[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF HEALTH]

EXPERIMENTS ON THE ISOLATION OF THE ANTINEURITIC VITAMIN

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In all methods used for the isolation of the antineuritic vitamin the losses of active material are so great that only meager amounts of the final concentrate are obtained. In many cases the quantities have not been sufficient even for preliminary chemical study.

Each advance in the purification of the active fraction demonstrates more clearly the minuteness of the dose of antineuritic vitamin capable of exerting a definite physiological effect. The most active preparations so far made are probably still far from chemical purity. Even the crystals isolated by Jansen and Donath[1] seem to owe their activity to admixed vitamin. This presumption is based on the fact that fractions active in distinctly smaller doses than the Jansen and Donath crystals[2] have been obtained by the procedure described in this and in previous publications[2,3] from this Laboratory.

The necessity for obtaining larger amounts of the most highly active fractions is apparent. Existing methods need to be improved both in the reduction of losses which occur at the several stages and in their application on an increasingly large scale. The experiments here recounted were made with this purpose in view.

For convenience of discussion the procedure may be subdivided into the following steps: (1) preparation of the "activated solid"; (2) extraction and concentration of the vitamin solution; (3) benzoylation and acetone precipitation of the salts; (4) extraction and acetone precipitation of the vitamin concentrate.

Preparation of the "Activated Solid."—This step of the process was first described some fifteen years ago[4] and since then various modifications of it have been introduced. The one to be described now was the result of efforts to prepare the product on a larger scale than had previously been possible. For the opportunity and equipment to do this grateful thanks are extended to M. H. Penau, Technical Director of Les Établissements Byla, located at Gentilly (Seine) near Paris, France.

[1] Jansen and Donath, "Med. van den Dienst der Volksgezondheid in Ned.-Indie," Weltevreden, Batavia, Java, Part 1 (1927).

[2] The results of tests of a sample of the Jansen and Donath crystals given in a previous publication [Seidell and Smith, *Pub. Health Repts.*, **45**, 3191–3200 (1930)] show the minimum curative dose for rats to be 0.04 mg., whereas repeated tests of the most active sample obtained in the present series of experiments showed it to be active in 0.03 mg. doses.

[3] Seidell, *J. Biol. Chem.*, **82**, 633–640 (1929).

[4] Seidell, *Pub. Health Repts.*, 31, 364 (1916).

One hundred and sixty kilograms of pressed brewer's yeast (equal to approximately 45 kg. of dry yeast) was added quickly to about 300 liters of rapidly stirred water heated to about 80°. The mixture, after cooling somewhat, was filtered through a large filter press. To the clear filtrate 15 kg. of fuller's earth was added and the mixture mechanically stirred for one hour. It was then allowed to stand for two hours for subsidence of the fuller's earth which had now become "activated solid." A sample of the mixture which stood in a glass cylinder showed that the supernatant liquid contained a considerable amount of material in suspension. Since this suspended colloidal matter might clog the filter press and would certainly contaminate the activated solid, it was decided to remove it by decantation. The necessities of the case, therefore, led to a modification of the procedure which subsequent experiments have shown to be of decided advantage. The final concentrate resulting from this experiment, when tested on pigeons and on rats by the Smith method,[5] was found to be considerably more active than any previously made from activated solid which had been separated from the yeast solution by means of a Sharples supercentrifuge. It is believed that this simple modification effects the removal of organic constituents which interfere with subsequent steps of the process. The supernatant liquid, therefore, should be decanted from the activated solid and this latter washed with slightly acidified (1.0 cc. of concd. hydrochloric acid per liter) water one or more times.

If the extraction of the activated solid is not to be made immediately it should be filtered or separated with a Sharples supercentrifuge and thoroughly dried or else simply immersed in about 20% alcohol to prevent decomposition of its adsorbed organic matter by organisms.

Although heating yeast in water to about 80° is a very simple and effective way of liberating the vitamins from the cells, an experiment was made in one case with the use of a liquefying agent for obtaining an aqueous solution of the vitamin.

