[CONTRIBUTION FROM THE HYGIENIC LABORATORY, UNITED STATES PUBLIC HEALTH SERVICE]

FURTHER EXPERIMENTS ON THE ISOLATION OF THE ANTINEURITIC VITAMIN[1]

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In a previous paper[2] on this subject a fractionation of a highly active yeast vitamin extract by precipitation with silver nitrate and ammoniacal silver nitrate was described. This work showed that the ammoniacal silver nitrate precipitate undoubtedly contained the antineuritic principle. Little information was, however, obtained as to the relative purity of the vitamin complex or the approximate quantitative distribution of the active material between the several fractions obtained in the process. This phase of the problem has, therefore, been studied in more detail and at the same time attention has also been directed towards improving the preliminary steps in the preparation of the vitamin extract used for the silver precipitations.

In general, the procedure for obtaining highly active vitamin fractions from fresh brewer's yeast, as developed in this laboratory, consists of the following steps: (1) the preparation of a clear aqueous solution of the vitamin originally present in the fresh yeast cells; (2) the formation of an adsorption compound of this vitamin and fuller's earth, that is, the preparation of the so-called vitamin "activated solid;" (3) the extraction of the vitamin from this product by means of saturated aqueous barium hydroxide solution; (4) the concentration of this dilute extract by rapid vacuum distillation, and (5) the fractionation of the resulting concentrated extract by silver precipitation.

The Vitamin Solution

The method originally followed for the preparation of a clear solution of the vitamin consisted in subjecting the fresh yeast to autolysis and filtering the thick slimy liquid thus obtained. This filtration was very slow and a considerable proportion of the vitamin was undoubtedly retained by the residue and thus lost. Futhermore, the soluble products formed in the autolysis entered the filtrate and introduced difficulties in the subsequent fractionation of the vitamin. One example of such a disturbing compound was found to be adenine.

It has been shown by Osborne and Wakeman[3] that when fresh yeast is added to boiling water and the mixture heated for a few minutes, the yeast

[1] Presented before the Division of Biological Chemistry at the 63rd Meeting of the American Chemical Society, April 3 to 7, 1922.

[2] Seidell, *J. Ind. Eng. Chem.*, **13**, 1111 (1921).

[3] Osborne and Wakeman, *J. Biol. Chem.*, **40**, 383 (1919).

cells are disrupted, the protein coagulated and the vitamin enters the aqueous solution. The separation of the solution from the coagulated protein is easily accomplished by filtration or more completely by centrifugation. The aqueous solution thus obtained undoubtedly contains a greater proportion of the total vitamin than is present in the filtrate from autolyzed yeast, and furthermore, is free from adenine and other products of the autolytic decomposition.

An experiment made for the purpose of showing the relative amount of dissolved solids in the clear solutions prepared, under comparable conditions, by the two methods, indicated that the quantity in the solution obtained by the boiling method is less then $^1/_2$ that in the filtrate from autolyzed yeast. An exact quantitative comparison, however, could not be made, since the allowance for diluting the original yeast before boiling and for the relative proportion of the soluble constituents retained by the insoluble material in both cases, were uncertain factors. The experiment, nevertheless, shows that far smaller amounts of interfering compounds will be present, for adsorption by fuller's earth, in a vitamin solution made by the boiling process, than in one made by the autolytic process. It would, therefore, be expected that the "activated solid" prepared by the new method would contain vitamin in a purer state than would the samples prepared by the old method. On this account the new method has now been adopted and all the experiments described in the present paper have been made with "activated solid" prepared by the new method. The details actually followed in the preparation of this improved product have been published elsewhere.[4]

The Extraction of Vitamin from "Activated Solid"

The adsorption of vitamin by fuller's earth takes place in acid solution; consequently, in order to perform the reverse operation, an alkaline medium must be employed. In the earlier experiments aqueous sodium hydroxide was used as the extracting agent, but the soluble sodium salts which remained after acidification were a source of annoyance in the subsequent purification steps. In order to overcome this difficulty aqueous barium hydroxide was used and, after acidifying with sulfuric acid, the barium was eliminated as the very insoluble sulfate.

