XCII. FURTHER DEDUCTIONS AS TO THE CHEMICAL NATURE OF VITAMIN B₁ FROM ULTRA-VIOLET ABSORPTION SPECTRA.

BY FRANCIS FARNHAM HEYROTH AND
JOHN ROBERT LOOFBOUROW.

*From the Basic Science Research Laboratory, University of Cincinnati,
Cincinnati, Ohio.*

(Received December 27th, 1935.)

IN 1932 we presented a comparison of the ultra-violet absorption spectra and biological assay values of a series of the most active vitamin B₁ concentrates then available [Heyroth and Loofbourow, 1932]. That work indicated the vitamin to have an absorption maximum at about 2600 Å. and led us to suggest that it might contain a pyrimidine derivative or one of ergothioneine. Several crystalline preparations have since been made available to us through the kindness of Dr M. I. Smith, of the National Institute of Health, Prof. R. A. Peters, Oxford, Dr A. G. Van Veen, Batavia, Java, and Profs. Suzuki and Ohdake, of Tokyo, some of whom [*e.g.* Peters and Philpot, 1933; Ohdake, 1932] have published data on the ultra-violet absorption of this vitamin. As in our earlier work, we first attempted to correlate the ultra-violet extinction values at various wave-lengths with the biological potencies of the preparations.

The study of these newer preparations indicated for them an absorption curve with two peaks at 2360 and 2650 Å. suggestive of that of cytosine [Heyroth and Loofbourow, 1934, 1], rather than a single peak at 2600 Å. The publication by Peters and Philpot [1933] of a curve with a single peak at 2470 Å. caused us to delay publication of our curves except in a preliminary note [Heyroth and Loofbourow, 1934, 2] until a cause of the discrepancy could be found. With three of the newer preparations we found very satisfactory agreement between absorption in aqueous solutions and biological potency, but in view of the unexpected variations in the absorption of the vitamin, we must regard this as in part the result of coincidence.

Recently Windaus *et al.* [1934] demonstrated the presence of two ring compounds, one a pyrimidine, in the vitamin. The other has been identified by Clarke and Gurin [1935] as a thiazole derivative. Recent spectrographic studies by Williams *et al.* [1935] on the pyrimidine and by Ruehle [1935] on the thiazole component afford a basis for a reconciliation of our curves for the vitamin (which have been confirmed by Wintersteiner *et al.* [1935]) with those of Peters and Philpot [1933] and Holiday [1935]. We therefore present, in leaving this field, our absorption curves and analyse them in terms of the separate contributions made, under varying conditions, by each of the two rings of the vitamin.

Absorption curves of crystalline vitamin B₁ of various workers.

The first crystalline samples made available to us (April, 1933) were three picrolonates of the vitamin isolated by Dr A. Seidell [Seidell and Smith, 1933] and furnished through the courtesy of Dr M. I. Smith, who also provided us with a solution which he had assayed of the vitamin hydrochloride, prepared

in the laboratory of Prof. Peters. It was thought to have been his preparation 53:A14.

From the absorptions of these picrolonate solutions it was necessary to deduct the absorptions of the amounts of picrolonic acid assumed from the biological potencies of the solutions to be present. The resulting curves for the absorption of the vitamin differed from the curve previously published by Windaus *et al.* [1932] in exhibiting two well-defined absorption bands with maxima at about 2350–2400 Å. and 2650–2700 Å. separated by a minimum at about 2550 Å. A certain measure of correlation between extinction and biological activity was found over the range 2600–2800 Å. The result indicated that the wave-length (2600 Å.) at which we had previously found the closest correlation does not correspond exactly with that of one of the maxima of the more highly purified vitamin.

The hydrochloride preparation of Prof. Peters in general confirmed this new type of absorption curve (Fig. 1, D). In addition to the maximum at 2650 Å. there is a stronger one situated at 2330 Å. This curve differed from the curves obtained from the picrolonate solutions only in having a more marked minimum.

