CCLXXXII. THE PROPERTIES OF BLUE FLUORESCENT SUBSTANCES FORMED BY OXIDATION OF VITAMIN B₁ (QUINOCHROMES).

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

From the Department of Biochemistry, Oxford.

(Received August 31st, 1935.)

It has been stated previously [Peters, 1935] that a blue fluorescent substance arises by oxidation of the non-fluorescent vitamin B₁. We have found that oxidation does not immediately destroy all properties of the vitamin, and we have accordingly explored in detail the changes in the formaldehyde azo-colour reaction [Kinnersley and Peters, 1934], in the state of the S and in the biological activity. New points have come to light. Windaus *et al.* [1932] had stated that permanganate oxidises the S in vitamin B₁ to —SO₄. Our work shows that oxidation in the cold does not necessarily destroy the biological activity or remove S from the molecule; we have further evidence that other forms of vitamin B₁ exist, one of which may be blue fluorescent. This latter is yellow in acid solution unlike vitamin B₁; the fluorescence disappears upon strong reduction and reappears upon reoxidation. We believe that these points are of immediate interest in relation to the problem of the grouping which is biologically active in this vitamin and of wider interest because they may shed some light upon the puzzle as to the function of the blue fluorescent substances in living tissues.

HISTORICAL.

During early attempts to concentrate vitamin B₁ in yeast extracts, we noticed their marked blue fluorescence. Bence-Jones [1866] had found blue fluorescent substances in animal tissues, which he called "quinoidine" owing to a blue fluorescence similar to that of quinine. In 1925, Kinnersley *et al.* [1925] confirmed the presence of quinoidine and its resistance to permanganate. They considered that several different blue fluorescent biochemical substances existed but could not find a pure biochemical substance showing this property among a large number tested. They produced evidence however suggesting that the blue fluorescent substance in urine was urochrome. Vitamin B₁ proved not to be a "quinoidine" [Kinnersley and Peters, 1928]. Rosenheim [1927] observed a blue fluorescence in ergosterol (from yeast) which he thought to be due to oxidation products. More recently interest in these substances has been revived by the discovery of the flavins [Warburg and Christian, 1933; Kuhn *et al.*, 1933], though at first there was no clear appreciation of the difference from these latter compounds. Euler and Adler [1934] have reinvestigated the distribution of blue fluorescent substances particularly in the eye. Some of the degradation products of the flavins and substances allied to them fluoresce blue under some conditions [Karrer *et al.*, 1934 (lumichrome, formula 1); Kuhn and Bär, 1934; Stern and Holiday, 1934]. As a general term for such blue fluorescent substances mentioned above, we suggest the word "quinochrome"; this brings the term into line with lyochrome. For a chronological account see Table I. From

Table I. *Observations on the quinochromes.*

Blue fluorescent substances of biochemical origin.

Author	Source of material <i>etc.</i>
1855 Helmholtz*	Human retina (solid)
1858 Regnault*	Lens of eye, sheep, dog, cat, rabbit
1859 Setchenow*	Lens of eye (like quinine)
1863 Schliess and Lowenfeld*	In sunlight (urine)
1866 Bence-Jones	Extracts from animal tissues called "animal quinoidine"
1922 Langecker*	Most urines
1925 Kinnersley, Peters and Squires	Animal quinoidine present in excreta, lens of eye <i>etc.</i>
1927 Rosenheim	Blue fluorescence of ergosterol from yeast due to oxidation products
1934 Euler and Adler	Extracts of the retina of fish eyes
1934 Karrer <i>et al.</i>	Degradation products of flavins, lumichrome and dimethylalloxazines
1934 Stern and Holiday	Synthetic alloxazines
1934 Kuhn and Bär	Synthetic dimethylalloxazines
1935 Peters	Oxidation product of vitamin B ₁
1935 Barger <i>et al.</i>	Thermal decomposition products of vitamin B ₁
1935 Kuhn <i>et al.</i>	Thiochrome from yeast, empirical formula similar to that of vitamin B ₁

* For references see paper by Kinnersley *et al.* [1925].

previous work upon the flavins, it might be thought that these were the major source of quinochromes in yeast. Since it has now been found that vitamin B₁ can give rise to blue fluorescent substances, quinochromes obviously can arise also from this vitamin. Recently Kuhn *et al.* [1935] have reported the isolation from the blue fluorescent substances in yeast of a sulphur-containing base of similar empirical composition to dehydrogenated vitamin B₁. They call this thiochrome¹ and believe it to be a degradation product of vitamin B₁. The inference that our oxidation products and thiochrome are intimately related is obvious; they are alike in several respects, but differ somewhat in solubility.

Synthetic work in collaboration with ourselves has been undertaken by Prof. Robinson (see Robinson and Tomlinson [1935] for the synthesis of the thiopyrimidazines III).

I. Lumichrome

II. Williams's formula for vitamin B₁, written to show relation to I and III

III. Tetrahydrothiopyrimidine

¹ After completion of this paper, Barger *et al.* [1935] described the preparation of thiochrome from vitamin B₁ by ferrieyanide.

The properties of vitamin B₁.

In Table II the properties of the vitamin are summarised particularly with the idea of showing the changes dependent upon variable p_H . The zones of p_H are only approximate and intended to bring out the broad differences. It is clear that the outstanding changes in its properties occur about p_H 7·0, which is striking in view of its biochemical activity; the properties to study from the standpoint of activity must be clearly those which change at this p_H . It will be noted that the effect of alkali upon the vitamin colour reaction [see Kinnersley and Peters, 1934] is irreversible, but upon the colour spectrum and titration curve it is reversible. But the spectrum from p_H 1 to 4 shows another complication. When dissolved in acid alcohol, the vitamin shows a band at $247 m\mu$ with a higher extinction coefficient than that of a specimen of vitamin which is acidified after being dissolved in 80% alcohol. This procedure would not inactivate the vitamin [Holiday, 1935]. In the case of the titration curve, differences occur with the hydrogen electrode as compared with the glass electrode. Both methods, however, indicate the presence of a pseudo-acid group in the vitamin. From the summarised evidence we can infer the independence of the band at $247 m\mu$ and the activity, and of the latter and the pseudo-acid group.

