FURTHER STUDIES OF ORGANIC FACTORS REQUIRED FOR PREVENTION OF ANEMIA IN CHICKS*

BY M. L. SCOTT, L. C. NORRIS, L. W. CHARKEY,† LOUISE J. DANIEL, AND G. F. HEUSER

(From the Agricultural Experiment Station and the School of Nutrition, Cornell University, Ithaca)

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The results of studies in several laboratories (1-3) have shown that folic acid is required for the prevention of anemia in the chick. The antianemic effect of β-pyrcalin lactone when administered together with the fermentation Lactobacillus casei factor (LCF), the folic acid conjugate of Hutchings, Stokstad, Bohonos, and Slobodkin (4), has been demonstrated by Scott, Norris, Heuser, and Bruce (5). In earlier studies reported by Scott, Norris, Heuser, Bruce, Coover, Bellamy, and Gunsalus (6) α-pyrcalin lactone was found to prevent anemia in chicks fed a diet containing factor S concentrate from yeast, described by Schumacher, Heuser, and Norris (7). Later it was found that the supply of factor S concentrate used in this study contained a larger amount of folic acid than usual, chiefly in the conjugate form.

Studies with factor S concentrate conducted in this laboratory by Hill (8) and by Scott and associates (5, 6) have consistently shown a slight but positive effect upon hemoglobin formation by this preparation, which at times could not be accounted for in terms of either folic acid or pyrcalin content of the preparation.

Since pyrcalin had been found to be active in the prevention of anemia in chicks when supplied together with folic acid conjugate, and since factor S appeared to have some effect upon hemoglobin formation in the chick, an investigation was undertaken to determine (a) whether pyrcalin is required for the prevention of anemia in the presence of free folic acid, and (b) whether factor S contains an antianemic factor not identical with folic acid or pyrcalin. The results of the investigation are presented in this report.

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† Present address, Department of Chemistry, Colorado State College, Fort Collins, Colorado.
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EXPERIMENTAL

White Leghorn male chicks were used in the experimental work. The chicks were fed a purified diet described earlier (5), except that phthalylsulfathiazole was substituted for the succinylsulfathiazole. The method of handling the diet was the same as that described in the earlier report (5).

Since crystalline folic acid was not available in sufficient quantities for use in this work, a liver folic acid concentrate which contained only free folic acid was used. This concentrate contained 1250 µg of folic acid per gm. by Streptococcus faecalis assay and 1300 µg per gm. by Lactobacillus casei, a synthetic folic acid being used as the standard. It was fed at a level which provided 100 µg of folic acid per 100 gm. of diet, β-pyracin at 100 µg per gm., and factor S concentrate at a level in the diet equivalent to 5 per cent yeast. These three supplements were included individually and in all possible combinations.

The quantities of folic acid and β-pyracin supplied in this study were in excess of the requirements. Robertson, Daniel, Farmer, and Norris have shown that the quantitative requirement of the chick for folic acid, when fed the diet used in this work, is 40 to 50 µg per 100 gm. of diet for optimum growth, 30 to 40 µg per 100 gm. for optimum hemoglobin formation, and 20 to 30 µg per 100 gm. for the prevention of mortality. Scott and associates (5) have shown 50 µg of pyracin to be sufficient for the prevention of anemia when supplied together with 50 µg of LCF per 100 gm. of diet.

The results presented in Table I show that in the presence of the folic acid concentrate the inclusion of β-pyracin had no effect upon hemoglobin level or growth, contrary to previous findings at this laboratory when conjugates were used as the source of folic acid. After 4 weeks on the experimental diets, however, the hemoglobin level of the chicks receiving the folic acid concentrate had not reached a maximum but was 1.3 gm. per 100 ml. of blood lower than the hemoglobin level of those chicks receiving a commercial chick diet. Factor S concentrate, when fed alone or in combination with β-pyracin, had little effect upon either chick growth or hemoglobin response, but, when it was supplied together with the folic acid concentrate, it maintained the hemoglobin level at values approximately equal to those obtained with the commercial diet.

