THE ANTINEURITIC VITAMIN

I. THE METHOD OF ASSAY, CONCENTRATION OF THE VITAMIN WITH SILVER UNDER VARIOUS CONDITIONS, AND ITS SOLUBILITY IN CERTAIN ORGANIC SOLVENTS*

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I. Method of Assay Used in These Investigations

In an attempt to repeat the study of Jansen and Donath (1927) on the isolation of antineuritic vitamin B we have made several observations of interest. Satisfactory description of this work necessarily involves the question as to the adequacy of the biological test employed in assaying the many fractions obtained. Inasmuch as there is no unanimity concerning the proper method to use when testing for antineuritic vitamin potency, it is necessary first of all to describe the particular technique which we have used in these investigations.

Plan

The assay in these studies is performed by a combination of weight-maintenance and curative techniques carried out on pigeons subsisting on the dietary régime below. The plan differs from that of Seidell (1917, 1922), Funk and Paton (1922), Kinnersley and Peters (1925), Williams and Waterman (1928), and others in one very important respect; namely, in that polished rice does not constitute the sole diet. It has been known for a considerable period that polished rice is not a complete food; that it is deficient not only with respect to antineuritic vitamin B, but protein, mineral nutrients, and fat-soluble vitamins as well. In view of

* The expenses of this research were defrayed in part by a grant from the Research Fund at Yale University School of Medicine.
this fact it appears logical for the investigator in this field who wishes to use polished rice in his diet to supplement it as well as possible so as to supply a food mixture deficient only with respect to the one variable of interest; namely, antineuritic vitamin B. This we have attempted to do. Our approach to the problem therefore differs from that of the workers mentioned above because these investigators experimented with polished rice supplemented simply by various vitamin B extracts in an endeavor to secure maintenance nutrition in the pigeon. Even Carter, Kinnersley, and Peters (1930, a) in a very recent study fed polished rice supplemented only with such products as cod liver oil and various extracts containing antineuritic vitamin. They concluded that antineuritic vitamin B alone as the supplement for polished rice does not afford maintenance. This may very well be the case. It is important in this connection to note, however, that Jansen and Donath (1926) found that their rice vitamin preparation did not effect weight maintenance in pigeons fed polished rice unless fed along with another substance (or substances?) which could be furnished in their experience by meat powder thoroughly extracted with boiling water. From this it appears obvious that the weight-maintenance technique with the pigeon may be of service in the assay for antineuritic vitamin, provided the birds also receive meat residue, cod liver oil, and a suitable salt mixture as part of their basal ration.

We believe that the inadequacies of a sole diet of polished rice are also revealed by the behavior of birds restricted to this ration for more than about a month. Carter, Kinnersley, and Peters (1930, a) comment on this fact. When, therefore, investigators feed birds for approximately 2 months on polished rice supplemented only by the products under assay, it is not surprising that the birds, although being relieved of their polyneuritic symptoms, fail to regain lost weight, an observation suggesting the existence of new hitherto unappreciated dietary essentials, as Williams and Waterman (1928) report.

The weight-maintenance technique used in the investigation reported in this paper is an extension of earlier studies conducted in this laboratory by Klotz (1926). The appearance of the subtle anorexia for a vitamin B-deficient diet is taken as the first sign that the organism's need for antineuritic vitamin is not being
met. This characteristic loss of the urge to eat is considered to be present in the pigeon subsisting on the dietary régime employed in this assay when the bird fails to ingest voluntarily enough calories to maintain normal weight. The bird is weighed daily except Sunday, and when a consistent steady decline in weight is noted over a period of at least 5 days, it is concluded that the characteristic anorexia has supervened, so that the bird is a proper subject upon which to test a preparation for content of antineuritic vitamin B. Recent researches have shown that the heat-stable vitamin G (or B$_2$) factor in the old undifferentiated vitamin B plays no rôle in maintenance of the urge to eat or the development of this subtle anorexia (Cowgill, Rosenberg, and Rogoff, 1931, a; Burack and Cowgill, 1931; Sherman and Sandels, 1931). The antineuritic vitamin B is undoubtedly the chief, if not the sole agent involved here. We conclude, therefore, that our technique determines the presence of the antineuritic component of the vitamin B complex.