Table II. p_H and properties of vitamin B₁.

Property	References	p_H 1-4	4-7	7-9	9-11
Colour	(1), (2)	Colourless	Colourless	Yellow fading to colourless. R.	
Spectrum	(3), (4), (5), (9)	$247 m\mu$	$234 m\mu$ and $268 m\mu$	Increase in absorption at $233 m\mu$ and appearance of broad band at $330-340 m\mu$, rapidly fading	
Titration	(6), (7)	Basic group p_K 3·4	Basic group p_K 4·8	Pseudo-acid group appears slowly with p_K 6·8. R.	
Azo colour reaction	(8)	Stable	Stable	Tending to become unstable and much lost in performing alkaline titration. I.	
—SH reaction	(2)	—	—	No	Present only after warming with strong alkali
Activity	(2)	Normal	Normal	Normal	Lost gradually on heating. I.

R = Reversible. I = Irreversible.

References: (1) van Veen [1933]; (2) Kinnersley *et al.* [1935]; (3) Peters and Philpot [1933]; (4) Smakula [1935]; (5) Holiday [1935]; (6) Birch and Harris [1935]; (7) Moggridge and Ogston [1935]; (8) Kinnersley and Peters [1934]; Wintersteiner *et al.* [1935].

The p_K values of the two basic groups at p_K 4·8 and 3·4 agree well with the p_H required to form the mono- and di-sulphate respectively [Kinnersley *et al.*, 1935]. Their interpretation upon the Williams formula is not obvious. Following observations of Levene and Simms [1926], who showed that pyrimidine amino-groups had p_K 4·0-4·6, Moggridge and Ogston [1935] concluded that the group at p_K 4·8 in the vitamin might be identified with the 6 —NH₂ group. They pointed out the difficulty that this would leave the quaternary N atom out of account in the titration, because the weaker basic group would have to correspond with one of the N atoms on the pyrimidine ring. This is possible; cyclic N in histidine has p_K 6·18 [Birch and Harris, 1930]. It seems to be more likely now that the S decreases the effective basicity of the quaternary N atom. It is known that S influences the precipitation of bases by phosphotungstates in this direction [Peters, 1930]; the precipitation point of thioglyoxalines is more

acid than that of the glyoxalines by some 4–5 p_H units. Further, Richardson [1933] determined the dissociation constants of *dl*-thiolhistidine, obtaining p_K 1.84, 8.47 and 11.4 for $-\text{COOH}$, $-\text{NH}_2$ and $-\text{SH}$ respectively. These are to be compared with histidine, p_K 1.77, 6.18, 9.18 for $-\text{COOH}$, cyclic N and $-\text{NH}_2$. Here S appears to have abolished the p_K of the cyclic N. If the S in the vitamin is having such an influence, then p_K 4.8 must be due to the N of the thiazole group and p_K 3.4 to the 6 $-\text{NH}_2$. Certainly the p_H at which the pseudo-acid group influences the titration would be consistent with this interpretation.

The amino-group. Williams and Gurin [1935] have stated recently that vitamin B_1 interacts slowly with nitrous acid to produce nitrogen. The reaction must be a very slow one; using crystalline vitamin B_1 , after a thorough nitrous acid treatment, the crystals were recovered in 75% yield and with no change in property (Exp. 5). Making allowance for the small scale of the work, the destruction could not have been more than 10%. This is entirely consistent with previous evidence upon impure vitamin as to stability to the action of nitrous acid.

The thiazole group. The recognition of a thiazole ring as a constituent of the vitamin B_1 molecule by Williams and his colleagues and the definite identification by Tomlinson [1935] of synthetic 4-methylthiazolecarboxylic acid with the nitric acid oxidation product of Windaus *et al.* [1934] does not solve all problems in relation to the state of the S in the vitamin. The S is not present as $-\text{SH}$ until after warming with alkali, to which the thiazole structure is usually stable. The vitamin must react with the ring intact and cannot function like glutathione by the $-\text{S}-\text{S}-$ to 2SH mechanism at a biochemical hydrogen ion concentration, owing to the relatively drastic treatment needed to free the S. Windaus *et al.* [1932] stated that warming with alkali splits off H_2S and also NH_3 . We have devoted a few experiments to this point (Exp. 6), and have clear evidence that S will appear as H_2S after heating with alkali under conditions which leave the N intact. Evidently the state of the S is different from that in insulin [Freudenberg and Wegmann, 1935] in which NH_3 and H_2S are stated to be formed simultaneously by heating with alkali. It is well known of course that insulin has no curative action upon vitamin B_1 symptoms, but in case anyone should think that vitamin B_1 might be the active group in insulin, it may be mentioned that an unpublished experiment by one of us (R. A. P.) showed that insulin after hydrolysis had no action as vitamin B_1 .

The formaldehyde azo-reaction. (Vitamin B_1 colour reaction.) This was described by Kinnersley and Peters [1934]. To a rather alkaline carbonate mixture are added diazotised sulphanilic acid, and after an interval the vitamin solution containing a drop of formaldehyde, added immediately before mixing. An immediate yellow colour appears which becomes pink after some 10 min. The maximum intensity is reached in an hour, and it will remain stable for many days.

It has not been mentioned so far that this reaction does not occur in the presence of reducing agents. It is not due to the presence of groups reacting as the S in thiolglyoxalines, because the vitamin does not give the Hunter reaction [1930]. It is given to some extent by acetone. The vitamin gives a reaction of Hunter for thymine,¹ in which after treatment in sodium carbonate with the diazo-reagent, strong NaOH and hydroxylamine are added, to yield a reddish colour. On the whole, the vitamin reaction seems to resemble most those given by certain oxygen-containing pyrimidines [Johnson and Clapp, 1908].

¹ We are indebted to Prof. G. Hunter for allowing one of us to see part of a thesis, containing a description of this reaction. In his classification, the vitamin colour reaction would be regarded as of the keto-enol type [cf. Koessler and Hanke, 1922.]

The special properties of vitamin B₁ which lend themselves to study are the catatorulin activity (brain enzyme test), the colour reaction, the liberation of H₂S on warming with alkali and the spectrum. This paper does not deal with the last.

