FURTHER STUDIES ON VITAMINS B_{10} AND B_{11} AND THEIR RELATION TO "FOLIC ACID" ACTIVITY*

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A previous report from this laboratory (1) demonstrated the existence of two water-soluble vitamins needed by the chick; namely, vitamin B_{10}, essential for proper feather formation, and vitamin B_{11}, necessary for growth. Both of these vitamins, present in a liver concentrate (Super Filtrol eluate), were shown to be distinct from "folic acid" which was also present in the liver concentrate. All three of these factors were adsorbed and eluted from norit and Super Filtrol but were partially separated by fractional precipitation with ethanol.

This paper presents further progress toward the separation and isolation of vitamins B_{10} and B_{11} and gives additional information on their chemical properties as they exist in impure concentrates. The relationship of these substances to "folic acid" is pointed out.

EXPERIMENTAL

To study the distribution and properties of vitamins B_{10} and B_{11} all liver preparations were assayed for feathering and growth activity with white Leghorn chicks (in groups of six) over a 4-weeks period. The experimental conditions and the basal ration, No. 486K, have been reported previously (1, 5). This ration consists essentially of dextrin, alcohol-extracted casein, gelatin, soy bean oil, salts, cystine, and ample levels of known crystalline fat- and water-soluble vitamins. The level of biotin has been raised from

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1 The term "folic acid" has been used in this paper to designate any substance necessary for the growth of Streptococcus lactis R and Lactobacillus casei when grown on a defined medium (2, 3). Mitchell, Snell, and Williams (4) first used this term for "an acid nutrient" which had been obtained nearly pure and which was necessary for the growth of Streptococcus lactis R. They reported that the substance stimulated the growth of Lactobacillus casei under similar conditions.
15 to 20 \( \gamma \) per 100 gm. of ration. Normal growth and feather formation are not attained by chicks fed this basal ration unless a source of the unknown vitamins is supplied.

Assays were made by incorporating each preparation uniformly in the basal ration and the response obtained compared to that of chicks receiving the basal ration with and without solubilized liver. A response in feathering, as determined by the use of the scale in Fig. 1, indicated the presence of vitamin B_{12}, while vitamin B_{11} was measured by an increase in growth.

"Folic acid" assays were performed either by the method of Mitchell and Snell (2) with Streptococcus lactis R as the test organism and solubilized liver as the standard or by the improved method of Luckey et al. (3) with both Streptococcus lactis R and Lactobacillus casei. Bacterial activity is expressed in terms of micrograms of "folic acid" by giving the standard, solubilized liver, a potency of 1 and "folic acid" an assumed potency of 40,000 (2). Thus, solubilized liver is given a value of 25 \( \gamma \) of "folic acid" per gm. We realize that this is purely an empirical method, because we do not know the exact amount of "folic acid" activity in solubilized liver and because the recent work of Stokstad (6) shows that crystalline liver "folic acid" has a relative potency of approximately 80,000 when compared to liver Fraction B. When the amount of "folic acid" activity in solubilized liver is known, the figures which we have used may be corrected to the true amount by use of a simple proportion.
Procedure for Making Liver Preparations—The Super Filtrol eluate is prepared according to the method of Hutchings, Bohonos, and Peterson (7). In brief, it is made by treating a solution (pH 3) of solubilized liver with norit and eluting the norit with a mixture of water, ethanol, and ammonia. Adsorption and elution are repeated with Super Filtrol in place of the norit and the eluate is concentrated under a vacuum. This preparation contains vitamin $B_6$, $B_{12}$, and “folic acid” activity and is used as the starting material for the majority of the preparations discussed in this paper. Approximately 20 gm. of dry matter are obtained from 1 kilo of the starting material by this procedure and the active factors present are concentrated about 20-fold (owing to about a 60 per cent loss).