To 7.0-kg. portions of pressed brewer's yeast there was added 700 cc. of a mixture prepared according to Pirie[6] in the ratio of 80 cc. of 81% ethyl alcohol, 20 cc. of concentrated sulfuric acid and 100 cc. of ether. The yeast rapidly liquefied and, after dilution with about three times its volume of cold water, was passed through the Sharples supercentrifuge. To the nearly clear effluent 500 g. of fuller's earth was added and the resulting activated solid after decantation of the supernatant liquid was washed twice by decantation with acidified water.

The combined activated solid thus prepared when used for the remaining steps of the process yielded an aqueous extract which contained an amount of dissolved solids greater than was present in the extracts from activated solid made by the heating method. This excess of solids was also found to interfere with subsequent steps of the process, resulting in a low yield of poor quality final concentrate.

Extraction and Concentration of the Vitamin Solution.[7]—As pointed out in previous papers the extraction of the activated solid is made by agitating it violently in approximately 0.4 to 0.5 normal sodium hydroxide solution for about five minutes and separating the alkaline liquid from the

[5] Smith, *Pub. Health Repts.*, **45**, 116–129 (1930).

[6] Pirie, *Biochem. J.*, **24**, 51 (1930).

[7] Appreciative acknowledgment is made for facilities placed at our disposal in Washington, D. C., by the Chemical and Technological Research unit of the Bureau of Chemistry and Soils, U. S. Department of Agriculture.

solid as quickly as possible by means of a Sharples supercentrifuge. The extract must be acidified quickly with sulfuric acid and adjusted to approximately P_H 3.0. In all cases just at this turning point a light, fluffy precipitate separates and serves as an indication that acidity has been reached. In one case a quantity of this precipitate was separated and tested for antineuritic vitamin. Its activity was found to be very little greater than the dry yeast from which it came. Since the amount of the precipitate was small the actual loss of vitamin was insignificant.

When the slightly acid extract is distilled under diminished pressure to about one-tenth its volume, a rather large amount of a brownish product separates. This is removed best by deposition in a large cup centrifuge and the nearly clear supernatant solution decanted. This, after seeding with sodium sulfate decahydrate, is kept in a cool place for crystallization of the large excess of this salt which is present.

The brown precipitate mentioned above has been found by Doctor M. I. Smith, in rat experiments,[8] to be very rich in the thermostable growth factor (B_2 or G) required as a supplement to the antineuritic vitamin for normal growth.[9] Dried samples of it are usually about five times as active as dried yeast. This product therefore furnishes a convenient source of the thermostable factor B_2 and by very simple means, designed to eliminate the sodium sulfate present, it can be concentrated to an activity of more than ten times that of the yeast from which it was derived.

The concentrated solution from which the excess of sodium sulfate decahydrate crystallizes should be treated with about an equal volume of methyl or ethyl alcohol in order to precipitate more of the inorganic salts and the organic impurities which are present.

It is a question as to just how far this alcohol precipitation should be continued. Of course, vitamin is lost if the alcoholic concentration is raised too high. Much material which interferes with subsequent purification of the vitamin is, however, eliminated and a much better final product may be obtained by sacrificing some of the vitamin at this point.

The clear approximately 50% alcoholic solution resulting from the above treatment usually contains about 30 g. of dissolved solids per liter, of which about 30% are inorganic salts. Some typical results obtained up to this point in the procedure are shown in the accompanying table (I).

It will be noted that the estimated quantity of dry yeast used per 5.0 kg. of activated solid, as shown in Table I, varied from 20.5 to 30 kg. This was due to the difficulty of estimating closely the amount of dry yeast corresponding to given amounts of fresh or even pressed yeast. There was also considerable variation in the amount of the thermostable growth vitamin (B_2) rich precipitate which separates during the distillation of the

[8] This work is still in progress and will be described later.

[9] Smith and Hendricks, *Pub. Health Repts.*, **41**, 201 (1926).