Vitamin B₁ quinochrome.

Before proceeding to detail, we shall draw attention to the broad facts by a recent experiment.

Exp. 1. A blue fluorescent solution was prepared from vitamin B₁ as follows:

0.92 mg. vitamin B₁.HCl (prep. 73.3) was dissolved in 0.82 ml. 15% ethyl alcohol (p_H about 5.5). 0.06 ml. MnO₂ suspension was added and the whole shaken at room temperature (23°) for 5 min.: 0.04 ml. *N*/20 NaOH was then added and the whole again shaken for 5 min. A blue fluorescence visible in daylight rapidly appeared after the addition of the alkali, p_H about 7.5. After standing for a further 5 min., the whole was reacidified with 0.08 ml. *N*/20 HCl to p_H 6.2; it stood 10 days at room temperature. The colour reaction was then nil, but there was a delayed azo-reaction (see below) of 20% of the original.

This solution was tested upon an avitaminous brain and gave the result that 0.61γ of the fluorescent products was equal in catatorulin activity to 0.52γ of the untreated vitamin, *i.e.* there was not more than 17% diminution in activity. Such fractions may also be curative to birds.

Is the fluorescence due to conversion of a small part of the vitamin into inactive fluorescent products, or is a blue fluorescent substance itself biologically active? The fluorescence is most intense in alcoholic solutions in the region of p_H 7.0, and solutions must be compared under these conditions. When acidified the solution becomes yellow, giving the whole a green appearance, which is stated to be also a property of thiochrome. We were early deceived by the extraordinary intensity of the fluorescence. Of our best fractions, such as that in Exp. 1, amounts of 0.5 mg. per ml. show marked fluorescence in daylight with a slight purple tint. As little as 0.5γ per ml. shows good fluorescence in the ultraviolet (Hg lamp) light, of the same order of intensity as 3γ per ml. of quinine sulphate (in *N*/80 H₂SO₄, 15% alcoholic solution). This is a conservative estimate; the colours are somewhat difficult to compare. The blue fluorescent products are certainly 6 times as fluorescent as quinine and more fluorescent than tetrahydro-alloxazine, for a specimen of which we are grateful to Prof. Robinson and Miss Tomlinson.

Hence by oxidation in this case a solution has been reached which is more fluorescent than some of the most fluorescent substances known, with a slight diminution in catatorulin activity almost within the experimental error. If the quinochrome were formed at the expense of 20% of the vitamin, it would have to be at least 30 times more fluorescent than quinine. As it is unlikely that any such substance exists, we conclude that the quinochromes themselves can be biologically active.

We have not obtained quinochromes by treatment with H₂O₂. In the first experiments they were produced by warming with traces of gold chloride (as we know now in very small amount). Maximum yields have been reached with permanganate and manganese oxides; the p_H is very important.

Formation of quinochromes from vitamin B₁ by manganese compounds.

The main points reached in a series of experiments can be summarised under 9 headings. In this work we have found it convenient to carry out the oxidations in small test-tubes, containing 1–2 ml. of concentration 1–2 mg. vitamin per ml.

As the solutions often stood for several days, pure redistilled ethyl alcohol was added to a concentration of 15% to keep down the growth of micro-organisms. Control experiments showed that this made no difference to the results. In order not to interfere with subsequent attempts to isolate the products, no buffers were added, and the p_H was estimated colorimetrically upon a white tile and is therefore only approximate.

Permanganate. 1. At p_H 5-5.5 oxidation with potassium permanganate ($N/100$) takes place very slowly at room temperature; 2-4 hours are needed for completion, the permanganate being added drop by drop. During the oxidation 1 mol. O_2 is taken up. Upon completion mere traces of fluorescence are present. The fluorescence may increase over several days, but is only a fraction of the total possible. Decoloration with permanganate will occur at as acid a reaction as p_H 2 but it may take 24 hours for completion. It is therefore favoured by the ionised molecule. Barium permanganate does not give such good results.

At this stage there seems to be no interference with the properties of the vitamin, though the catatorulin activity may be a little diminished.

We do not understand what is happening in this step, possibly $(6)NH_2 \rightarrow :NH$. The permanganate is not oxidising traces of possible alcohol left in the crystals or traces of HCl; this was excluded by controls. Assuming a definite oxidation the step must be reversible, because after removing the oxides of manganese, crystals of vitamin $B_1 \cdot HCl$ have been recovered with all the usual properties, m.p., colour reaction and activity. They can further be re-subjected to a fresh oxidation with permanganate.

2. After 1, if the solution is brought to p_H 7-7.5, the fluorescence gradually increases during 10 days to a maximum. Heating the solution increases the rate of formation with a decrease in the total quinochrome yield. The pseudo-acid group is still present after 1, because the p_H has to be stabilised by addition of traces of alkali. During the course of this the azo-reaction tends to disappear and to be replaced by a delayed colour reaction, in which the solution after mixing with the diazo-reagent shows no colour for an hour or more. The catatorulin activity is present though variable.

3. With addition of more alkali to p_H 8.0, less fluorescence is produced; a further amount of permanganate can be taken up at this stage, up to 2 mols. of O_2 being recorded in one experiment. There appears to be destruction or oxidation beyond the fluorescent stage.

4. An oxidation started at p_H 8.0 produces a poor yield. The changes detailed in 1-3 can be completed much more quickly by adding the brown oxides of manganese (thoroughly washed) formed in an oxidation of oxalic acid. Hence there are really two steps in 2 and 3, the last being due to the oxides formed in the first.

5. *Oxides of manganese.* Addition of the oxides of Mn at p_H 5-5.5 gives practically no fluorescence even upon standing for 30 min.

6. After 5, if the p_H is taken to 6.8 (short of p_H 7.0) there is an immediate fluorescence, visible to the naked eye. At first there is little change in colour reaction or catatorulin. After standing for a few days the colour reaction changes to the delayed type, and there is an improved yield of quinochrome. In a particular case the fluorescence increased as follows, 10 being considered to be maximum, 30 min. 0.5, 2 hours 1.0, 1 day 5.0. At this p_H , 6.5-7.0, more fluorescent substance has been formed than in any other way.

