CXLII. CRYSTALLINE TORULIN (AS VITAMIN B₁) AND THE INTERNATIONAL VITAMIN B₁ STANDARD.

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THE work described in this paper originated in 1931 with tests upon an acid clay supplied by Prof. Jansen as a preliminary trial for the international standard. It was continued with the actual international standard, and extended to include trials of extracts made from the international standard and of crystalline vitamin B₁. Both of the latter can be compared by injection and by the mouth. Some rather remarkable discrepancies have appeared between tests with our crystalline vitamin [Kinnersley *et al.*, 1935] and a vitamin concentrate.

EXPERIMENTAL.

Tests have been made by the technique previously described with little change. Birds have been left for at least 4 hours after dosing with sugar solution to minimise the number of pseudo-cures. Samples of clay or extracts have been given in approximately 5 ml. water, the latter being neutralised where necessary immediately before use.

We have made 3 types of comparison, (1) tests carried out over different parts of the year, (2) serial tests, in which a group of standard or other tests is sandwiched in between groups of other tests, and (3) some simultaneous tests, in which alternate birds are dosed with the preparations to be compared. Though the latter may give more confidence as a control, we are inclined to think that, owing to the particular way in which birds have to be prepared for these tests, the extra gain in control over the serial method is illusory [*v. Kinnersley et al.*, 1935, p. 707]. So far as we can tell, the variations as between one time of the year and the other lie within the error of the method. Following our usual convention we have not included in our reckoning those birds which were not cured for 1 day. Contrary to previous practice we have included in our average even very high tests, because it is difficult to exclude these; owing to the large error of the method big groups have been used.

Table I gives results for the solids, preparation 2 being the international standard. It is remarkable to note how consistent the values were for the groups of tests in 1934. The average value for the whole series of 50 mg. tests gives "10 mg. equal to 1.12 pigeon day doses in our terms".

Comparing Exps. 1-4 with Exps. 5 and 6, we see that high doses of the clay tend to give erroneous results. This is probably due to failure upon the part of the gut to elute the vitamin from the clay in the course of passage through the animal. R. B. Fisher (private communication) has found that food passes through the gut of pigeons in a few hours. Indirect evidence was obtained in some other experiments that this might happen.

There arises at once the question whether some of the variations in the figures of Table I arise from the uneven liberation of vitamin from the clay by the

Table I. *Jansen's activated clay.*

Exp.	Prep.	Year	Month	Dose mg.	No. birds	No days cured* av.	Not cured† %	Day dose mg.	Individual tests days
1	1	1931	Jan.-March	250	2	11.5	0	22.0	—
2		1931	Jan.-March	200	4	9.0	0		
3		1931	Jan.-Feb.	100	9	7.4	30	12.8	10, 4, 7, 6, 6, 13, 9, 7, 5 4, 5, 6, 7, 8, 15, 13
4		1931	March	100	7	8.3			
5		1931	May-June	50	5	5.6	20	9.8	6, 6, 6, 8, 2 5, 4, 5, 3, 2, 3, 5, 11, 9, 2, 4
6		1932	Oct.-Nov.	50	11	4.8			
7	2(i.s.)	1932	Oct.-Nov.	50	8	6.5	—	7.7	4, 6, 6, 5, 7, 10, 10, 4
8		1934	28/29 May	50	10	5.5	9	9.1	10, 6, 2, 2, 3.5, 2, 15, 5, 5, 5
9		1934	Sept.	50	14	5.6	7	8.9	5, 5, 5, 3, 12, 2, 6, 5, 4, 8, 5, 9, 5, 4
10		1934	28 Oct.-Nov.	50	18	5.3	—	9.4	5, 9, 10, 2, 5, 7, 4, 3, 5, 3, 6, 11, 2, 6, 9, 2, 3, 3
Average				50	5.6			8.9	1.12 average day doses 1.09 if 9.2 taken for 1934 figures

* Cure and protection.

† Not cured for 1 day; not included in results.

Preparation 2 is the International Standard (i.s.).

Serial tests. Exps. 6 and 7. Exps. 8 and 30-10 [*v. Kinnersley et al.*, 1935]: 34.3 immediately succeeded Exp. 10.

digestive juices. The matter has been tested by obtaining data with an eluate from the clay made with baryta. If the clay is merely shaken with the baryta as in the Jansen and Donath method, quantitative extraction from the clay does not take place. If, however, it is ground with baryta as is usual at the phosphotungstate stage in our preparation of vitamin B₁, results indicate that quantitative elution takes place.¹

Standard baryta extracts of the acid clay.