Preparation 52A (Containing Vitamins $B_6$, $B_{12}$, and “Folic Acid” Activity)—This fraction is made by adding 1500 cc. of absolute ethanol to 500 cc. of the Super Filtrol eluate equivalent to 1 kilo of solubilized liver and after this has stood in the cold the filtrate is acidified to pH 3 with sulfuric acid according to the technique of filtering, etc., outlined previously (1). The filtrate (Preparation 52A) is concentrated under a vacuum to the desired volume and neutralized. Further inactive material may be removed by adding acetone to the acid filtrate before concentration until a flocculent precipitate is formed. The filtrate is collected and concentrated in the same manner. Although we have used this same concentrate extensively, the procedure produces somewhat variable results.

Alcohol-Soluble Butyl Ester—The starting material (generally Preparation 52A), equivalent to 1 kilo of solubilized liver, is dried carefully and placed in 5 liters of normal butyl alcohol (which then contains approximately 2 mg. of dry matter per cc.) and the solution is kept under nitrogen at 60° with constant stirring for 4 to 5 hours. The entire mixture is concentrated to dryness under a vacuum, extracted with 5 liters of absolute ethanol for 3 hours, and filtered. The alcohol-soluble portion is evaporated to dryness and extracted with 2 liters of water. The water-insoluble portion, containing the activity, is hydrolyzed under pressure, with 5 liters of a 1.5 per cent solution of ammonium hydroxide in a boiling water bath. The hydrolyzed material, now water-soluble, represents a concentration of vitamin $B_6$ approximately 140-fold from solubilized liver, and slightly less for vitamin $B_{12}$; however, an appreciable amount of “folic acid” activity was lost. When this preparation was fed to two different groups of chicks receiving the basal ration at a level equivalent to 8 per cent of solubilized liver (supplying 14 mg. of dry matter per 100 gm. of ration), normal growth and good feathers were produced (see Group 11, Table I).

Preparations 183C and 183D (Containing Principally Vitamin $B_{10}$)—

*This procedure may be found in detail in E. B. McQuarrie’s Master’s thesis, University of Wisconsin, 1943.*
Preparation 133C is a 75 per cent ethanol precipitate (pH 7) of the 85 per cent ethanol precipitate (Preparation 2 of our previous report) of a modified Super Filtrol eluate. Preparation 133D is the precipitate obtained by acidifying to pH 3 the filtrate left from making Preparation 133C. Both of these precipitates are dissolved in water and made slightly basic with ammonium hydroxide and stored under toluene in the cold (as are all other preparations described in this paper). These fractions contain a large amount of vitamin B₁₀ activity in comparison to their content of vitamin B₁₁ and "folic acid."

**Dialysis Procedure (Separation of Vitamins B₁₀ and B₁₁ from "Folic Acid" Activity)—**Dialysis may be performed by placing the solution to be dialyzed in a closed cellophane bag in a large Soxhlet extractor with water. This permits the dialysate to be collected and concentrated and at the same time allows the bag to come in contact repeatedly with a new supply of hot distilled water. Treatment of the material to be dialyzed with taka-diastase (20 mg. of taka-diastase for each gm. of dry matter and incubation at 37° for 24 hours under toluene) gives better separation. After dialysis "folic acid" activity occurs principally in the dialysate, while the greater part of vitamin B₁₀ and vitamin B₁₁ activity remains in the dialysis residue. (Growth results with such fractions are presented later in the paper.) This simple procedure concentrates vitamin B₁₀ and B₁₁ activity 4-fold from the Super Filtrol eluate. Dialysis may be carried out with similar results in cold running water if the dialysate is not desired.

**Preparation 159R (Vitamin B₁₀ and B₁₁ Concentrate)—**This preparation is the dialysis residue (see the dialysis procedure) of a calcium hydroxide filtrate of the Super Filtrol eluate. It contains nearly all of the vitamin B₁₀ and B₁₁ activity contained in the eluate but less than half of the "folic acid" activity.

**Results**

The results are given in Table I and are arranged in ascending order of vitamin B₁₀ activity to facilitate study of the table. Each entry is an average of results obtained with six chicks, except for the basal and control groups, which are an average of many groups. For the sake of brevity and simplicity, only about one-fourth of the results which we have obtained since our previous paper is presented. Groups of chicks whose response did not show a separation of any of the factors are omitted from Table I but the chemical properties of the factors as learned from such groups are presented later.

