THE DIFFERENTIAL EXTRACTION FROM DRIED BREWERS' YEAST OF THE ANTINEURITIC (VITAMIN B₁) AND GROWTH-PROMOTING (VITAMIN B₂) VITAMINS AND THEIR BIOLOGICAL STANDARDIZATION

WITH A NOTE ON THE RELATION OF HEMIN TO VITAMIN B₂

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Since the demonstration of the dual nature of vitamin B in 1926 (1, 2) which has been amply confirmed in recent years (3-5), there has been no satisfactory method for the separation of the heat-labile antineuritic (vitamin B₁) from the more heat-stable growth-promoting (vitamin B₂) component of the vitamin B complex. There are numerous experiments recorded in the literature indicating that at least partial separation of the two vitamins might be effected either by differential solubility in certain solvents or by differential adsorbability on certain adsorbents. Actual attempts in this direction made upon material rich in the two vitamins, such as yeast, have not met with much success (6-11).

It appears from the foregoing that for practical purposes the only reliable source of vitamin B₂, nearly if not completely free from vitamin B₁, is still an autoclaved yeast or yeast extract, as first suggested in 1926 (1). To get further information on the relation of vitamins B₁ and B₂ in the nutrition of the white rat and their rôle in the production of experimental polyneuritis in this animal, it appeared desirable to attempt to obtain a vitamin B₂ fraction by removing intact the vitamin B₁ component from material containing the complex rather than destroying it by heat as is done in the autoclaving process. The advantages of such a procedure are too obvious for discussion. The present work deals
with the results of such experiments, which aimed at a separation of vitamins $B_1$ and $B_2$ by their differential solubility in certain solvents.

Methods

Dried brewers' yeast was used as the sole source of the vitamin B complex in all this work. The yeast was obtained from a local brewery. The bulk of the water having been removed by means of a hydraulic press, the expressed yeast was then dried in a current of warm air and ground to 40 or 60 mesh powder. This was then extracted by percolation with a definite volume of the particular solvent in question. A weighed quantity of the powdered dried yeast was moistened with the solvent, packed in a suitable percolator, and after maceration overnight was percolated with the solvent at such a rate that from 1500 to 2000 cc. of the percolate were collected in the course of the day.

The extract and the air-dried extracted material were then each tested for vitamin $B_1$ and vitamin $B_2$, respectively. In addition to these tests the extracted material was also fed to young rats at a 10 per cent level incorporated in a basal ration free from the vitamin B complex but adequate in other respects. This was fed over a period of 50 days or more in order to ascertain the effect of such a diet on the growth curve and to determine whether or not such a ration could produce the experimental vitamin $B_1$ deficiency disease in the rat.

The tests for vitamin $B_1$ on the extracts were conducted according to the method described in 1930 (12). A definite volume of a given extract was evaporated to dryness in vacuo, the residue was taken up in a suitable volume of physiological saline, filtered, and neutralized with sodium bicarbonate if necessary, and the minimal amount thereof required to cure the specific experimental polyneuritis in the majority of the animals treated was ascertained. A given dose usually supplied injections for three to four rats. The number of rat units in the extract was then calculated therefrom.

The tests for vitamin $B_2$ were carried out upon the air-dried extracted material. The technique employed is based on the observation that when young rats are maintained on a basal diet free from the vitamin B complex and are treated with an adequate dose
of the antineuritic vitamin at 5 or 6 day intervals, the growth curve remains stationary; and that any change in the growth curve that follows the administration of material containing vitamin B₂ is quantitatively proportional to the vitamin so administered. The basal diet free from vitamin B complex differs from the polyneuritis-producing diet in that it contains no autoclaved yeast. It consists of

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>Purified casein</td>
<td>18</td>
</tr>
<tr>
<td>McCollum's Salt Mixture 185</td>
<td>4</td>
</tr>
<tr>
<td>Cod liver oil</td>
<td>2</td>
</tr>
<tr>
<td>Olive oil</td>
<td>8</td>
</tr>
<tr>
<td>Corn-starch</td>
<td>68</td>
</tr>
</tbody>
</table>