5.0 g. of the acid clay are ground in a 100 ml. centrifuge-tube with an equal volume of solid powdered baryta and 25 ml. distilled water for 3 min. at room temperature, 15 ± 3.0°. The whole is centrifuged and the extract filtered through a small filter-paper, the filtrate being made acid immediately with 20% H₂SO₄ avoiding excess. The residue is ground twice more with 25 ml. water, and the process repeated, in all 3 extracts being made. The combined filtrates are adjusted to *p*_H 3.0, warmed, centrifuged and made up to a convenient volume containing 20% ethyl alcohol; it is suitable if 1.0 ml. is equal to 50 mg. of original clay.

Table II. *Extracts of activated clay.*

Exp.	Prep.	Year	Month	Dose mg.	No. birds	No. days cured Av.	Not cured %	Day dose mg.	Individual tests
11	1	1933	Jan.-Feb.	67	10	6.1	17	11.0	8, 14, 7, 7, 3, 2, 7, 4, 4, 5
12		1933	March-April	67	10	5.45	6.2	10.8	4, 4.5, 7.5, 5, 2.5, 6, 4, 9, 5, 7 6, 3, 2, 7, 10, 6, 7, 9, 9, 3, 6, 2, 9, 5, 13, 10
13		1933	18 April-May	67	16	6.7			
14		1934	July 14-17	50	6	4.8	14	10.6	9, 4, 8, 2, 3, 3,
15		1932/33	Nov.-Feb.	45	3	4.3	—	10.5	4, 4, 5
16		1932/33	Nov.-Feb.	22.5	5	3.8	—	5.9	2, 2, 5, 2, 8
15	2(i.s.)	1932/33	Dec.-Jan.	72.6	7	7.3	—	9.9	6, 5, 10, 9, 2, 10, 9
16		1932/33	Dec.-Jan.	54.5	7	7.0	12	7.8	4, 5, 9, 11, 9, 5, 6
17		1932/33	Dec.-Jan.	36.3	4	7.2	20	5.0	4, 4, 5, 16
18		1932/33	Dec.-Jan.	18.2	6	2.8	—	6.5	4, 2, 2, 5, 1, 3
19		1934	June	50	15	6.0	18	8.3	3, 10, 5, 2, 5, 11, 1, 10, 5, 11, 6, 7, 4, 5, 5

¹ We do not know whether this has advantage over the quinine method of Williams *et al.* [1934].

In Table II are given all the results obtained with the extracts; in Exps. 15, 16, 17, 18, there are not enough birds for the results to be more than suggestive, but there is a tendency for high results to be obtained with amounts below the equivalent of 50 mg. In our hands, less than 50 mg. did not cure many birds when it was given as the solid. There is no more than this in favour of the improved rate of absorption of the extract; for in Table III it will be seen that the results obtained with the solid and with the extract are remarkably alike and well within the limits of error.

Table III. *Comparison of tests with solid and extracts. (Activated clay.)*

Prep.		Amount mg.	No tests	Day dose mg.
1	Solid	50	16	9.8
	Extract	50-67	22	10.7
2, i.s.	Solid	50	50	8.9
	Extract	50-73	29	8.5

It may be enquired further whether the injected extract of the clay compares favourably with the extract given by the mouth.¹ The experiments are shown in Table IV. Experiments with the i.s. were sufficient to show the correspondence.

Table IV. *Comparison of injection with oral administration (50 mg. dose).*

Exp. Prep.		Year	Month	No birds	No. days cured, av.	Not cured %	Day dose mg.	Individual tests	
14	1	Mouth	1934	July	6	4.7	14	10.6	See Table II
20		Injection	1934	July	6	3.5	Nil	14.0	4, 4, 3, 4, 3, 3
19	2	Mouth	1934	June	15	6.0	18	8.3	See Table II
21		Injection	1934	June	22	6.05	—	8.3	6, 4, 3, 8, 10, 6, 4, 3, 4, 5, 4, 1, 11, 4, 5, 6, 7, 6, 4, 13, 13, 6

In each case tests were simultaneous.

This is important because it gives confidence in the oral route for administration; but further it makes it possible with the baryta extracts to have a soluble standard for injection, made quantitatively from the i.s. So far the impossibility of injecting the i.s. of vitamin B₁ has been an objection. It may be significant that all birds were cured with injected vitamin, as compared with 84 and 88% by mouth.

