LXXXIX. ANTINEURITIC POTENCY OF SYNTHETIC AND NATURAL CRYSTALLINE VITAMIN B_1 AS DETERMINED BY THE "BRADYCARDIA" METHOD

WITH A STATISTICAL STUDY OF THE DEGREE OF ACCURACY OF THE "BRADYCARDIA" METHOD

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WITH the isolation of pure crystalline vitamin B_1, and more lately its successful synthesis in the laboratory, it becomes of importance to know as precisely as possible its antineuritic activity in terms of the present international standard. The "bradycardia" method as used in this laboratory [Birch & Harris, 1934; Harris, 1934; Harris & Leong, 1936] and elsewhere [e.g. Baker & Wright, 1935] offered a convenient means of assay which possesses a degree of accuracy rather unusual among biological methods. During the course of the present work, four specimens have been examined by this method, three being natural and one a synthetic material: all have been found to show the same activity (within the small experimental error).

EXPERIMENTAL

Technique. The details of the method adopted were essentially the same as those described in an earlier paper [Birch & Harris, 1934]. The principle of the method is to take rats suffering from a low rate of heart beat, as brought about by a diet deficient in vitamin B_1. Single graded doses of the substance under test are administered, whereupon the condition is benefited in proportion to the amount of vitamin given. The period of time is noted which elapses until the heart is again beating at the same slow rate as when the dose was given. The following technical points may be specially noted. (1) Rats taken for test (piebald animals only) should be between 45 and 55 g. body weight. (2) In order, as far as possible, to minimize coprophagy a wide (e.g. $\frac{1}{2}$ in.) mesh-bottom should be fixed in the cages—all rats being kept in separate cages.[1]

RESULTS

In Tables I–IV are assembled the results of the various tests. As will be seen, a simultaneous comparison was made of three specimens of crystalline vitamin B_1 hydrochloride (one synthetic, and two natural) with the international

[1] Surprisingly enough, the tying down of the rat preparatory to the measurement of its heart rate and the insertion of the electrode needles have a negligibly small effect on the rate of its heart beat. Nevertheless a practice has been made, after the rats are securely fastened on the board, of stroking them gently for about 10 sec. before the electrocardiogram is taken. More recently, some measurements have been taken without the rat being tied down. The animal is held in the hand and the electrodes inserted in the usual positions. The heart rate was found to be the same when taken by either method.

standard during September to October 1936 (Table I). Another specimen of natural crystalline vitamin B_1-HCl was examined in January to March 1936 (Table II), and almost continuous tests on the international standard were made from May 1935 to October 1936 (Tables I and III). Since the responses produced by a given dose of international standard were found to be of the same average magnitude and to have approximately the same frequency-distribution curve on the various occasions, it can be concluded that there was little or no change in

Table I. *Simultaneous comparison of international standard with specimens of synthetic and natural crystalline vitamin B_1-HCl*

Date	Material	Amount given	Days cured: Individual animals*	Days cured: Mean
Sept.–Oct. 1936	International standard	10 mg.	2, *3*, *3*, 3	2·75
		20 mg.	*4*, 4, 4, 4, *5*, *5*, 5, 5	4·50
		30 mg.	6, *7*, *7*, 7	6·75
		40 mg.	7, 8, 8, 8	7·75
Sept.–Oct. 1936	Crystalline vitamin B_1-HCl, natural, specimen No. II	2·5γ	*2*, *3*, 3, 3	2·75
		5·0γ	*4*, *4*, 4, 4, 4, *5*, *6*	4·43
		7·5γ	5, 5, 6, 7	5·75
		10·0γ	6, 6, 7, 8	6·75
Sept.–Oct. 1936	Crystalline vitamin B_1-HCl, natural, specimen No. III	2·5γ	3, *3*, 3, 3	3·00
		5·0γ	3, *4*, *4*, 4, 4, 4, 4, *5*, 5, 6	4·30
		7·5γ	*5*, *5*, 6, 7	5·75
		10·0γ	6, 7, 7, 7	6·76
Sept.–Oct. 1936	Crystalline vitamin B_1-HCl, synthetic	2·5γ	*2*, *3*, *3*, 3	2·75
		5·0γ	3, *4*, 4, 4, 4, *5*, *5*, 5, 5, 6	4·50
		7·5γ	*5*, *5*, 6, 7	5·75
		10·0γ	*7*, *7*, 7, *9*	7·50

* For meaning of italicized numerals see footnote, p. 678.