**Separation of Vitamin B₁₀ Activity from Vitamin B₁₁ Activity—**By comparing Column 4 with Column 5 (Table I) it is evident that, in confirmation of our previous results, the Super Filtrol eluate and certain liver fractions...
contain at least two vitamins, vitamin B<sub>12</sub> for proper feather formation and vitamin B<sub>11</sub> for growth (note Groups 3 and 19, 9 and 10, 11 and 13, etc.). Since maximum feathering was not obtained without at least some growth

**Table I**

*Results of Feeding Various Supplements to Basal Ration 486K (Given in Ascending Order of Feather Formation)*

<table>
<thead>
<tr>
<th>Preparation No. and level fed equivalent to solubilized liver</th>
<th>Description of supplement (made from Preparation 52A* unless otherwise indicated)</th>
<th>Vitamin B&lt;sub&gt;6&lt;/sub&gt; activity (feather formation)†</th>
<th>Vitamin B&lt;sub&gt;11&lt;/sub&gt; activity (growth)</th>
<th>“Folic acid” activity, per 100 gm. ration</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>(2)</td>
<td>(3)</td>
<td>(4)</td>
<td>(5)</td>
</tr>
<tr>
<td>1 Basal ration</td>
<td>No supplement</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 128 ♠10%</td>
<td>Methanol extract of Preparation 58 (cf. Briggs et al. (1))</td>
<td>35</td>
<td>-21</td>
<td>17</td>
</tr>
<tr>
<td>3 120R ♠10%</td>
<td>Hot ethanol (100%) residue</td>
<td>40</td>
<td>70</td>
<td>7.5</td>
</tr>
<tr>
<td>4 101 ♠5%</td>
<td>Methanol extract</td>
<td>45</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>5 120E ♠10%</td>
<td>Hot ethanol (100%) extract (see Group 3)</td>
<td>47</td>
<td>41</td>
<td>25</td>
</tr>
<tr>
<td>6 143F ♠5%</td>
<td>Norit filtrate (pH 3) of Super Filtrol eluate*</td>
<td>50</td>
<td>55</td>
<td>9</td>
</tr>
<tr>
<td>7 113 ♠5%</td>
<td>Norit (pH 3) eluate</td>
<td>60</td>
<td>-19</td>
<td>15</td>
</tr>
<tr>
<td>8 102 ♠5%</td>
<td>Methanol residue (see Group 4)</td>
<td>60</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>9 133D ♠5%</td>
<td>See text</td>
<td>70</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>10 111 ♠5%</td>
<td>Super Filtrol (pH 3) eluate</td>
<td>70</td>
<td>96</td>
<td>16</td>
</tr>
<tr>
<td>11 107 ♠8%</td>
<td>Hydrolyzed butyl esters* (2 groups of chicks)</td>
<td>75</td>
<td>110</td>
<td>8.3</td>
</tr>
<tr>
<td>12 139E ♠5%</td>
<td>Norit (pH 10) eluate of Super Filtrol eluate</td>
<td>75</td>
<td>55</td>
<td>17.5</td>
</tr>
<tr>
<td>13 133C ♠5%</td>
<td>See text</td>
<td>75</td>
<td>32</td>
<td>0.6</td>
</tr>
<tr>
<td>14 78R ♠5%</td>
<td>Made same as Preparation 50 (cf. (1))</td>
<td>75</td>
<td>101</td>
<td>5</td>
</tr>
<tr>
<td>15 161P ♠10%</td>
<td>Lead ppt. of Preparation 159R*</td>
<td>93</td>
<td>65</td>
<td>6</td>
</tr>
<tr>
<td>16 30-2 ♠8%</td>
<td>Made same as Preparation 30 (cf. (1))</td>
<td>95</td>
<td>115</td>
<td>8</td>
</tr>
<tr>
<td>17 162P ♠10%</td>
<td>Zinc ppt. of Preparation 159R*</td>
<td>95</td>
<td>69</td>
<td>5</td>
</tr>
<tr>
<td>18 138R ♠5%</td>
<td>Hot ethanol (95%) residue of Super Filtrol eluate</td>
<td>95</td>
<td>87</td>
<td>40</td>
</tr>
</tbody>
</table>