Shortly after we had obtained this curve, Peters and Philpot [1933] presented absorption curves of a number of samples which differed both from that reported by Windaus *et al.* [1932] and from that which we had just found in having one somewhat asymmetrical band with its maximum between 2450 and 2500 Å. To aid in elucidating the discrepancy, Prof. Peters kindly sent us a solution of his sample 64:19 in alcohol about $N/200$ in hydrochloric acid. Repeated determinations of the absorption of this sample diluted with about 25 to 50 volumes of either water or alcohol showed that the solvent was not responsible for the discrepancy (Fig. 1). Van Veen's preparation gave the same curve with water as with alcohol as solvent (Fig. 2). This curve agreed in general with the more recent ones of Wintersteiner *et al.* [1935], in both solvents.

Vitamin B₁ hydrochlorides.

Fig. 1. Preparations of Peters: A — 64:19 in water; B — 64:19 in alcohol; C — 64:19 in water; D — 53:A14 in water.

Fig. 2. Preparations of Van Veen: A — in water; B — in alcohol.

Fig. 3. A — Average of two yeast oryzanin curves; B — Average of two rice oryzanin curves; C — Preparation 64:19 of Peters in water.

Fig. 1 indicates also a lack of reproducibility of the absorption curves in water; our curve in alcohol also differs from that of Holiday [1935], who has attributed the variations to changes in p_H between 5.5 and 7.0. It is unlikely,

however, that the vitamin undergoes tautomeric change with alteration of p_H since the curves of Peters and Philpot [1933] under extremely varied conditions of acidity and alkalinity failed to exhibit the two-banded type of absorption.

Holiday [1935] also thought that when dissolved in acid alcohol various samples of the vitamin, including recent ones of Windaus and Van Veen, gave absorption of the Peters and Philpot type with considerable uniformity. Before the appearance of his paper we had also found a similar uniformity in the two-banded absorption of aqueous and alcoholic solutions of preparations by Ohdake (Fig. 3, A and B) and Van Veen (Fig. 2), which agreed with the curves of Seidell's samples and with those of Peters (Fig. 3, C) under certain conditions, as well as with those later reported by Wintersteiner *et al.* [1935]. In 1934 we published a note [Heyroth and Loofbourow, 1934, 2] comparing the absorptions of Ohdake's preparations (Fig. 3, A and B) from rice polishings and from yeast with that of one curve of Peters 64:19 (Fig. 3, C). The rat day doses of these three preparations were found to be [Heyroth and Loofbourow, 1936] of the order of 100, 85, 91 for the (64:19) yeast and rice preparations, respectively. The molecular extinction values at 2600 Å. were found to be 7400, 5700, 6200, in very close agreement with this ratio of potencies. However, had we chosen one of the other curves for Peters's preparation (Fig. 1), the agreement would have been less apparent. It is obvious that the variability of the absorption of the vitamin greatly lessens the value of any correlation of potency and absorption of this material, unless the comparison be restricted to curves of the same form.

The limitations of biological assay render the slight variations in potency of crystalline preparations, as at present prepared, somewhat uncertain, and this, together with the variations in their absorption, makes any agreement between potency and absorption appear fortuitous. The general agreement that the crystals obtained by most workers represent the very nearly pure vitamin, moreover, makes unnecessary the continuation of such studies.

Interpretations of the two types of vitamin B₁ ultra-violet absorption curves.

In a communication to Prof. Peters cited by Holiday [1935], Windaus has stated that his more recent preparations yield curves of the Peters-Philpot type. It remains now to correlate the recent work on the constitution of the vitamin with its ability to exhibit either one-banded absorption as found by Peters and Philpot or the two-banded curve first observed by us.

After cleavage of the vitamin by the action of sodium sulphite, Williams *et al.* [1935] isolated two products, one of which they believed to be the sulphonic acid of an ethyl- (or dimethyl-) aminopyrimidine. After deamination it yielded the sulphonic acid of an ethyl- (or dimethyl-) oxypyrimidine.