A statistical analysis of the hemoglobin values by "Student's" method for unpaired data was conducted to determine whether the increase in hemoglobin was biologically important. The t value obtained for the significance of the difference was 8.52. With approximately 60 values

per group, the odds are 9999:1 when t equals 5.1. The results showed, therefore, that the increase in hemoglobin level of approximately 1 gm. per 100 ml. of blood was highly significant.

Microbiological assays of the factor S concentrate used in these studies showed that, even after incubation with liver enzymes, the amount of folic acid in this preparation was low. It contributed less than 5 γ of folic acid per 100 gm. of diet when fed at the level equivalent to 5 per cent yeast. Since, in the lots receiving factor S concentrate alone, or with pyracin, approximately 75 per cent of the chicks died in 4 weeks, further evidence is provided that the factor S concentrate contained little folic acid. In view of the fact that the factor S concentrate was found to be a poor source of folic acid, and because the liver folic acid concentrate provided almost

<table>
<thead>
<tr>
<th>Supplement</th>
<th>No. of chicks surviving at 4 wks.</th>
<th>Average chick weight at 4 wks. (gm.)</th>
<th>Average Hb at 4 wks. (gm. per 100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6 (15)*</td>
<td>94</td>
<td>2.3</td>
</tr>
<tr>
<td>Factor S</td>
<td>4 (15)</td>
<td>93</td>
<td>5.0</td>
</tr>
<tr>
<td>β-Pyracin</td>
<td>8 (30)</td>
<td>103</td>
<td>3.5</td>
</tr>
<tr>
<td>Factor S + β-pyracin</td>
<td>7 (30)</td>
<td>112</td>
<td>3.7</td>
</tr>
<tr>
<td>Folic acid</td>
<td>15 (15)</td>
<td>282</td>
<td>8.5</td>
</tr>
<tr>
<td>&quot; &quot; + β-pyracin</td>
<td>45 (45)</td>
<td>276</td>
<td>8.6</td>
</tr>
<tr>
<td>Factor S + folic acid</td>
<td>14 (15)</td>
<td>282</td>
<td>9.7</td>
</tr>
<tr>
<td>&quot; &quot; + &quot; &quot; + β-pyracin</td>
<td>44 (45)</td>
<td>287</td>
<td>9.5</td>
</tr>
<tr>
<td>Commercial chick diet</td>
<td>15 (15)</td>
<td>291</td>
<td>9.8</td>
</tr>
</tbody>
</table>

* The figures in parentheses indicate the number of chicks started.

double the amount of this vitamin shown to be required by Robertson and associates,1 the effect of the factor S concentrate upon hemoglobin formation cannot be explained in terms of additional folic acid and, therefore, must have been produced by some other factor present in the factor S concentrate.

Since the folic acid was supplied to the chicks in this study in the form of a liver concentrate, the possibility existed that the liver concentrate contained pyracin and, therefore, additional pyracin was not required for prevention of anemia. In view of this a study was undertaken to ascertain whether or not β-pyracin was present in the concentrate.

Huff and Perlzweig (9) have pointed out that the lactone of 4-pyridoxic acid (β-pyracin lactone), when irradiated with ultraviolet light, emits a strong blue fluorescence, the intensity of which varies with the pH of the
lactone solution. They have also reported that the acid form of this compound can be converted into the lactone by boiling in a mineral acid. Accordingly, 0.1 gm. of liver folic acid concentrate was refluxed in 50 ml. of 0.1 N HCl for 15 minutes. The solution was neutralized with sodium hydroxide and its fluorescence intensity determined at a level of 20 \( \gamma \) of the concentrate per ml. over a pH range from 6.5 to 10.0. The fluorescence intensity of this solution, which served as the control, is shown by Curve A in Fig. 1.