*Fuc acid* activity per 100 gm. ration

- Stirpe- 
- Lactis R (6) 
- Lac- 
- bacillus casei (7)
TABLE I—Concluded

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Preparation No. and level fed equivalent to solubilized liver</th>
<th>Description of supplement (made from Preparation 52A unless otherwise indicated)</th>
<th>Vitamin B12 activity (feather formation)†</th>
<th>Vitamin B11 activity (growth)</th>
<th>&quot;Folic acid&quot; activity, per 100 gm. ration</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>79 ±5% 1st norit eluate of solubilized liver</td>
<td>100 42 42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>150 ±10% Butanol residue</td>
<td>100 102 25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>146 ±10% 75% ethanol (pH 3) residue of Super Filtrol eluate</td>
<td>100 128 15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>52A ±5% See text</td>
<td>100 102 24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Control ration, 2% solubilized liver</td>
<td>100 100 50</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* See the text for information regarding the starting material.
† 0 = very poor; 25 = poor; 50 = fair; 75 = good; 100 = very good.
‡ Gm. gain over basal × 100

The average control weights at 4 weeks range from 200 to 275 gm. and the average basal weights range from 100 to 150 gm., depending on the batch of chickens.

increase, we cannot definitely conclude that vitamin B10 is not utilized in part for growth as well as for feathering. Likewise, since maximum growth was not obtained without at least "good" feathers, it is impossible to decide at the present time whether or not vitamin B11 also improves feather formation to some extent (even in the absence of vitamin B10). Thus, although the principal effects of the two vitamins are entirely different, it is conceivable that there may be some overlapping in their action (especially if bacterial synthesis within the intestine is considered).

Separation of Vitamin B10 and B11 Activity from "Folic Acid" Activity—
In confirmation of our earlier results, the two vitamins are distinct from "folic acid" activity (compare Columns 4 and 5 with Columns 6 and 7). Maximum or near maximum feather formation could be obtained (Groups 13 to 17) with lower amounts of "folic acid" activity than those which gave little feather formation (Groups 2 to 6). Likewise normal or near normal growth could be obtained (Groups 11, 14, and 16) with lower amounts of "folic acid" than those which gave only small increases in growth (Groups 2, 4, 5, 7, 12, and 19). We recognize the fact that there is a general tendency for supplements which have high vitamin B10 and B11 activity to have high "folic acid" activity; however, such supplements are usually those that have undergone little purification.

Separation of Streptococcus lactis R from Lactobacillus casei Activity—
When Column 6 is compared with Column 7, it is evident that "folic acid" activity as measured by the two organisms does not correlate. (Compare Groups 2 and 3, 6 and 16, 20 and 21, etc.) Nor does the activity of either one correlate any better than the other with growth or feathering. Thus, there are at least four biologically active compounds in the Super Filtrol eluate of liver, the significance of which will be discussed later in the paper.

In regard to the requirement of the chick for "folic acid," we have stated in our previous paper (1) that "maximum growth was not obtained in chicks unless levels were fed equal to, or above, 17.5 γ of folic acid per 100 gm. of ration." In Table I it is seen that maximum growth may be obtained with smaller amounts of "folic acid" than this; namely, 5 γ per 100 gm. of ration as measured by Streptococcus lactis R (Group 14) or 3 γ per 100 gm. of ration as measured by Lactobacillus casei (Group 16). Near maximum feather formation may be obtained with similar amounts of "folic acid" activity (see Groups 16 and 17). On the other hand various supplements which supplied greater amounts of "folic acid" were low or devoid of growth and feathering activity (Groups 2 to 5 and Group 7). Thus, we may conclude, that liver "folic acid" as we measure it is not needed per se by the chick for growth or feathering unless in very small amounts.