7. After 5, if the p_H is brought to 7.6 approximately, there is again immediate fluorescence, but the colour reaction is less stable and the total yield less, unless the p_H is brought back to 6.

Table III. *Progress of azo-reaction and blue fluorescence in oxidised vitamin solutions.*

Prep. p_H	Days after start											
	2	3	4	5	6	7	8	9	10	11	12	15
73-1 7.5	—	—	—	—	—	—	$\frac{5}{0::1+}$	—	—	—	$\frac{0.1}{0::3.5}$	—
73-2 7.6 to 4.5	—	—	—	—	—	—	$\frac{1}{4::6}$	—	—	—	$\frac{0.5}{4::4}$	—
73-3 7.2 to 4.5	—	—	—	—	$\frac{10}{3::4}$	—	—	—	$\frac{10}{0.5::2}$	—	—	—
73-4 6.9	—	—	—	—	—	$\frac{10}{2::4}$	$\frac{10}{0::1}$	$\frac{10}{0::0}$	—	—	$\frac{10^*}{1.3::1.8}$	0 :: Trace
74-1 7.5	—	—	—	$\frac{3 \pm 5}{0::6}$	$\frac{5}{0::1.7}$	—	—	—	—	—	—	—
74-2 7.2	—	$\frac{3 \pm 0.5}{3::8}$	$\frac{5}{0::2}$	$\frac{5}{0::3}$	—	—	—	$\frac{7.5}{0::2.5}$	—	$\frac{9.5}{0::2.5}$	—	—
74-3 —	—	$\frac{0.5}{0::2}$	$\frac{1.5}{0::—}$	—	—	$\frac{1+}{0::1.7}$	—	—	—	$\frac{1}{0::1.5}$	—	—
74-4 6.5	$\frac{5}{6::8}$	$\frac{7.5}{4::4}$	—	$\frac{7.5}{4::4}$	$\frac{10}{—::1.3}$	—	—	—	$\frac{10}{2::1.5}$	—	—	—

Numerator is blue fluorescence in terms of maximum 10 $\equiv$ at least 60 quinine sulphate.
 Denominator gives azo-reaction Normal :: Delayed in terms of maximum 10.
 For details of the preparations see Experimental, and Table V.

* 30 γ taken for the test. On 8th and 9th days 10 γ gave negligible reaction.

8. When brought back to p_H 4.0 there is an immediate change of the quinochrome solutions to a yellow colour with change in the tint of fluorescence.

9. Treatment with the oxides abolishes the pseudo-acid shift. There is negligible formation of $-\text{SO}_4$ during these oxidations; the end result is a solution which gives no reaction for $-\text{SH}$ on warming with alkali, or even on fusion with Na or K in amounts which for the vitamin would give a strong reaction. However, the S can be converted into sulphate and so identified by Carius oxidation with nitric acid in a sealed tube. That the vitamin S can reach such a state may excuse previous failures to find it, but it is clear that these properties indicate a substance of different chemical behaviour from the usual vitamin B_1 .

Relation of blue fluorescence and the azo-reaction.

The question may now be asked as to what is the relation between the different variables. That between the blue fluorescence and azo-reaction is treated in Table III, between the former and the nitroprusside reaction on warming with alkali in Table IV, and the exploratory experiments upon the biological activity in Table V. Records of the normal and delayed colour reactions are given in Table III as well as the blue fluorescence in terms of a standard at intervals after treatment with MnO_2 . The fluorescence once formed is stable under these conditions and even remains stable if the p_H is shifted to 10 in the dark. There is no relation between the azo-reaction delayed or otherwise and the amount of the fluorescence. For instance, we have 73.1, Fl 1 azo nil; 73.3, Fl 10, while azo-reaction falls from 3:4 to 0.5:2, and 74.4 Fl 5-7.5 while azo-test is 6:8 and 4:4. There is a tendency for the azo-reaction to fall off more quickly when the p_H is greater than 7 (*cf.* 74.2 against 74.4). There is a general correlation between the loss of the property of forming $-\text{SH}$ on warming with alkali and the appearance of quinochrome (Table IV). The provisional conclusion is that azo-reactions and formation of quinochrome are independent reactions, but that the latter is closely related to disappearance of $-\text{SH}$.

Table IV. $-\text{SH}$ reaction with hot alkali, and blue fluorescence.

Preparation	10 = maximum. $-\text{SH}$	Fluorescence
Before treatment	10	Nil
73.1	10	1
74.3	4	1
74.2	2	5 -
74.4	2	10 -
73.4	Trace	10

Biological activity.

Information upon this is comprised in some 20 catatorulin tests upon the brain enzyme system [Passmore *et al.*, 1933; Kinnersley *et al.*, 1935] and 2 sets of curative tests upon pigeons. The curative tests prove that there can be still much biological activity left in specimens of vitamin treated with permanganate or manganese oxides and containing much quinochrome. The value of 4γ per day dose must be accepted with the reserve imposed by the recent finding that crystalline vitamin does not give satisfactory tests [see Kinnersley *et al.*, 1935]. The preparation might have seemed more active with a lower dose.

The catatorulin tests give more precise results and must be accepted as proof of action upon a specific enzyme system. The values have been obtained in media containing pyrophosphate, and as usual the relative maintenance of

respiration is the standard. They must be regarded as preliminary because in some cases it appeared that the shape of the curve relating dose to O_2 uptake was changed in the oxidised samples.

Table V. *Biological tests.*

Fluorescence 10 maximum $\equiv$ at least 60 quinine sulphate. () indicates the number of birds upon which test is based. N, normal; D, delayed.

A. Curative pigeon tests.

Preparation	Dates	Azo-reaction		Blue fluorescence	Catatorulin	Dose given	Day dose	No. of birds
		N	D					
57.7	iv. 35	0	:: 4	[5]*	9 -	20 γ	3.7 γ	(9)
59	iii. 35	2	:: 3.5	[5]*	6	20 γ	2.9 γ	(2)
74.2	vii. 35	0	:: 2.5	10	6	15 γ	2.7 γ	(9)

* Blue fluorescence not accurately measured in the first two; the value in square brackets is judged from that reached in other experiments.