Table II. *Data for natural crystalline vitamin B_1-HCl, specimen No. I*

Date	Material	Amount given, γ	Days cured: Individual animals	Days cured: Mean
Jan.–March 1936	Crystalline vitamin B_1-HCl, natural, specimen No. I	2·5	2, 2, 2, 3	2·25
		5·0	3, 4, 4, 4, 4, 4, 4, 4, 4, 4, 5, 5, 5	4·15
		7·5	4, 4, 5, 5, 5, 6, 6, 7, 7, 7, 7	5·75
		10·0	7, 8, 9, 9	8·25

Table III. *Data for international standard*

Date	Material	Amount given mg.	Days cured: Individual animals	Days cured: Mean
May 1935–July 1936	I.S.	10	2, 2, 2, 2, 2, 2, 2, 2, 2, 2, 2, 2, 2, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 4, 4, 4, 4, 4, 5, 5	2·91
		20	2, 2, 2, 3, 3, 3, 3, 3, 3, 4, 4, 4, 4, 4, 4, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 6, 6, 6, 6, 6, 6, 6, 6, 6, 6, 6, 7, 7, 8	4·74
		30	4, 4, 4, 4, 5, 5, 5, 5, 5, 5, 6, 6, 6, 6, 6, 6, 6, 6, 6, 6, 6, 7, 7, 7, 7, 7, 7, 7, 7, 8, 8, 9, 9, 9, 9, 9, 9, 9, 9, 9, 9, 9	6·72
		40	5, 5, 6, 6, 6, 7, 7, 7, 7, 7, 8, 8, 8, 9, 9, 9, 9, 9, 10, 10, 10, 13, 13	8·17

Table IV. *Data for international standard, combined results (May 1935–October 1936), for construction of standard reference curve*

Dose given I.U.	Days cured	Frequency	No. of observations	Mean	Twice standard error of mean, 2ϵ*
1	2	18	51	2·82	±0·21 (= ±7·6 %)
	3	26			
	4	5			
	5	2			
2	2	3	51	4·67	±0·36 (= ±7·7 %)
	3	7			
	4	11			
	5	17			
	6	10			
	7	2			
	8	1			
3	4	4	49	6·74	±0·44 (= ±6·5 %)
	5	6			
	6	13			
	7	13			
	8	2			
	9	11			
4	5	2	27	8·11	±0·76 (= ±9·5 %)
	6	3			
	7	6			
	8	6			
	9	5			
	10	3			
	13	2			

* $\epsilon = \frac{\sigma}{\sqrt{n}}$, where σ = standard deviation $= \sqrt{\frac{\Sigma d^2}{n-1}}$, where d = deviation from mean, n = number of observations.

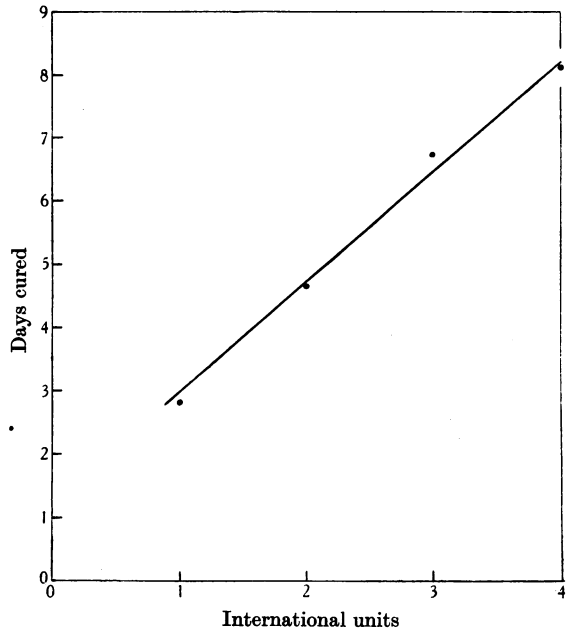

Fig. 1. Dose-response curve with international standard.