Very recently, Clarke and Gurin [1935] have identified the sulphur-containing ring as 4-methyl-5- β -hydroxyethylthiazole and Williams [1935] views the vitamin as a quaternary compound of the thiazole with a chloride of the aminopyrimidine. Ruehle's curves [1935] for the absorptions of the free thiazole and its quaternary methiodide and those for the pyrimidine [Williams *et al.* 1935] now afford a basis for making tentative pyrimidine-thiazole summation curves, which indicate a probable explanation of the variations in absorption exhibited by the vitamin.

Two processes require consideration, a deamination of the vitamin, analogous to that demonstrated by Williams *et al.* [1935] for the pyrimidine component, and the breaking of the quaternary linking resulting in a dissociation of the vitamin into its components, a change shown by Williams to occur in the

presence of sodium sulphite which forms a relatively insoluble pyrimidine sulphonic acid.

To prepare summation curves covering various stages of these two possibilities, there are required the absorptions of the tertiary and quaternary forms of the thiazole and those of the free and bound oxy- and amino-pyrimidines, six curves in all.

The absorption of the tertiary thiazole has been given by Ruehle [1935]; his curve for the thiazole methiodide may be employed as an approximation to the contribution to the vitamin absorption made by the thiazole when in quaternary combination with the pyrimidine.

The vitamin curves of Peters and Philpot [1933] and of Holiday [1935] in acid alcohol agree in the location of their maxima with that of the absorption of the sulphonic acid of the aminopyrimidine, and may be considered as summations of the absorptions of the quaternary thiazole with that of the aminopyrimidine when bound to thiazole. Deduction of the curve of the thiazole methiodide from

Fig. 4. Curves used in constructing Figs. 7 and 8. A — Absorption of the aminopyrimidine when bound to thiazole. B — Absorption of the oxypyrimidine when bound to thiazole. C — Absorption of free aminopyrimidine. D — Smakula's curve for Windaus's ethoxypyrimidine replotted with molecular extinction coefficients as ordinates. E — Sulphonic acid of the aminopyrimidine. Curve of Williams *et al.* and Ruehle.

Fig. 5. Hypothetical curves of various stages of dissociation of the aminovitamin. A — 10%, B — 30%, C — 50%, D — 70%, E — 90%, dissociated.

the Holiday curve gives an approximation to the absorption of the aminopyrimidine as it may be modified by linking with thiazole. It resembles (Fig. 4, A) the absorption of the aminopyrimidinesulphonic acid (Fig. 4, E) with greatly increased extinction values.

Smakula [1934] has given the absorption of a "hydrolysis product" of the vitamin, the analysis of which agrees with the result of deamination of the

vitamin (Windaus formula). His curve may be presumed to represent the summation of the absorptions of the quaternary thiazole and of the oxypyrimidine when bound to the thiazole. Deduction of the absorption of the thiazole methiodide gives the curve of the bound oxypyrimidine (Fig. 4, B).

In obtaining these curves, it has been assumed that in each case the vitamin curves represented the undissociated complex.

There is no assurance that the absorptions of the amino- and oxy-pyrimidines are similar to those of their sulphonic acids. For the free oxypyrimidine we have employed, for reasons stated in the next section, the curve (Fig. 4, D) given by Smakula [1934] for a pyrimidine obtained by Windaus *et al.* [1934] by oxidative cleavage of the vitamin with nitric acid. To obtain the absorption of the free aminopyrimidine (Fig. 4, C), we deducted the absorption of tertiary thiazole from the Holiday curve for the vitamin in neutral alcohol on the assumption that this curve, which has very low extinction values, represents the vitamin after complete dissociation into its aminopyrimidine and thiazole components. The assumption that the pyrimidine has suffered little if any deamination is based upon the observation of Holiday that on acidifying a solution of the vitamin in neutral alcohol, its absorption increased and tended towards the form of curve exhibited when the vitamin is originally dissolved in acid alcohol. As the addition of acid could not introduce an amino-group, it is evident that the pyrimidine in the Holiday neutral alcohol curve could not have been an oxypyrimidine. The addition of acid apparently leads to the recombination of the dissociated components into a quaternary thiazole.