Further Description of Deficiency Symptoms and Other Results—When chicks are about 1 week of age, the innermost secondary wing feathers begin to curl toward the body and the wing coverts (the feathers covering the quills) begin to curl outward, giving the chick a ruffled appearance. The shafts of many wing feathers appear narrower than normal shafts and the feathers are frequently broken off. By the time the chick is 4 weeks old, the wing feathers remaining are either stunted or have scanty barb formation. They are curled outward, and frequently have a malformed shaft (often completely twisted around). Body and tail feathers are slow in appearing and are of abnormal structure (see Fig. 1).

An anemia (macrocytic), as mentioned previously (8), occurs in chicks on the basal ration. Any supplement which improves growth or feathering when fed with the basal ration likewise appears to correct the anemia partially; so it is possible that both vitamins are necessary for proper blood formation. As will be seen in Table II, the anemia-preventing activity can be separated from "folic acid" activity as measured with both Streptococcus lactis R and Lactobacillus casei. Preliminary studies showed that deficient chicks also have leucopenia. The paralysis and the perosis, as observed previously (1) in chicks receiving the basal ration, still occur occasionally.

Among the various compounds which have been tested for vitamin $B_{10}$ and $B_{11}$ activity and found to be inactive are $dl$-lysine, $l$-tryptophane, $d$-glutamic acid, $l$-aspartic acid, asparagine, glutamine, pimelic acid, yeast
nucleic acid, xanthine, guanine, uracil, orotic acid, adenine, adenylic acid, xanthopterin, adenylthiomethylpentose, and m-aminobenzoic acid. The level of known vitamins was doubled in the ration with no effect. Likewise, the addition of small amounts of inorganic salts of cobalt, boron, silicon, nickel, molybdenum, and aluminum was ineffective, as was the ash of solubilized liver.

The following substances have varying growth-promoting or feather-forming activity, but because they occur in only trace amounts in the eluate they cannot be either vitamin $B_6$ or $B_12$: $p$-aminobenzoic acid (5 to 10 mg. per 100 gm. of ration) (5), ascorbic acid (100 mg. per 100 gm. of ration) (9), thymine (200 $\gamma$ per 100 gm. of ration), and thymus nucleic acid (20 mg. per 100 gm. of ration). We do not routinely add any of these compounds to our ration because their action may be indirect and would con-

TABLE II

<table>
<thead>
<tr>
<th>Supplement to basal Ration 486K</th>
<th>No. dead at 4 wks. (24 chicks at start) per group</th>
<th>Vitamin $B_6$ activity (feather formation)*</th>
<th>Vitamin $B_12$ activity (growth)*</th>
<th>&quot;Folic acid&quot; activity, per 100 gm. ration</th>
<th>Hemoglobin (12 chicks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Staphylococcus pseudomonas</td>
<td>Lactobacillus casei</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>gm. per 100 cc.</td>
<td>gm. per 100 cc.</td>
</tr>
<tr>
<td>Dialysate (average of 4 preparations)</td>
<td>1</td>
<td>38</td>
<td>39</td>
<td>18.4</td>
<td>11.5</td>
</tr>
<tr>
<td>Dialysis residue (average of 4 preparations)</td>
<td>0</td>
<td>82</td>
<td>79</td>
<td>13.1</td>
<td>10.1</td>
</tr>
</tbody>
</table>

*See Table I.

fuse the results. The effects obtained by feeding sulfasuxidine to chicks receiving this ration have been presented (5).

Properties of Vitamin $B_6$ and Vitamin $B_12$—The properties of these two vitamins are considered together, since they are similar and separated only with difficulty. Unless otherwise indicated, all tests were made on fractions obtained from solubilized liver which were concentrated 20 to 100 times. (The Super Filtrol eluate was used most generally.) All fractions were tested for "folic acid" activity but, since descriptions of many properties of "folic acid" have been published (4, 7, 10), only those results will be given which have a direct bearing on the immediate problem. It is important to bear in mind that properties of the vitamins may vary, depending on the concentration of the solution used.

