LXXXI. CRYSTALLINE VITAMIN B₁.

By HENRY WULFF KINNERSLEY, JOHN RICHARD O'BRIEN
AND RUDOLPH ALBERT PETERS.

From the Department of Biochemistry, Oxford.

(Received February 1st, 1935.)

CRYSTALS with vitamin B₁ activity isolated from baker's yeast by Kinnersley *et al.* [1932; 1933] differed from those of others in certain respects. Further work failed to resolve differences [Kinnersley *et al.*, 1934]. But lately, co-operation with Prof. Windaus and collaboration with Dr Holiday [Holiday *et al.*, 1934] have led to a revision of some of the facts published from other laboratories, which eliminates the main differences between ourselves and them. Valuable help has also been obtained from X-ray analysis methods. So that in our opinion, there is now no valid reason for questioning the view that vitamin B₁ has been isolated, though certain details have not yet been settled.

HISTORICAL.

Jansen and Donath [1926] were the first to isolate crystalline vitamin B₁ preparations. These were not quite pure; they contained substances precipitable by mercuric sulphate in acid solution, which is not a property of the vitamin [Kinnersley and Peters, 1928; Van Veen, 1932]. Much later, Ohdake [1932]; Van Veen [1933]; Windaus *et al.* [1932]; ourselves [1934]; Williams *et al.* [1934] obtained crystals, which were claimed to be of higher activity; this issue of activity was however somewhat blurred. Windaus *et al.* [1932] stated that their crystals reached an activity (pigeon dose) of 2.4 γ per diem under the conditions of their tests (injection of 6 γ). Later it was concluded by Van Veen that the Windaus preparation had the same activity as that of Jansen and Donath [1926]. Since the activity of a sample of this latter had been determined previously in this laboratory [Jansen *et al.*, 1930], and had been found to be less active, there were grounds for thinking that our own preparations actually had a higher potency. Such a view was confirmed by a parallel test upon some crystals (Windaus 1) sent by Prof. Windaus and some of our own; ours proved more active than theirs. The difference between the two crystalline samples was also found to persist for two other new methods of test devised in this laboratory, namely the catatorulin test upon brain enzyme systems [Passmore *et al.*, 1933] and the formaldehyde azo-colour test [Kinnersley and Peters, 1934]. In the meanwhile Seidell and Smith [1933] reported for our crystals as tested upon rats an activity of 80 γ as against 120 γ for a sample from Windaus. They did not consider the difference to be significant. Van Veen [1934] however reported that our crystals were more potent; and later he claimed to have made a preparation of higher activity than ours. We hesitated to accept this because he mentioned that his most active sample contained 500 units of vitamin B₁ per mg. which was very little different from our estimates. In fact a new specimen of his vitamin B₁ was not distinguishable from ours by animal test, though this specimen might have become less active in transit. Our conclusion had to be that there was no difference between our preparation and that of Van Veen, but that the Windaus 1 sample was not the same as these two.

The differences between the products of this laboratory and those of others were reflected in other attributes of the crystals. All seemed to be agreed that the formula was either $C_{12}H_{18}O_2N_4S$ or $C_{12}H_{16}ON_4S$ for the base. Bernal and Crowfoot [1933] stated in a report on the X-ray analysis that the crystals from different laboratories were the same. On the other hand we found slightly different analytical figures, in particular a C content of 42.2 against 40.6 %, and slightly higher S and Cl values. There was also a marked difference in the ultraviolet absorption spectra; the band reported by Windaus *et al.* was at $260 m\mu$ in alcoholic solution, whereas that of ours was at $247-249 m\mu$ [Peters and Philpot, 1933]; (see Holiday [1935] for a discussion of this point).

Convinced that our facts were correct, and that these differences were therefore real, we searched for a more active vitamin component. It was found possible to refractionate the vitamin crystals with phosphotungstate, which has proved a fractional method of attack at earlier stages. By collection of the precipitates at different p_H values however, no certainly more potent sample was reached.

Fractionation experiments with phosphotungstate were carried out by taking the crystals and refractionating within narrow limits. In the later experiments formalin was added. Though the question has not been properly investigated there is some evidence in our hands that formalin shifts the points of precipitation of some bases more than others; in this case it should prove a refinement of phosphotungstate fractionation. Theoretically, it should influence the point of precipitation by interfering with the basicity of a group such as $-NH$ [*cf.* Barnes and Peters, 1932]. In its presence vitamin B_1 is stable provided that the p_H is not made greater than 6.0; the precipitation point is shifted towards a more acid reaction. The claim by Malcolm [1933] that formaldehyde destroys vitamin B_1 cannot readily be reconciled with our observations. A typical formalin experiment is as follows:

Exp. 1. A solution of about 50 mg. of the vitamin B_1 crystals in water was adjusted to p_H 7 and an equal volume of 40 % formalin added—volume 35 ml. 1.6 ml. of 10 % 24-phosphotungstic acid were added. The p_H was now 5.6–6. The precipitate was collected and washed with $N/10$ sulphuric acid and the washings were added to the filtrate. The precipitate was ground with solid baryta in the usual way and worked up into the crystals. By the addition of 1.0–1.5 ml. $N/10$ sulphuric acid and phosphotungstic acid as required, precipitates were collected in order at about p_H 4 A, B, C and D, and crystals were prepared from each fraction. The p_H was not determined exactly, the aim being to collect what appeared to be zonal precipitations. The total volumes used in the precipitation were 6 ml. $N/10$ sulphuric acid and 3 ml. 10 % sodium phosphotungstate. The precipitates C and D were too small for analysis. For A and B, we have (10 γ doses given):

	Wt. of crystals recovered (mg.)	Birds	Day-dose γ
A	11.4	12	2.17
B	17.5	16	2.00

The colour reactions were of equal intensity. There is no evidence of increased activity in either A or B which might have been expected if the crystals were a mixture.