Extended experience has led to the following procedure for the extraction.

One hundred g. of "activated solid" is violently agitated with 1000 cc. of saturated barium hydroxide solution for 3 minutes. The solid is then promptly removed by a rapidly acting Büchner funnel or, better, by means of a De Laval cream separator No. 4, with the cones removed from the bowl. The nearly clear liquid is immediately acidified with about 8 cc. of conc. sulfuric acid. The extracted solid is then again shaken with about 300 cc. of water and the mixture separated as before. The combined aqueous extract is brought to distinct acidity with further additions of sulfuric acid, after which an excess of barium carbonate is added and the milky mixture repeatedly shaken. This

[4] Seidell, *U. S. Pub. Health Repts.*, **37**, 801 (1922).

serves to remove the excess of sulfuric acid. After the liquid becomes neutral to litmus, it, together with two or more similar extracts from 300g. portions of "activated solid," is filtered from the barium sulfate and excess of barium carbonate.

The clear neutral aqueous extract, prepared in this way, contains the vitamin and the accompanying bases as sulfates. Attempts to concentrate the solution by vacuum distillation were uniformly unsuccessful on account of the excessive foaming. It has been found that this foaming can be overcome by addition of sufficient hydrochloric (but not acetic) acid. The conc. extract thus obtained contains the bases as hydrochlorides. Although this procedure has several good points, it is believed that a better one is as follows.

Experience has shown that if the neutral aqueous extract, filtered from the barium sulfate and carbonate, is subjected to precipitation with saturated lead acetate ($Pb(C_2H_3O_2)_2.3H_2O$) solution, and the excess of lead removed by hydrogen sulfide from the filtrate from this precipitate, the clear solution thus obtained can then be distilled under diminished pressure, at a very rapid rate, without any foaming whatever. This lead acetate precipitation converts the sulfates of the vitamin and other bases to acetates and probably also removes some constituent responsible for the troublesome foaming. This procedure has, therefore, been adopted for all the fractionations described in the following pages.

Concentration of the Vitamin Extract

This concentration is accomplished by rapid distillation at a moderate temperature under diminished pressure.

The approximately 4 liters of clear solution, obtained by the extraction of three 100g. portions of "activated solid," is reduced to about 100 cc. before any precipitation occurs. At this point an amorphous white compound begins to separate and its amount increases during the further reduction of the volume to 30–50 cc. The conc. mixture is then transferred to a centrifuge tube and cooled in ice water. It is centrifuged and the clear liquid decanted from the white solid. This latter is washed once by stirring with a small amount of water and centrifuging. The further evaporation of the decanted liquid is accomplished in a vacuum desiccator containing sulfuric acid. Another quantity of the white precipitate may be obtained and this is likewise removed and washed by centrifugation. The dry weight of the combined separated white solid varies, in different lots, from about 1.5–2.5 g. After removal of this less soluble fraction, the evaporation of the liquid is continued in a vacuum desiccator. The residue becomes more and more viscous until, in its final dried condition, it is a resinous, transparent, hygroscopic, sticky film. The weight obtained from 300 g. of "activated solid" is usually between 8 and 10 g.

In regard to the antineuritic activity of this solid vitamin extract, numerous experiments have shown that when samples of it are dissolved in water and these used to activate fuller's earth, the protective dose for pigeons on polished rice,[5] calculated to the solid extract, is usually 10 mg.

[5] For details regarding this test see "A Physiological Test for the Activity of Vitamine Preparations," Seidell, *U. S. Pub. Health Repts.*, **37**, 1519 (1922).

given on alternate days. Doses as low as 7.5 mg. have, in some instances, been found adequate.

If we take the lower figure and estimate on the basis of a yield of 10 g. of solid extract, the total vitamin recovered in the form of solid extract from 300 g. of "activated solid" would correspond to $10.0 \div 0.0075 = 1{,}333$ protective doses. Since the "activated solid" itself protects in doses of 0.1 g., 300 g. of it corresponds to 3,000 protective doses. Consequently, by the alkaline extraction a recovery of less than 50% of the active vitamin is accomplished. It is probable that a recovery of about $^1/_3$ is nearer the correct figure.