Many workers, notably Kinnersley and Peters (1928), have preferred the curative test when assaying materials for antineuritic vitamin. On the basis of our experience with different species of animals it is difficult to decide when the polyneuritic condition in a given animal is of a severity equal to that of a companion individual. Our objections to the pigeon curative technique as the sole method of assay are essentially those summarized by Smith (1930) and therefore need not be repeated here. We have gained the impression that the curative method, although valuable because of its objective character, is not as well suited as a weight-maintenance technique for detection of moderate or slight differences in potencies of vitamin preparations, such as an investigator desires when endeavoring to isolate the antineuritic factor. This is supported by the report of curative tests of the Jansen-Donath vitamin crystals with the Kinnersley and Peters technique, concerning which Jansen, Kinnersley, Peters, and Reader (1930) remarked, "It is to be noted that there is a rather considerable variation in the pigeon tests, all of which have been here included. The variation is larger than that to which we are accustomed with yeast preparations." Even with these objections in mind there can be no doubt as to the advantage of an objective demonstration of antineuritic vitamin potency, such as a cure represents, over
any indirect method. Therefore, in our assay, tests by the weight-
maintenance method are supplemented by trials on polyneuritic
birds for curative power, and the latter regarded as confirmatory,
and quantitative only in a rough way.

At least three pigeons are used for each assay. The usual pro-
cedure has been to determine by preliminary trials on one or
more birds the approximate daily dose required and then to con-
firm this and determine the required dose more accurately on at
least two other birds. The larger changes in dose made in an en-
deavor to approximate the daily minimum are thus made on one
pigeon, and slight changes on the confirmatory birds. This re-
results in considerable saving of time when numerous fractions are
at hand to be tested. The results are expressed in terms of the
pigeon unit, which may be defined as the amount required to main-
tain the body weight constant over a period of from 10 to 14 days in
the case of a 300 gm. pigeon fed according to the method described in
this paper. When the weight of a given bird differs markedly
from 300 gm, the measured dose is corrected by a calculation based
on the relationship between body weight and vitamin minimum
discovered by Cowgill and Klotz (1927); namely, vitamin = \(K\)
weight\(^5\) (see Table II) in which \(K\) is merely an equating constant.
The agreement of assays on several birds of approximately 300 gm.
of body weight with those for larger or smaller pigeons after correc-
ting for the difference in size has proved to be good, and has served
further to strengthen our conviction concerning the validity of
the relationship stated above. The assays are reported in terms
both of mg. of solids and mg. of nitrogen per pigeon unit.

*Care of Birds*

The pigeons are housed in small cages which necessarily re-
strict movement somewhat and thus reduce the metabolism oc-
casioned by exercise to a minimum fairly constant amount. The
work of Cowgill, Rosenberg, and Rogoff (1931, b) on the effect
of exercise on the vitamin B requirement may be cited as evidence
of the importance of reducing the exercise factor to a minimum.
For this reason the practice of caging numerous birds in the same
pen, where considerable flying is possible, should be avoided.
When cages about 18 inches long, 10 inches wide, and 12 inches
deep are used, there appears to be no disadvantage in housing
two birds together. Cages suitable for metabolism work with rabbits have proved to be serviceable, because the solid wall rising about 6 inches from the bottom of the cage serves to confine within the cage any rice scattered by the birds. Each cage is provided with containers for water, polished rice, and stone grit.

Diet

Polished rice is offered *ad libitum* and is intended to serve as the chief source of calories. The birds also receive daily a forced administration of commercial meat residue\(^1\) as a source of good protein and heat-stable vitamin G (B\(_2\)) factor relatively free from antineuritic vitamin (Osborne and Mendel, 1917; Cowgill, 1926–27), the Osborne-Mendel salt mixture (1917) designed to furnish mineral nutrients, and cod liver oil, intended as a source of fat-soluble vitamins. These supplements are placed in a No. 000 gelatin capsule and one capsule is given daily to each bird. This feeding technique is very similar to that used by Jansen and Donath, a fact that we learned after our first experiments (Cowgill and Klotz, 1927) had been completed. In our method, however, inorganic nutrients also are supplied.