Procedures which would inactivate the vitamin were not valid, and benzylation is eliminated by the well-known fact that the vitamin escapes this treatment [Seidell, 1929]. Attempts to fractionate by use of lead acetate also gave no success. Since such a procedure separates some nucleotides, substances of pyrimidine type might have been thus removed. More success attended the preparation of the nitrate and the sulphate, which gave opportunity for further tests and for observation by X-ray analysis. No more active vitamin was however reached.

At this stage, Dr Holiday reinvestigated the ultraviolet absorption curve of some of our sulphate and hydrochloride and also of the hydrochloride of Van Veen. The results in acid alcoholic solution reproduced those obtained earlier by Peters and Philpot. A second sample from Prof. Windaus (Windaus 2), which he stated to have been many times recrystallised as the chloroaurate, showed the same spectrum and molecular extinction coefficient as our own. Holiday [1935] has also found that the spectrum of vitamin B₁ changes profoundly according to the conditions of solution and p_H .

This accounts for puzzling points in the curves of Heyroth and Loufbarrow [1934]. It seems clear that all active specimens of vitamin B₁ have the same absorption band with the peak at 247–249 $m\mu$ when tested in acid alcoholic solution. Preparation 2 of Windaus showed the same biological activity, catatorulin activity and intensity of formaldehyde-azo-reaction as our own; hence biological differences have disappeared. Furthermore in private communication Prof. Windaus has told us that intensive drying in high vacuum at 80° raises his values for C content to 42.2 %¹, in substantial agreement with our own which were obtained from material dried in a vacuum desiccator; and his values for absorption bands are now in agreement with ours. There are therefore no longer grounds for a belief that the purest crystalline preparations of vitamin B₁ from yeast and rice polishings are substantially different. Since we have been unable to prepare a more potent fraction in spite of strenuous endeavour, we must conclude that the crystals are actually vitamin B₁.

Though the problem as to identity of the crystals with vitamin B₁ may be considered to be settled, we do not feel that all questions connected with the formula are satisfactorily answered; we propose to give our data pointing out discrepancies. Doubtless, determination of the vitamin constitution by other methods will lead to their resolution.

Preparation of salts. The hydrochloride was prepared as before; nitrates and sulphates as follows. The sulphates prepared have the approximate composition $2X(SO_4)_3$ low M.P. and $2X(SO_4)_4$ high M.P. (X = base).

Vitamin B₁ sulphate. On converting the hydrochloride into the sulphate, two compounds are obtained with melting-points of 203°² and 276 to 278° respectively. The salt of low M.P. is obtained at p_H 3, that of high M.P. at p_H 1.

Low M.P. sulphate (Fig. 1). To 20 mg. vitamin B₁. HCl a hot saturated solution of Ag₂SO₄ is added until a precipitate of AgCl is no longer obtained (about 2 ml.). Absolute alcoholic H₂SO₄ (p_H 3) is added, the precipitate centrifuged off and washed with 1 or 2 drops of water and absolute alcoholic H₂SO₄ (p_H 3)³. The filtrate is concentrated on the water-bath, first in a glass evaporating basin and then in a test-tube, and any inactive precipitate obtained is centrifuged off. Addition of absolute alcohol is

Fig. 1. Sulphate of low M.P. $\times 200$.

¹ It is to be noted that some of the analyses of Jansen *et al.* and Ohdake show figures for C content higher than 40.6 %.

² Values for M.P. corrected throughout.

³ Prepared by adding 1–2 drops concentrated H₂SO₄ to 50 ml. redistilled absolute alcohol (not dried) so that a drop tested on a white plate with bromophenol blue shows approximately the colour for p_H 3.0.

made from time to time until most of the water has been removed and absolute alcoholic H_2SO_4 is added if necessary.

A solution is finally obtained which inclines to be opaque and from which a precipitate settles out on cooling while it redissolves on warming. If the solution is left overnight in the cold chamber, there is obtained a clear solution with sulphate crystals round the sides of the test-tube.

Sulphate of high m.p. To 20 mg. vitamin B_1 .HCl a drop of $N \text{ H}_2\text{SO}_4$ is added before decomposing the hydrochloride with Ag_2SO_4 . It is then worked up as above. The addition of $N \text{ H}_2\text{SO}_4$ to p_{H} 1 is only made in the last stages in order to avoid the risk of inactivation by concentrating at this acid p_{H} .

Nitrate of vitamin B_1 crystals. To 10 mg. vitamin B_1 .HCl is added 1 ml. 1 % AgNO_3 (theory: no excess); it is warmed upon the water-bath, 4 ml. absolute alcoholic HNO_3 , p_{H} 2, are added, the solution is centrifuged and the precipitate of AgCl washed with 1 drop of water and absolute alcohol.

The filtrate is then concentrated on the water-bath to remove most of the water and worked into about 97 % alcohol at p_{H} 3 in the usual way. A yellow inactive precipitate which comes out is discarded. Yield 10.7 mg.

Melting-points. Various observers state that vitamin B_1 hydrochloride melts with decomposition; Windaus *et al.* [1932], 250° "unter Zersetzung"; Ohdake [1934], 249° (uncorrected) with decomposition; Van Veen [1933], 250° (corrected); Williams *et al.* [1934], 249 – 250° .

In spite of much attention to this point, we have never obtained melting-points as high as 250° for our hydrochlorides. We usually determine m.p. in small open tubes in Mason's melting-point apparatus under microscopic observation; we have satisfied ourselves that these results do not differ from those in sealed tubes. The m.p. for the hydrochloride is not sharp and is usually 230 ± 1 – 2° . It makes little difference whether the compound is heated up slowly or quickly to a temperature of 200 – 220° . It is best to allow the temperature to rise very slowly for the last 5° , taking 3 or 4 mins. for this period. After a definite change at about 230° , no further alteration is noticed up to 270° . In marked contrast to the hydrochloride the sulphates melt sharply, that of lower m.p. at 203° , and that of the higher m.p. at 274 – 276° . The nitrate decomposes sharply at 164 – 165° .