Two series of hypothetical absorption curves of various proportions of free and bound aminopyrimidine and of free and bound oxypyrimidine were prepared. Summations of the absorption due to various ratios of quaternary and tertiary thiazole were then made with the corresponding proportions of free and bound pyrimidines of each type, yielding two families of curves indicative of the effect of dissociation of the pyrimidine-thiazole complex, one for the amino- and the other for the oxy-pyrimidine (Figs. 5 and 6). At each level of dissociation it should be possible to construct a series of curves showing the effect of deamination. In preparing these summation curves, errors are introduced at wave-lengths shorter than about 2400 Å. because the effects of negative absorptions or apparent fluorescences of the bound forms of the pyrimidines have been neglected.

We have compared several vitamin absorption curves with the most similar of these hypothetical curves in Figs. 7 and 8 of which the legends are self-explanatory. Obviously, the Holiday acid alcohol curves, the Holiday neutral alcohol curve and that of the Smakula deamination product are also accounted for, since the hypothetical curves were derived from these. No quantitative significance is to be attached to the figures stated for the extent to which each of the processes has occurred in the cases cited; they are to be regarded merely as roughly indicative of the changes which may have occurred in the very dilute solutions employed for the measurements. In all these comparisons the disagreements are similar in type. The hypothetical curves usually have too little absorption in the short-wave region and too much in the longer. The error is probably chiefly the result of the assumption of too small a contribution from quaternary thiazole. The agreement appears close enough to indicate that the two assumed reactions (reversible dissociation of the quaternary thiazole and deamination of the pyrimidine component) are sufficient to account for the diversity of curves published for the vitamin.

From these considerations it appears that the most nearly correct curves for the absorption of the aminopyrimidine-thiazole compound are those of Peters

and Philpot [1933] or of Holiday [1935] in acid alcohol. When the compound is largely dissociated into its components the absorption assumes the two-banded form obtained by Holiday in neutral alcohol. Subsequent acidification increases the absorption by inducing a recombination. When dissociation is relatively

Fig. 6. Hypothetical absorption curves for the deaminated vitamin in various stages of dissociation. — 10%, — — — 30%, - - - - 50%, - · - · - 70%, · · · · · 90%, dissociated.

Comparisons of observed and hypothetical absorption curves.

Fig. 7. — Vitamin B₁ dissolved in neutral alcohol and re-acidified (curve of Holiday). - - - - Hypothetical curve for amino-vitamin 50% dissociated. — — — Vitamin B₁ (curve of Smakula). - · - · - Hypothetical curve of undissociated vitamin, 50% deaminated.

Fig. 8. — Vitamin B₁ curve of Wintersteiner *et al.* - - - - Hypothetical curve for the oxy-pyrimidinethiazole complex, 30% dissociated. — — — Vitamin B₁ prepared by Van Veen. - · - · - Hypothetical curve for vitamin 70% dissociated and 50% deaminated.

slight and accompanied by some deamination, curves of the Smakula [1934] form result. Further dissociation, accompanied probably by variable amounts of deamination, yields curves of the type obtained in neutral alcohol or water by Wintersteiner *et al.* [1935] for their product, and by ourselves for samples obtained from Prof. Peters, from Dr Van Veen and from Profs. Suzuki and Ohdake.

The pyrimidine component and its absorption.

If the free vitamin base corresponding to the hydrochloride is formed in a manner analogous to the thiazole methiodide, the parent pyrimidine according to Williams's formula is 5-hydroxy-6-ethyl-4-aminopyrimidine. The assumption of a hydroxy-group in the parent pyrimidine, rather than the very unlikely chlorine, would resolve the most serious discrepancy between the empirical formulae of Williams's pyrimidine (analysed as sulphonic acid) and the pyrimidine, C₇H₁₀O₂N₂ of Windaus *et al.* [1934]. The presence of but two nitrogen atoms in the latter indicates deamination of the original pyrimidine to have occurred during the action of nitric acid on the vitamin.