The day dose method and crystalline vitamin B₁.

The international Committee has asked us (and others) to determine the relation between the activity of crystalline vitamin B₁ and the i.s., for which 1 unit is 10 mg. We did not anticipate that this would prove an awkward task, but actually it is beset by considerable difficulty with the pigeon, to which some brief allusion has already been made [Kinnersley *et al.*, 1935]. The experiments in this laboratory have been based upon the assumption that there is a satisfactory relation between the time of cure and the dose given. Others are in agreement with this view among whom we may mention Coward *et al.* [1933], Birch and Harris [1934] and Ammerman and Waterman [1935]. The tests in Tables I and II do not bring this out very clearly though the rule seems to be

¹ Our previous view that injected vitamin is more potent than vitamin by mouth is not supported in this paper for these extracts; but for crystals there was slight evidence in its favour [Kinnersley *et al.*, 1935].

followed roughly if we exclude the very high and very low doses. Two further series of tests (see Table V) have been made with one of the 50 % alcoholic extracts of charcoal prepared by us. The experiments in the first series tested were not simultaneous and not numerous, but the second series was done under

Table V.

Exp.	Year	Month	Dose ml.	No. of birds cured	No. days cured av.	Not cured	Day dose per 1-01 ml.	Individual tests	
A. Vitamin B ₁ concentrate 71 A.									
22	1932	April-May	0.05	8	4	1/9	8	2, 3, 1, 5, 6, 5, 6, 4	
			0.075	7	6		8	8, 13, 6, 3, 2, 4, 6	
			0.1	6	6		6	5, 7, 12, 3, 4, 4	
	1932	Feb.	0.25	9	9.3		3.7	10, 8, 10, 12, 9, 6, 10, 10, 9	
23	1935*	Mar.-July	0.05	36	4.9	8/44	9.8	Mar. 5, 5, 6, 4, 3, 2, 5, 5, 2, 5, 3 May 7, 11, 11, 4, 8, 2, 3, 4, 6, 6, 5, 3, 1 June 5, 5, 3, 10, 7, 6, 3, 3, 7, 5, 2, 4	
			0.1	37	7.85	9/46(av.)	7.85	Mar. 2, 6, 1, 4, 4, 9, 8, 5, 9, 5, 11 May 7, 5, 6, 11, 10, 16, 7, 3, 10, 17, 13, 6, 10 June 6, 4, 4, 6, 3, 6, 16, 5, 7, 13, 6, 23, 6	
			B. Crystalline vitamin B ₁ injected, 30-10.						
			γ				γ		
24	1934*		5	27	5.2	—	0.96†	10, 4, 5, 13, 4, 9, 8, 9, 5, 7, 3, 3, 6, 10, 3, 2, 3, 6, 2, 3, 1, 3, 3, 7, 2, 2, 6	
			10	23	5.7	—	1.6	11, 14, 5, 10, 4, 3, 10, 7, 2, 3, 7, 3, 11, 3, 3, 4, 3, 3, 12, 2, 1, 3, 8	
25	1934*	Sept-Nov.	10	37	4.54	—	2.2	4, 2, 2, 3, 5, 6, 5, 3, 3, 5, 2, 5, 2, 2, 4 10, 7, 3, 3, 2, 6, 4, 7, 7, 2, 6, 5, 4, 3	
			Nov. 2, 3, 12, 7, 10, 1, 9						
	1934	Sept.-Nov.	15	42	4.55	—	3.3	7, 7, 5, 4, 4, 3, 3, 2, 5, 5, 8, 3, 4, 3, 5, 4, 4, 8, 8, 4 3, 2, 3, 4, 4, 5, 6, 4, 4, 5, 5, 3, 5, 4, 4, 4, Nov. 5, 3, 7, 8, 3, 5	
			C. Crystalline vitamin B ₁ by mouth.						
26	1936	Jan.-Mar.	7	30	5.0	10	—	Jan. 3, 3, 0, 2, 3, 0, 7 2, 4, 3, 5, 0, 0, 0 Mar. 2, 2, 0, 4, 4, 2, 2, 2, 2, 6, 2, 0, 3, 0, 0, 18, 0, 15, 6, 2, 15, 7, 1, 4, 6, 13	
	1936	Jan.-Mar.	14	37	5.8	4	—	Jan. 0, 3, 2, 4, 6, 3, 2, 5, 3, 0, 6, 5, 0, 9, 0 Mar. 6, 16, 2, 5, 4, 5, 7, 3, 1, 17, 7, 2, 4, 6, 6, 5, 2, 15, 5, 9, 3, 8, 3, 13, 10, 2	

* Simultaneous dosing.