Inasmuch as the administration of these supplements is designed to meet the deficiencies of the polished rice, it is pertinent to consider whether the quantities of the respective supplements contained in each capsule are sufficient. About 7 per cent of polished rice is protein.\(^2\) From the weights of numerous filled capsules and the nitrogen content of the meat residue it is estimated that each capsule furnished about 0.9 gm. of meat-residue protein. It is assumed that the birds will adjust their energy intake according to their energy demands (Cowgill, 1928). Therefore pigeons ranging from 250 to 600 gm. of body weight will consume from 52 to 93 calories per day, or from 14 to 25 gm. of rice, and receive from 1 to 1.8 gm. of rice protein per day. Therefore the total amount of protein ingested daily will range from 1.9 to 2.7 gm. On this basis the protein calories constitute from 15 to 12 per cent of the total, the higher figure corresponding to the smaller birds which are the ones most commonly used. Students of nutrition are aware that this level of protein intake is quite sufficient. From

\(^1\) From the Valentine Meat Juice Company, Richmond, Virginia.

this it is evident that our feeding technique guards against any loss of body weight being due to shortage of protein.

The requirement for inorganic nutrients was met by means of the Osborne-Mendel salt mixture. This material filled the concavity of the end of the capsule and weighed on an average from 0.075 to 0.1 gm. Fat-soluble vitamins A and D were supplied daily by 2 drops of tested cod liver oil administered either directly to the bird by medicine dropper or contained in the capsule along with the other supplements.

The question arises as to whether this scheme of feeding prevents any possible weight loss by the pigeon due to lack of the heat-stable vitamin G (B_2) factor. Inasmuch as both muscle (Hoagland and Snider, 1930; Goldberger, Wheeler, Lillie, and Rogers, 1926) and a meat-residue preparation even more highly extracted than the product used in our experiments (Vars, 1931) have been found to contain fair amounts of this heat-stable substance, it must be concluded that this method of feeding supplies some vitamin G (B_2). At the present time there does not appear to be any satisfactory method by which one may calculate the vitamin G (B_2) requirement in order to determine whether the amount of meat residue given our birds daily supplies adequate amounts of this factor. Another pertinent fact is the recent finding (Cowgill, Rosenberg, and Rogoff, 1931, a; Burack and Cowgill, 1931; Sherman and Sandels, 1931) that vitamin G (B_2) does not play a rôle in the development of the anorexia characteristic of lack of undifferentiated vitamin B. In view of these considerations we feel justified in concluding that loss of weight by adult pigeons fed according to the technique described in this paper cannot be attributed to shortage of heat-stable vitamin G (B_2).

Williams and Waterman (1928) and, more recently, Eddy, Gurin, and Keresztesy (1930) have brought forward evidence interpreted to mean that the bird requires a third hitherto unrecognized factor believed to be present in undifferentiated vitamin B. According to the nomenclature favored by the English workers, this factor has been called vitamin B_3. Jansen and Donath (1927) who, like ourselves, gave their birds polished rice supplemented with meat residue and cod liver oil, obtained no evidence that this hypothetical third factor was lacking. Williams and Waterman explain this failure to confirm their finding by the assumption
that the new factor is present in either the meat residue or the cod liver oil. The same explanation might be offered for our results. Another reason may be cited for our view that, if birds require this hypothetical substance, it must be present in our basal diet. We have successfully fed pigeons on our basal diet supplemented by a concentrate of antineuritic vitamin for from 60 to 100 days with remarkable constancy of body weight during the entire period. The amount of solids furnished daily by the vitamin B\textsubscript{1} concentrate used in these particular instances was of the order of about 1.5 mg. Furthermore, the preparation of this concentrate involved procedures which, according to Eddy, Gurin, and Keresztesy, should have destroyed or inactivated the new vitamin B\textsubscript{3} factor. Evidently, then, the basal diet was supplying this needed nutrient.

Concerning the suggestion (Peters, 1929, 1930) that pigeons may also require for maintenance Reader's (1929, 1930) B\textsubscript{4} factor, or the more recently discovered alleged factor B\textsubscript{5} (Carter, Kinnersley, and Peters, 1930, b) we have no evidence from our own work to offer. We can only cite again the investigations of Janssen and Donath, who secured maintenance and protection against polyneuritis by feeding a basal diet of polished rice, meat residue, and cod liver oil, supplemented by very minute amounts of their crystalline vitamin preparation. Evidently these additional factors are contained in the meat residue or the cod liver oil.