TABLE I

EXPERIMENTAL DATA

Expt.	Brewer's yeast used	Est. amt. dry yeast present, kg.	Amt. activated solid made, kg.	(B_2) rich brown precipitate, g.	(B_1) rich 50% alc. soln., liters	Total grams dissolved solids in (B_1) solution Organic	Inorganic
23	Fresh	28.0	5.0	230	7.0	Not detd.	Not detd.
24	Fresh	42.0	7.5	844	8.0	Not detd.	Not detd.
25	Fresh	25.0	5.0	377	7.0	Not detd.	Not detd.
27	Pressed	23.6	5.0	112	12.2	186	205
28	Com. dry (H)	22.6	5.0	300	10.5	222	83
29	Fresh	30.0	6.0	425	5.8	144	61
30	Com. dry (W)	20.5	5.0	434	5.0	121	46
31	Pressed	33.0	5.5	604	7.0	128	53

aqueous extract of the activated solid. This is due not only to a variation in the quantity of this insoluble material but also to the presence of variable amounts of sodium sulfate and other salts which separate together with it.

The volume of the alcoholic antineuritic (B_1) vitamin solution depends upon the degree to which the concentration by distillation has been carried and this cannot be accurately controlled in the large enamel lined vacuum distilling apparatus which was used.

The extent of the recovery of the antineuritic vitamin itself is not shown in the table, but estimates from experiments on pigeons made with certain of the fractions indicated that between 5 and 20% of the original vitamin of the yeast is present in the alcoholic solution. There is consequently a loss of 80 to 95% of the antineuritic vitamin up to this point. Some of this loss may be ascribed to variations in the vitamin content of different samples of brewer's yeast but, of course, the larger part is simply due to the imperfections of the fractionation procedure. In this connection it should be pointed out that the actual yield is not such an important matter as the quality of the final product obtained. Accumulated experience seems to show that the quality and yield of the final concentrate obtained by the subsequent steps of the process vary inversely with the percentage of the original vitamin present at the 50% alcoholic solution stage. The most active concentrate which has so far been made was obtained from an alcoholic solution in which there was only about 5% of the antineuritic vitamin of the yeast from which it was prepared. Apparently the removal of inactive constituents can be effected only with simultaneous considerable losses of the active compound, especially in the earlier stages of the process.

Benzoylation and Acetone Precipitation of the Salts.—The 5 to 12 liter quantities of alcoholic vitamin solution obtained by the procedure described above were usually divided into three equal portions and these separately distilled down and benzoylated. In the case of Experiments 23, 24 and 25 it was observed that the final products obtained from the three

fractions of each of these solutions differed considerably in activity even though the benzoylation and acetone precipitations were conducted as nearly alike as possible. In an attempt to ascertain the effects of slight variations of reagents and technique upon the character of the final product, a series of experiments was made with the alcoholic solutions obtained in Experiments 27–31. Each of these was divided into three equal portions which were separately distilled to small volumes. They were each centrifuged to remove slight amounts of insoluble material, and the clear supernatant solutions diluted with water to the volumes shown in Table II. The quantities of sodium carbonate and benzoyl chloride recorded in the table were quickly added and the mixtures stirred with a thermometer. The highest temperature reached during the reaction was noted and about an hour allowed for the mixtures to cool. They were then extracted with chloroform and the resulting aqueous layers poured slowly into 10–15 times their volume of acetone. The weights of the precipitated salts and their nitrogen contents are recorded in the table.

Extraction and Acetone Precipitation of the Vitamin Concentrate.— The vitamin salts were extracted by rotating for twenty-four hours with a mixture of three volumes of normal propyl alcohol and one volume of concentrated hydrochloric acid, using about 3 cc. of this mixture per 1.0 g. of salts. The extract was separated by centrifugation and the residue extracted a second time in the same manner. The two extracts were distilled under diminished pressure to about 50 cc. This concentrated liquid was centrifuged to remove a small amount of salts which separate during the distillation. The clear solution was then added dropwise to about 1600 cc. of acetone. The resulting precipitate was separated by centrifugation and dissolved in about 15 cc. of methyl alcohol and this added dropwise to about 800 cc. of acetone. The precipitate is now white and in a voluminous flaky condition. It is centrifuged and then washed with acetone by centrifugation and finally dried in a vacuum. The quantities obtained in the several experiments are shown in Table II.