Animals of about 30 days of age, weighing usually 50 to 60 gm., are placed on this diet and in about 7 to 10 days receive an intravenous injection (in the tail vein) of 1 cc. containing 1 mg. of an antineuritic concentrate of the type described by Seidell and Smith (14). Such concentrates have an activity of from 0.05 to 0.1 mg.; i.e., a rat with experimental polyneuritis will recover from a single injection of such a dose and the polyneuritis will not recur in less than 5 days. It will be observed that rats to be used for the vitamin B₂ test receive 10 to 20 times or more their minimal requirement of this vitamin with no appreciable effect on their weight curves. The injection of the vitamin is repeated at 4 to 6 day intervals throughout the course of the experiment. When the weight curve has remained stationary for at least 10 days the material to be tested is administered daily as a supplement apart from the basal ration for a period of 10 days and the weight increment during this period is noted. This is compared with the corresponding weight increment produced under the same conditions by a known supplement of dried brewers' yeast which is used as the standard, and therefrom the vitamin B₂ activity of the material tested is computed as per cent of the standard.

Chart 1 shows the daily weight increment over a period of 10 days of rats treated with graded supplements of the dried brewers' yeast used in this work as the standard. The crosses in the chart indicate the individual experiments and the circles represent the averages for the group. It will be seen that there is a rapid in-

¹ Further details may be found in a previous publication (12).
crease in the daily weight increment with increasing doses from 0.2 to 0.7 gm. of yeast per day, and then the curve flattens out. Hence, when a sample of material is tested for vitamin B₂ potency, a dose is sought which will yield a daily weight increment to correspond to that produced by 0.2 to 0.5 gm. of the standard. The

**Chart 1.** Daily weight increments of rats receiving as vitamin B₂ supplement graded doses of dried brewers' yeast. Basal Ration 250 devoid of vitamin B complex, and intravenous injections of an antineuritic concentrate every 5 to 6 days were given. The crosses indicate individual experiments and the circles represent the averages for the group.

effect produced by a given dose is usually quite uniform and three or four animals on a given dose have been found to be generally sufficient.

By way of illustration an actual experiment may be cited in which the vitamin B₂ potency was ascertained in the autoclaved brewers' yeast, which is regularly included to the extent of 10
per cent in our experimental polyneuritis-producing diet. After a preliminary period of 20 days two groups of three rats each were administered daily supplements of this material in 300 and 600 mg. doses respectively. At the end of the 10 day observation period the animals of the first group showed a daily increment of 1.4, 1.2, and 1.3 gm. or an average of 1.3 gm. per day. The animals of the second group showed an increment of 2.0, 2.4, and 2.9 or an average of 2.4 gm. per day (Curves 250-A and 250-B, Chart 2). Reference to the standard curve of Chart 1 shows that a daily increment of 1.3 gm. corresponds to about 180 mg. of dried brewers’ yeast and a daily increment of 2.4 gm. corresponds very nearly to 380 mg. of dried brewers’ yeast. The first value indicates a vitamin B₁ activity for the autoclaved yeast of 60 per cent and the second value 63 per cent as compared with the dried brewers’ yeast. Evidently the autoclaving process not only destroys most, if not all the vitamin B₁ factor, but it also destroys some 40 per cent of vitamin B₂. This is in agreement with similar conclusions recorded in the literature and arrived at from somewhat different observations (15, 16).

The third test routinely performed was to ascertain the effects of the extracted material on prolonged feeding. For this purpose rats of a similar age and weight, and from the same colony, were placed on the same synthetic ration to which the material to be tested had been added in the proportion of 10 per cent, replacing an equivalent amount of starch. Four to six animals were used in a group, and the feeding continued usually for a period of at least 50 days or longer if it seemed desirable. Two things were noted in these experiments, first the weight curve, and secondly whether or not experimental polyneuritis resulted. Whenever experimental polyneuritis had developed on such rations, the curative effect of an antineuritic concentrate of known potency was examined in order to make certain the specific nature of the polyneuritis. Such tests invariably indicated that whenever polyneuritis had developed we were actually dealing with the specific deficiency disease.