Solubility—In general, both vitamins $B_6$ and $B_12$ are insoluble in common organic solvents such as ether (neutral or pH 3), butyl alcohol (neutral,
pH 10, or pH 3), acetone, ethyl acetate, and 95 per cent and absolute (cold) ethanol. If hot absolute ethanol is used as the solvent, "folic acid" activity for *Streptococcus lactis* R is largely removed, leaving vitamin B$_{11}$ mainly in the residue, while vitamin B$_{10}$ and "folic acid" activity for *Lactobacillus casei* are in both extract and residue. The two vitamins are less soluble in 90 per cent ethanol (pH 3) and 85 per cent ethanol than is "folic acid" activity. Also, at such concentrations (75 to 85 per cent) vitamin B$_{10}$ tends to be more insoluble in ethanol than vitamin B$_{11}$. Both vitamins are soluble in water and glacial acetic acid. Vitamin B$_{11}$ is slightly more soluble in methanol than vitamin B$_{10}$; however, both vitamins are less soluble than "folic acid" activity.

**Stability**—Vitamins B$_{10}$ and B$_{11}$ as they occur in the eluate are completely destroyed by autoclaving at 15 pounds pressure in 2 N hydrochloric acid for 1 hour, although they remain stable when autoclaved at pH 3 for the same time. Autoclaving with 1 N sodium hydroxide for 30 minutes destroys little vitamin B$_{10}$ and B$_{11}$ activity, while preparations autoclaved at pH 10 or 7 for 30 minutes retain all measurable amounts of activity. Both vitamins and "folic acid" are stable to dry heat (110°) for 24 hours.

Oxidation with a 2 per cent solution of hydrogen peroxide for 2 hours (hot) destroys most of the vitamin B$_{10}$ and "folic acid" activity but about 50 per cent of the vitamin B$_{11}$ activity remains. Bubbling hydrogen through a solution of the vitamins for 10 hours causes no destruction of any of the three factors. Nitrous acid treatment (5 per cent solution of nitrous acid at room temperature for 12 hours) causes destruction of nearly all biological activity, indicating the presence of active amino groups in all of these compounds.

**Other Properties**—Both vitamins are adsorbed to the greatest extent in acid solution (pH 3 is used) on fuller's earth, norit, and Super Filtrol. Vitamin B$_{10}$ is adsorbed on norit more completely than vitamin B$_{11}$ and it is also eluted more rapidly (with the alcohol, ammonia, and water mixture), which suggests the possibility of separating these two vitamins from each other by chromatographic adsorption.

Both vitamins and "folic acid" activity are precipitated completely by lead acetate and zinc chloride and partially with silver nitrate and barium hydroxide (the water-soluble barium salts are insoluble in 80 per cent ethanol). The activity is not precipitated by calcium hydroxide.

The butyl esters of both vitamins can be made by the procedure described in this paper, which indicates that both of these vitamins have acid groups. That acid groups are present and may even predominate is also suggested by the fact that both vitamin B$_{10}$ and vitamin B$_{11}$ migrate toward the anode during electrophoresis. The activity of all three factors was concentrated in an acid cell (pH 2.1) of a five cell electrodialysis apparatus.
Dialysis procedures have produced very interesting results, which are given in Table II. It can be seen that both vitamins $B_{10}$ and $B_{11}$ (and antianemic activity) do not pass readily through cellophane membranes. On the other hand “folic acid” activity for both bacteria occurs principally in the dialysate. Results are especially clear cut when the fraction has been previously treated with taka-diastase (see the dialysis procedure).

DISCUSSION

The importance of vitamin $B_{10}$ and vitamin $B_{11}$ in the nutrition of other animals besides the chick has not been determined as yet. However, the Super Filtrol eluate, containing both of these vitamins (as well as “folic acid” activity) has been shown to be important for the nutrition of the rat (11–13), dog, and monkey (14). The growth activity of this fraction has been largely attributed to its “folic acid” content but our results with the chick suggest that vitamins $B_{10}$ and $B_{11}$ may be as important or more so.