Illustration of Method

In Table I are shown the actual assay data obtained with three pigeons tested with Concentrate I-A. It will be noticed that a curative test was performed with Pigeon 86. The data for body weight and measured dose shown in Table II were taken from Table I. The figures in the last column of Table II are doses corrected for a 300 gm. pigeon by the Cowgill-Klotz (1927) formula. In the case of the tests with Concentrate I-A it will be noticed that all three of the birds weighed more that 300 gm. In the case of the pigeons used in the assay of Concentrate I-B-4, two of the birds weighed less than 300 gm., and the actual assays, treated without regard to the weights of the individual birds, showed a variation of 14 per cent of the mean; when corrected to apply to the 300 gm. pigeon, the doses showed a variation of only 8 per
Antineuritic Vitamin. I

**TABLE I**

*Assay Data for Antineuritic Vitamin B Concentrate I-A*

<table>
<thead>
<tr>
<th>Dose</th>
<th>Pigeon 80</th>
<th>Pigeon 86</th>
<th>Pigeon 88</th>
</tr>
</thead>
<tbody>
<tr>
<td>cc.</td>
<td>gm.</td>
<td>cc.</td>
<td>gm.</td>
</tr>
<tr>
<td>0</td>
<td>311</td>
<td>3.0*</td>
<td>238</td>
</tr>
<tr>
<td></td>
<td>310</td>
<td></td>
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<tr>
<td>Sunday</td>
<td>2.0</td>
<td>251</td>
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</tr>
<tr>
<td>296</td>
<td>287</td>
<td>1.0</td>
<td>260</td>
</tr>
<tr>
<td>0.5</td>
<td>(287)†</td>
<td>0.5</td>
<td>272</td>
</tr>
<tr>
<td>289</td>
<td>286</td>
<td></td>
<td>305</td>
</tr>
<tr>
<td>298</td>
<td>301</td>
<td>Sunday</td>
<td>310</td>
</tr>
<tr>
<td>313</td>
<td>319</td>
<td></td>
<td>320</td>
</tr>
<tr>
<td>Sunday</td>
<td>317</td>
<td></td>
<td>317</td>
</tr>
<tr>
<td>0.2</td>
<td>(314)†</td>
<td>0.2</td>
<td>(317)†</td>
</tr>
<tr>
<td>322</td>
<td>324</td>
<td>Sunday</td>
<td>327</td>
</tr>
<tr>
<td>319</td>
<td>323</td>
<td></td>
<td>336</td>
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<td>328</td>
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<tr>
<td>Sunday</td>
<td>330</td>
<td></td>
<td>326</td>
</tr>
<tr>
<td>0.1</td>
<td>(Sunday)</td>
<td>343</td>
<td>328</td>
</tr>
<tr>
<td>324</td>
<td>327</td>
<td>Sunday</td>
<td>328</td>
</tr>
<tr>
<td>327</td>
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<td></td>
<td>328</td>
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<tr>
<td>327</td>
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<td>328</td>
</tr>
<tr>
<td>327</td>
<td>0.1</td>
<td>(Sunday)</td>
<td>328</td>
</tr>
<tr>
<td>—</td>
<td>344</td>
<td></td>
<td>328</td>
</tr>
<tr>
<td>—</td>
<td>347</td>
<td></td>
<td>328</td>
</tr>
<tr>
<td>Sunday</td>
<td>343</td>
<td></td>
<td>328</td>
</tr>
<tr>
<td>327</td>
<td>347</td>
<td></td>
<td>328</td>
</tr>
<tr>
<td>338</td>
<td>350</td>
<td></td>
<td>328</td>
</tr>
</tbody>
</table>

* Dose administered to cure neuritic symptoms.

† Each weight given is that measured on the day following the administration of a given dose; if the dose had any effect, one would expect to notice this on the following day rather than the same day. The weight enclosed in parentheses is that for the same day the new dose was begun; this weight was taken just before administering the test material.
TABLE I—Concluded

<table>
<thead>
<tr>
<th>Pigeon 80</th>
<th>Pigeon 86</th>
<th>Pigeon 83</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (cc)</td>
<td>Dose (cc)</td>
<td>Dose (cc)</td>
</tr>
<tr>
<td>Daily body weight (g)</td>
<td>Daily body weight (g)</td>
<td>Daily body weight (g)</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>0</td>
<td>(338)†</td>
<td>Sunday</td>
</tr>
<tr>
<td>315</td>
<td></td>
<td>337</td>
</tr>
<tr>
<td>313</td>
<td></td>
<td>342</td>
</tr>
<tr>
<td>309</td>
<td></td>
<td></td>
</tr>
<tr>
<td>305</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sunday</td>
<td>0</td>
<td>(342)†</td>
</tr>
<tr>
<td></td>
<td>332</td>
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</tr>
<tr>
<td></td>
<td>332</td>
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<tr>
<td></td>
<td>323</td>
<td></td>
</tr>
<tr>
<td></td>
<td>325</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sunday</td>
<td>311</td>
</tr>
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<td></td>
<td></td>
<td>304</td>
</tr>
<tr>
<td></td>
<td></td>
<td>297</td>
</tr>
</tbody>
</table>