Results

The total series of experiments comprised a number of extractions with three different solvents, methyl and ethyl alcohols and
TABLE I
Differential Extraction of Vitamins B₁ and B₂ from Dried Brewers' Yeast with Certain Solvents, and Effect of Feeding Extracted Material to Rats

| Ration No. | Solvent | Volume | Vitamin B₁ removed, units per gm. yeast | Vitamin B₂ left, per cent of original | No. of rats and sex | Experimental polyneuritis | Growth | Average weight
<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>251</td>
<td>Methyl alcohol + 5 per cent HCl</td>
<td>5</td>
<td>4 M.</td>
<td>None</td>
<td>Slight</td>
<td>45</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>267</td>
<td>&quot; + 5 &quot; + 5 &quot;</td>
<td>10</td>
<td>0 &quot;</td>
<td>None</td>
<td>&quot;</td>
<td>55</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>269</td>
<td>70 per cent methyl alcohol + 3 per cent HCl</td>
<td>5</td>
<td>0 &quot;</td>
<td>5 F.</td>
<td>&quot;</td>
<td>45</td>
<td>†</td>
<td></td>
</tr>
<tr>
<td>255</td>
<td>95 &quot; ethyl &quot; + 1 &quot; &quot; glacial acetic acid</td>
<td>8</td>
<td>6 M.</td>
<td>None</td>
<td>&quot;</td>
<td>46</td>
<td>152</td>
<td></td>
</tr>
<tr>
<td>260</td>
<td>95 per cent ethyl alcohol + 5 per cent HCl</td>
<td>8</td>
<td>9 70</td>
<td>4 F.</td>
<td>&quot;</td>
<td>Subnormal</td>
<td>48</td>
<td>107</td>
</tr>
<tr>
<td>262</td>
<td>&quot; &quot; &quot; &quot; + 5 &quot; &quot;</td>
<td>10</td>
<td>11 50</td>
<td>6 M.</td>
<td>&quot;</td>
<td>Slight</td>
<td>45</td>
<td>80</td>
</tr>
<tr>
<td>274</td>
<td>76 &quot; &quot; &quot; &quot;</td>
<td>10</td>
<td>16 100</td>
<td>5 &quot;</td>
<td>Subnormal</td>
<td>50</td>
<td>115</td>
<td></td>
</tr>
<tr>
<td>277</td>
<td>76 &quot; &quot; &quot; &quot; + 1 &quot; &quot; &quot;</td>
<td>10</td>
<td>19 63</td>
<td>5 F.</td>
<td>In all, 60-80 days</td>
<td>Subnormal</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>270</td>
<td>70 &quot; &quot; &quot; &quot; + 3 &quot; &quot; &quot;</td>
<td>5</td>
<td>18 33</td>
<td>5 &quot;</td>
<td>None</td>
<td>Slight</td>
<td>42</td>
<td>58</td>
</tr>
<tr>
<td>279</td>
<td>67 &quot; &quot; &quot; &quot; + 1 &quot; &quot; &quot;</td>
<td>10</td>
<td>21 46</td>
<td>4 &quot;</td>
<td>In 3, 40-50 days.</td>
<td>Normal</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>273</td>
<td>57 &quot; &quot; &quot; &quot;</td>
<td>5</td>
<td>14 70</td>
<td>6 &quot;</td>
<td>None</td>
<td>Subnormal</td>
<td>51</td>
<td>118</td>
</tr>
<tr>
<td>264</td>
<td>50 &quot; &quot; &quot; &quot;</td>
<td>5</td>
<td>50 5 M.</td>
<td>In 3. 2 died</td>
<td>Normal</td>
<td>50</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>272</td>
<td>70 acetone</td>
<td>10</td>
<td>4 95</td>
<td>5 &quot;</td>
<td>None</td>
<td>Normal</td>
<td>50</td>
<td>155</td>
</tr>
<tr>
<td>271</td>
<td>70 &quot; &quot; &quot; &quot; + 1 per cent HCl</td>
<td>8</td>
<td>60 5 F.</td>
<td>In all, 30-40 days</td>
<td>Normal</td>
<td>48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>275</td>
<td>&quot; &quot; &quot; &quot; + 1 &quot; &quot; &quot;</td>
<td>8</td>
<td>16 95</td>
<td>4 M.</td>
<td>&quot; &quot; 75-85 &quot;</td>
<td>Normal</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>268</td>
<td>&quot; &quot; &quot; &quot; + 3 &quot; &quot; &quot;</td>
<td>5</td>
<td>40 5 F.</td>
<td>None</td>
<td>Subnormal</td>
<td>47</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>276</td>
<td>&quot; &quot; &quot; &quot; + 3 &quot; &quot; &quot;</td>
<td>8</td>
<td>21 35</td>
<td>5 &quot;</td>
<td>Subnormal</td>
<td>50</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>278</td>
<td>60 &quot; &quot; &quot; &quot; + 1 &quot; &quot; &quot;</td>
<td>10</td>
<td>20 31</td>
<td>5 M.</td>
<td>In all, 40-50 days</td>
<td>Subnormal</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

