

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF PHYSICIANS AND SURGEONS, AND DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, TEACHERS COLLEGE, BOTH OF COLUMBIA UNIVERSITY]

## Studies of Crystalline Vitamin B<sub>1</sub>.<sup>1</sup> II. Elementary Composition and Ultraviolet Absorption

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In spite of the fact that salts of vitamin B<sub>1</sub> have been obtained in crystalline condition and analyzed by a number of investigators, there is still a lack of unanimity as to its elementary composition. This may be partly explainable by certain analytical difficulties which the substance seems to offer, especially in regard to the correct estimation of the nitrogen content. On the other hand, since in the absence of suitable physical criteria the purity of the vitamin hydrochloride is difficult to ascertain, the presence of varying amounts of impurities might account for the discrepancies in the analytical findings. We have now analyzed a number of carefully purified crystalline preparations of the vitamin hydrochloride. The crude crystalline material has been made available in comparatively large amounts by the method of Williams, Waterman and Keresztesy.<sup>2</sup> This material was recrystallized several times by dissolving it in a minimum amount of water and adding hot absolute alcohol to the solution to make the final alcohol concentration 95%. Later it was found advantageous, in a series of recrystallizations, to decrease the ultimate alcohol concentration from 95 to 93 and finally 85%. This procedure entails, of course, a greater loss of material in the mother liquors but leads to colorless preparations of an apparently greater degree of purity but without detectable change in curative potency. In some cases the vitamin was also recrystallized by addition of petroleum ether to its solution in 95%, or stronger, alcohol. The crystalline preparations were dried for analysis to constant weight over calcium chloride in a partial vacuum at 55°.

In Table I we give the results of our microanalyses (Pregl) of five different preparations obtained by recrystallization by the methods outlined above.

### Discussion

The water content of the air-dried crystals of the vitamin hydrochloride showed no constancy

(1) The first paper in this series by Williams, Waterman and Keresztesy, "Larger Yields of Crystalline Antineuritic Vitamin," appeared in THIS JOURNAL, 56, 1187 (1934).

(2) R. R. Williams, R. E. Waterman, and J. C. Keresztesy, *ibid.*, 56, 1187 (1934).

with different preparations, and in the same preparation it apparently varies with changes in atmospheric conditions. Determinations of moisture were made by drying to constant weight at 55° *in vacuo* over calcium chloride. The highest figure obtained was 4.66%, representing a state of equilibrium with a humid summer atmosphere. Standing over calcium chloride at room temperature reduced the water content in one case to 0.55%. A certain degree of opacity develops on drying which may indicate that at least a part of the water is present in form of water of crystallization. The samples after drying are slightly hygroscopic and take up varying amounts of water when allowed to stand in the open.

The values for the carbon content show satisfactory agreement in all cases. The average figure, 42.75, is higher than those reported by all other investigators. The hydrogen figures average 5.35% if the last two analyses in the table, which were performed on a very hot and humid summer day, are discarded.

The determination of the nitrogen content, however, seems to offer difficulties. The combustion method of Dumas yielded figures in the neighborhood of 16%. These values are probably somewhat too low. Windaus, Tschesche and Ruhkopf<sup>3</sup> remark on the difficulties in obtaining correct nitrogen figures. The Kjeldahl method was therefore tried, but the variability under apparently identical conditions during digestion was very great. Digestion in the presence of about 100 mg. of glucose invariably leads to values which are obviously much too low, probably due to the loss of sulfuric acid by reduction. Higher figures were generally obtained by treatment of the sample with hydriodic acid in the bomb tube at 300° prior to digestion, following the method of Friedrich,<sup>4</sup> but here also the agreement of the results left much to be desired. It was found finally that by prolonged digestion with twice the volume of sulfuric acid customary in the Pregl method, more consistent figures, at least

(3) A. Windaus, R. Tschesche and H. Ruhkopf, *Nachr. v. der Ges. der Wiss. Göttingen III*, Chemie No. 22, 342 (1932).