Another 0.1 gm. sample of the concentrate was refluxed in 50 ml. of 1.0 N hydrochloric acid for 2 hours, cooled, and neutralized. Curve B in Fig. 1 shows the fluorescence intensity obtained with this solution of hydrolyzed folic acid concentrate at a level of 20 \( \gamma \) of the concentrate per ml. over the same pH range. Curve C in Fig. 1 shows the pH-fluorescence curve produced by the addition of 20 millimicrograms of pure \( \beta \)-pyracin lactone per ml. of control solution. The two latter curves are almost superimposed. This is evidence that the liver folic acid concentrate contains pyracin. The amount of \( \beta \)-pyracin, calculated from these results, was approximately 1 mg. per gm. of folic acid concentrate. Thus the folic acid concentrate contributed about 90 \( \gamma \) of \( \beta \)-pyracin per 100 gm. of diet at the level fed to the chicks.

By means of ether extraction at pH 6.5 of an acid-hydrolyzed solution of 30 gm. of the folic acid concentrate, followed by adsorption and elution from Super Filtrol and subsequent purification by sublimation under re-
duced pressure, 5 mg. of a crystalline substance were obtained. The pH-fluorescence curve of the crystals was identical with that of pure synthetic β-pyracin lactone at the same concentration.

Since these findings strongly indicate that pyracin was present in the liver concentrate, no conclusions could be drawn from this study as to whether or not pyracin is required for prevention of anemia in the presence of free folic acid.

Therefore, when synthetic folic acid became available later, a second experiment was undertaken. The chicks were fed the purified diet supplemented with factor S concentrate equivalent to 5 per cent yeast. Synthetic folic acid and β-pyracin were supplied singly and in combination at levels of 100 γ per 100 gm. of diet. The liver folic acid concentrate was also included in this experiment at a level to provide 100 γ of folic acid per

### Table II
**Results Showing Prevention of Anemia by Synthetic Folic Acid in Absence of Pyracin**

<table>
<thead>
<tr>
<th>Supplement</th>
<th>No. of chicks surviving at 4 wks *</th>
<th>Average chick weight at 4 wks.</th>
<th>Average Hb at 4 wks.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor S</td>
<td>0</td>
<td>gm.</td>
<td>gm. per 100 ml.</td>
</tr>
<tr>
<td>&quot; &quot; + β-pyracin</td>
<td>4</td>
<td>103</td>
<td>2.4</td>
</tr>
<tr>
<td>&quot; &quot; + synthetic folic acid</td>
<td>17</td>
<td>252</td>
<td>9.5</td>
</tr>
<tr>
<td>&quot; &quot; + &quot; &quot; + &quot; &quot; + β-</td>
<td>17</td>
<td>241</td>
<td>9.6</td>
</tr>
<tr>
<td>pyracin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor S + liver folic acid conc.</td>
<td>16</td>
<td>241</td>
<td>9.5</td>
</tr>
</tbody>
</table>

* Each lot was started with seventeen white Leghorn chicks of mixed sex.

100 gm. of diet, so that the results could be compared with those obtained in the previous experiment. The results of this study are presented in Table II. They show that the synthetic folic acid promoted a hemoglobin level which was not increased by the addition of pyracin to the diet.

**DISCUSSION**

From the data presented in this report, it is evident that factor S concentrate contains an antianemic factor which is effective in raising the hemoglobin level in chicks above that obtained with folic acid.