The empirical formula of vitamin B_1 .

For the hydrochloride Windaus *et al.* [1932] proposed two alternative formulae (Nos. 1 and 2, Table I) differing by H_2O . Their hydrochloride agreed with No. 1, and their picrolonate with No. 2. Ohdake [1934] considers that the formula is $\text{C}_{12}\text{H}_{16}\text{O}_2\text{N}_4\text{S} \cdot 2\text{HCl}$, Van Veen $\text{C}_{12}\text{H}_{20}\text{O}_2\text{N}_4\text{S} \cdot 2\text{HCl}$, and Jansen *et al.* [1933] support the Windaus formula No. 1.

Our own analyses give as average the following (see Appendix I):

	C	H	N	S	Cl	
Hydrochloride	42.2	5.7	15.1	9.5	20.9	(Schoeller) previous value with addition of one further S analysis = 9.42 %
	42.1	6.2	15.6	9.75	20.94	(Kinnersley and O'Brien, see Appendix I)
Sulphate	C	H	N	S	SO_4	
High m.p.	30.33	5.8	12.33	18.11	40.1	
Low m.p.	35.00	5.50	13.38	18.51	34.7	

The possible formulae based upon $\text{C}_{12 \pm 1}$ are calculated in Table I.

Table I. *Possible formulae for vitamin B₁ hydrochloride.*

No.	Formula	C	H	N	S	Cl	Total mol. wt.	Base only
1	C ₁₂ H ₁₆ O ₂ N ₂ S ₂ ·2HCl	40·6	5·7	15·8	9·0	20·0	355	232
2	C ₁₂ H ₁₆ ON ₄ S ₂ ·2HCl	42·7	5·34	16·6	9·5	21·05	337	264
3	C ₁₂ H ₁₆ ON ₄ S ₂ ·2HCl	42·45	5·9	16·5	9·44	20·92	339	266
4	C ₂₄ H ₃₄ O ₂ N ₈ S ₂ ·4HCl	41·6	5·5	16·2	9·25	20·5	346*	273*
5	C ₂₄ H ₃₆ O ₂ N ₈ S ₂ ·4HCl	41·4	5·76	16·1	9·24	20·45	347*	274*
6	C ₂₄ H ₃₆ O ₂ N ₇ S ₂ ·4HCl	42·4	5·9	14·4	9·43	20·9	340*	267*
7	C ₂₄ H ₃₄ O ₂ N ₇ S ₂ ·4HCl	42·5	5·61	14·45	9·46	20·95	339*	266*
8	C ₂₄ H ₃₄ O ₄ N ₇ S ₂ ·4HCl	41·5	5·48	14·1	9·22	20·45	347*	274*
8a	C ₂₄ H ₃₂ O ₂ N ₈ S ₂ ·4HCl	43·6	5·46	14·85	9·7	21·5	330*	257*
9	C ₂₃ H ₃₄ O ₂ N ₇ S ₂ ·4HCl	42·4	5·84	15·1	9·85	21·8	325*	252*
10	C ₂₅ H ₃₆ O ₄ N ₇ S ₂ ·4HCl	42·4	5·65	13·8	9·04	20·1	354*	281*
11	C ₂₅ H ₃₆ O ₃ N ₈ S ₂ ·4HCl	42·5	5·66	15·85	9·08	20·1	353*	280*

* = half mol. wt. values.

The most satisfactory fit for the analytical figures is evidently formula 9, but this must be excluded upon the results of X-ray analysis.

By crystallographic methods Bernal and Crowfoot [1934] obtain the values given in Table II; double the values given are not excluded by X-ray analysis. The value for the hydrochloride is the most accurate.

Table II. *Possible mol. wt. (lowest formula wt. for base).*

	Min.	Prob.	Max.
Hydrochloride	270	278	286
Sulphate* (low M.P.)	249	270	286
Nitrate*	263	274	286

* Personal communication.

Values below 263 are certainly excluded and below 270 doubtful. Hence 8a and 9 are eliminated. 1, 4, 5, 8 can be excluded upon C, Cl, S values, 10, 11 upon Cl, S values. The mol. wt. is not consistent with 8a, 9, or really with 2, 3, 6, 7.

No formula therefore agrees with the analyses and mol. wt. estimations by X-ray analysis. If we consider that the X-ray analysis is some 2 % in error, then formulae 2, 3, 6 and 7 are possible. The nitrogen determinations fit none of these, being intermediate in value. There is some fundamental difficulty about nitrogen estimation in the hydrochloride to which Windaus *et al.* [1932] have already drawn attention. Jansen *et al.* [1933] obtained values nearer 16 % by treating with potassium chlorate. For the sulphates, evidently the difficulty does not arise. It should be mentioned that the nitrogen analyses in the laboratory were controlled by estimations of known substances, such as sulphanilic acid, which gave theoretical results under the same conditions within an error of 0·5–1 %

In our view, the high C values mean that No. 1, the formula proposed by Windaus *et al.* and supported by the others mentioned, is not the simplest vitamin B₁ formula. We favour the second of their proposals Nos. 2 or 3, as a working hypothesis. But in doing so, it must be recognised that there are still unreconciled data. Possibly it is not water but —OH which is the difference between the two formulae; in this case the vitamin would exist in two states of oxidation, and act as an —OH donator. We are not inclined to favour the idea that the vitamin exists as an N₃ compound as well as N₄. This idea could be reconciled with the mol. wt. but not with the nitrogen determinations [*cf.* Heyroth and Loufbarrow, 1934]. A mixture however would explain these.

Biological activity.