(4) A. Friedrich, *Z. physiol. Chem.*, 216, 68 (1933).

TABLE I  
RESULTS OF MICROANALYSES  
(K) indicates Kjeldahl method; (D) Dumas method

Preparation	C	H	N	S	Cl
A. Large colorless needles, recrystallized three times from alcohol-petroleum ether; somewhat sticky; from 180-mg. batch	42.77 42.86	5.31 5.37	16.07 (D) 16.00 (D) 16.41 (K) 16.22 (K)	9.87 9.97	20.79
B. Long colorless needles, from mother liquor of A; sticky				10.07	
VD-4. Large plates, slightly colored; recrystallized four times from 95% alcohol	42.74	5.47	17.00 (K) <sup>a</sup> 15.87 (K)	10.00	20.90 <sup>b</sup> 21.12
VD-3a-1. Small colorless needles, from mother liquor from third recrystallization of VD by addition of petroleum ether; then recrystallized once from alcohol-petroleum ether	42.66	5.32	16.30 (K) <sup>a</sup>	9.33 9.88	20.90
VD-3a-2. Small colorless needles, from mother liquors from third and fourth recrystallizations of 500 mg. batch + petroleum ether	42.80	5.27	15.76 (K) <sup>a</sup> 16.06 (K)	9.27 9.67	20.88 20.79
W. Colorless plates, recrystallized from 95, 93, 85% alcohol	42.60 42.83 <sup>c</sup>	5.93 <sup>d</sup> 5.95 <sup>c,d</sup>	15.83 (D) 16.02 (K) <sup>a</sup> 16.42 (K) <sup>a</sup> 16.31 (K) 16.55 (K)	9.52 9.53	20.78

<sup>a</sup> Treated with hydriodic acid before digestion. <sup>b</sup> Chloride precipitated directly from solution, not by combustion. Dried at room temperature over phosphorus pentoxide at 3 mm. <sup>d</sup> High values due to atmospheric conditions.

TABLE II

Calcd. for	C	H	N	S	Cl
C <sub>12</sub> H <sub>18</sub> ON <sub>4</sub> S·2HCl	42.71	5.38	16.62	9.51	21.03
C <sub>12</sub> H <sub>18</sub> ON <sub>4</sub> S·2HCl	42.46	5.94	16.52	9.45	20.91
C <sub>12</sub> H <sub>18</sub> O <sub>2</sub> N <sub>4</sub> S·2HCl (Ohdake)	40.78	5.14	15.87	9.08	20.08 <sup>5</sup>
C <sub>12</sub> H <sub>18</sub> O <sub>2</sub> N <sub>4</sub> S·2HCl (Jansen)	40.55	5.68	15.78	9.03	19.97 <sup>6</sup>
C <sub>12</sub> H <sub>20</sub> O <sub>2</sub> N <sub>4</sub> S·2HCl (Van Veen)	40.32	6.21	15.69	8.98	19.85 <sup>7</sup>
Found (av.)	42.75	5.35	16.26 (K)	9.51	20.88

on the same sample, could be obtained. Only the latter and those obtained after treatment with hydriodic acid are included in Table I. The average value of all the Kjeldahl determinations recorded there is 16.26%.

Comparatively large variations, exceeding the precision of the method, were obtained with the sulfur determinations. The average of ten determinations is 9.51%. High values close to 10% were given by samples A and B. These samples were obtained from crude crystalline material that was more difficult to purify than the subsequent batches. However, for preparation VD-4 which also gave a high value, a better starting material was used. This sample had been recrystallized four times from 95% alcohol.