The relationship of these two vitamins to other unknown chick factors has been discussed in our previous paper (1). However, work done since that time changes the picture slightly. Pfiffner et al. (15) reported the isolation from liver of an antianemia factor for chicks, called vitamin $B_{0}$, in yellow crystalline form. The method of isolation and rations used are unpublished at the present time. The vitamin prevented anemia in chicks and caused the chicks to “grow normally” when fed at a level of 250 $\gamma$ (probably an excess) per 100 gm. of ration. The authors state that they “demonstrate conclusively the identity of Hogan’s antianemia factor and Peterson’s ‘eluate factor’” (16) and state that these factors are probably the same as “folic acid.” If this is true, the results presented in this paper indicate that the crystalline vitamin $B_{0}$ of Pfiffner et al. is distinct from true vitamins $B_{10}$ and $B_{11}$ as they occur in the Super Filtrol eluate. Furthermore, it is also important to note (Table II) that the antianemia activity in our work remains in the undialyzable portion and does not follow liver “folic acid” activity. Later, a report by O’Dell and Hogan (17) presented some chemical properties of vitamin $B_{0}$ (in impure concentrates) and gave a technique for its assay. They do not give, however, the effects of their factor upon growth or feathering when fed in addition to their improved purified diets. From the properties ascribed to vitamin $B_{0}$ by O’Dell and Hogan (such as solubility in methanol, incomplete adsorption on fullers’ earth at acid pH, and stability to oxidation, etc.) it would appear that their vitamin is more similar to vitamin $B_{11}$ than to vitamin $B_{10}$.

A review of the literature on “folic acid” has been made by Luckey et al. (3) but it is important to mention the report of Stokstad (6) who isolated

a compound from liver (thought to be identical with the crystalline compound of Pfiffer et al. (15)), and also isolated a different compound from yeast. This latter compound had about one-half the activity for *Streptococcus lactis* R as the liver compound but about the same amount of *Lactobacillus casei* activity. We have not as yet tried either of these isolated substances for vitamin B₁₀ or vitamin B₁₁ activity. However, from the results which we have presented in this paper we would expect the liver compound to be largely inactive. In this connection Almquist has recently reported (18) that a purified fraction of the “folic acid” for *Lactobacillus casei* obtained from Stokstad had little, if any, activity for the chick and states, in agreement with our previous results (1), that “at least one unknown member of the B-complex is required by chicks” other than the *Lactobacillus casei* factor. It is interesting that Daft and Sebrell have reported (19) that the several “folic acids” (crystalline) had activity in the prevention of granulocytopenia and leukopenia in rats fed sulfonamides.

The results which we have obtained with the dialysis procedures may indicate that the various “folic acids” as measured by *Streptococcus lactis* R and *Lactobacillus casei* are fragments of a large molecule, or molecules, which are needed by the chick in the intact form for true vitamin B₁₀, vitamin B₁₁, and antianemia activity. Thus, the possibility exists that vitamins B₁₀ and B₁₁ may have some “folic acid” activity. Similarly it is entirely possible that pure compounds showing high activity for either *Streptococcus lactis* R or *Lactobacillus casei* may have some vitamin B₁₀ or vitamin B₁₁ activity but the action may be indirect. Work to answer this question and to separate and concentrate vitamins B₁₀ and B₁₁ further is in progress at the present time.

**SUMMARY**

Various chemical properties for vitamins B₁₀ and B₁₁ as well as methods for their partial separation are described.

At least four substances with biological activity, namely vitamin B₁₀ (necessary for feather formation in chicks), vitamin B₁₁ (necessary for growth), and two factors necessary for *Streptococcus lactis* R and *Lactobacillus casei*, are present in the Super Filtrol eluate of solubilized liver. The significance of this is discussed.

“Folic acid” activity has been separated, at least in part, from vitamin B₁₀, B₁₁, and antianemia activity and does not appear to be necessary *per se* for the chick unless in small amounts.

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FURTHER STUDIES ON VITAMINS B\textsubscript{10} AND B\textsubscript{11} AND THEIR RELATION TO "FOLIC ACID" ACTIVITY

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