In order to gain some light on the question of where the loss occurs in the process, a sample of "activated solid" which had been extracted in the usual manner with barium hydroxide solution, was in one case quickly shaken with sufficient dil. hydrochloric acid to neutralize the excess of alkali and then filtered, washed and dried. Using this material it was found that protection was afforded pigeons on rice by doses of 0.4 g. instead of 0.1 g. of the unextracted solid. This result shows that about 25% of the vitamin is not removed by the alkaline extraction as at present conducted.

By means of nitrogen determinations it was found that whereas the "activated solid," before extraction with barium hydroxide solution, contained 1.9% of nitrogen, the same sample after extraction still retained 1.1% of nitrogen. This shows that a considerable proportion of the non-vitamin nitrogenous compounds, adsorbed by fuller's earth, are not removed by the alkaline extraction.

The Distribution of Solids and of Antineuritic Activity Obtained by Silver Fractionation

As referred to above, previous work showed that the precipitate obtained by ammoniacal silver nitrate undoubtedly contained significant amounts of antineuritic vitamin. The experiments, however, did not show what proportion of the total vitamin was present in either the ammoniacal silver nitrate precipitate or the other fractions. It was, therefore, decided to attempt to follow the distribution of the active vitamin in the several fractions by approximately quantitative estimations. For this purpose the activity was in every case referred to the number of milligrams of solid which, under the conditions of the physiological test, just protect pigeons from loss in weight on a diet of polished rice. This standard may, for convenience, be referred to as the unit of active vitamin. It is that quantity of vitamin, given to a normal pigeon on each alternate day, which is just sufficient to replace the vitamin deficiency of an exclusive diet of polished rice.

The vitamin extract from 300 g. of "activated solid," is dissolved in water and diluted, usually to 50 cc. This is designated as Fraction I.

An aliquot of it is evaporated in a platinum dish and the total solids and ash determined. On the basis of the total solids determination, another aliquot is diluted to about 300 cc. with water, and such an amount of fuller's earth added that each 0.1 g. corresponds to a definite number of milligrams of the solids present. The fuller's earth-vitamin complex thus prepared is used for the physiological tests.

To the remainder of the 50 cc. of solution, nearly saturated silver nitrate solution is carefully added until no further precipitation occurs. The mixture is subjected to centrifugation, and the supernatant liquid and one wash solution of the precipitate are reserved for ammoniacal silver nitrate precipitation. The once-washed silver nitrate precipitate is suspended in a small amount of water and hydrogen sulfide introduced. The precipitated silver sulfide is removed by centrifugation, and the clear supernatant liquid filtered and diluted to 50 cc. This is designated as Fraction II. An aliquot of this solution is used for total solids and ash determination, another aliquot for the preparation of the fuller's earth adsorption complex for the physiological test.

The supernatant liquid from the silver nitrate fraction is then precipitated in exactly the same manner with a concentrated solution of ammoniacal silver nitrate. This precipitate, after removal by centrifugation and washing once, is likewise decomposed with hydrogen sulfide and the silver-free solution diluted to 50 cc. This is designated as Fraction III, and an aliquot is used for total solids determination and another for the preparation of the fuller's earth adsorption complex for physiological tests.

The supernatant liquid from the ammoniacal silver nitrate precipitate, which gives no further precipitate with either silver nitrate or ammoniacal silver nitrate, is treated with hydrogen sulfide and the resulting silver sulfide removed by centrifugation. The filtered solution is diluted to a given volume and designated as Fraction IV. The total solids in it are determined as usual. The result, however, does not represent the true value for solids not precipitated by silver, since ammonium nitrate and possibly other compounds, derived from the reagents, will be present. The calculation for the preparation of the fuller's earth adsorption complex for physiological tests will, therefore, be only approximate. The solids can, however, be estimated by difference and this value used. In any event, considering the limitations of the physiological tests, the final results for units of active vitamin are only a rough approximation.