Restoration of the amino-group and saponification of the ethoxyl group, which was introduced during the isolation, would make the formula for the original pyrimidine C₅H₇ON₃, which differs from the pyrimidine of the Williams formula only in lacking a methyl group. During the action of nitric acid this might have been removed by oxidation and loss of carbon dioxide.

Esterification of the carboxyl formed by oxidation of the methyl group can be eliminated as it would yield a product containing more oxygen than Windaus found. The hydroxyl introduced by deamination would in the acid solution be expected to undergo a lactim-lactam rearrangement so that it would not be available for replacement by ethoxyl. It is therefore likely that the esterification in Windaus's pyrimidine occurred on the hydroxyl initially present on the carbon atom which becomes linked to thiazole in the vitamin.

The absorption of Windaus's ethoxy-derivative of the oxypyrimidine gives some information as to the allocation of this hydroxyl group. If the ethoxyl occurred in the 2-position, the saponified product would be a methylthymine, and both it and its ethoxyl derivative should exhibit one absorption band [Heyroth and Loofbrourow, 1934, 1; Austin, 1934]. As the Windaus product showed two bands, a 2-ethoxyl structure appears unlikely.

The absorption of the free oxypyrimidine cannot be determined from the vitamin curves. When bound to thiazole, its absorption (Fig. 4 B) differs from that of its sulphonic acid in lacking the short-wave band. This is understandable in view of the possibility of a lactam-lactim rearrangement. It was therefore necessary to choose for the curve to represent the free oxypyrimidine in the comparisons of the preceding section between a one-banded and a two-banded curve.

Actually, two sets of the summation curves were prepared, using for the absorption of the free oxypyrimidine as a one-banded curve the absorption of thymine, and as a two-banded curve, that of the Windaus ethoxy-derivative. The curves based upon the former failed to give agreement with the observed vitamin curves. This affords further support for the view that position 2 is not the site of the hydroxyl group in the free pyrimidine, and leaves only positions 5 or 6 available for it. It is interesting to note here that 5-hydroxypyrimidine derivatives, *e.g.* isobarbituric acid, are easily oxidised to dipyrimidines, fluorescent in alkaline solution. This may point to a mechanism for the production of fluorescent compounds on oxidation of the vitamin [Peters, 1935].

SUMMARY.

1. Holiday's observation of variations in the ultra-violet absorption spectrum of vitamin B₁ prepared by Peters has been confirmed.

2. Samples of the vitamin prepared by Seidell, Suzuki and Ohdake, and Van Veen have in aqueous solutions ultra-violet absorptions similar to those reported by Wintersteiner, Williams and Ruehle.

3. A correlation between biological activity and ultra-violet absorption has been demonstrated for three crystalline preparations, but because of the ease with which the absorption is altered must be considered merely as a coincidence.

4. The diversity of ultra-violet absorption spectra reported for the vitamin is attributed to two independent processes occurring to varied extents: (a) reversible dissociation of the vitamin into an aminopyrimidine derivative and a thiazole derivative and (b) deamination of the aminopyrimidine.

5. The curve presented by Peters and Philpot for acid alcohol solutions most nearly represents the vitamin. Holiday's curves in neutral alcohol represent the result of the breaking of the quaternary linkages of the thiazole. Other curves represent intermediate stages, accompanied by some deamination of the pyrimidine.

6. A consideration of the possible structures indicated by the analysis of the pyrimidines isolated from the vitamin by Windaus and by Williams leads to the

conclusion that the pyrimidine component of the vitamin has one amino, one hydroxyl and two methyl (or one ethyl) groups as substituents. The spectrographic evidence suggests that the hydroxyl group is probably not in the 2-position.

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