the "uncontrolled variables" of the experimental procedure, and hence for more adequate statistical treatment the results obtained with the international standard on the different occasions may be combined and considered together. These combined results for the international standard are summarized in Table IV.[1]

Construction of dose-response curve. The arithmetical means of the responses, observed at each level of dosage of international standard, were used to construct a reference curve (Fig. 1). It will be seen that the relation is very nearly linear, within the particular ranges of doses under examination. Therefore to avoid unnecessary complication a straight line has been drawn, fitted to the observed means by the method of least squares.

Calculation of activity of the various preparations. By using this reference curve, one may determine the number of international units corresponding with each of the responses produced by the different doses of crystalline vitamin B_1-HCl, as given in Tables I and II. The findings are collected in Table V, and for each specimen a final value for the activity is obtained by calculation of the weighted means.

Table V. *Comparison of various preparations of crystalline vitamin B_1-HCl against international standard*

Material	Dose given γ	No. of observations	Days cured (mean)	I.U. equivalent to dose given (read from curve, Fig. 1)	Amount of specimen, in γ, equivalent to 1 I.U.	
					Individual values	Weighted mean
Natural, specimen No. I	2·5	4	2·25	0·60	4·17	3·02
	5·0	13	4·15	1·68	2·97	
	7·5	11	5·75	2·60	2·88	
	10·0	4	8·25	4·05	2·47	
Natural, specimen No. II	2·5	4	2·75	0·88	2·84	2·87
	5·0	7	4·43	1·84	2·72	
	7·5	4	5·75	2·60	2·88	
	10·0	4	6·75	3·18	3·14	
Natural, specimen No. III	2·5	4	3·00	1·03	2·42	2·83
	5·0	10	4·30	1·76	2·84	
	7·5	4	5·75	2·60	2·88	
	10·0	4	6·75	3·18	3·14	
Synthetic	2·5	4	2·75	0·88	2·84	2·75
	5·0	10	4·50	1·87	2·67	
	7·5	4	5·75	2·60	2·88	
	10·0	4	7·50	3·60	2·74	

Conclusions. It will be seen from the last column of Table V that almost identical values are obtained for all four specimens of crystalline vitamin B_1-HCl, viz. 2·8–3·0, with a good mean value of 2·9. (These values are expressed as γ of crystalline vitamin B_1-HCl per 1 I.U.) We may reasonably conclude that the differences found are not statistically significant: the number of observations made on each specimen varied from 19 to 32, and, from the data calculated below, twice the standard error of the mean under these circumstances should be about $\pm 10\%$, i.e. the error is within the observed variation.

[1] In ordinary routine assays for vitamin B_1 it is, in our opinion, always advisable to carry out simultaneous tests on both unknown and standard (preferably with at least two levels of each since, under varying conditions, the dose-response curve may alter appreciably from one experiment to another.

A combined dose-response curve for the data of Table I, given in Fig. 2, illustrates the precision of the method and the agreement between the different specimens.