† [Kinnorsley *et al.*, 1935].

the best conditions of modern control. Doses of 0.05 ml. and of 0.1 ml. of the concentrate were compared over a period from March to July with simultaneous dosing. Though the relation is not rigid, the figures support the assumption which we have always made that vitamin B₁ in these concentrates shows a rough relation between dose and activity. Therefore it is all the more surprising to find that this relation does not hold at all for crystalline vitamin B₁; Table V B shows simultaneous tests, with different doses of injected hydrochloride.

The fact is that upon the average injected crystalline vitamin B₁ in our hands does not give a longer period of cure with increasing dose. Supposing that we are correct in believing that such a relation is shown by the concentrates, there are two possible explanations: (1) it is due to the route of administration or (2) it is due to some chemical difference, the impurities are having some adjuvant

action. We have tried to obtain light upon (1) by carrying out a series of simultaneous tests by the mouth with 7 γ and 14 γ vitamin, Table V c. In these again there is no clear relation between time of cure and dose, though there is a distinct tendency for the low dose not to cure the animals (so that the oral route is better for test). It may be interpreted that the difference is due to (2) and that there is some factor in the crude preparations which alters the effect of the vitamin B₁. This would be consistent with the suggestion of vitamin B₅, which has previously been made [Carter *et al.*, 1930], but the data are not decisive.

Meanwhile, it seems certain that the pigeon day dose is no use for deciding differences in potency between injected specimens of vitamin B₁. Fortunately this does not now matter because there are available both the colour reaction, formaldehyde-azo-test [Kinnersley and Peters, 1934], and the catatorulin test, as well as the bradycardia test which is stated to give good results.

Catatorulin tests (Appendix I) were made upon the extract of the international standard clay, comparing this with crystalline vitamin B₁. They functioned efficiently, and showed definitely that an amount of the international standard extract equal to 10 mg. of the original clay (1 unit) was equivalent in effect to 1.99 γ vitamin HCl.

Statistical considerations

As previously stated, a rough plotting of our figures shows that they have a skew distribution. If the values are plotted logarithmically, *i.e.* dose against the logarithm of the number of days of cure, there is a nearer approximation to the normal distribution. When the logarithmic averages are calculated with the standard deviation for some of the principal groups in this paper, the values of Table VI are obtained. These averages make no substantial difference in the

Table VI. *Results for the most significant experiments calculated logarithmically.*

Exp.	Dose	No. of birds	Mean (logarithmic) as days	Mean times lying between 21/22 days
7, 8, 9, 10	Activated clay 50 mg.	50	4.91	4.10-5.87
19	Activated extract 50 mg.	15	5.07	4.50-5.71
23	Vit. B ₁ concentrate	0.05 ml. 36	4.31	3.60-5.19
		0.1 ml. 37	6.67	5.43-8.18
24	Crystalline vit. B ₁ injected	5 γ 27	4.28	—
		10 γ 23	4.13	—
25	Crystalline vit. B ₁ injected	10 γ 37	3.85	3.22-4.60
		15 γ 42	4.3	3.84-4.81
26	Crystalline vit. B ₁ by mouth	7 γ 30	3.76	2.96-4.78
		14 γ 37	4.68	3.76-5.82

Note. Figures in last column obtained by finding standard deviation (σ) for the logarithmic mean $\sqrt{\frac{\sum d_2}{n-1}}$ and then $\epsilon = \frac{\sigma}{\sqrt{n}}$. Actual figures = antilog mean $\pm 2\epsilon$.

conclusions, but the main contention is brought out more sharply so far as the difference between the crystalline vitamin and the 50% alcohol concentrates is concerned. The differences in Exp. 23 are significant. An application of Fisher's [1929] *t* test¹ (difference of means) gave $t=3.1516$ from which the probability that random sampling could give the differences is considerably less than 1 in 100. Compared with this in Exp. 26 (crystalline vitamin) $t=1.285$ giving a probability of 1 in 5. Provided that the statistical formulae apply to the logarithms, this may be considered to prove the presence of a chemical difference in the impure concentrate.

¹ We are grateful to Dr R. B. Fisher for this calculation.

DISCUSSION.