B. Catatorulin tests.

Exp.	Preparation	Days after oxidation	Azo-reaction		Blue fluorescence	Catatorulin activity	Treatment
			N	D			
			(a) Oxidised by manganese oxides.				
915	73.3	0	10	:: —	Trace	8	Shaken with MnO ₂ at p _H 5.0 only
914	73.2	0	6.7	:: —	[1]	10 +	p _H 7.5 and brought back to p _H 4.5 after 45 min.
926	73.3	10	Trace	:: 2	10	8.3	p _H 7.5 for 5 min. and then back to acid p _H
917	73.4	2	8	:: —	[3-5]	10	Kept at p _H 6.9
921	73.4	4	5	:: —	[10]	7	
924	74.2	13	28	:: 8 +	3	4.2	Kept at p _H 7.2
928	74.2	11	0	:: 2.5	10	6	—
923	74.3	3	0	:: 2.5	0.5	4.3	Oxidised in 90% alcohol at p _H 7.0. (Exp. 2.)
928	74.4	10	2	:: 1.5	10	3.3	p _H 6.5

For data on some of these tests, see Exp. 7.

(b) Oxidised by permanganate.

820	57.7	22		7	[5]	8-10 +	Oxidised and kept at p_H 7.5
821	57.7	27		4	[5]	8-9 +	
826	59	22	5	:: —	[5]	10 +	
829	59	24		—	[5]	5	
830	59	25	2	:: 3.7	[5]	7	—
831	60	1	10	:: —	Trace	4	Oxidised and kept at p_H 5.5 approx.

In the experiments marked 10 + there was a definite increase in the catatorulin effect above the maximum possible with addition of normal vitamin B_1 . Square brackets means that the fluorescence was judged from other experiments.

Exps. 820, 926, 921, 928 show 5-10 fluorescence and high catatorulin activity.

Exps. 830, 918, 926, 928, 923 show poor azo-reaction and high catatorulin activity.

Exp. 928 shows high fluorescence and low catatorulin activity.

There seems to be a super-catatorulin effect amounting to some 10% in several of the specimens; this is marked in the table as 10 + (see also Exp. 8 for tests upon a worked oxidised fraction). It means that the catatorulin action of these specimens exceeds the maximum value possible with excess of vitamin B_1 . Accepting ± 1 (10%) as the limits for the tests (in many cases they are more precise than this), the following is shown.

1. Specimens with high catatorulin activity, may show maximum fluorescence.
2. Complete loss of colour reaction is not necessarily attended with complete loss of activity.
3. An entirely oxidised specimen, from which all traces of vitamin B₁ proper have been removed, will still show catatorulin activity.
4. The conditions for forming quinochrome without loss of biological activity are not completely understood.

In interpreting these results, we must remember that much trouble has been taken in establishing in previous papers from this laboratory the fact that azo-reaction and catatorulin tests are satisfactory indicators of activity in crystalline vitamin B₁. The general conclusion must be that other forms of the vitamin exist than the one which gives the normal azo-reaction, and that it is unlikely that there is not a quinochrome form of vitamin B₁ with different properties, at least as far as the S is concerned.

From the above work, it follows that there can be no direct relation between the group responsible for biological activity and that giving the azo-reaction, or between the structure especially responsible for the blue fluorescence and the activity; in view of the former conclusion this must include the state of the S.

In order to explain the experiments, there must be postulated as separate entities the quinochrome substance still giving the full azo-reaction, that giving the delayed reaction, and the fluorescent and non-fluorescent degradation products. In addition there is the elusive first product of permanganate oxidation. For a summary of the oxidation stages, see Table VI.

Table VI.

Oxidation stages	Azo-test	Catatorulin	Pigeon	—SH	—S fusion	Fluorescence	Colour in acid	Pseudo-acid
Vitamin B ₁	+	+	+	+	+	—	0	+
↓								
Intermediate product	+	+	+	+		—	0	+
↓								
Quinochrome A	±	+	+	Nil	Nil	+	Yellow	—
↓								
Quinochrome B ? thiochrome	?	—	—	—	—	+	Yellow	—
↓								
Non-fluorescent degradation products								

The formation of the intermediate product does not stop the pseudo-acid change, but is essential to production of the fluorescence (notes 6 and 7). The formation of A completely abolishes the pseudo-acid change, and acidification then gives the yellow colour at p_H values more acid than p_H 6.0, in strong solution visible at 7.0.

Properties. After removal of manganese oxides and evaporation to dryness with alcohol to remove the last traces of salt, the substances present in the blue fluorescent solutions form an oil and will not crystallise, nor will the addition of light petroleum to the alcoholic solution bring out crystals as with the pure vitamin. Mixtures of other solvents have so far not succeeded. The substances are soluble in ethyl and methyl alcohol and water, but only in traces in lipid solvents.¹ Solubility in chloroform is very low, and this seems to be a marked difference from thiochrome. No insoluble picrate or picrolonate could be made,

¹ Rangier [1935] states that urochrome, containing 2% S, is soluble in H₂O and dilute alcohol but not in organic solvents.

nor silver salt at acid p_H values. The only promising precipitant was mercuric chloride with sodium acetate, both in excess. Fractional precipitation from any unchanged vitamin can be carried out with this reagent, because unchanged vitamin requires less of the reagents for precipitation. It has not proved possible to purify the mercuric chloride compound upon the small scale. Analyses upon 3 different samples, gave $HgCl_2$ 70 % (Hg 60 % approx.), N 5–6 % and C 9–13 %. The C/N ratios were nearest to $C_{12}N_4$ ($C_{10}N_4$ in 2 of the samples). $C_{9-12}N_3$ was excluded by the analyses.

Treatment of the mercuric chloride complex with H_2S precipitates the quinochrome for the most part as an adsorption complex with the sulphide, from which it can be freed with hot water. In its ease of adsorption, it resembles rather closely the flavins, and it is very readily adsorbed upon vitamin B_1 hydrochloride crystals. The loss of biological activity at the gold stage in the preparation of crystals of vitamin B_1 is associated with the adsorption of blue fluorescent substances upon the gold sulphides from which they can be extracted by pyridine-methyl alcohol-water, following the technique of Kuhn *et al.* for flavins. In this connection it may be mentioned that Schopfer [1934] states that certain apparently pure specimens of flavin still contained traces of vitamin B_1 , sufficient to affect the growth of micro-organisms. It seems most likely that this was the quinochrome form.