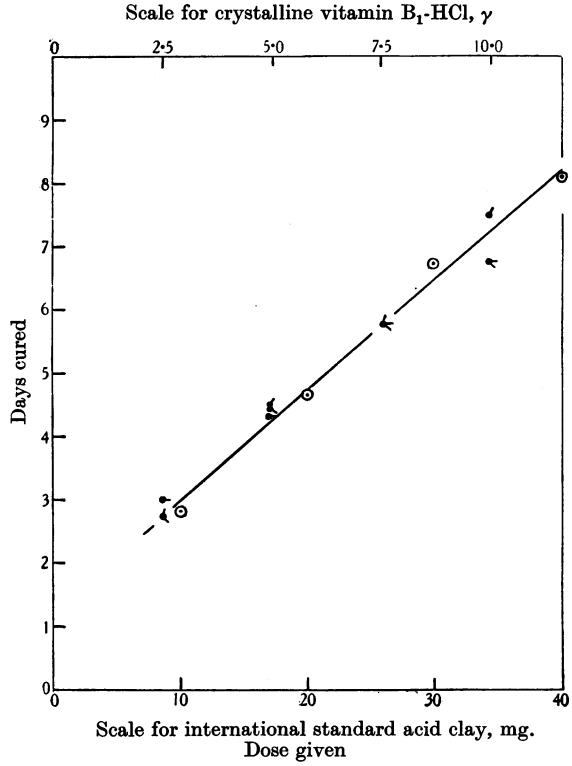

Fig. 2. Simultaneous comparison of I.S. and synthetic and natural specimens of crystalline vitamin B_1-HCl.

The scales are adjusted so that 10 mg. I.S. (i.e. 1 I.U.) = 2·9γ crystalline vitamin B_1-HCl. ⊙ International standard; ◐ Natural, specimen No. II; ● Natural, specimen No. III; ◐ synthetic.

Comparison with results of other workers. The values we have obtained are compared with the recent findings of other workers in Table VI.

Table VI

Authors	Amount in γ equivalent to 1 I.U.	Method used
Ohdake & Yamagishi [1935]	1·6	Pigeon curative
Waterman & Ammerman [1935]	5	Rat growth
Kinnersley & Peters [1936]	2	Pigeon curative "Catatorulin" tests
	1·99	
Jansen [1936]	3	Cure of convulsions in rats
Leong & Harris:		
Natural, specimen No. I	3·0	"Bradycardia"
Natural, specimen No. II	2·9	
Natural, specimen No. III	2·8	
Synthetic	2·8	

A comparison of various natural preparations was made by Heyroth [1936], using the method based on the cure of convulsions in a rat. He found values of 80·7 and 91·3 for the relative potencies of specimens (supplied by Ohdake) from yeast and rice respectively, taking a preparation obtained from Peters as 100. He adds: "the differences between them [the three specimens] appear to be only slightly beyond the limits of accuracy of the method".

From the results of the present tests, 1 I.U. of the standard acid clay as at present issued may be taken as approximately equivalent to 3γ of crystalline vitamin B_1-HCl.

Statistical study of accuracy of the method

From the collected results for the international standard material given in Table IV it will be seen that the distribution of responses to a given test dose forms a curve of frequency of approximately normal type (within the limits of the number of observations made). The same is true of crystalline vitamin B_1-HCl (Tables I and II). It is convenient therefore to base the calculations as to the degree of accuracy of the method on the collected results for the standard acid clay, as a large number of tests have been made with this material. (The results with the various specimens of crystalline vitamin B_1-HCl have also been submitted to a different method of statistical treatment by Dr J. Wishart (see below). The conclusions reached are identical.)

(1) *Calculation of standard error of mean.* In Table IV the *standard error of the mean* has been calculated for the mean response observed at each level of dosing. The last column gives the corresponding values of $\pm 2\epsilon$. To take an example: with a level of dosage of 1 I.U., the standard error of the mean of the results of 51 tests is $\pm 0{\cdot}107$, or twice the standard error is $\pm 0{\cdot}21$, i.e. 7·6 %. This latter statement means that the probability is 21/22 that the mean obtained is within $\pm 7{\cdot}6$ % of the true value.

It will be noticed that very similar standard errors are obtained at all levels of dosing when they are expressed on a percentage basis.