Hill (8) has shown that factor S is not removed from solution by adsorption with activated charcoal (Darco G-60) at pH 3.0. In the course of his studies with factors R and S of Schumacher and associates (7), Hill found that he could obtain the factor R fraction free of factor S by treating the former concentrate with charcoal, thus leaving factor S in the fil-
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trate. This adsorbate of factor R contained most of the folic acid present in the original factor R concentrate. After treatment of factor S concentrate with charcoal, he found that the non-adsorbable fraction contained factor S, but was free of folic acid. When the adsorbate of factor R was supplied to chicks fed a purified diet, the average hemoglobin level in the chicks at 5 weeks was 6.8 gm. per 100 ml. Adding the folic acid-free factor S concentrate alone to the diet failed to promote any increase in hemoglobin level over that of the control chicks. The hemoglobin levels for these lots averaged 3.0 and 3.5 gm. per 100 ml., respectively. However, when the adsorbate of factor R and the folic acid-free factor S concentrate were fed together, the average hemoglobin level in the chicks at 5 weeks was 8.5 gm. per 100 ml. of blood.

Campbell, Brown, and Emmett (1) have demonstrated that crystalline vitamin B₁₂ (folic acid), when added to a purified diet very similar in composition to the one used in the present investigation, is almost, but not quite, as effective as a normal ration in preventing anemia in chicks up to 4 weeks of age. Vitamin B₁₂ at a level of 100 γ per 100 gm. of diet promoted a hemoglobin level of 7.6 gm. per 100 ml. 4 times this amount of vitamin B₁₂ resulted in a hemoglobin level of only 7.7 gm. per 100 ml., while the chicks receiving a normal broiler ration had an average hemoglobin of 8.7 gm. per 100 ml. of blood. The hemoglobin results which they reported are, therefore, strikingly similar to the ones reported here for the liver folic acid concentrate. The fact that the normal broiler ration promoted a hemoglobin level approximately 1 gm. per 100 ml. of blood higher than the plateau response obtained with vitamin B₁₂ may mean that the normal broiler ration contained factor S, which was lacking in their purified diet.

As a result of studies on vitamins B₁₀ and B₁₁ and related substances, Briggs, Luckey, Elvehjem, and Hart (10) have suggested the existence of a new hemoglobin factor. They found that liver fractions low in vitamin B₁₂ (folic acid) activity raised the hemoglobin values in chicks, and that other fractions rich in either vitamin B₁₀ or B₁₁ did not completely prevent anemia. They showed that α-pyracin lactone was not identical with the suggested new antianemic factor. It is possible that this antianemic activity was due to factor S, as indicated in this report.

Daniel, Scott, Norris, and Heuser (11) have shown that pyracin is necessary in order to convert LCF into folic acid and thus make it available to Streptococcus faecalis. From their results, they concluded that the action of pyracin in promoting an increased production of folic acid from LCF is caused either by conjugation with LCF to form folic acid, or by the fact that pyracin functions in an enzyme system required for the breakdown of LCF and the setting free of folic acid. It has been announced by Jukes
and Stokstad\textsuperscript{2} that LCF appears to be a folic acid conjugate. Therefore, the latter explanation of Daniel and associates is probably the correct one.

The studies presented in this report have shown that pyracin is not required for hematopoiesis in the chick when the diet contains free folic acid. On the other hand, since pyracin is required with certain folic acid conjugates for the prevention of anemia in chicks, further evidence is provided that it enters into an enzyme system required for the breakdown of folic acid conjugates. This suggests the possibility that \(\beta\)-pyracin is the prosthetic group of an enzyme.

**SUMMARY**

The hemoglobin level in chicks has been significantly increased by the addition of factor S from dried brewers' yeast to a diet containing adequate amounts of folic acid and \(\beta\)-pyracin. This demonstrates that factor S possesses antianemic activity.

Pyracin has been found not to be required for the prevention of anemia in chicks when the diet contains free folic acid. Since \(\beta\)-pyracin is necessary with certain folic acid conjugates for the prevention of anemia in chicks, further evidence is provided that it functions in an enzyme system required for the breakdown of folic acid conjugates and setting free of folic acid.

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**BIBLIOGRAPHY**


\textsuperscript{2} Reported at the Informal Poultry Nutrition Conference, Meetings of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, March, 1946.
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