The activities of the 16 precipitates made in this manner were determined by Dr. M. I. Smith, using his rat method.[5] The activities of the alcoholic vitamin solutions of Experiments 28–31 were also determined in the same manner and found to correspond, respectively, to 116, 83, 55 and 87 thousand vitamin units, or minimum curative doses.

Discussion

An examination of the results in Table II shows that unaccountable variations in the yield and quality of the final acetone precipitate frequently occur. In the case of Experiment 28(a) the yield of vitamin was only about 11%, while in the case of Experiment 31(a) it was about 70%. The only intentional difference in the two cases was that the proportion of

TABLE II

EXPERIMENTAL DATA

Expt.	Organic solids present, g.	Vol. of aqueous soln., cc.	—Benzoylation reagents— Na_2CO_3, g.	C_6H_5COCl, cc.	Max. temp. of reaction, °C.	Pptd. vitamin salts, g.	N in vitamin salts, %	Final acetone pptd. vitamin, g.	N in acetone pptd. vitamin, %	Minimum curative dose (rats) mg.	Total vitamin, units
27a	63	500 (8.0)	100 (1.6)	180 (1.8)	50	119	0.54	1.19	10.5	0.1	11,900
27b	63	500 (8.0)	75 (1.2)	180 (2.4)	45	100	.69	2.01	11.4	.25	8,000
27c	60	500 (8.3)	75 (1.2)	135 (1.8)	50	97.5	.98	0.79	10.0	.3	2,630
28a	74	500 (6.8)	150 (2.0)	275 (1.8)	60	132	.49	1.30	12.6	.3	4,333
28b	74	500 (6.8)	150 (2.0)	275 (1.8)	25	143	.59	1.58	12.0	.5	3,160
28c	74	500 (6.8)	150 (2.0)	275 (1.8)	30	120	.61	2.03	12.4	.5	4,070
29a	46	364 (8.0)	77 (1.7)	133 (1.7)	32	72	.9	2.47	12.1	.3	8,233
29b	46	288 (6.2)	86 (1.8)	155 (1.8)	73	99.5	.23	0.675	9.3	.06	11,250
29c	46	240 (5.0)	86 (1.8)	155 (1.8)	60	84	.50	1.06	11.5	.15	7,070
30a	40	241 (6.0)	72 (1.8)	130 (1.8)	60	70	.42	1.09	10.9	.15	7,270
30b	40	241 (6.0)	72 (1.8)	130 (1.8)	61	69.5	.37	1.09	10.0	.17	6,400
30c	40	280 (7.0)	72 (1.8)	130 (1.8)	58	76.0	.52	1.83	10.4	.25	7,320
31a	42.7	299 (7.0)	77 (1.8)	138 (1.8)	62	77.5	.75	1.64	13.1	.08	20,500
31b	40.0	240 (6.0)	68 (1.7)	116 (1.7)	61	69.0	.59	1.09	11.9	.10	10,900
31c	40.0	280 (7.0)	80 (2.0)	144 (1.8)	67	77.0	.31	0.70	8.9	.8	8,750
22	94 (Est.)	675 (7.2)	150 (1.6)	275 (1.8)	50	160.0	.06	a 0.074	8.2	...	....
								b 0.120	7.0	0.03	400

Of the figures in parentheses the first in each experiment shows the ratio of solution to organic solids; the second shows the ratio of sodium carbonate to organic solids, and the third the ratio of benzoyl chloride to sodium carbonate.

sodium carbonate used in the benzoylation was slightly higher in the first case than in the second. A similar diminution with increase of sodium carbonate is noted in Expt. 30(c) but in this case the quality of the final precipitate was not affected but only the quantity obtained. It is apparent that the exact ratio of reagents to organic solids in the solution being benzoylated must be very carefully controlled in order to get the best results. The optimum ratios appear to be approximately seven times as much aqueous solution as there are organic solids, 1.8 times as much sodium carbonate as organic solids and 1.8 times as much benzoyl chloride as sodium carbonate.

