LXXXII. THE BIOLOGICAL DETERMINATION OF CRYSTALLINE VITAMIN B₁

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KINNERSLEY & PETERS [1936] stated that, although they had always found a rough relation between effect and dose of vitamin B₁ when this was given in the form of a food or concentrate, this relation did not hold at all when crystalline vitamin B₁ was given. They measured effect by the duration of the cure of "retracted neck" in pigeons. Doses of concentrates were given by mouth but doses of crystalline vitamin B₁ were given by injection. When they gave doses of crystalline vitamin B₁ by mouth, the effect, as measured by the duration of cure, still bore no relation to the size of dose given, but when measured by the percentage of birds cured (7 μ g. cured 75 % and 14 μ g. cured 90 %) then a relation between effect and size of dose became apparent. They therefore considered the oral route the more satisfactory way of giving doses, but concluded finally that the "day-dose" method of evaluating results from pigeons is not satisfactory for assay of injected crystalline vitamin B₁ though useful in assaying impure preparations of the vitamin.

We had ourselves by that time obtained one result showing a relation between effect and dose of crystalline vitamin B₁, the effect being measured by the percentage of birds cured and doses being given orally. There was also a difference in the effects as measured by the average duration of the cure but it was not a large one (Table Ia). We decided, therefore, to carry out further tests, as it seemed probable that crystalline vitamin B₁ would shortly take the place of the adsorbate of that factor which had been used as the International Standard for several years. We wished also to obtain further information on the accuracy of the pigeon test, for it has the distinct advantage of being specific for vitamin B₁ while the "increase in weight" method is not.

EXPERIMENTAL

We always use pigeons suffering from their first attack of "retracted neck". Pigeons which have been cured in one experiment are never used a second time. As the birds become ready for test they are distributed in rotation to the various groups arranged for the comparison, so that as nearly as possible equal numbers of birds are assigned to each group and given their doses on the same day.

Our first experiment, mentioned above, with graded doses of the crystalline vitamin B₁, was a comparison between the crystalline preparation destined for the new International Standard and the current International Standard of vitamin B₁ adsorbate. In all experiments doses were given orally. In this one, doses of 5 and 10 μ g. crystalline vitamin B₁ were tested in series with a dose of 3 units (0.03 g.) of the International Standard. The dose of 5 μ g. produced 33.3 % of cures in 15 birds and the dose of 10 μ g. produced 66.7 % of cures in 15 birds. This was very clear evidence of a graded response to graded doses of the vitamin (Table Ia).

Table I. *Response of pigeons to graded doses of crystalline vitamin B₁ given orally. Criteria: percentage of birds cured and duration of cure*

Exp.	Dose μg.	No. of birds dosed	% birds cured	Av. duration of cure of birds, days
a	5.0	15	33.3	3.6
	10.0	15	66.7	4.3
b	4.5	22	54.5	6.6
	9.0	22	81.8	5.5
	13.5	22	90.9	6.2
c	3.0	22	59.1	6.2
	6.0	22	50.0	8.7
	9.0	22	77.3	7.4
d	1.0	25	8.0	5.5
	3.0	25	28.0	5.6
	9.0	25	56.0	5.4

Our second experiment was performed merely to investigate the nature of the relation between the dose of crystalline vitamin B₁ given by mouth and the percentage of birds cured. Doses of 4.5, 9.0 and 13.5 μg. of crystalline B₁ were given to different groups, each group consisting of 22 birds. The percentages of birds cured were 54.5, 81.8 and 90.9 respectively, again a graded response to graded doses given (Table Ib).

In our third experiment doses of 3, 6 and 9 μg. respectively were given to different groups with 22 birds in each group. The percentages of birds cured were 59.1, 50.1 and 77.3 respectively. If one may judge from the other tests, it is the group given the lowest dose which is out of line in this series (Table Ic).