(2) *Calculation of standard deviation (σ) of a single observation.* In Table VII the standard deviations of the mean responses observed have been calculated

Table VII. *Standard deviation of a single observation*

No. of tests	Dose of standard acid clay (I.U.)	Days cured mean	Standard deviation $\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$
51	1	2·78	$\pm 0{\cdot}77$ ($= \pm 28$ %)
51	2	4·67	$\pm 1{\cdot}31$ ($= \pm 28$ %)
49	3	6·73	$\pm 1{\cdot}55$ ($= \pm 23$ %)
27	4	8·11	$\pm 1{\cdot}98$ ($= \pm 24$ %)
Weighted mean	—	—	($= \pm 26$ %)

for each level of dosing. In order to afford a proper comparison, the results of this calculation have also been expressed as a percentage of these mean responses. The average (weighted) standard deviation for a single test is seen to be about ± 26 %. (There is little variation from this percentage value at different levels of dosing.)

This finding means that two out of every three observations may be expected to give a response lying within ± 26 % of the mean value.

(3) *Calculation of degree of accuracy attainable with different numbers of tests.* Fig. 3 shows how the degree of accuracy which may be expected in an assay increases as an increasing number of animal tests are made. To obtain this curve the standard error of the mean $\left(\epsilon, \text{ where } \epsilon = \frac{\sigma}{\sqrt{n}}\right)$ has been calculated for different values of n, and the results plotted.

To cite some concrete examples: If only three tests are made (i.e. three doses fed once to three animals) the probability is 21/22 that the result obtained will be within $\pm 30\%$ of the true value. Or, if five tests are made, the mean result will be within $\pm 22\%$ of the true value 21 times out of 22. With 20 tests the accuracy has so increased that the error is only $\pm 12\%$ 21 times out of 22.

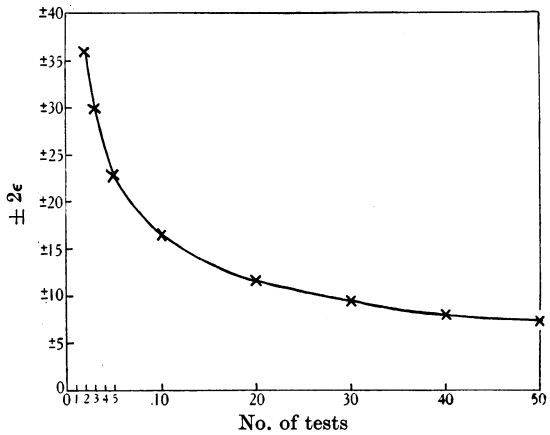

Fig. 3. Accuracy obtainable with different numbers of tests. The ordinates represent $\pm 2\epsilon$, where ϵ = the standard error of the mean (expressed as a percentage) obtained with different numbers of tests (one "test" means one animal given one dose on one occasion).

In actual practice, the "20 tests" referred to in the last sentence will often consist not of 20 such tests of response all at the same level of dosing of one substance, but (say) of five tests of standard at two levels each, plus five tests of unknown at two similar levels of activity. Under such circumstances, i.e. if a standard reference curve be not used, the total error of the test would be worked out as follows.

Since the percentage standard error of the mean of the response at the level of (say) 1 I.U. = that at 2 I.U. = that at 3 I.U. = that at 4 I.U. (see Table IV), hence the S.E. with two levels of dosing involving five tests at each level is approximately the same as the S.E. of the mean of ten tests. From Fig. 3 it will be seen that $2\epsilon = \pm 16\%$. Approximately the same error will be expected with the 5+5 tests of unknown, i.e. $\pm 16\%$.

Hence the total error $= \pm\sqrt{(\epsilon_1^2+\epsilon_2^2)} = \sqrt{(16^2+16^2)} = 22\%$.

Dr J. Wishart in the following appendix has been kind enough to submit our figures to an independent statistical analysis, which has yielded similar conclusions to those of our own method of calculation.

Appendix

By J. WISHART

Calculations on the data of Table I[1] are tabulated below. The principal conclusions to be drawn are:

(1) There is no difference of statistical significance between the various specimens of crystalline vitamin B_1-HCl.

[1] In Table I the duration of cure in days is given to the next largest integer. Thus a cure of over 2 days but less than 3 is counted as 3 days. The numerals shown in italics in Table I represent examples of such cures of intermediate duration. For the purposes of the present calculations a cure of 3–4 days is counted as 3·5 days, 4–5 as 4·5, etc., P. C. L., L. J. H.