The curative pigeon day dose method with injection of vitamin has been used in the majority of tests; it is not very satisfactory for the crystalline vitamin. The relation between dose and time of cure has broken down in some cases. In Table III are given the average values for injection of 10 γ . The final values

Table III. *Curative pigeon test with vitamin B₁ salts.*10 γ doses injected.

Salt	Preparation	Year	Month	No. of birds	Av. cure days	γ /day dose	Colour reaction	Remarks
HCl	12. 7	1933	—	8	5.0	2.0	5.8	(HgSO ₄ technique)
	17. 0	1933	—	7	6.0	1.67	5.0	—
	92 A 50	1933	Sept.-Oct.	9	5.0	2.0	5.0	—
		1934	Mar.-Apr.	12	3.8	2.63	—	—
		1934	July	13	5.4	1.85	—	—
	19 A	1934	Feb.	6	4.7	2.13	6.0	—
		1934	Feb.	8	4.8	2.08	4.0	High m.p. SO ₄ to HCl
	18	1934	Feb.	5	6.2	1.61	6.0	Low m.p. SO ₄ to HCl.
	93	1933	Sept.-Nov.	10	4.3	2.33	—	—
	30. 10	1934	May	20	6.3	1.59	5.0	—
	34 A 3	1934	July	15	4.9	2.04	5.8	—
				113	5.093	1.96		

For the whole 113 tests, the mean duration of cure will only exceed 5.093 ± 0.250 once in 100 times. The average value for the day dose of free base is 1.54 γ .

HCl	V. Veen (1)	1933	Sept.	11	4.0	2.5	6.2	—
	V. Veen (2)	1934	Jan.-Feb.	15	5.3	1.89	6.0	—
	Windaus (2)	1934	June	12	6.1	1.64	4.9	—
HNO ₃	7	1933	Nov.	9	5.1	1.96	4.0	—
H ₂ SO ₄	19 A	1934	Feb.-Mar.	11	5.2	1.92	3.0	High m.p.
	19	1934	Feb.	9	6.3	1.59	5.5	Low m.p.
	19	1934	Feb.-Mar.	9	4.0	2.5	4.5	Low m.p.
	24 α	1934	Mar.-Apr.	10	4.8	2.08	4.0	—
	24 β	1934	Mar.-Apr.	8	5.0	2.0	4.0	—

Examples of tests.

30. 10. May, 1934. Days cure. 10, 9.5, 13, 5, 10, 4, 3, 10, 7, 2, 3, 7, 3, 11, 3, 3, 4, 3, 3, 12.

92 A 48. Mar.-Apr. Days cure. 4, 4, 2, 3, 3, 2, 6, 8, 3, 4, 2, 4.

reached are controlled (a) by an integration of tests made throughout the year to avoid seasonal changes and (b) by simultaneous tests; 92 A 50, 34 A 3 for instance in July 1934 were made by the method of alternate dosing; these give results near the average.

The sulphate from the average figures appears more active, but the difference is not statistically significant. It is clear that by this test the preparations Van Veen (1) and (2) and Windaus (2) are identical within the limits of error.

Table IV.

			Birds	Days	Day cure
30. 10	June, 1934	Per os	14	4.35	2.3 [s]
	May	Injection	—	6.1	1.64
Windaus	June	Per os	9	4.3	2.32 [s]
	June	Injection	12	6.1	1.64

[s] simultaneous test.

Administration by mouth and by injection have also been compared in simultaneous tests. With extracts of the International Standard, results by the two routes are practically identical (see a paper in course of preparation).

In each case the result by injection is higher; this has been found previously. No stress can be laid upon it owing to the variation.

The average estimate of activity is 1.96 γ per day dose for the hydrochloride, and $1.17^1 \times 1.96\gamma$ per international unit = 2.29 γ or 437 units/mg. The limits outside which the average for such a series of tests could be expected to fall less than 1 in 100 times will be 490–380 units/mg.

These values lie sufficiently close to those of others (estimated at 500 units/mg.) to give confidence that substantially the same activity has been reached.

The above tests have all been made with doses of the same amount. The values obtained agree well with the colorimetric tests and also fairly well with the catatorulin tests and ultraviolet absorption methods. There seems little doubt that they are substantially reliable.

Some discrepancies. Nevertheless, the curative pigeon test is not satisfactory with injection of crystalline vitamin. In previous work from this laboratory, with less pure vitamin given by mouth, it has been found that curative tests by the day dose method gave reliable answers. This was supported by Burn *et al.* [1933] in a comparison of their testing methods with our own. Further the day dose technique is also substantially supported by Birch and Harris [1934]. On the other hand, Williams and Eddy [1933] have criticised our methods in relation to the tests upon preparation Windaus 1 in view of the difference in dose used. This particular criticism was not well founded because with the smaller dose of 10 γ in the case of Windaus 1 (by mouth) proper cures could not be effected; there was other evidence too of a lower activity in this sample. The criticism that wide variations occur by the day dose method for injected vitamin receives support from some recent comparisons of our own.

Table V. *Injections of vitamin B₁; simultaneous tests in each case.*

Preparation	Time	Amount	No. of birds	Average days of cure
52 A 15	May, 11–14	5	4	4.2
		10	4	3.7
30. 10	June, 14–16	5	24	5.2
		10	19	6.3
Windaus 2	June, 2–6	5	11	3.3
		10	12	6.1

Upon these figures for 5 γ injections, the activity for hydrochloride 30. 10 might be considered to be of the order of 1 γ . Small doses injected are evidently liable to give inaccurate results.

We have evidence here that the curative test does not work for injected crystalline vitamin. The matter justifies caution and requires more investigation. In our present opinion, the most reliable biological test for crystalline vitamin will ultimately prove to be the catatorulin test upon avitaminous brain, in which the individual variation is eliminated.

Method of testing and errors.