Blank readings indicate that the vitamin was not determined.

* Died within 40 days. † Died within 30 days.
acetone with varying percentages of water. Hydrochloric acid from 1 to 5 per cent was used in many of these experiments. The aim has been to find a suitable solvent to remove vitamin B1 as completely as possible and to leave vitamin B2 as much as possible unextracted. The results of these experiments are summarized in Table I and illustrated in Chart 2.

Analysis of these data seems to indicate clearly that methyl alcohol with hydrochloric acid is not a suitable solvent for the separation of vitamins B1 and B2, as it appears to remove both vitamins at about the same rate, while ethyl alcohol and acetone in suitable concentrations with or without hydrochloric acid show a sufficient degree of preferential solubility for vitamin B1 to make it possible to effect a separation of the two vitamins to the extent of removing most if not all of the vitamin B1 factor, leaving behind the greater part of vitamin B2.

Is the Thermostable Growth-Promoting (Vitamin B2) Factor Identical with the Antidermatitis or Pellagra-Preventive Factor?

In 1928 Goldberger, Wheeler, Lillie, and Rogers (17) stated in reference to this question as follows: "It [the black tongue preventive factor] can not be identified with any of the older well-recognized dietary essentials, but is believed to be identical with the thermostable [growth-promoting] substance of Smith and Hendrick." Other writers have generally assumed and tacitly accepted this suggested identity as a proved fact. More recently, however, there has been presented some evidence casting considerable doubt on the identity of the thermostable growth factor (referred to in this paper as vitamin B2) and the antidermatitis factor (18, 19).

Kollath (20) advanced the hypothesis that hemin which normally occurs in yeast is the antidermatitis factor. This suggestion was based on the observation that rats on a basal ration free from the vitamin B complex developed experimental polynoeritis but failed to develop skin lesions if the diet was supplemented with 0.5 mg. of hematin daily. The weight curves given by Kollath on this diet indicate very little effect on growth. This it seemed, if it could be corroborated, might yield further evidence on this question of identity or non-identity of the factors referred to. Moreover, if Kollath's suggestion concerning the relation of hema-
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![Chart 2](chart2.png)

1. Chart 2 illustrates the changes in Gm. over a period of 10 days for various samples.
2. The chart shows the progression of different samples labeled A, B, C, and D, indicating their Gm. levels at different time points.
3. Sample A shows a steady increase in Gm. from day 0 to day 10.
4. Sample B starts with a high Gm. level and drops significantly by day 10.
5. Sample C exhibits a fluctuating pattern with peaks and troughs, reaching a high of 150 Gm. by day 10.
6. Sample D has a consistent level throughout the 10 days, with a slight decrease at the end.

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tin to the antidermatitis factor proved correct, it would shed further light on Bliss' recent suggestion to the effect that pellagra is an iron deficiency disease (21).

In the present work there were two series of experiments. All the rats were kept on our basal diet minus the vitamin B complex (Ration 250), and the antineuritis requirements were amply met by intravenous injection of an active concentrate every 5 to 6 days. Hemin, prepared from beef blood, was administered separately in daily doses of 5 mg. in the first series and 50 to 100 mg. in the second series. The first series consisted of six controls and five treated animals, and the second series of six controls and an equal number of treated animals, three of which received 50 mg. daily and three 100 mg. daily. In the first series the hemin, which had not been recrystallized, was administered as hematin in aqueous solution with Na$_2$CO$_3$. In the second series the hemin, which had been recrystallized from pyridine-chloroform, was administered as such.

