$2^1/_2$ hours after injection	$3^1/_2$ hours after injection		$^1/_2$ hour before injection	$4^1/_2$ hours after injection	$^1/_2$ hour after injection	$1^1/_2$ hours after injection	$2^1/_2$ hours after injection	$3^1/_2$ hours after injection
82	100	96	15/4-26	100	96	82	85	89	94
85	96	94	—	96	94	71	91	98	96
60	96	92	—	98	96	46	64	85	92
78	96	94	—	96	100	78	87	94	96
100	101	100	—	110	105	100	92	100	103
82	82	87	12/4-26	89	91	49	64	71	85
74	78	85	—	103	100	46	56	85	96
67	85	89	—	101	100	50	53	96	100
74	89	96	—	100	96	53	64	78	96
71	94	92	—	92	96	47	64	89	96
773	917	925		985	974	622	720	885	954
Sum: 3352				Sum $2\times = 3918$		Sum: 3181			
ence: 454				Difference: 737					

$\frac{454}{737} = 0{,}615$

be seen from table III, where a series of double estimations is presented.

Table III.

	1,33	1,21	1,09	1,08	1,09	1,14	1,24	1,08	1,16	1,03	1,17	1,23	1,19
	1,30	1,18	1,05	0,97	1,06	0,93	1,22	0,98	0,96	1,02	0,85	1,04	1,12
Diff....	0,03	0,03	0,04	0,11	0,03	0,21	0,02	0,10	0,20	0,01	0,32	0,19	0,07

By means of the difference between double estimations we can make out the mean error of a single estimation by the formula $\mu = \sqrt{\frac{[\Delta\Delta]}{2s}}$, where Δ is the difference between a pair of estimations, s the number of differences. The mean error is found to be 0,099. In the cases presenting very large divergence there has possibly been a different varia-

tion in sensitivity in the two groups of rabbits used for one or both of the estimations.

As a rule we use the average of two estimations for a complete assay, and accordingly 4 experimental days will be required for it.

2. Mouse convulsion test.

The mouse test used by us has already been published (3), and we therefore only recapitulate the chief points of it.

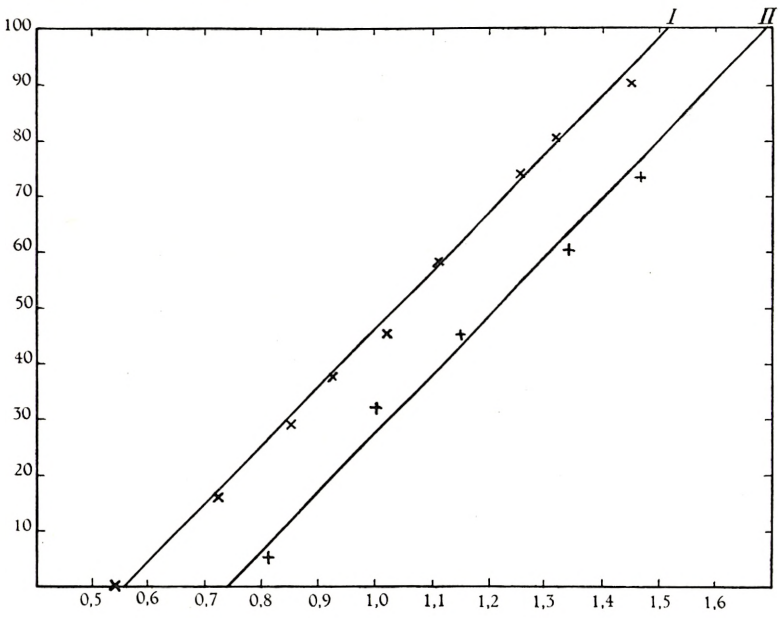
The mice are fed on a standard diet and starved $1^1/_2$ hour before the test. For the test each mouse, regardless of the weight, is subcutaneously injected with $^1/_4$ cc of a suitably diluted solution of the insulin and placed in a cylindrical glass jar (battery jar 16 cm high and 10 cm in diameter) on the shelves of a large incubator with glazed doors so as to allow regular inspection. The air in the incubator is mixed by an electric fan so as to maintain the same temperature (within about 1°) throughout. 30° is selected as being high enough to allow the convulsions to develop, while not itself visibly affecting the mice.

Mice, which are affected (*A*), generally become very quiet, sitting still most of the time with curved back and drooping head. The legs, and especially the hind legs, become slightly paralysed, they slide out from the body on the smooth bottom of the glass jar, and remain for considerable periods in unnatural positions. At this stage the eyes are usually widely open, and some exophthalmus is a frequent symptom. This is usually even more pronounced in the second stage (*A A*), when the mouse is sprawling on the belly with almost completely paralysed hind legs, and often with the tail elevated to a more or less vertical position. This stage may, usually after some re-

missions, pass directly into a stage of collapse (*Cl*), in which the mouse lies motionless on the belly or on the side, and is unable to raise itself when put on its back, but more often the collapse is broken at intervals or on any provocation (e. g. attempts to move) by violent convulsions (*Cn*). When a mouse is collapsed or convulsions appear, a subcutaneous injection of $^1/_2$ cc 10 per cent glucose is given. By this treatment very few mice are lost, and the same animals can be used for three days in the week over a period of 1 or 2 months.