The probable value of the mean has been calculated for all the hydrochloride results pooled and is given in Table III together with the standard deviation of the figures considered as a whole. This value is considerably higher than that

¹ 1.17 day dose = 1 international unit upon average.

calculated for groups such as the 20 animals of 30. 10. We have refrained from discussing the individual averages further, because we are doubtful whether the standard formulae for a normal distribution apply to this case. At Mr R. B. Fisher's suggestion, we have found that the results when plotted form a skew-shaped distribution curve, and we wish to analyse this further.

It will be noticed that some comparisons of activity have been made by the method of alternate dosing in agreement with the latest modern practice [Burn *et al.*, 1933]. Though the general value of this method of control is indisputable, in the particular case here considered, there is much to be said for an average of tests made throughout the year. In our practice, successive batches of birds are put down weekly, 40 or 60 in number, birds from which develop symptoms in 18–30 days. Some of these will show temporary cures; by the time that birds are ripe for test we are really presented with a random selection of animals, which have been on the diet for variable periods. Thoroughly shuffled results are equally likely to be reached by alternating batches of control and batches of unknown birds as in the method of strict alternate dosing, provided that such tests are made within a reasonable period of time.

Catatorulin tests.

These were done by the method of Passmore *et al.* [1933] as modified (see Appendix II).

Table VI. *Catatorulin tests.*

Unknown vitamin preparation	Exp.	Standard (s) equivalent	Average
Van Veen 1	505	$0.6\gamma = 0.65\gamma$	10 γ Van Veen 1 = 11.5 γ (s)
	510	$0.4\gamma = 0.45\gamma$	
	513	$0.4\gamma = 0.5\gamma$	
Van Veen 2	547	$0.4\gamma = 0.58\gamma$	10 γ Windaus 2 = 10.8 γ (s)
Windaus 2	623	$0.35\gamma = 0.34\gamma$	
	627	$0.5\gamma = 0.6\gamma$	

The standard preparations were 64. 19 for 505, 510, 513; 92 A 50 for 547; 30. 10 for 623, 627. Na pyrophosphate (p_H 7.4) was used in experiments 510, 623, 627. The constants employed for calculation were $R = 20 \times 10^{-2}\gamma$ in all experiments; $k_c = 10 \times 10^{-2}\gamma$ in experiments 505, 513, 547; in experiment 623, $k_c = 30 \times 10^{-2}\gamma$, and in experiment 627 $20 \times 10^{-2}\gamma$; values in experiment 510 were read from an empirical curve.

Exp.	Oxygen uptakes, $\mu\text{l./g./hr.}$ Standard		Unknown
	0.3 γ	1.0 γ	
505	955	1090	1045
510	1100	1320	1175
513	700	800	748
547	1120	1268	1205
623	776	1025	810
627	1034	1255	1165

The results leave no doubt that the preparations have substantially the same activity; there is a tendency for the Van Veen material to give a higher value, which is interesting in view of his claim that his preparation is more active. One unreliable test had to be rejected for Van Veen 2.

The effect of crystalline vitamin B₁ on the growth of rats.

The potency of crystalline vitamin B₁ preparations has also been determined by the effect upon the growth of young rats. The rats weighing 30–40 g. were fed on a pure synthetic diet (rice starch 70, caseinogen 20, salt mixture 5, agar-agar 3

and cod-liver oil 3) supplemented with the other factors of the vitamin B complex in the form of autoclaved alkaline marmite. On such a diet, the rats show an initial rise in weight followed by a rapid decline culminating in convulsions and death. When in the convulsive state or on the verge of convulsions, the rats were given the dose of vitamin B₁. The effect of varying doses of the vitamin on the growth of rats is shown in Table VII.

Table VII. *Effect of vitamin B₁ on young rats*.*

Vitamin B ₁ dose per day	No. of rats	Duration of Exp. (days)	Average growth per week (g.)	σ
1.0	5	18-21	4.64	2.55
1.5	7	17-19	5.24	2.47
2.5	18	17-28	12.7	2.26
5.0	20	14-35	13.94	2.85
6.0	4	10-14	17.5	2.88
10.0	7	21-28	16.34	3.85

* Tests by J. R. O'Brien.

σ = Standard deviation.

The results indicate that doses of vitamin B₁ of the order of 1 γ suffice to maintain the young rat; after 2-3 weeks on a 1 γ dose of vitamin B₁, the rats tend to weaken and to become moribund. Doses of 2.5-5 γ are, however, adequate to promote practically normal growth in the young rat. Continued daily dosing of the rats leads to regular growth (Fig. 2). From Table VII, it is evident that a

Fig. 2. This shows the effect of varying amounts of crystalline vitamin B₁ hydrochloride upon the growth of young rats of the same sex. Curve 1 shows the rise and decline of weight of the control rat which went into convulsions and died at the point C. Curves 1, 2 and 3 are weight curves for rats of the same litter, 4 and 5 for rats of another litter to which vitamin B₁ was administered at the time marked by the arrow. The control of these two latter behaved like that of 2 and 3.

daily dose of 2.5-5 γ of crystalline vitamin B₁ is necessary to promote growth up to maximum weight in rats supplied with the other factors of the vitamin B complex under our experimental conditions. It is also clear that doses in excess of this amount have relatively little extra growth-promoting effect.

These figures mean that 1.3 pigeon doses (1.1 international unit) suffices for nearly maximum growth and 2.6 pigeon doses produce improvement after 90 g. weight. These figures are in good agreement with the values of Reader [1930] who

usually gave 2-3 pigeon doses to each rat to get normal growth. The lower estimate of 1γ for healthy growth in young rats (by Ohdake [1934]) does not necessarily indicate greater activity of his preparation as compared with ours, because the diet was different and reference to the curves shows that 1γ did not maintain growth well beyond 80-90 g. Ohdake's estimate for pigeons agrees substantially with our own.