The oxidation-reduction properties of the quinochrome are rather negative as for the flavins. It is not reduced by H_2S or ascorbic acid. No change in fluorescence was seen upon incubation anaerobically in a Thunberg tube with an active succinoxidase solution. But sodium hydrosulphite readily reduces quinochrome to a leuco-compound, which again becomes fluorescent upon re-oxidation. The implied stability to sulphite is interesting in view of the ease with which vitamin B_1 itself has been shown by Williams *et al.* [1935] to be degraded by sulphite. In neutral or acid solution the fluorescence is stable to strong light (Exp. 4). After reading the statement of Kuhn *et al.* [1935] as to the instability of thiochrome upon illumination in alkaline solutions, we have found that this is also a property of our solutions.

DISCUSSION.

The empirical formula and blue fluorescence of the vitamin oxidation products together with their general adsorption behaviour led to the hypothesis [Peters, 1935] that vitamin B_1 might be a thiopyrimidine (hexahydrothiolumichrome). In support of this, it was found that the thiopyrimidines made by Robinson and Tomlinson could be readily converted into quinochromes by permanganate oxidation about p_H 7.0, with removal of S as SO_4 . This view is not now tenable for at least 3 reasons; vitamin B_1 appears to be a pyrimidinethiazole compound [Williams, 1935]: blue fluorescence arises without removal of S as SO_4 as we have shown here: and thiochrome [Kuhn *et al.* 1935], a S-containing substance, has a blue fluorescence. But the idea that the blue fluorescent substance may be produced by ring formation between the $-NH_2$ at 6 and the $-CH_3$ [Robinson and Tomlinson, 1935] is still tenable. From the present work it appears that formation of quinochrome is coincident with loss of the pseudo-acid shift and loss of the $-SH$ reaction upon warming. Change in the valency of the N atom from quaternary to tertiary might well account for this and might accompany the closure of another ring. Evidently the critical p_H for the formation of active quinochrome with permanganate is about 7.0, which coincides with the p_H at which the basic groups become practically un-ionised and with the start of the pseudo-acid shift. The immediate appearance of some

10% at least of the fluorescence at room temperature is curious. The substances formed certainly resemble the thiopyrimidazines in colour, but not in ease of crystallisation or in solubility in aqueous solutions. Formation of a third ring by closure in the 6 position should be accompanied by a slight change in basicity which might be revealed by titration. Upon the views here expressed there should be a change in the alkaline spectrum after oxidation to quinochrome A. Some preliminary results have already been obtained in this direction by our colleague Dr Holiday, whose work will be reported later; it will be noted that the spectrum of thiochrome shows no alkaline change.

The question arises as to how far the Williams formula accounts for the groups responsible for the colour reaction and the biological activity. The brain enzyme work published from this laboratory implies that the vitamin acts as an acceptor or donator of —H or —OH in some oxidation-reduction system. We can readily picture the changes upon oxidation and closure of the ring. Closure of the ring and change in the S must be simultaneous. Reasons have been given against any belief that we are dealing with a sulphhydryl-disulphide system; and the independence of the S behaviour and the biological activity do not suggest a direct relation with this part of the molecule. Our provisional conclusion is that both the biological activity and the colour reaction are connected with the —NH_2 group at 6, which would therefore be capable of existing in two states of oxidation —NH_2 and =NH .¹ Upon such a view the nitrogen atom in the closed azine ring might be still able to function as H acceptor and donator. It is indeed quite conceivable that the blue fluorescent oxidised form may represent the actual biologically active state. This would explain the excess catatorulin activity noticed in several experiments.

The connection of the colour reaction with the —NH_2 at 6 would explain the strict relation between colour reaction and activity in the normal vitamin and the changes in the colour reaction which take place during gradual closure of the ring. If the first stage of oxidation is from —NH_2 to =NH , we can picture the intermediate stage as follows:

We have often been driven in our work to a belief that more than one form of vitamin exists [Kinnersley and Peters, 1928]. It is satisfactory to have more direct evidence for this. Some of the properties of the more impure vitamin concentrates, which are known to differ from those of the crystals, may be explained by the presence of these modifications of the vitamin. The matter is now under investigation.

EXPERIMENTAL.

Note. Comparison of the intensities of the fluorescence of different samples has been made with the aid of quinine standards.

Exp. 2. Oxidation of vitamin B₁ in concentrated alcoholic solution by manganese dioxide. Previous to the observation that vitamin B₁ on oxidation formed quinochrome, it had been noticed that alcoholic solutions of vitamin B₁ on standing in the cold or at room temperature for variable periods up to several months contained a blue fluorescent substance. (Care must be taken to use extracted corks since a blue fluorescent substance is freed from cork by alcohol.) A quinochrome is formed in small amounts and is rather insoluble in water.

¹ This would be consistent with the change of the p_K 3.4 group when titrating with the hydrogen electrode.

At 0° it is produced more rapidly the nearer the p_H is to 7, whilst very little of it is formed at p_H 2. The substance is made more quickly by the addition of manganese dioxide in 85% alcoholic solution and an adjustment of the p_H to approximately 7. In prep. 74·2 for instance the fluorescence produced was not by any means maximum but was almost instantaneously formed; after oxidation, about half the catatorulin activity was left with no colour reaction. (See Table V, B (a).)

Exp. 3. *Comparison of the rates of formation of fluorescence at p_H 5·5 and p_H 7·5.* To 1·14 mg. vitamin HCl in 1 ml. 15% alcohol was added an excess of MnO_2 . The mixture was shaken for 5 min. adjusted to p_H 7 and again shaken for 15 min. after which the p_H was brought back to 5·5.

A similar solution of 1·19 mg. vitamin HCl was treated in the same way. The p_H was, however, maintained at 7. Comparison of the fluorescence of these two solutions showed that there was ultimately no difference in the intensity, but in the acidic solution the fluorescence tended to develop more slowly.