The analytical data so far accumulated conform best with the composition C<sub>12</sub>H<sub>18</sub>ON<sub>4</sub>S·2HCl (Table II). This formula is in agreement with the one adopted for the base by Windaus, Tsche-

sche and Ruhkopf.<sup>3</sup> It must, however, be pointed out that this formula was calculated from the analysis of the picrolonate rather than of the vitamin hydrochloride. On analysis of the hydrochloride obtained by decomposition of the picrolonate, the above authors found figures agreeing with the formula C<sub>12</sub>H<sub>18</sub>O<sub>2</sub>N<sub>4</sub>S·2HCl, which differs from the above formula by one H<sub>2</sub>O. Also the rufanate prepared by Windaus analyzed in agreement with the formula C<sub>12</sub>H<sub>18</sub>O<sub>2</sub>N<sub>4</sub>S for the base. To explain these findings, Windaus and his collaborators assumed that the vitamin hydrochloride as well as the rufanate contain one molecule of water of crystallization, absent in the picrolonate. They dried the vitamin hydrochloride for analysis *in vacuo* over phosphorus pentoxide, presumably at room temperature. The carbon content of such preparations was found to be 40.42 on the average. Under the same conditions of drying, however, we obtained a carbon value of 42.83, which agrees very well with those of all our other analyses. It should

(5) S. Ohdake, *Proc. Imp. Acad. Tokyo*, **10**, 95 (1934).

(6) B. C. P. Jansen, *Rec. trav. chim.*, **52**, 366 (1933).

(7) A. G. Van Veen, *Z. physiol. Chem.*, **208**, 125 (1932).

also be mentioned here that Ohdake, who dried his preparation (oryzanin from yeast) for thirty minutes at 160–170°, found that no water was given off under these conditions, but in spite of this he also obtained lower values than ours for carbon (41.15, 41.35). This discrepancy in the findings regarding the molecule of water has yet to be explained.

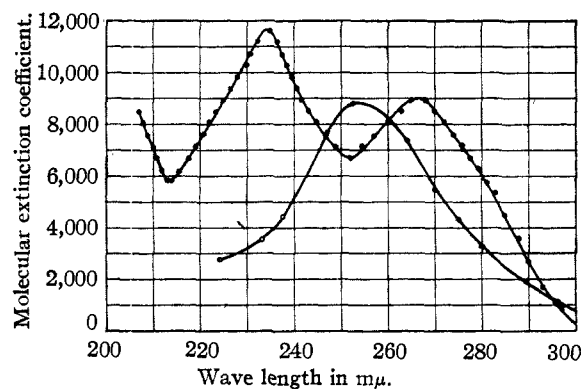


Fig. 1.—Ultraviolet absorption of vitamin: ●, sample VD-3a-2 in water; ○, Windaus' results in alcohol.

Of all the workers in the field only Kinnersley, O'Brien and Peters<sup>8</sup> report analytical figures which come close to ours. Their preparations were dried *in vacuo* at 80°. The values found average C, 42.2; H, 5.7; N, 14.9; S, 9.5; Cl, 20.9. The authors do not claim purity for their crystals and refrain from calculating a formula from their analyses. They also report a lower melting point for the hydrochloride ranging as low as 221° as compared with 250° observed by other workers including ourselves.

**Ultraviolet Absorption.**—The absorption of vitamin B<sub>1</sub> in the ultraviolet has recently been studied by Ohdake,<sup>9</sup> by Windaus, Tschesche, Ruhkopf, Laquer and Schultz,<sup>10</sup> by Peters and Philpot,<sup>11</sup> and by Heyroth and Loofbourow,<sup>12</sup> but without entire agreement. Windaus *et al.* reported a single absorption band at 260 mμ; Peters and Philpot a single intense band at 245. Both these groups of workers used alcoholic solutions. On the other hand Ohdake found two bands at 239 and 270, respectively, in a water solution. Heyroth and Loofbourow in their most

(8) W. K. Kinnersley, J. R. O'Brien and R. A. Peters, *Biochem. J.*, **27**, 232 (1933).

(9) S. Ohdake, *Bull. Agri. Chem. Soc. Japan*, **8**, 113 (1932).

(10) A. Windaus, R. Tschesche, H. Ruhkopf, F. Laquer and F. Schultz, *Z. physiol. Chem.*, **204**, 123 (1932).