Vitamin B₂ concentrates prepared from lead acetate precipitates.

In some cases, with complete success, we have substituted for alkaline auto-claved marmite a vitamin B₂ concentrate prepared from the lead acetate precipitates (a by-product in our large scale process [Kinnersley *et al.*, 1933]) by hydrolysis with acid. The vitamin B₂ complex was shown to be stable to acid by Chick and Roscoe [1929] and Narayanan and Drummond [1930].

Preparation. 600 g. moist lead acetate precipitate [Kinnersley *et al.*, 1933] are treated with 600 ml. H₂O and 300 ml. 20% sulphuric acid. The whole is boiled in a 1500 ml. beaker for 1½ hours with stirring. It is cooled and filtered from the lead sulphate. 20% NaOH is then added to p_H 3.0 and the mixture is treated with H₂S and filtered. It is usually stored in the dark in cold store until required; any sodium sulphate crystallising out is removed. Before using, H₂S is removed by boiling. The usual volume is 900 ml. for 600 g. precipitate ($\approx$ 10 kg. original yeast).

1 ml. of this vitamin B concentrate gives maximum effects, 0.5 ml. suffices as a source of all B₂-factors. This concentrate has been both mixed with the diet and given by mouth with identical results. Together with crystalline vitamin B₁, it has cured 23 rats of the dermatitis symptoms of vitamin B₂ deficiency. This concentrate is now being used in attempts to elucidate the problem of factor B_{4a}.

The problem of vitamin B₄.

The very definite results obtained by addition of pure crystalline vitamin B₁ alone raise acutely the question of the meaning of the previous work upon vitamin B₄. The following alternative explanations are possible: (1) Vitamin B₁ in excess can act as a substitute for vitamin B₄. (2) Vitamin B₁ must be present either in the diet or in the rat, *i.e.* by storage. So far as can be ascertained, no change has been made in the experimental diet except for possible variations in the marmite; to the breeding rats, smaller amounts of liver have been given, otherwise the diet has been as before, mixed corn, milk and lettuce twice weekly. Of course, there is no guarantee that climatic conditions have not influenced the composition of the corn *etc.* in some unknown way. (3) The explanation of the previous experiments [Reader, 1929; 1930] must have been in error. If we examine carefully the evidence for vitamin B₄ presented by Reader, we find in reality that two vitamin B₄ factors have been proposed. B_{4a}, the original, was a factor necessary for the growth of young rats; like the flavins, it was precipitated by mercuric sulphate. Believing that no change had been made in the factor studied, a reasonable view at the time, Reader [1930] changed the test and finally worked with a curative test for adult rats. The factor B_{4b} so studied was found in the same crude acid charcoal concentrates as B_{4a}. There is however no real guarantee that the factors B_{4a} and B_{4b} were identical. Considering the factor B_{4a}, the important point of the original work was that some factor other than vitamins B₁ and B₂ (as then understood) was essential for the rat. This point is abundantly supported by recent work; many investigators have recognised a need for a B₄-factor among whom may be quoted Moore *et al.* [1932], Halliday [1934] (rat), Keenan *et al.* [1933] (young chickens).

ERRATUM

Vol. XXIX, page 711, in Summary, Par. 1

for $\text{C}_{12}\text{H}_{16}\text{ON}_4\text{S}_3 \cdot 2\text{HCl}$ *read* $\text{C}_{12}\text{H}_{16}\text{ON}_4\text{S} \cdot 2\text{HCl}$

It is now generally believed that flavin is part of the original vitamin B₂ [György *et al.* 1934; György, 1934; Chick and Copping, 1934]; but it is not excluded that the factor B_{4a} might have been some manifestation of flavin and factor Y either separately or together; especially as these are found in crude vitamin B₄ concentrates¹. The explanation of the factor B_{4b} is more obscure. Vitamin B₁ concentrates were originally obtained which failed both to cure the special symptoms and to increase the weight of rats. Such concentrates cannot now be prepared. The ataxic symptoms described by Reader in the adult rat (not the young rat) have been reproduced and cured by crystalline vitamin B₁ [O'Brien, 1934]. But the condition of pink feet has been of late uniformly absent. This may be a deciding factor; until it can be reproduced, a considered judgment upon the problem of factor B_{4b} must be suspended. It may be noted that Halliday described ataxic symptoms in adult rats upon milk diets which were attributed to vitamin B₄ deficiency. The pink paws are suggestive of some features of the egg-white syndrome [Boas-Fixsen, 1931]. We cannot exclude the possibility that the symptoms had a toxic origin and that factor B_{4b} may be an antitoxic agent; though we are loth to complicate the problem with such a view at the present stage.

SUMMARY.

The information in this and the accompanying paper by Holiday [1935] can be summarised in tabular form (Table VIII).

Table VIII.

Method of test	Our HCl	Windaus II	Van Veen I	Van Veen II
Pigeon (day dose)	1.96γ	1.64γ	2.5γ	1.9γ
Catatorulin test	10γ	9.9γ	11.5γ	15γ
ε/c spectroscopic measurement (Holiday)	45	45.6	39.3	40.4
Colour test	10γ	9.6γ	12.4γ	12γ

1. Vitamin B₁ hydrochlorides from different sources are shown to be identical by various methods of test. An average activity of 380–490 international units/g., average 437, has been found. Analyses agree best with the formula C₁₂H₁₈ON₄S₃·2HCl, but they are not quite consistent with this.

2. One nitrate and two sulphates of vitamin B₁ have been prepared with distinct and sharp melting-points.

We are indebted to the Medical Research Council for personal grants and for grants for expenses, and to M. Kempson and R. W. Wakelin for skilful assistance.

¹ György's vitamin B₆ is here considered to be identical with factor Y.

APPENDIX I.