**TABLE II**

Assay Data for Antineuritic Vitamin B Corrected to Apply to a 300 Gm. Pigeon

<table>
<thead>
<tr>
<th>Test product</th>
<th>Pigeon No.</th>
<th>Average body weight over test period (g)</th>
<th>Minimum dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Measured</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>cc.</td>
</tr>
<tr>
<td>Concentrate</td>
<td>89</td>
<td>328</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>86</td>
<td>344</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>83</td>
<td>319</td>
<td>0.1</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentrate</td>
<td>89</td>
<td>281</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>300</td>
<td>1.4</td>
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<td></td>
<td>63</td>
<td>277</td>
<td>1.0</td>
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<td></td>
<td>86</td>
<td>299</td>
<td>1.5</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>1.27</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>0.18</td>
</tr>
<tr>
<td>Average deviation</td>
<td></td>
<td></td>
<td>14</td>
</tr>
</tbody>
</table>

Corrected dose taken for comparison with results of assays of other preparations: 1.5

THE JOURNAL OF BIOLOGICAL CHEMISTRY, VOL. XCV, NO. 3
cent of the mean. These results are typical of many that might be cited.

II. Concentration of Antineuritic Vitamin by Silver under Various Conditions

Jansen and Donath (1927) have renewed interest in the chemistry of the antineuritic vitamin by the report of the isolation of a potent crystalline substance. This material prevented the appearance of polyneuritis in bondols (rice birds) in amounts of from 2 to 4 micrograms per day. The precipitation of the active material with little loss of potency was reported to take place between pH 4.5 and 6.5 in the presence of silver nitrate and barium hydroxide. In an attempt to repeat this work, we observed a considerable loss of activity at this point. Therefore, it was hoped that a study of the conditions of precipitation by silver and alkali would be useful. Various silver salts have been employed as a precipitant for the vitamin by many workers, but in so far as we are aware, there has been no estimation of the comparative efficiency of various combinations of acids, silver salts, and bases by one group of workers.

Funk (1911) reported that the antineuritic vitamin from rice polishings was precipitated by barium hydroxide in the presence of silver nitrate. The same author (1912-13) found that the vitamin extracted from yeast was not precipitated by silver nitrate-barium hydroxide unless the yeast had been previously hydrolyzed. An active fraction from wheat bran was obtained by Sullivan and Voegtlin (1918) by precipitating with silver acetate and barium hydroxide. Yeast vitamin was precipitated by the same reagents by bringing the reaction to pH 8 to 9 (Myers and Voegtlin, 1920). Kinnersley and Peters (1925) obtained a vitamin concentrate from yeast by adsorption on and elution from norit followed by an extensive alcoholic fractionation. The active substance in this concentrate was not precipitated by silver sulfate and sulfuric acid but was by silver nitrate and ammonia. A potent concentrate from rice polishings and yeast is obtained by treatment with silver picrate (Funk, 1927). Guha and Drummond (1929) found that if a vitamin concentrate from wheat germ is treated with silver oxide at pH 7 or by silver nitrate and barium hydroxide at pH 4.5 to 6.5, the vitamin is precipitated. Silver nitrate-barium
hydroxide between the pH values of 4.5 and 6.5 is a good precipi-
tant for the antineuritic substance from rice polishings but not for
that obtained from yeast (Williams, Waterman, and Gurin, 1930).

The plan of this research was to acidify the vitamin solution to
pH 4, add an excess of a soluble silver salt, and remove the result-
ing precipitate. This precipitate, as was expected, contained
little or no activity and was discarded. The filtrate was then
brought to about pH 7.0 by the careful addition of a strong base,
and the precipitate which formed at this point was removed. This
second precipitate, after removal of the silver, was tested quanti-
tatively for the antineuritic vitamin by the pigeon technique de-
scribed in Part I of this paper. The assays were conducted as a
separate investigation by one of us (G. R. C.) who was not in-
formed concerning the details of the preparation of the products
submitted for test.