(2) The error as determined by this alternative procedure is virtually the same as that calculated in the body of the paper. The further conclusions given above therefore hold good.

The data have been treated to an analysis of variance. For this purpose the figures for "days cured" with international standard were first scaled down from the reference curve to be equivalent to 2·5, 5, 7·5 and 10γ. We then have 16 samples of variable size, representing four quantities of four treatments, and the data can be analysed as follows:

	Degree of freedom	Sum of squares	Mean squares
Amount given	3	180·31	60·10
Kind of material	3	0·44	0·15
Interaction	9	4·14	0·46
Error	67	36·40	0·54
	82	221·29	

The first two items are (1) the weighted sums of squares of deviations from the general mean of the means for all materials at the 2·5, 5, 7·5 and 10γ levels, and (2) the same calculation for the four materials, the samples being bulked for all amounts given. The sum of squares with 82 D.F. is the sum of squares of deviations of all individual figures from the common mean. The part for interaction + error (76 D.F.) is then obtained by subtraction. But the error alone (67 D.F.) can be got directly by taking the sum of squares of deviations from each sample's mean and adding all the results together. It is assumed that the numbers of observations at different levels of dosage are in proportion for all materials, which is nearly correct.

The mean square for "kind of material" is only 0·15, less than error 0·54. Thus there is no difference between the various materials and international standard.

The standard error of a single observation is $\sqrt{(0{\cdot}54)} = 0{\cdot}73$. The S.E. of the mean of 51 observations is $\frac{0{\cdot}73}{\sqrt{51}} = 0{\cdot}102$, compared with 0·107 obtained from the more extensive international standard data only.

A further analysis of the error (67 D.F.) is illuminating. This can be calculated either (*a*) within each material (bulking for amount given) or (*b*) within amounts given (bulking for materials). The results are as follows:

	D.F.	Sum of squares	Mean square	Standard deviation
(*a*) International standard	16	1·48	0·092	0·30
Crystalline vitamin B_1-HCl, natural, specimen No. II	15	9·86	0·657	0·81
Crystalline vitamin B_1-HCl, natural, specimen No. III	18	11·02	0·612	0·78
Crystalline vitamin B_1-HCl, synthetic	18	14·04	0·780	0·88
	67	36·40		
(*b*) 2·5γ	12	3·34	0·279	0·53
5·0γ	31	15·67	0·505	0·71
7·5γ	12	11·16	0·930	0·96
10·0γ	12	6·23	0·519	0·72
	67	36·40		

The values obtained for the standard deviations in (*b*) above may be compared with those given in Table VII, viz. 0·77, 1·31, 1·55 and 1·98. The standard deviation as here calculated is lower, but so is the mean (see Table VII).

SUMMARY

1. The "bradycardia" method has been used to make a precise assay of the antineuritic potency of crystalline vitamin B_1 hydrochloride. Three specimens from natural sources, together with one synthetic preparation, have been examined, and all agree in showing a potency of 2·8–3·0γ per 1 I.U. (This variation is within the probable experimental error of the method: twice the standard error of the mean, under the conditions of the test, being approximately $\pm 10\%$.)

2. A statistical analysis has been made of the data obtained in experiments in which graded amounts of international standard and "unknown" were administered, involving a total of upwards of 270 separate tests. It is shown that the accuracy of the method is such that the probability is 21/22 that the mean result will be within $\pm 22\%$ of the true value when five "tests" are made, or within $\pm 16\%$ for ten tests, $\pm 12\%$ for 20 tests, $\pm 9\%$ for 30 tests, or $\pm 8\%$ for 40 tests (each "test" means one dose given on one occasion to one rat). An appendix (by J. Wishart) confirms these calculations by an independent method of analysis.

We are grateful to the Vitamin B_1 Sub-Committee of the Accessory Food Factors Committee of the Medical Research Council, to Dr van Veen of the Geneeskundig Laboratorium, Batavia, and Messrs Merck of Darmstadt for the gift of specimens of the materials tested.

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