The experiments have definitely demonstrated the advantage of higher temperatures for the reaction. In the cases of experiments 28(b) and (c) and 29(a) the mixtures were immersed in ice water to prevent a rise in temperature, and in each case the activity and yield of the final precipitate was of a low order. The effect of higher temperatures than result spontaneously during the reaction has not as yet been studied.

The results in the table also show that the lower the percentage of nitrogen in the precipitated vitamin salts, the higher the activity of the final acetone precipitated vitamin. Attention should therefore be directed continually toward conducting the benzoylation in such a manner that as little nitrogenous material as possible remains in the acetone precipitated salts.

It is noteworthy that the samples of final acetone precipitated vitamin except those from solutions cooled during the benzoylation vary in activity, as measured by minimum curative dose, from 0.03 mg. to 0.3 mg. It will be noted that in general the more active samples have the lower nitrogen content. There is, however, no close parallelism between nitrogen content and activity. Hence, considerable amounts of non-vitamin nitrogenous compounds are probably still present even in the more active samples.

In conclusion, attention is directed to the fact that the procedure here described does not involve the use of any of the precipitating agents which form so important a part of most other methods for the concentration of the antineuritic vitamin. Even without the use of platinum precipitation, a concentrate distinctly more active than the crystals made with such extraordinary expenditure of effort by Jansen and Donath has been obtained. Furthermore, fairly large quantities may be prepared. Under favorable conditions about 1.0 g. of a product approximately equal in activity to the Jansen and Donath crystals should be obtained from each 10 kg. of dried brewer's yeast. It is hoped that with further intensive study this yield can be increased; also, that it will be possible to prepare considerably larger amounts of the product curative in 0.03-mg. doses which has, so far, been obtained only once.

Summary

The procedure involving adsorption of the antineuritic vitamin upon fuller's earth and subsequent extraction, benzoylation and acetone precipitation has yielded, without the use of any precipitating agents, final products the most active of which was curative in 0.03-mg. doses, by the Smith rat method. This is an activity about one-fourth greater than that of the Jansen and Donath crystals. It is more than twice as great on the nitrogen basis. Products of somewhat lower activity were obtained in considerably larger yields. Apparently small variations of conditions at certain stages of the process may affect greatly the quantity and quality of the final product. Especial attention has been given to the effect of variation of ratio of the benzoylating reagents and of the temperature of the reaction upon the quality and yield of the final concentrate.

WASHINGTON, D. C.

[CONTRIBUTION NO. 75 FROM THE COBB CHEMICAL LABORATORY OF THE UNIVERSITY OF VIRGINIA]

NEW ALKAMINES IN THE TETRAHYDRONAPHTHALENE SERIES[1]

BY ERICH MOSETTIG AND ALFRED BURGER

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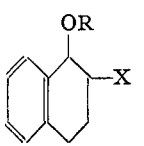
In the classic investigations of partially hydrogenated naphthalene derivatives carried out by Bamberger[2] over forty years ago, the fact was established that alicyclic β-tetrahydronaphthylamine and its derivatives exert to a considerable degree a specific pharmacological action. After studying a number of compounds, Bamberger stated the rule that this property is exhibited only when the substituted nucleus is hydrogenated, and only when the basic substituent occupies the β-position. In more recent years, this observation was confirmed through the investigations of von Braun[3] on alicyclic amino alcohol derivatives of tetrahydronaphthalene. He was able to show that only compounds of type I have pharma-



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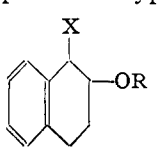

II

X = NH_2, $HNCH_3$, $N(CH_3)_2$, etc. R = H or alkyl

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[1] This investigation was supported by a grant from the Committee on Drug Addiction of the National Research Council from funds provided by the Bureau of Social Hygiene, Inc.

[2] E. Bamberger and W. Filehne, *Ber.*, **22**, 777 (1889).

[3] Von Braun, Braunsdorf and Kirschbaum, *ibid.*, **55**, 3648 (1922); v. Braun and Weissbach, *ibid.*, **63**, 3052 (1930); *cf.* Strauss and Rohrbacher, *ibid.*, **54**, 40 (1921).