In such an experiment there are three major variables, the acid,
the silver salt, and the base. Sulfuric, nitric, or lactic acid was
used as the source of hydrogen ions; barium or sodium hydroxide
was employed to bring the reaction from pH 4 to pH 7.0; and to
introduce silver we made use of silver lactate or nitrate. Each
of these reagents has distinctly different properties because of the
effect of the negative ion in the case of sulfuric acid, etc., and of
the positive ion in sodium hydroxide, etc. The degrees of con-
centration of the active principle effected by the various combina-
tions of these reagents are summarized in Table III.

Chemical Procedures

The material used in these studies was kindly furnished by Eli
Lilly and Company and was prepared by them in the following
manner. Rice polishings were thoroughly extracted with acidu-
lated dilute ethyl alcohol. The alcohol was removed by concen-
tration in vacuo, and the vitamin adsorbed on Lloyd’s reagent.
The activated earth was washed with water, and the vitamin
eluted with sodium hydroxide. The alkaline solution was im-
mediately acidified with sulfuric acid, and all the free sulfuric
acid was neutralized by sodium hydroxide. A large part of the
resulting sodium sulfate was precipitated by alcohol. The alco-
holic vitamin solution was concentrated to a syrup and preserved
with chloroform. In this condition it can be kept at room tem-
perature for many months without apparent deterioration.
Antineuritic Vitamin. I

This vitamin concentrate was diluted until the amount of total solids (dried overnight at 100°) was about 30 per cent, and an aliquot part of this solution containing 2500 pigeon units (see Part I of this paper) was acidified with concentrated nitric acid to pH 4 (Congo red paper), and an excess of silver nitrate was introduced. The resulting precipitate was washed by centrifuging, and the combined washings concentrated in vacuo (maximum temperature 30°) to the same volume as originally used. Hot saturated barium hydroxide was now added to about pH 7.0 as tested by brom-thymol blue. The precipitate was centrifuged off and

* Corrected to apply to a 300 gm. pigeon after the formula of Cowgill and Klotz (1927). The pigeon unit is the amount required to maintain the body weight constant over a period of from 10 to 14 days in the case of a 300 gm. pigeon fed according to the method described in this paper.

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**TABLE III**

Concentration of Antineuritic Vitamin B with Silver under Various Conditions

<table>
<thead>
<tr>
<th>Combination of reagents used</th>
<th>Biological assay</th>
<th>Degree of concentration calculated from</th>
<th>Vitamin recovered in active fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver</td>
<td>Acid</td>
<td>Alkali</td>
<td>Mean weight of birds</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>HNO₃</td>
<td>Ba(OH)₂</td>
<td>295</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>NaOH</td>
<td>292</td>
</tr>
<tr>
<td>&quot;</td>
<td>H₂SO₄</td>
<td>Ba(OH)₂</td>
<td>315</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>NaOH</td>
<td>290</td>
</tr>
<tr>
<td>&quot;</td>
<td>Lactic acid</td>
<td>Ba(OH)₂</td>
<td>280</td>
</tr>
<tr>
<td>Ag lactate</td>
<td>H₂SO₄</td>
<td>Ba(OH)₂</td>
<td>285</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>NaOH</td>
<td>352</td>
</tr>
<tr>
<td>&quot;</td>
<td>Lactic acid</td>
<td>Ba(OH)₂</td>
<td>298</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>NaOH</td>
<td>319</td>
</tr>
</tbody>
</table>

---

2 Excess silver was tested for (1) by the brown spot test with barium hydroxide, (2) by adding a drop of hydrochloric acid to the clear solution, and (3) by demonstration of silver in the filtrate after precipitation at pH 7.0.
washed\textsuperscript{4} once by centrifuging with a little ice water. The precipitate was suspended in dilute hydrochloric acid (pH 1) and decomposed by boiling for $\frac{1}{2}$ hour. The precipitation and washing of the vitamin never took more than 10 minutes. The silver chloride was removed and washed with warm water. The washings were concentrated to dryness \textit{in vacuo}, and the residue was dissolved in about 100 cc. of water. Dilute sulfuric acid was added to precipitate all of the barium, and the barium sulfate centrifuged and washed. The washings were concentrated to dryness \textit{in vacuo}, and the residue diluted to the same volume as had been originally employed. Aliquots of this solution were removed to determine total solids and nitrogen. The remainder was preserved with a trace of chloroform, and used for the feeding tests. The other experiments listed in Table III were carried out in essentially the same manner.