Table IX. *Analyses of vitamin B₁ hydrochloride by H. W. Kinnersley and J. R. O'Brien.*

Date	Preparation	Amount mg.	% C H N S Cl				
			C	H	N	S	Cl
24. v. 33	57. 7	2.218	42.33	5.88	—	—	—
20. v. 33	57. 7	1.805	—	—	14.6	—	—
25. v. 33	57. 7	1.647	—	—	—	—	21.0
18. i. 34	68 A 11	2.108	—	—	15.56	—	—
			—	—	15.13	—	—
19. x. 33	68 A 11	7.21	—	—	—	9.82	—
	69. 32	22.72	—	—	—	—	20.7
24. v. 33	78. 33	1.718	42.06	7.02	—	—	—
20. v. 33	80. 37	1.817	—	—	15.15	—	—
23. v. 33	80. 37	2.214	—	—	—	9.03	—
24. v. 33	83. 7	2.067	41.6	5.97	—	—	—
21. ix. 33	70 A 13	1.792	—	—	—	9.80	—
	93 A 47	13.2	—	—	—	—	20.66
21. ix. 33	93 A 47	2.670	—	—	—	9.77	—
4. x. 33	93 A 47	5.166	—	—	—	10.00	—
9. x. 33	93 A 47	6.09	—	—	—	10.12	—
14. x. 33	93 A 47	6.64	—	—	—	9.72	—
20. x. 33	92 A 50	5.60	—	—	—	9.72	—
10. x. 33	74. 26	4.25	—	—	—	—	21.14
2. v. 34	70 A 6	6.53	—	—	—	—	21.2
27. i. 34	68 A 11	2.550	42.94	5.6	—	—	—

Analyses of vitamin B₁ hydrochloride obtained by conversion of the sulphates.

From the sulphate of low M.P.:

13. ii. 34	19 A 51	1.998	—	—	15.92	—	—
14. ii. 34	19 A 51	2.815	41.68	5.45	—	—	—
28. iii. 34	18. 51	1.653	—	—	15.92	—	—
		1.897	42.27	8.0	—	—	—

From the sulphate of high M.P.:

28. iii. 34	18. 37	3.415	41.9	5.53	—	—	—
		1.851	—	—	17.32	—	—
27. iv. 34	18 A 3	2.250	—	—	15.37	—	—
		3.43	41.34	8.4	—	—	—

Average values

Kinnersley and O'Brien	...	41.9	6.6	15.6	9.75	20.94
Schoeller	...	42.2	5.7	15.1	9.5	20.92

All values are corrected for water content and residue of vitamin. Sulphur values were determined by modified Pregl sulphur analysis [Kinnersley and O'Brien, 1934].

APPENDIX II.

Notes upon the catatorulin test for vitamin B₁ by R. A. Peters.

In this test, the power of vitamin B₁ to restore a specific *in vitro* respiration in avitaminous brain tissue is used. Experience with the method of Passmore *et al.* [1933] has led to the introduction of changes. Working with lactate only, the differences between 0.3 γ and 1 γ vitamin B₁ are often obscured by the

variation in duplicate samples. It is better to take advantage of the magnification in the catatorulin effect induced (*a*) by the use of pyrophosphate [Peters and Sinclair, 1933] and (*b*) by warm mincing [Peters *et al.*, 1935]. The latter requires considerable modification of the empirical equation, previously suggested. Details of procedure must be controlled at each point, otherwise unsatisfactory duplicates are obtained.

Procedure. Pigeons are cured at least once for periods over two days with a known or experimental sample of vitamin B₁. They are rendered more free from vitamin B₁ in this way. When symptoms reappear, the head is removed by guillotine, excess blood allowed to drain from the head upon filter-paper for 2–3 mins. The brain is then exposed by cautious excision of the top of the skull. The excised brain is placed upon a glass water-jacketed funnel ($37^{\circ} \pm 2^{\circ}$) and the required parts, usually cerebrum, optic lobes and lower parts, minced with a bone spatula for 2–3 mins. and thoroughly mixed. Quantities of about 100 mg. are selected and placed successively in each tared bottle containing the Ringer-lactate-pyrophosphate solution. It is so arranged that one half of the bottles are filled before the other half. In this way, differences occurring as the tissue stands are minimised. The tissue is allowed to remain without disturbance in a small ball; the bottles are then re-weighed. When the weighing is complete, the tissue is divided into small fragments with a glass rod or crusher; care is taken to remove minimum amounts of fluid upon withdrawing the crusher. The divided tissue is allowed to stand for 1–2 mins. and addition of vitamin B₁ is then made. The vitamin B₁ is prepared by evaporation to dryness of an acid alcoholic solution in a small tube *in vacuo*; the dry vitamin is dissolved in the Ringer-phosphate-lactate-pyrophosphate¹ medium.

Bottles are then twice evacuated to 70 mm. and filled with oxygen; duplicates are evacuated in different batches again to control changes occurring upon standing. As each batch is filled with oxygen the taps are turned and the apparatus briefly shaken to saturate the Ringer with oxygen. The apparatus are all together quickly placed in the bath and shaken for 12 mins. before taking the first reading.

Readings can be taken at half-hour periods as before. In some cases, the first period has been 15 mins. and the subsequent readings for half-hourly periods.

Exp. 761 shows an experiment with pyrophosphate, the values being the mean of duplicates. The figures represent O₂ uptakes (μ l.).

Period Vitamin ...	Period hrs.				
	1	2	3	4	5
	0– $\frac{1}{4}$	$\frac{1}{4}$ – $\frac{1}{2}$	$\frac{1}{2}$ – $1\frac{1}{4}$	$1\frac{1}{4}$ – $1\frac{1}{2}$	$1\frac{1}{2}$ – $2\frac{1}{4}$
Nil	2060	1650	1360	1045	850
0.5 γ	2350	2175	1900	1645	1460
1.0 γ	2400	2300	2040	1860	1645
2.0 γ	2410	2250	2100	1870	1695

Within the first hour, $\frac{1}{4}$ – $1\frac{1}{4}$, the differences are of the same order as formerly. We can calculate *V* from the Michaelis equation originally given by Passmore *et al.* [1933], and, as in this case, we often get fair agreement.