(11) R. A. Peters and J. St. L. Philpot, *Proc. Roy. Soc. (London)*, **B113**, 48 (1933).

(12) F. F. Heyroth and J. R. Loofbourow, *Nature*, **134**, 461 (1934); *Bull. Bas. Sci. Res.*, **4**, 35 (1932).

recent report also show two bands, at 235 mμ and 265 mμ, respectively, for water solutions of two samples furnished them by Ohdake as well as of a sample furnished by Peters. However, the curve for Peters' material has a shallower valley between the peaks than the Ohdake preparations.

Our experience which is illustrated by Fig. 1 is that two bands are present at 235 mμ and 267 mμ, respectively, whether the solvent is alcohol or water. With ultraviolet sensitized plates or with long exposures of ordinary plates, the second band appears with alcoholic solutions and the presence of the beginnings of a third band in the 200 mμ region is indicated when observations are made in water solution. Our curves were obtained with a Hilger E316 spectrograph and a Hilger rotating sector. The source used was a high tension spark between electrodes of tool steel. The cells were of the Hilger B type with quartz end plates.

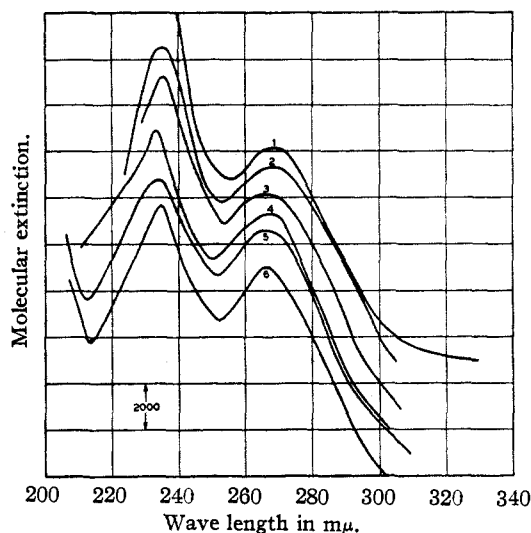


Fig. 2.—Consistency of absorption results. Each successive curve is displaced vertically by 1000 units to avoid confusion of lines.

Curve	Prep. no.	Solvent
1	VD-3a-2	Alcohol
2	W	Alcohol
3	VD-4	95% Alcohol
4	VD-4	Water
5	W	Water
6	VD-3a-2	Water

The consistency of our results obtained with different lots of our crystalline material in both solvents may be judged by comparison of the curves in Fig. 2. The absorption which we observe is also fairly consistent with the results of Ohdake and of Heyroth and Loofbourow. Discrepancies with those of other workers may be due in part to compositional differences and in part to

differences in method of observing absorption. Below 250  $m\mu$  discrepancies have been observed for other materials<sup>13</sup> between spectra obtained by different methods in common use. Considering the possibility that the discrepancy in Windaus' observed absorption<sup>14</sup> may be due to technique and in view of the fact that Windaus' deductions from later analyses agree with our own, we attach little weight to Heyroth and Loofbourow's suggestion that Windaus' preparation differs from others in respect to the pyrimidine nucleus present.

It should be noted that the absorption for Peters' material as published by Heyroth and Loofbourow differs somewhat from that of all preparations by other workers and also that the absorption which Peters and Philpot report is radically different from Heyroth and Loofbou-

(13) E. C. C. Baly, R. A. Morton and R. W. Riding, *Proc. Roy. Soc. (London)*, **A113**, 709 (1927); H. Von Halban and J. Eisenbrand, *ibid.*, **A116**, 153 (1927).

(14) Very recently A. Smakula reports an error in Windaus' absorption results and finds two bands in his material, *Z. physiol. Chem.*, **230**, 231 (1934).

row's curve on material furnished them by Peters. In this connection it should be pointed out that Peters' material also differs from that of all other workers in that its melting point is much lower and more variable<sup>4</sup> so that Peters' substance should be regarded as different in some respect from that of others.