\textbf{III. Solubility of the Antineuritic Vitamin in Certain Organic Solvents}\textsuperscript{5}

Since the report on the concentration of antineuritic vitamin B by fractional precipitation with ethyl alcohol by Osborne and Wakeman (1919), many attempts have been made to purify the vitamin by means of this solvent. There have been reports that the vitamin is not soluble in 99 to 100 per cent ethyl alcohol (Seidell, 1926; Seidell and Smith, 1930; Levene and van der Hoeven, 1925, 1926; Levene, 1928). Other investigators have reported solution of the active substance in absolute alcohol (Funk, 1911; Abderhalden and Schaumann, 1918; Kinnersley and Peters, 1927; Jansen and Donath, 1927; Guha and Drummond, 1929; Williams, Waterman, and Gurin, 1930). Van Veen (1931) attempted to concentrate the vitamin in the following ingenious fashion. He dried an active solution on powdered quartz and thoroughly extracted the finely ground sand with boiling absolute alcohol. However, this treatment failed to remove any appreciable amount

\textsuperscript{4} A biological assay of this washing showed no vitamin present.

\textsuperscript{5} Part of the work reported in Part III was done in the laboratories of the College of Physicians and Surgeons, Columbia University. One of us (R. J. B.) is indebted to Dr. Hans T. Clarke for the privilege of working in these laboratories and for assistance in securing special apparatus through a grant from the Chemical Foundation.
of the active substance. These seemingly contradictory results have been tentatively explained by Kinnersley and Peters (Sherman and Smith, 1931) thus: In the presence of some alcohol-insoluble impurities and at certain acidities, the vitamin is thrown out upon alcoholic precipitation; after the removal of these substances, it is soluble in alcohol of high concentration. The writers believe this suggestion to be incorrect because of the results of the experiments described below.

The object of these experiments was to prepare a solution of antineuritic vitamin free from inorganic salts. We therefore attempted to remove these salts from a Lloyd’s reagent concentrate prepared for us by Eli Lilly and Company according to the procedures described above in Part II. It was noticed that on adding alcohol to the aqueous solution a large, flocculent gummy precipitate appeared. This precipitate could not be washed quantitatively and so resulted in a loss of from 50 to 60 per cent of the active material. However, it appeared to us that if the concentration of alcohol in the vitamin solution could be raised very gradually from 50 to 100 per cent, there should be a complete precipitation of the inorganic salts and other material insoluble in absolute alcohol, the vitamin remaining in solution. This hypothesis was substantiated experimentally in the following manner.

**Solubility in Ethyl Alcohol-Carbon Tetrachloride**

Through the kindness of Eli Lilly and Company, we obtained three vitamin concentrates. Two of these were prepared from rice polishings and had been eluted from Lloyd’s reagent with sodium hydroxide and the alkali neutralized to pH 4 with sulfuric acid. The third (Concentrate III-A) was obtained from yeast and had been eluted with sodium hydroxide and neutralized with hydrochloric acid. The concentrates were diluted until the total solid content was approximately 30 per cent. Hydrochloric acid was added to bring the reaction to pH 1 and any sulfuric acid present was removed by addition of barium chloride. The solution was heated on the steam bath and 5 volumes of a 1:1 solution of 95 per cent ethyl alcohol and carbon tetrachloride were added;

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*For a more detailed description of the preparation of these concentrates see Part II of this paper.*
the water was then removed by conducting a distillation in the apparatus described in “Organic syntheses” (Adams, 1921). To compensate for the alcohol and carbon tetrachloride lost in the “water” layer, 10 cc. of carbon tetrachloride and 65 cc. of absolute alcohol were added for every 100 cc. of “water layer” distillate. The extraction was continued until no more water came over. The reaction flask was allowed to stand in the ice box overnight and the heavy precipitate filtered off. The filtrate was concentrated to dryness, a little water added and the solution again evaporated to dryness, this process being repeated several times in order