In our fourth experiment a greater spread of doses was chosen, namely 1, 3 and 9 μg., a series nearer the lower end of the curve. The percentages of birds cured were 8.0, 28.0 and 56.0 respectively, again a graded response to graded doses given (Table Id).

Thus it is evident that when the percentage of birds cured is used as the criterion of activity the response of pigeons is graded to the dose given.

The average duration of the cure of these birds was also noted, but many of the birds that had been cured died suddenly without developing retracted neck again. However, the cure was counted as lasting up to the day of death. It may be seen from Table Ia, b, c, d, that the duration of cure of birds given different doses of crystalline vitamin B₁ by mouth is not graded to the dose given. Yet Coward *et al.* [1933] had obtained responses which, when measured as duration of cure, were graded to the dose of vitamin B₁ given by mouth when the vitamin was contained in (a) an acid clay adsorbate, (b) dried yeast, (c) a concentrate, (d) an extract of rice polishings and (e) a soft extract of yeast. Thus our experience with regard to the "duration of cure" criterion for measuring vitamin B₁ has been similar to that of Kinnersley and Peters. With regard to the use of "percentage of birds cured" as criterion we consider we have ample evidence that the response thus measured is graded to the dose of crystalline vitamin B₁ given orally.

The accuracy obtainable by this method (% of birds cured)

The examination of the figures obtained in these tests shows that we did not attain a much greater degree of accuracy than that calculated from our figures by the Sub-Committee on the Accuracy of Biological Assays, Pharmacopoeia

Commission [1936]. The accuracy of a determination based on an "all or none" reaction may be calculated from the following equation:

$$\lambda_M^2 = \left(\frac{1}{S(B'n')} + \frac{1}{S(B''n'')} + \frac{(\bar{y}' - \bar{y}'')^2 \sigma_b^2}{b^2} \right) \times \frac{1}{b^2},$$

in which λ_M is the standard deviation of the logarithm of the ratio of the activities,

n' , n'' are the numbers of animals in the groups given a dose of Standard and test substance respectively,

B' , B'' are the corresponding "weight factors" which depend on the observed percentage response,

y' , y'' are the normal equivalent deviations corresponding to the percentage of birds cured,

b is the slope of the curve relating y to the log of the dose to the base 10,

σ_b is the standard deviation of b .

The values for y' , y'' are found directly from tables [Gaddum, 1933; Bliss, 1935; Pearson, 1930 (Table I)]. The values for B' , B'' are found from the equation $B = z^2/pq$, in which z is found from Pearson's Table II, x in this table being y of the present calculation, p is the proportion of birds cured and q the proportion not cured.

It is obvious from the form of the equation that (a) the larger the number of animals used, (b) the larger the values of B (i.e. the more nearly the number of birds cured approaches 50 %) and (c) the steeper the curve of response, the more accurate will the determination be.

In order to gain a general idea of the accuracy obtainable by using a reasonable number of birds in this test, the Sub-Committee on the Accuracy of Biological Assays (Brit. Pharm. Commission) chose the values $n' = n'' = 10$, $B' = B'' = 0.5$ (which corresponds to about 79 or 21 % of birds cured in each group), $y' = y''$ (which makes the last fraction in the bracket of the equation vanish, and $b = 2.00$ (the average of the 6 values, 1.80, 2.64, 1.50, 1.11, 1.25 and 4.0 already available from 6 different experiments). Thus estimated, λ_M was equal to ± 0.3162 . By multiplying this by 2.576 and taking the antilogs, the limits of error ($P = 0.99$) were estimated as 15 and 650 %.