The assay is carried out by a series of tests, in each of which about 160 mice with a weight dispersion of 4 gr. are used. One half of the number are injected with various doses of the standard preparation, while the other half receive doses supposed to be of the same strength of the substance under assay. These tests are repeated until the accuracy deemed necessary has been obtained. The number of mice showing unmistakable symptoms in each test are noted. Most of these attain the stage of collapse or convulsions, but also those recovering after manifestation of the other unmistakable symptoms are taken into account. The result is obtained as follows. The percentage numbers of mice showing unmistakable symptoms at each concentration employed are worked out and plotted as in the figure with the logarithms of the doses as abscissæ and the percentage of mice affected as ordinates. The relation is seen to be linear. Through the points determined for the standard preparation only one straight line can be drawn, and the points obtained from the unknown sample are very well represented by the straight line given. If the assumption regarding the strength of the preparation under test had been correct, the two lines should be identical. In

the example here given, we have on purpose taken a case, in which the assumption (20 units per mg.) is very far from being correct, and as we must expect a constant proportion between the doses assumed for the unknown preparation



I, Curve of Standard Preparation. II, Curve of unknown Preparation assumed to be 20 units per mg.

and those giving the identical percentage number of mice affected in the standard line, the same percentage number of mice affected must be represented by a constant logarithmic difference, that is the two straight lines must be parallel.

The horizontal distance between the two lines is log $x = 0{,}175$, and we obtain the strength of the unknown by subtracting log. $20 - \log x = 1{,}301 - 0{,}176 = 1{,}125$; Antilog. $1{,}125 = 13{,}34$ units per mg.

The mean error of this result can be made out in the

usual way by measuring the horizontal distance of each point from the corresponding line. When we have the mean error on the standard line ε and on the line for the preparation under assay ε_1, the mean error of the result is $e = \sqrt{\varepsilon^2 + \varepsilon_1^2}$. In the case under discussion we have $\varepsilon = 0{,}006$, $\varepsilon_1 = 0{,}014$ and $e = 0{,}015$. This figure is a logarithm, and we have the number of units per mg for the unknown preparation: Antilog $1{,}125 \pm 0{,}015 = 13{,}34 \pm 0{,}46$, that is a mean error of $\pm 3{,}5$ per cent.

Instead of using the graphical method here described, the parallel lines and their horizontal distance can be worked out arithmetically by the method of least squares.

When changes in the sensitivity of the whole stock of mice make their appearance during a determination of this kind, the single tests must be treated separately in a way similar to that described, and the average of these single determinations is calculated. The mean error of a single test usually is about 12 per cent, but varies according to the dispersion of sensitivity, which is graphically expressed by the inclination of the straight line. When the mean error μ of the single determinations is varying, allowance must be made for this when calculating the average by giving the determinations a weight p proportional to $\frac{1}{\mu^2}$.

Furthermore the mean error of the average must be calculated from the formula $\frac{1}{\sqrt{\left[\frac{1}{\mu^2}\right]}}$ or $\sqrt{\frac{[p \cdot d^2]}{[p]\,(n-1)}}$ where d is the deviation of a single determination from the average. The mean error calculated from the two formulas may differ in the special case, but comparisons from a large series of determinations show, that they are identical on an average.

The demonstration that the relation of frequency of symptoms to dose injected is practically logarithmic, corresponds closely with the middle portion of the dose-mortality curves of rats found by VOEGTLIN (4) and the dose-convulsion curves of mice given by TREVAN and BOOCK (5 and 6). We consider the regions of the ordinate next to 0 and 100 per cent less suitable for the mathematical treatment, and accordingly they have been discarded from our curves. The assertion of TREVAN and BOOCK that the slope of the dose-convulsion curve is constant regardless of the variations of the average sensitivity of the mice is not supported by our experience. The very flat curve published by TREVAN and BOOCK at 29° is not found in our experiments except in cases of very large dispersion.

3. Comparison of the methods.

It has been asserted by LAQUEUR and GREVENSTUK (7) that the incidence of convulsions in rabbits depends not only on the insulin concentration, but also on the purity of the preparation, in the sense that impure preparations producing the same lowering of the blood sugar are more liable to produce convulsions. A relation of this kind might conceivably be present also in mice, and be the source of grave systematic errors making the purest preparations relatively too strong, as regards their therapeutic effect on the blood-sugar. We have therefore instituted a series of comparisons between the two methods described in this paper. A preparation which had been compared with the international standard by means of the mouse test, and made to correspond to the international standard with a mean error of ± 5 per cent, was compared also in five experiments on two to four rabbits each, and found to be

94, 95, 96, 96 and 99 per cent of the international unit, with an average of $96 \pm 3{,}5$ per cent. We have, further, compared a pure preparation of 17 units per mg. with the corresponding concentrated extract before final purification and found, that the blood-sugar method on rabbits showed the same relative strength for the extract in proportion to the pure powder as the mouse method. In a final test we have compared the primary extract of the glands with the concentrated and purified extract. In this case the concentrated extract, which contains only a fraction of the impurities of the primary, did show a slightly higher relative concentration according to the blood-sugar method than according to the convulsive-dose method, but the difference was well within the limits of error of the determination. We can safely conclude, therefore, that there is no systematic difference, depending on purity, between the two methods.

In another paper dealing with the destructive action of temperature on insulin solutions, the concordance of the results obtained from the two methods will be demonstrated in a different way (8).

LITERATURE

1. Publications of the League of Nations III. Health 1926. III. 7. p. 57.
2. Biochem. Zeitschr. 135. p. 46 1923 and 137. p. 92. 1923.
3. Publications of the League of Nations III. Health 1926. III. 7. p. 40.
4. Public Health Reports, 1924. 39. p. 1935.
5. Publications of the League of Nations III. Health 1926. III. 7. p. 47.
6. Proc. Roy. Soc. B. 1927. 101. p. 483.
7. GREVENSTUK, A. and LAQUEUR, E. "Insulin" p. 171. München, 1925. I. F. BERGMANN.
8. KROGH and HEMMINGSEN. 1928. Bioch. Journal (in press.).

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