A large number of experiments have been made according to the scheme outlined above and various slight modifications of it, and results which are in general agreement have been obtained.

A typical example of such results, obtained in Expt. 37, is shown in Table I.

The outstanding conclusion from this and many similar experiments is that only about $1/3$ of the solids is precipitated by both silver nitrate and

ammoniacal silver nitrate, and these silver precipitates carry somewhat more than $^1/_2$ of the total active vitamin. The most plausible explanation of the non-precipitation by silver of so large a proportion of vitamin appears to be the rather high solubility of the silver-vitamin complex. A considerable loss of active material by the silver method is, therefore, inevitable under the conditions of the fractionation as now conducted.

TABLE I

EFFECT OF FRACTIONATION ON VITAMIN ACTIVITY

Fraction	Description	Solids present G.	Minimum protective dose of the solids Mg.	Calculated "Units of vitamin" present
I	Original dried vitamin extract	9.71	7.5	1,295
II	$AgNO_3$ precipitate	2.76	5.0	551
III	Ammoniacal $AgNO_3$ precipitate	0.59	3.0	197
IV	Non-precipitable by silver	6.37(dif.)	15.0(?)	424

As compared with the results reported in a former paper on this subject,[2] the activity of the silver nitrate precipitate is much higher than previously found. The most probable explanation of this is that in the previous case the vitamin extract, used for the silver precipitations, contained considerable amounts of adenine which formed a predominantly large part of the silver nitrate precipitate, greatly reducing the apparent activity of this fraction.

It may also be noted in this connection, that whereas considerable oxidation, as expressed in rapid darkening of the silver precipitate, occurred during the precipitations described in the previous paper, little trouble on this score has been experienced with the vitamin extracts prepared from the "activated solid" made by the new method. Thus, in addition to adenine, another troublesome factor has been eliminated by the new procedure for preparing "activated solid."

The Stability of the Vitamin Complex

Considerable experience with the vitamin fractions obtained from various active extracts by silver precipitation shows them to be quite stable. The silver precipitates themselves, when brought to a dry condition, appear to lose a part of their activity but when, before drying, the silver is removed by means of hydrochloric acid or hydrogen sulfide, the residues may then be kept indefinitely in a dry condition or in aqueous solution without appreciable loss of activity.

In one case the unused aqueous solutions of Fraction IV obtained in 5 different experiments (32, 34, 35, 36 and 37) were mixed and a calculated amount of fuller's earth added to the resulting mixture. The several fractions had been kept in the laboratory for various lengths of time from a few weeks to 4 months. The dissolved solids and physiological activity

of each had been determined at the time of its preparation. On the basis of these figures there were approximately 15.5 g. of solids present and the activity corresponded to almost 1,200 "units of vitamin." The fuller's earth-vitamin complex prepared from the combined Fractions IV, when tested on pigeons, was found to possess an activity which agreed satisfactorily with the calculated vitamin content. Thus a noteworthy deterioration had not occurred. A nitrogen determination in this sample showed 0.42% and it protected in doses of 0.2 g. Thus on the nitrogen basis each protective dose contained 0.84 mg. of nitrogen.

In regard to the keeping qualities of the dried silver-free residues, unused portions of Fractions II and III of Expt. 37 were evaporated to dryness and kept in a vacuum desiccator containing stick sodium hydroxide, as the drying agent, for one month, and portions of each then removed and used for the preparation of new samples of the fuller's earth adsorption complex. On testing these samples no diminution of vitamin activity could be detected. The results for these determinations are given in brackets in Table II.

The Dialyzability of the Vitamin Complex

Although it is well recognized that the antineuritic vitamin dialyzes comparatively easily, the completeness of the process has probably not been fully realized. On this account the following experiment is believed to be of considerable interest.