It seems from the catatorulin test that one i.u. (10 mg.) $\equiv$ 2 γ vitamin B₁ hydrochloride; a similar value is obtained with formaldehyde-azo-tests, using the extract of the i.s. This value can be used to interpret the pigeon data. From Table VI we have 5 i.u. (50 mg.) $\equiv$ 5.0 pigeon day doses, 5–15 γ B₁ by injection and 7–14 γ B₁ by mouth $\equiv$ 4.13 and 4.38 day doses respectively. An approximately correct value is obtained for the crystals, if we assume that 10 γ vitamin B₁ give the correct value. In this case 2.5 γ B₁ would be equivalent to 1 i.u. In the past we have used mainly 10 γ , and we have always quoted 1 i.u. as approximately equivalent to 2.0 γ vitamin B₁. Hence the present work entails no modification of previous data. But it is now clear that the values reached previously with injected crystalline material were actually empirical.

In a recent paper from Suzuki's laboratory, Ohdake and Yamagashi [1935] have found for their vitamin hydrochloride 1.6 γ $\equiv$ 1 i.u. At first sight this indicates a greater potency than ours. Examination of their protocols shows that they had a day curative dose for the i.s. of 15 mg., i.e. 50 % more than needed by our pigeons. Their figures were (Table I, p. 111),

International Clay dose mg.	No. of birds	Average day dose mg.
150	7	18.6
50	4	11.2

They averaged these figures in the belief that this was permissible with the adsorption product. From our Table I, the higher amount of 100 mg. gave a larger average dose. If their results for 50 mg. are taken, they are so close to our own that there is no reason to believe in a higher potency. 0.0023–0.0025 mg. was their actual day dose. That the crystals from Prof. Suzuki's laboratory are not more potent is suggested by two further facts. Heyroth [1936] has concluded that our preparation 64.19 by a rat technique is slightly more potent than one sent by Ohdake. We have carried out one catatorulin test (see Appendix) and colour tests upon a specimen of oryzanin fortior (Suzuki); the potency was identical with the specimen of our crystals which was used.

The biological explanation. A curious dilemma is raised by the finding that doses of 5–15 γ vitamin show equal biological effects. The fact is difficult to reconcile with the well-known belief that utilisation of vitamin is proportional to the metabolic rate; for instance Cowgill's [1934] calculations for vitamin B₁ requirement are based upon the formula $\frac{\text{vitamin}}{\text{calories}} = k \cdot \text{weight}$. The explanation is likely to prove interesting.

SUMMARY.

1. One international unit of vitamin B₁ (10 mg. Jansen Clay) is approximately equivalent to 2 γ crystalline vitamin B₁ hydrochloride.
2. Extracts of the International Standard clay of full activity can be made by a standard method and injected. The same result is obtained as by mouth.
3. The day dose method for pigeons is not satisfactory for assay of injected crystalline vitamin B₁, though useful for impure vitamin.

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APPENDIX I.

Catatorulin tests.

These were done as described by Kinnersley *et al.* [1935], with the following changes: the Ringer-phosphate, p_H 7.3, contained Na pyruvate approximately 0.034 *M*. This was added immediately before tissue division with the glass

Table VII. *Catatorulin tests.*

Exp.	989	μ l./g./hr.	992	1057
Vitamin B ₁ *	0	658	886	1018
	0.5 γ	1020	1270	1540†
	1 γ	1220	1322	—
I.S. extract	$\equiv$ 0.167 unit	923	—	—
	$\equiv$ 0.25 unit	—	1243	—
	$\equiv$ 0.333 unit	1088	—	—
Vitamin B ₁ (Suzuki)	0.5 γ	—	—	1533†

By inspection of curve 0.167 I.U. = 0.35 γ or 2.1 γ per unit
 0.333 I.U. = 0.65 γ or 1.95 γ per unit
 0.25 I.U. = 0.48 γ or 1.92 γ per unit
 Average = 1.99 γ

* Vitamin B₁ (Kinnersley, O'Brien and Peters).

† Average of triplicate, rest duplicate.

crusher. Na pyruvate was made freshly before use by cautiously neutralising a solution of pyruvic acid (approx. 5 *M*) obtained from crystalline acid and kept in cold store. The reason for using pyruvate alone will be given in a subsequent paper by R. A. P.

Four experiments were made of which the details of two are given. The two not quoted were less satisfactory but in substantial agreement. The average O₂ uptake for the period $\frac{1}{2}$ –2 hours is given in Table VII.

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