The results already described in this paper can be used to gain further information on the accuracy of the pigeon method (% cure of birds). The same conditions of experiment as in the B.P. estimation of the accuracy of the method may be assumed, i.e. $n' = n'' = 10$ and $B' = B'' = 0.5$ (79 or 21 % of the birds were cured in the two groups given Standard and test substance respectively). The only difference lies in the slopes of the curves of response. These were calculated as regression lines relating the normal equivalent deviation (determined from tables from the percentages of birds cured) and the log to the base 10 of the dose of crystalline vitamin B₁ given. The 4 values for b found from the 4 experiments were 2.87, 2.57, 1.85 and 2.95 respectively; the mean, weighted according to the number of birds used in constructing each curve, was 2.53, not greatly different from the mean 2.00 used in the B.P. estimation of the accuracy of the test. λ_M was calculated for each of the 4 experiments as ± 0.2204 , ± 0.2458 , ± 0.3419 and ± 0.2145 respectively. The limits of error ($P = 0.99$) were found to be 27-370, 23-430, 13-760, 28-357, mean 21-466, not very different from the first estimate of 15-650.

The probability that there has been a gain in accuracy in the last 4 experiments is very small indeed. Since the slopes of the curves are the determining factors in these calculations of the limits of error, the value of $t = \frac{M_1 - M_2}{\sqrt{(\epsilon_1^2 + \epsilon_2^2)}}$ has been determined for the two average slopes (2.00 for the first set of 6 figures, used by the Sub-Committee on the Accuracy of Biological Assays, and 2.53 for the 4 experiments described in this paper). t was found to be 1.145, which is not large enough to indicate a significant difference between two averages of 6 and 4 observations respectively. Hence, unless some means of obtaining a much steeper curve of response can be discovered, it seems unlikely that the test can be made to give more accurate results than have been already obtained.

Table II. *The accuracy obtainable in pigeon tests in which $n' = n'' = 10$, $b = 2.00$; the percentage of birds cured is the same in both groups (on Standard and test substance respectively) and ranges from 50 to 5 or 95 %*

% birds cured	λ_M	$\lambda_M \times 2.576$	Limits of error $P = 0.99$
50	± 0.28027	± 0.7220	19-527
40 or 60	± 0.28362	± 0.7306	19-538
30 or 70	± 0.29517	± 0.7604	17-576
20 or 80	± 0.31954	± 0.8230	15-665
10 or 90	± 0.38228	± 0.9848	10-966
5 or 95	± 0.47272	± 1.2177	6-1650
0 or 100	α	α	0- α

A calculation has been made of the accuracy obtainable in similar pigeon tests with $b = 2.00$, $n' = n'' = 10$ and B calculated from the assumption that both groups had equal percentages of birds cured, ranging from 50 to 5 or 95 %. It is summarized in Table II. It is evident that the accuracy does not vary much when the results lie between 30 and 70 % or even between 20 and 80 %, but beyond those limits the accuracy decreases very rapidly indeed, and, very obviously, no faith whatever could be placed in a result in which the doses of Standard and test substance respectively cured 100 % of birds in both groups.

SUMMARY

Four experiments have been carried out in which it has been found that the responses of pigeons were graded to the dose of crystalline vitamin B₁ given. The criterion used was the percentage of birds cured of retracted neck; the doses were given orally. These findings confirm and greatly augment the evidence for the statement of Kinnersley & Peters that a relation between effect and size of dose of crystalline vitamin B₁ given orally becomes apparent when the effect is measured by the percentage of birds cured.

When the response was measured by the duration of the cure of those birds which were cured, the response bore no relation to the size of dose of crystalline vitamin B₁ given. This confirms the statement of Kinnersley & Peters that the "day-dose" method of measuring the response of pigeons to doses of crystalline vitamin B₁ is useless.

Conditions for obtaining the greatest accuracy possible in this method are discussed.

The apparently greater degree of accuracy which seemed to be obtainable after an examination of the figures obtained in these experiments, when compared with the original estimate of the accuracy of the pigeon test, was found not to be significant. The pigeon test, even using the percentage of birds cured,

therefore remains the least accurate of those methods of vitamin assay whose accuracy was investigated by the Sub-Committee on the Accuracy of Biological Assays, British Pharmacopoeia Commission.

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