An extract of 100 g. of "activated solid" was prepared according to the procedure described in a preceding section of this paper and was reduced by distillation to approximately 200 cc. This was placed in a collodion sack and suspended in about 700 cc. of distilled water. After about 18 hours the sack was suspended in another 700cc. portion of distilled water for about 4 hours, and finally it was placed in a third portion of about 600 cc. of distilled water for 18 hours. The contents of the collodion sack were then transferred to a graduated flask and diluted to 1000 cc. The combined diffusates were diluted to 2000 cc. One-tenth of each solution was evaporated to dryness and the total solids weighed. The results showed the presence of 0.025 g. in the dialyzate and 2.58 g. in the diffusate. The remaining $^9/_{10}$ of each solution was shaken with 25 g. of fuller's earth and the samples thus prepared used for tests on pigeons. The results showed that no appreciable protection was afforded by 0.3- or 0.6g. doses of the sample prepared from the dialyzate, but that 0.1g. doses of the sample prepared from the diffusate sufficed to replace the deficiency of the rice diet. The latter dosage corresponds to about 10 mg. of dialyzed solids and is in fair agreement with the amount of dried vitamin extract, found in other experiments, to replace the deficiency of a rice diet.

The experiment, therefore, shows that both the vitamin and other bases present in a vitamin extract from "activated solid" diffuse through a collodion membrane rapidly and practically completely. This indicates that the vitamine molecule is of relatively simple constitution and, therefore, furnishes renewed hope that the pure compound may eventually be isolated.

Nitrogen Content as a Criterion of the Purity of Vitamin Fractions

There appears to be no reasonable doubt that vitamin contains one or more atoms of nitrogen in its molecule. Therefore, for the purpose of controlling fractionation procedures, it should be possible to use nitrogen determinations as a measure of the unknown vitamin. Obviously, if reasonably accurate comparisons of nitrogen content and antineuritic activity can be made, the result need only be multiplied by a factor, which may be determined later, to be converted to vitamin.

An effort has accordingly been made to determine the smallest quantity of nitrogen corresponding to that activity previously defined as a "unit of vitamin." With such a standard, estimates can be made of the relative purity of various fractions which protect in quantities of nitrogen greater than the standard. In these cases the excess of nitrogen above the established minimum will represent other nitrogenous bases than vitamin. In the case of Expt. 37 it was found that the fuller's earth preparations made from Fractions I, II, III and IV gave the following values for nitrogen content and milligrams of nitrogen corresponding to a unit of vitamin.

TABLE II

VITAMIN ACTIVITY IN RELATION TO NITROGEN CONTENT

Fraction	Protective dose of fuller's earth preparation	Nitrogen in fuller's earth preparation	Calculated N contained in a protective dose
	G.	%	Mg.
I	0.3	0.17	0.51
II	0.25 [0.3]	0.20 [0.16]	0.5 [0.48]
III	0.3 [0.2]	0.10 [0.13]	0.3 [0.26]
IV	0.3+	0.33	1.0+

Duplicate determinations with other samples of fuller's earth prepared from portions of the dried solids of Fractions II and III, which had been kept in a desiccator for one month, are enclosed in brackets.

From these results it appears that in the case of Fraction III a smaller amount of nitrogen was required for protection than in the case of the other fractions. Thus in Fraction I, and especially in Fraction IV, a considerable proportion of the nitrogen must represent other bases than vitamin. The figures also indicate that by ammoniacal silver precipitation a partial concentration of the vitamin has been effected. This conclusion is emphasized by the apparently large amount of inactive nitrogen present in Fraction IV.

In regard to the lowest amount of nitrogen, corresponding to a "unit of vitamin," so far obtained, a comparison of a large number of determinations showed that in one case a fuller's earth sample prepared from an ammoniacal silver nitrate fraction obtained after silver acetate precipitation, protected in doses of 0.1 g. and contained 0.08% of nitrogen. This sample, therefore, on the nitrogen basis was nearly four times as active as Fraction

III shown above. From this result it may be concluded that the nitrogen in the present Fraction III corresponds to about 25% of the pure vitamin base and 75% of other nitrogenous compounds.