Preparation	Time				
	2 hr.	3 days	4 days	6 days	
p_H 5·5	Yellow	5	5	10	Units of fluorescence
p_H 7·0	Blue	5	10	10	

10 regarded as maximum.

Exp. 4. *Effect of sunlight upon quinochrome.* (a) Comparison was made between two 15% alcoholic solutions of quinochrome each equivalent to 0·250 mg. original vitamin B_1 ; in one case the p_H was adjusted to 7 and in the other to 9. After standing for three days, no difference was observed in their fluorescence. Exposure of the solution at p_H 9 to sunlight for 1·25 hr. caused a 95% diminution in the intensity of fluorescence. No change was observed in the solution at p_H 7.

(b) 0·25 mg. vitamin HCl in 0·25 ml. H_2O (p_H approximately 5·5) was oxidised by the addition of 0·25 ml. $N/100 KMnO_4$ drop by drop. On exposure to sunlight for 1 hr., no diminution in fluorescence was observed.

Exp. 5. *The action of nitrous acid on vitamin B_1 .* 12·01 mg. of vitamin HCl were treated with 8·2 mg. barium nitrite in 0·45 ml. H_2O and 0·05 ml. HCl and allowed to stand for 3 hr. when a further 8 mg. of barium nitrite were added. The solution was thoroughly acid and the evolution of gas complete. After heating on the water-bath the solution was treated with 0·05 ml. baryta, saturated at room temperature and the whole worked into absolute alcohol at p_H 3. By colour reaction, 4000 doses of vitamin were present corresponding to 8·0 mg. After removing the inactive precipitate of $BaCl_2$ which gave no colour reaction, 8·8 mg. of vitamin B_1 crystals were isolated giving normal colour reaction and catatorulin activity.

The recovery was therefore at least 75% not allowing for certain unworkable residues, so that not more than 10% was destroyed.

Exp. 6. *Liberation of sulphur from vitamin B_1 by treatment with alkali.* 10·83 mg. of vitamin HCl were dissolved in 1·5 ml. H_2O and added to 150 mg. $Ba(OH)_2$. The mixture was heated on a water-bath for a total period of 1·25 hr. Volatile gases were expelled by blowing nitrogen through the mixture and collected in 7·55 ml. $N/100 HCl$ which at the end of the experiment required exactly 7·55 ml. $N/100 NaOH$ for neutralisation. We concluded that no ammonia had been liberated from the vitamin under these conditions. The mixture was acidified with 2 ml. $2N H_2SO_4$ and again heated. The liberated H_2S was expelled by a stream of nitrogen into a 12·5% solution of lead acetate

containing 1 or 2 drops of acetic acid and weighed as lead sulphide. The total amount of sulphur converted into H_2S was approximately 40% of that present in the original vitamin.

The residual solution after the above treatment, brought to p_H 3, showed a strong blue fluorescence. Like quinochrome, this blue fluorescent substance is precipitated by mercuric chloride and sodium acetate. In view of the fact that only part of the sulphur was removed by alkali treatment it seems probable that the fluorescence is due to quinochrome itself since the sulphur in this compound is not very labile to alkali.

In Table VII the different amounts of H_2S formed by alkali in several experiments are given. In no experiment have we been able to detect the liberation of ammonia. In some experiments attempts were made to collect volatile mercaptans by alcoholic mercuric chloride solutions. No evidence was obtained of sulphur produced in this form from the vitamin by action of alkalis.

Table VII.

Exp.	Wt. of vitamin mg.	Reagent	Amount of NH_3 liberated	Amount of S liberated (%)
1	11.63	N NaOH	Nil	58.8
2	5.42	"	"	15.4
3	3.4	"	"	47.5
4	2.4	"	—	74.5
5	10.83	10% " baryta	Nil	40.0

Sulphur was determined as lead sulphide except in the case of Exp. 4 where it was weighed as barium sulphate.

Exp. 7. *Catatorulin tests.* The catatorulin tests (see Table V) were made for the most part on the cerebrum of the avitaminous pigeon in head retraction. (The cerebrum gives better duplicate values.) Several had to be rejected owing to insufficient depletion of the cerebrum in vitamin. Pyrophosphate was present in the medium and the technique was essentially that described in a previous paper by Kinnersley, O'Brien and Peters [1935, Appendix]. The data for O_2 uptakes for three typical experiments for a period of respiration of 1–2 hr. are given in Table VIII. It must be remembered that 1.0 γ normal vitamin gives practically maximum increase in respiration.

Table VIII.

O_2 uptake for 1–2 hr. period expressed as $\mu l./g./hr.$

Normal vitamin			Unknown		
	Weight of vitamin (γ)	O_2 uptake	Preparation	Weight (γ)	O_2 uptake
914	2.28	$1142 \pm 3.5\%$	73.2	0.76	$1048 \pm 4\%$
	0.38	$848 \pm 1\%$		0.253	$791 \pm 2.5\%$
921	2.5	$1505 \pm 1.2\%$	73.4	5.0	$1360 \pm 1.7\%*$
	0.67	$1287 \pm 1.0\%$		1.0	$1335 \pm 0.5\%$
926	3.0	$1550 \pm 2\%$	73.3	5.0	$1522 \pm 1\%$
	0.3	$1246 \pm 0.5\%$		0.61	$1407 \pm 3\%$

NOTE. The mean is recorded with $\pm$ deviation of the duplicates from the mean.

* Not used in computing the results because the respiration decreased at an abnormal rate.

Exp. 8. *The preparation of blue fluorescent material free from vitamin B_1 .* 20.63 mg. vitamin B_1 HCl were oxidised with MnO_2 at p_H 7.5 for 5 days. Fluorescence appeared rapidly and at no time was there evidence of the formation of sulphate during the oxidation. The colour reaction gradually changed to

the delayed type and on the third day was 0 : 4. The whole was concentrated *in vacuo* at p_H 7 and worked into 1 ml. absolute alcohol at p_H 7; 3 ml. light petroleum were added which removed traces of insoluble material. The filtrate was further concentrated to 0.3 ml. and 0.7 ml. light petroleum was added. A few very fine needles separated after standing 2 days in the cold room. At this stage any unchanged vitamin would have precipitated. The filtrate from the needles was very yellow and fluorescent; to it were added 2 similar fractions from 21.5 mg. and 4.93 mg. amounts of vitamin.