	<i>v</i>	<i>V</i>
For 0.0 γ	1505	2260
0.5 γ	2037	2330
1.0 γ	2170	2350

During the second hour, pyrophosphate gives a largely increased vitamin effect, *i.e.* 66 % over the control for 1 γ vitamin as compared with 44 % in the first hour. This increase varies to some extent with the condition of the tissue. The reactions are complicated and do not lend themselves at present to simple

¹ In some cases, Na pyruvate, 1 mg./3 ml. has been added, but it is not certain that this is an improvement.

conceptions (*cf.* Peters *et al.* [1935] for a partial analysis of this). I believe it best to use a frankly empirical equation to fit individual cases. In Exp. 761 and some others, the points: 0.0, 0.5 γ and 1.0 γ are fitted approximately by the Michaelis equation if $kc=30$ and $R=20$.

For the second hour, $kc=30$, $R=20$.

	v	V
0.0 γ	950	2370
0.5 γ	1550	2220
1.0 γ	1750	2190

1 γ as in the original work evidently gives approximately maximum effects so that the curve is discontinuous after 1 γ , *i.e.* 2 $\gamma \equiv 1\gamma$.

In the cases which have been investigated, the approximate answer is not usually in any doubt. With pyrophosphate and warm mincing a quite satisfactory comparison of activity can often be obtained with the mean of duplicate values, owing to the much larger relative change in the oxygen uptake for different vitamin concentrations. I regard this as a most promising method of comparing the strengths of vitamin solutions, though more work is required to determine the most suitable equation for the new conditions.

REFERENCES.

- Barnes and Peters (1932). *Biochem. J.* **26**, 2203.
 Birch and Harris (1934). *Biochem. J.* **28**, 602.
 Bernal and Crowfoot (1933). *Nature*, **131**, 911.
 ——— (1934). *Nature*, **134**, 809.
 Boas-Fixsen (1931). *Biochem. J.* **25**, 597.
 Burn, Coward, Ling and Morgan (1933). *Biochem. J.* **27**, 1719.
 Chick and Copping (1934). *Chem. and Ind.* **53**, 874.
 ——— and Roscoe (1929). *Biochem. J.* **23**, 506.
 György (1934). *Nature*, **133**, 498.
 ——— Klaveren, Kuhn and Wagner-Jauregg (1934). *Z. physiol. Chem.* **223**, 236.
 Halliday (1934). *J. Biol. Chem.* **106**, 29.
 Heyroth and Loufbarrow (1934). *Nature*, **134**, 461.
 Holiday (1935). *Biochem. J.* **29**, 718.
 ——— Kinnersley, O'Brien and Peters (1934). *Chem. and Ind.* **53**, 1024.
 Jansen and Donath (1926). *Proc. K. Acad. Wetensch. Amsterdam*, **29**, 1390.
 ——— Kinnersley, Peters and Reader (1930). *Biochem. J.* **24**, 1824.
 ——— Wibaut, Hubo, and Wierli (1933). *Rec. Trav. Chim. Pays-Bas*, **52**, 366.
 Keenan, Kline, Elvehjem and Hart (1933). *J. Biol. Chem.* **103**, 671.
 Kinnersley and O'Brien (1934). *Chem. and Ind.* **53**, 830.
 ——— and Peters (1932). *J. Physiol.* **75**, 17 P.
 ——— (1933). *Biochem. J.* **27**, 232.
 ——— (1934). *Chem. and Ind.* **53**, 452.
 ——— and Peters (1928). *Biochem. J.* **22**, 419.
 ——— (1929). *Biochem. J.* **23**, 1126.
 ——— (1930). *Biochem. J.* **24**, 711.
 ——— (1934). *Biochem. J.* **28**, 667.
 Malcolm (1933). *Quart. J. Exp. Physiol.* **23**, 83.
 Moore, Plymate, Andrew and White (1932). *Amer. J. Physiol.* **102**, 566.
 Narayanan and Drummond (1930). *Biochem. J.* **24**, 24.
 O'Brien (1934). *Chem. and Ind.* **53**, 452.
 Ohdake (1932). *Proc. Imp. Acad. Japan*, **8**, 179.
 ——— (1934). *Bull. Agric. Chem. Soc. Japan*, **10**, 71.

- Passmore, Peters and Sinclair (1933). *Biochem. J.* **27**, 842.
Peters and Philpot (1933). *Proc. Roy. Soc. Lond. B* **113**, 48.
—— and Sinclair (1933). *Biochem. J.* **27**, 1910.
—— Rydin and Thompson (1935). *Biochem. J.* **29**, 53.
Reader (1929). *Biochem. J.* **23**, 689.
—— (1930). *Biochem. J.* **24**, 77, 1827.
Seidell (1929). *J. Biol. Chem.* **82**, 633.
—— and Smith (1933). *J. Amer. Chem. Soc.* **55**, 3380.
Van Veen (1932). *Rec. Trav. Chem. Pays-Bas*, **51**, 265, 269.
—— (1933). *Z. Physiol. Chem.* **208**, 125.
—— (1934). *Natur*, **133**, 137.
Williams and Eddy (1933). *Carnegie Inst. Year Book*, 320.
—— Waterman and Keresztesy (1934). *J. Amer. Chem. Soc.* **56**, 1187.
Windaus, Tschesche and Ruhkopf (1932). *Nachricht. Göttingen*, 342.
—— ——— Laquer and Schultz (1933). *Z. physiol. Chem.* **204**, 123.