We owe our thanks to Mr. F. Rosebury for some of the spectrograms and to Mr. W. Saschek for many of the analyses. We also wish to acknowledge the financial aid of the Carnegie Corporation of New York through the Carnegie Institution of Washington.

### Summary

1. Analyses of the vitamin hydrochloride agree best with the formula  $C_{12}H_{16}N_4OS$ , as calculated for the hypothetical free base.

2. Ultraviolet absorption occurs in two bands at 235  $m\mu$  and 267  $m\mu$ , respectively.

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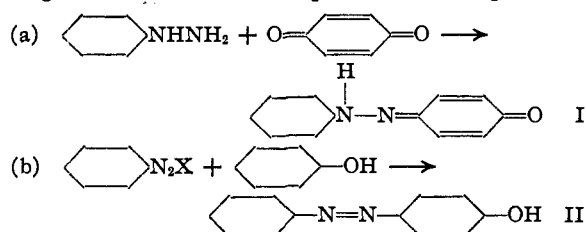
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[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY OF THE UNIVERSITY OF MINNESOTA]

## The Structure of Hydroxyazo Compounds<sup>1</sup>

BY WALTER M. LAUER AND SIDNEY E. MILLER

There are two general methods for the preparation of hydroxyazo compounds: (a) the action of phenylhydrazines on quinones and (b) the coupling of diazonium compounds with phenols.



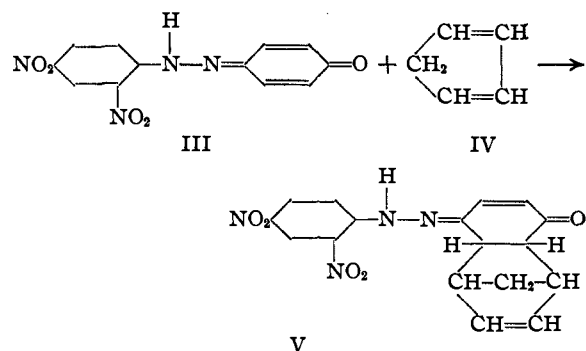
These two reactions yield the same product, so that the problem of structure immediately presents itself. This question has concerned a number of investigators<sup>2</sup> and certain relevant

(1) From a thesis submitted by Sidney E. Miller to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the Ph.D. degree, July, 1934.

(2) In addition to references cited later on in this paper, see: Meldola *et al.*, *J. Chem. Soc.*, **55**, 114 (1889); **59**, 710 (1891); **63**, 923, 930 (1893); **65**, 835 (1894); Goldschmidt *et al.*, *Ber.*, **23**, 487 (1890); **33**, 1098 (1905); C. Smith and Mitchell, *J. Chem. Soc.*, **93**, 843 (1908); v. Auwers *et al.*, *Ber.*, **33**, 1923 (1900); **40**, 2154 (1907); **41**, 403, 420 (1908); **47**, 1297 (1914); *Ann.*, **359**, 336 (1908); **360**, 1, 11 (1908); **365**, 291 (1909); **369**, 209 (1909); **373**, 210 (1910); **487**, 79 (1931); **505**, 283 (1933); Burawoy and Markowitsch, *Ann.*, **503**, 180 (1933); **504**, 71 (1933); Burawoy, *ibid.*, **509**, 60 (1934).

methods of approach will be introduced in the discussion of the results of the present study.

The fruitful "Diene Synthesis" of Diels and Alder<sup>3</sup> suggested a method of detecting quinoidation, for if the hydroxyazo compounds can exist in a tautomeric quinoid form (I) they should add a 1,4 conjugated diene, since a number of such dienes add to quinones. Accordingly, the addition of cyclopentadiene to various hydroxyazo compounds was investigated. It was found that



(3) For an excellent review see Abderhalden's "Handbuch der biologischen Arbeitsmethoden," Abt. I, Teil 2, p. 3079-3192, Urban and Schwarzenberg, Berlin, 1933.