An 18 γ sample of the total filtrates containing 35 mg. of oxidation products gave a colour reaction: normal, nil, delayed 8, and fluorescence not more than 2. The catatorulin activity of these fluorescent products is shown in Exp. 918 where comparison is made between the effect of normal vitamin B₁ and that of the fluorescent substances on the respiration of avitaminous pigeon brain. It is seen that the oxidation products gave an increased catatorulin effect, greater than the maximum of the normal vitamin.

Exp. 918.

Normal vitamin		Fluorescent preparation	
Weight (γ)	O ₂ uptake μ l./g./hr.	Weight (γ)	O ₂ uptake μ l./g./hr.
1	1506 $\pm$ 3.7 %	10	1617 $\pm$ 3 %
0.33	1090 $\pm$ 1 %	1	1515 $\pm$ 1.6 %

Note the increased activity over 1 γ normal vitamin.

Exp. 919 shows the increase in O₂ uptake with increasing concentrations of the fraction.

Weight of fraction (γ)	O ₂ uptake μ l./g./hr.
Nil	1000 $\pm$ 8.1 %
0.5	1142 $\pm$ 4.3 %
1.0	1390 $\pm$ 1.2 %
5.0	1637 $\pm$ 3.0 %

From these experiments, there is no doubt that a fluorescent fraction from which all normal vitamin was removed is still biologically active.

SUMMARY.

1. Blue fluorescent oxidation products of pure vitamin B₁ are produced at room temperature very slowly by the action of permanganate and manganese oxides at p_H values more acid than p_H 6.0, more rapidly about p_H 7.0. The substances formed are yellow in acid solution. Sulphur is not split off as sulphate but the state of combination of S changes. The vitamin B₁ colour reaction (formaldehyde-azo-reaction) diminishes during oxidation, but not as in the case of alkali-labile S in proportion to the development of the fluorescence.

2. The fluorescent oxidation products show biological activity even in absence of the normal colour reaction; hence other forms of vitamin B₁ exist than the one at present isolated.

3. The sulphur is split off as H₂S from vitamin B₁ by a treatment with hot alkali which leaves the nitrogen intact.

4. Pure vitamin B₁ reacts very slowly if at all with nitrous acid.

5. These changes are discussed in relation to the chemical constitution and biological activity.

We are indebted to the Medical Research Council for grants in aid of the research and for personal grants to two of us, also to R. W. Wakelin for skilful assistance.

REFERENCES.

- Barger, Bengel and Todd (1935). *Nature*, **136**, 259.
 Bence-Jones (1866). *Chem. News*, **13**, 197.
 Birch and Harris (1930). *Biochem. J.* **24**, 564.
 ——— (1935). *Nature*, **135**, 654.
 Euler and Adler (1934). *Z. physiol. Chem.* **228**, 1.
 Freundenberg and Wegman (1935). *Z. physiol. Chem.* **233**, 159.
 Holiday (1935). *Biochem. J.* **29**, 719.
 Hunter (1930). *J. Chem. Soc.* 2343.
 Johnson and Clapp (1908). *J. Biol. Chem.* **5**, 163.
 Karrer, Salomon, Schopp, Schlitter and Fritzsche (1934). *Helv. Chim. Acta*, **17**, 1010.
 Kinnersley, O'Brien and Peters (1935). *Biochem. J.* **29**, 701.
 ——— and Peters (1928). *Biochem. J.* **22**, 419.
 ——— (1934). *Biochem. J.* **28**, 667.
 ——— and Squires (1925). *Biochem. J.* **19**, 404.
 Koessler and Hanke (1922). *J. Biol. Chem.* **50**, 235.
 Kuhn and Bär (1934). *Ber. deutsch. chem. Ges.* **67**, 898.
 ——— György and Wagner-Jauregg (1933). *Ber. deutsch. chem. Ges.* **66**, 317.
 ——— Wagner-Jauregg, van Klaveren and Vetter (1935). *Z. physiol. Chem.* **234**, 196.
 Levene and Simms (1926). *J. Biol. Chem.* **70**, 229.
 Moggridge and Ogston (1935). *Biochem. J.* **29**, 866.
 Passmore, Peters and Sinclair (1933). *Biochem. J.* **27**, 843.
 Peters (1930). *Trans. Far. Soc.* **26**, 797.
 ——— (1935). *Nature*, **135**, 107.
 ——— and Philpot (1933). *Proc. Roy. Soc. Lond. B* **113**, 48.
 Rangier (1935). *Bull. Soc. Chim. Biol.* **17**, 502.
 Richardson (1933). *Biochem. J.* **27**, 1036.
 Robinson and Tomlinson (1935). *J. Chem. Soc.* 467.
 Rosenheim (1927). *Biochem. J.* **21**, 1335.
 Schopfer (1934). *Arch. Mikrobiol.* **5**, 549.
 Smakula (1935). *Z. physiol. Chem.* **230**, 231.
 Stern and Holiday (1934). *Ber. deutsch. chem. Ges.* **67**, 1104, 1352, 1442.
 Tomlinson (1935). *J. Chem. Soc.* 1030.
 van Veen (1933). *Z. physiol. Chem.* **208**, 125.
 Warburg and Christian (1933). *Biochem. Z.* **266**, 377.
 Williams (1935). *J. Amer. Chem. Soc.* **57**, 229.
 ——— Buchmann and Ruehle (1935). *J. Amer. Chem. Soc.* **57**, 1093.
 ——— and Gurin, unpublished observation, quoted by Williams *et al.* (1935).
 Windaus, Tschesche and Crewe (1934). *Z. physiol. Chem.* **228**, 27.
 ——— and Ruhkopf (1932). *Nachr. Ges. Wissen. Göttingen*, **111**, 343.
 Wintersteiner, Williams and Ruehle (1935). *J. Amer. Chem. Soc.* **57**, 517.

Note added September 29th, 1935. Further work shows that large amounts of CHCl_3 extract the blue fluorescent substance from aqueous solution and that the fluorescence of extracts in some cases exceeds the previous [maximum estimate, *i.e.* > 6 times quinine.