Reasoning in this manner it may be concluded that the extracts prepared from "activated solid" are mixtures of vitamin with one or more other nitrogenous bases. By silver fractionation the proportion of vitamin may be increased to a certain extent, but a considerable degree of purification cannot be effected by this method. In general it has been found that the higher the purity of the fraction, the smaller the amount of it obtained.

The Character of the Fractions Obtained by the Silver Method

When the evidence in regard to the large proportion of non-precipitable vitamin (Table I, Fraction IV, last column) was first obtained, a doubt arose as to whether the precipitates contained a silver compound of vitamin, or consisted of silver compounds of other bases to which the vitamin was attached by adsorption. Brief consideration shows, however, that this latter assumption requires that, of the mixture of bases in the original extract, vitamin, in spite of exhibiting characteristics identical with the others up to this point, now differentiates itself from them by failure to combine with silver. This appears to be an entirely unwarranted assumption. Consequently, a doubt as to the chemical union of silver and the vitamin base does not appear justified.

Furthermore, the assumption that both the vitamin and non-vitamin bases are simultaneously precipitated by silver furnishes a reasonable explanation of the failure to secure a noteworthy concentration of vitamin by the silver method. The very slight purification which was actually effected is exhibited by the figures given in Table I. Thus, on the basis of the minimum protective dose, which may be taken as a criterion of the purity of the vitamin fraction, there was a reduction of from 7.5 to 5 mg. obtained by the use of silver nitrate, and a further reduction to 3 mg. by ammoniacal silver nitrate.

Additional evidence that, after precipitation, the ratio of the components of the mixture is not greatly changed is obtained from the outward appearance of the residues after removal of the silver. These residues exhibit no noteworthy differences and in each case consist of viscous hygroscopic material of a character such as would be expected of a complex mixture.

The conception to be gained from the above discussion is that the vitamin fractions so far obained are mixtures of two or more analogous bases. The chemical similarity of these constituent bases is exhibited in their common adsorbability on fuller's earth, ease of common extraction by aqueous alkali and common precipitation by both neutral and ammoniacal silver nitrate. It is, of course, a question whether the antineuritic activity resides in a single member of the mixture, or is a function of the comple-

mentary action of two or more individuals. The universal experience is that no single isolated compound has ever been found to exhibit unmistakable antineuritic activity. To what extent this evidence supports the latter view cannot be stated.

This apparent complex character of all vitamin fractions, so far obtained, accounts for the exceptional difficulty encountered in all efforts to identify the active substance.

Summary

A method is described for the isolation of highly active vitamin fractions from yeast by utilizing the adsorptive power of fuller's earth. The "activated solid" thus obtained is extracted with barium hydroxide solution and the barium eliminated by acidifying with sulfuric acid. The extract, after concentration by vacuum distillation, is precipitated successively with silver nitrate and ammoniacal silver nitrate. Approximately $^1/_3$ of the solids of the extract is precipitated as silver compounds and these contain somewhat more than $^1/_2$ of the antineuritic vitamin. The incomplete precipitation of the vitamin base is believed to be due to the considerable solubility of its silver compound.

The vitamin fractions were found to be quite stable both in solution and in the dried condition. They dialyze almost completely through a collodion membrane, and physiological tests showed that all of the vitamin is in the diffusate, thus indicating that the vitamin molecule is of relatively simple constitution. Using nitrogen determinations as a criterion of purity, it was concluded that the highly active fractions contain vitamin and one or more analogous nitrogenous bases, and these cannot be advantageously separated by silver precipitation.

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A SULFONATED NAPHTHYLARSINIC ACID

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In the course of a certain chemotherapeutic research at this laboratory, it seemed advisable to prepare a compound similar in properties to the α-naphthylarsinic acid described by W. Kelbe[1] but more soluble in water. It was reasonable to suppose that this might be obtained by sulfonating the original α-naphthylarsinic acid. A careful review of the literature leads us to believe that only one compound containing both sulfonic and arsenic acid groups on the same nucleus has been prepared. This is the trisulfonic

[1] Kelbe, *Ber.*, **11**, 1503 (1878).