<table>
<thead>
<tr>
<th>Alcohol used</th>
<th>Total solids per pigeon per day</th>
<th>Degree of concentration of vitamin</th>
<th>Vitamin recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl</td>
<td>48 mg.</td>
<td>3.5 ×</td>
<td>85</td>
</tr>
<tr>
<td>“</td>
<td>11 mg.</td>
<td>3.0 ×</td>
<td>100</td>
</tr>
<tr>
<td>“</td>
<td>7 mg.</td>
<td>4.8 ×</td>
<td>100</td>
</tr>
<tr>
<td>“</td>
<td>20 mg.</td>
<td>2.0 ×</td>
<td>85</td>
</tr>
<tr>
<td>n-Propyl</td>
<td>40 mg.</td>
<td>1.0 ×</td>
<td>20</td>
</tr>
<tr>
<td>Isopropyl</td>
<td>26 mg.</td>
<td>1.5 ×</td>
<td>50</td>
</tr>
<tr>
<td>Allyl</td>
<td>23 mg.</td>
<td>1.7 ×</td>
<td>100</td>
</tr>
<tr>
<td>n-Butyl</td>
<td>18 mg.</td>
<td>2.4 ×</td>
<td>100</td>
</tr>
<tr>
<td>Tertiary butyl</td>
<td>24 mg.</td>
<td>1.5 ×</td>
<td>25</td>
</tr>
<tr>
<td>“ amyl</td>
<td>100 mg.</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE IV
Solubility of Antineuritic Vitamin in Binary Mixtures of Alcohol and Carbon Tetrachloride

Solubility in Other Binary Mixtures

On account of the good results obtained with ethyl alcohol, it was considered advisable to attempt to concentrate the vitamin by employing other alcohols that form low boiling ternary mix-
tures with water and carbon tetrachloride. The following alcohols were tried: n-propyl, isopropyl, allyl, n-butyl, tertiary butyl, and tertiary amyl. The results, summarized in Table IV, indicate that only n-butyl and allyl alcohols are as good as or better than ethyl alcohol as a means of concentrating the antineuritic vitamin by this carbon tetrachloride process. Since the use of allyl alcohol is inadvisable on account of its high cost and its lacrimatory properties, ethyl and n-butyl alcohols remain as efficient solvents for concentrating the vitamin according to this technique.

**Solubility in Certain Mixtures of Organic Solvents**

The vitamin solution, which was purified according to the ethyl alcohol-carbon tetrachloride technique, was dissolved in absolute methyl alcohol. The alcoholic solution was then poured into 10 volumes of acetone, allyl, or amyl alcohol, and allowed to stand in the ice box for 24 hours. The distribution of the vitamin and the total solids in the filtrate and precipitate were then determined. The results indicate that no one of these mixtures can be used to advantage in concentrating the vitamin.

**SUMMARY AND CONCLUSION**

The method of assay for antineuritic vitamin B used in this investigation is described. It is a combination of weight-maintenance and curative techniques carried out on pigeons given a diet of polished rice *ad libitum* supplemented daily with meat residue, cod liver oil, and Osborne-Mendel salt mixture.

Ten experiments were carried out on the concentration of the antineuritic vitamin by means of silver. As sources of the silver ion, the nitrate and lactate salts were used; nitric, sulfuric, or lactic acid was employed as source of the hydrogen ion; and barium or sodium hydroxide was used for alkalizing. Silver nitrate, lactic acid, and barium hydroxide gave the most favorable purification with the least loss of activity; treatment with silver lactate, lactic acid, and sodium hydroxide resulted in the greatest increase of potency but in a poor yield of the vitamin.

The behavior of antineuritic vitamin in the ternary mixture of water, ethyl alcohol, and carbon tetrachloride was studied. Whereas the inorganic salts are precipitated as the water is re-
moved and the concentration of the alcohol approaches 100 per cent, it was found that the vitamin remains in the liquid phase from which it may be recovered quantitatively. Solubility of the vitamin in other binary mixtures was studied. Six other alcohols were substituted for ethyl alcohol in the carbon tetrachloride procedure; of these only n-butyl and allyl proved to be as good as ethyl alcohol for concentrating the vitamin.

The solubility of the antineuritic vitamin in mixtures of methyl alcohol with acetone, allyl, and amyl alcohols was studied. No one of these mixtures can be used profitably to concentrate the vitamin.

**BIBLIOGRAPHY**


Adams, R., Organic syntheses, New York, 1, 68 (1921).


Klotz, B. H., Determination of the vitamin B minimum in the pigeon, Thesis, Yale University (1926).


Peters, R. A., *J. State Med.*, 37, 653 (1929); 38, 3, 63 (1930).


Vars, H. M., (1931) personal communication.


THE ANTINEURITIC VITAMIN: I. THE METHOD OF ASSAY, CONCENTRATION OF THE VITAMIN WITH SILVER UNDER VARIOUS CONDITIONS, AND ITS SOLUBILITY IN CERTAIN ORGANIC SOLVENTS

Richard J. Block, George R. Cowgill and Benjamin Howard Klotz

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