The results of this experiment were as follows:

1. The six controls of the first series all developed typical skin lesions in from 30 to 60 days. Administration of autoclaved yeast to these animals promptly restored growth and cleared up the lesions.

2. The five hematin-treated animals of this series all developed typical skin lesions in from 40 to 100 days. At this time the

CHART 2. Typical weight curves of groups of rats on Rations 250, 244, 271, 264, 267, 252, 255, 252, and 269. D indicates death of an animal and P polyneuritis. Curves 250-A and 250-B illustrate the effect of graded doses of autoclaved yeast as used for its vitamin B$\alpha$ assay. At A 300 mg. were administered per day and at B 600 mg. per day. Curve 250-H-A$y$ shows effect of daily doses of 300 mg. of hemin (at H) and 500 mg. of autoclaved yeast (at A$y$) on growth and skin lesions of a group of three rats on Ration 250 supplemented with intravenous injections of an antineuritic concentrate every 5 to 6 days. Curve 244 shows the average weight gains of four rats on the polyneuritis-producing diet containing no vitamin B$_1$ and 10 per cent autoclaved yeast as a source of vitamin B$\alpha$. Curve 252 is the average of six rats fed a similar diet with 10 per cent autoclaved yeast which had previously been extracted with 5 volumes of 5 per cent hydrochloric acid in methyl alcohol. This removes vitamin B$_1$; and Curve 252 is identical with one obtained on the basal ration free from the vitamin B complex. The other curves refer to the rations described in Table I.
hematin was discontinued and autoclaved yeast substituted with the same result as in the control group.

3. Of the six rats in the second series treated with 50 to 100 mg. of hemin daily, three developed typical lesions at the end of 60 days, two showed minor lesions, and one appeared normal.

4. In the control group of six rats in this series three developed typical lesions at the end of 60 days and three showed but slight if any involvement. To the first group of three rats with typical lesions 300 mg. of hemin were given daily for a period of 2 weeks without any effect. Autoclaved yeast was then substituted for the hemin in 500 mg. doses per day for a period of 12 days, whereupon the skin lesions cleared up and growth resumed as shown in Curve 250-H-Ay.

The conclusion to be drawn from this experiment is that hemin or hematin administered to rats on a vitamin B-free ration adequately supplemented with vitamin B1 does not promote growth and it does not prevent the onset of dermatitis; nor is it capable of curing such dermatitis as typically occurs in rats when fed a diet lacking in the antidermatitis factor, which may or may not be identical with the thermostable growth factor. These experiments are cited not as evidence for or against the view of identity of the antidermatitis and growth factor, but as failing to confirm the suggestion by Kollath (20), which if confirmed might be construed as offering evidence in this direction.

SUMMARY AND CONCLUSIONS

The differential extraction of vitamins B1 and B2 by percolation of dried brewers' yeast with several different solvents has been studied. In each case vitamin B1 was estimated in the extract by a method previously described and vitamin B2 was estimated in the extracted residue by a technique described herein.

Three solvents were studied in some detail, methyl and ethyl alcohols and acetone with varying percentages of water and hydrochloric acid.

Methyl alcohol with 5 per cent hydrochloric acid is not a suitable solvent for differentiating vitamins B1 and B2 since it removes the two vitamins with nearly equal facility.

76 per cent ethyl alcohol and 70 per cent acetone plus 1 per cent hydrochloric acid may be used to remove some 80 per cent or more
of the available vitamin B\textsubscript{1} without removing appreciable quantities of vitamin B\textsubscript{2}. The addition of hydrochloric acid to the ethyl alcohol or its further dilution with water increases its solubility for vitamin B\textsubscript{1} but it also increases its solubility for vitamin B\textsubscript{2}. The same may be said of acetone.

These experiments confirm the view previously expressed that the successful production of the deficiency polyneuritis in the rat is conditional upon two factors, (1) the liberal inclusion of the thermostable growth accessory vitamin B\textsubscript{2} in the experimental diet, and (2) the rigorous exclusion therefrom of the thermolabile antineuritic vitamin B\textsubscript{1}.

Hemin or hematin has no relation to the thermostable growth factor (vitamin B\textsubscript{2}) or the antidermatitis factor, which are often referred to as one and the same thing, though their identity has recently been questioned and certainly never definitely established.

**BIBLIOGRAPHY**

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