Gard[2] for a strain of Theiler's mouse virus. While this conclusion was suggested by one of Bourdillon's[3] experiments in which an older dialyzed, purified preparation showed double refraction of flow, the results published by Jungeblut and Bourdillon[4] show a remarkable agreement between purified SK and our purified Lansing preparations. The purified SK samples in contrast to those from unpurified tissue cultures showed the presence of "spherical or elliptic bodies" having a diameter between 25 and 30 mμ. It is noteworthy that particles of the same relative size and shape have been obtained, therefore, from 2 different strains of poliomyelitis virus, both of which were isolated originally from infectious human material. As it might be expected that different human strains would have similar physical properties, the results from the 2 laboratories confirm the fact that human poliomyelitis virus as cultivated in mice and in cotton rats consists of relatively spherical particles of about 25 mμ diameter.

2 Gard, S., *Acta Med. Scand.*, Suppl., 1943, **143**, 173p.

3 Bourdillon, J., *Arch. Biochem.*, 1943, **3**, 285.

4 Jungeblut, C. W., and Bourdillon, J., *J. Am. Med. Assn.*, 1943, **123**, 399.

## 15713

## Further Studies of Factor R.*

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Bauernfeind, Schumacher, Hodson, Norris, and Heuser[1] announced the existence of a new factor required for growth and reproduction in the domestic fowl. Schumacher, Heuser, and Norris[2] presented evidence that the factor consisted of 2 components; factor S precipitated from an acid solution by alcohol, and factor R precipitated by alcohol from neutral solution.

While these studies on factors R and S were in progress, evidence of the existence of a factor required for the growth of *L. casei* and *S. faecalis* was presented by various laboratories. This factor is now generally referred to as folic acid. Briggs, Luckey, Elvehjem, and Hart[3] reported that chicks grew normally on a diet containing no more than 8 μg of folic acid per 100 g of diet. Hill, Norris, and Heuser[4] concluded that factor R was not identical with folic acid, and that if folic acid was required by the chick, the amount needed was less than 15 μg per 100 g of diet.

Binkley and associates[5] reported the presence of vitamin $B_c$ (folic acid) conjugate in yeast. This substance was highly active for the chick, but only slightly active for *S.*

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1 Bauernfeind, J. C., Schumacher, A. E., Hodson, A. Z., Norris, L. C., and Heuser, G. F., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 108.

2 Schumacher, A. E., Heuser, G. F., and Norris, L. C., *J. Biol. Chem.*, 1940, **135**, 313.

3 Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1945, **158**, 303.

4 Hill, F. W., Norris, L. C., and Heuser, G. F., *J. Nutrition*, 1944, **28**, 175.

5 Binkley, S. B., Bird, O. D., Bloom, E. S., Brown, R. A., Calkins, D. G., Campbell, C. J., Emmett, A. D., and Pfiffner, J. J., *Science*, 1944, **100**, 36.

*faecalis* and *L. casei*. Concentrates of this substance, however, became active for the microorganisms after enzymatic digestion.

In view of the results of Binkley and associates,[5] further work on factor R was undertaken in this laboratory to determine if its activity for chicks could be accounted for in terms of folic acid, as measured by microorganisms after enzymatic conversion of the conjugate to folic acid.

While the study reported in this paper was in progress, Pfiffner and associates[6] reported the isolation of vitamin $B_c$ conjugate in crystalline form from yeast. The vitamin $B_c$ present in the conjugate was found to be available to the chick, and became available to the microorganism after digestion with hog kidney conjugase.

*Experimental.* The experimental procedure consisted of treatment of factor R with folic acid liberating enzymes, determination by microorganisms of the folic acid in the treated preparations, and comparison with the amount of folic acid present as evidenced by growth and hemoglobin formation in the chick.

*Microbiological Work.* Folic acid assays were made by a modification of the procedure of Luckey, Briggs, and Elvehjem[7] using *S. faecalis*. The standard of comparison was either synthetic folic acid of Angier and associates‡,[8] or a folic acid concentrate supplied by Dr. R. J. Williams. One μg of synthetic folic acid was found to be equivalent to 11.5 μg of Williams' folic acid. Values, obtained when Williams' folic acid was used as the standard, were converted to synthetic folic acid by means of this factor.

The amount of free folic acid in factor R was determined following either incubation of the sample with takadiastase and papain or steaming in phosphate buffer at pH 7 for 15 minutes. Both methods of extraction gave comparable values. The results showed that there was not sufficient folic acid present as such to account for the growth response of the chick to factor R. Therefore, other enzymatic digestions were undertaken to determine the presence of potential or conjugated folic acid that was microbiologically inactive, but available to the chick.

In preliminary work the most effective procedure found was one involving a 24-hour predigestion at 37°C with malt diastase, followed by a 24-hour incubation at 37°C with fresh rat liver suspension. The entire procedure was carried out in citrate-phosphate buffer at pH 4.5. This treatment was applied to factor R and to a Superfiltrol eluate of factor R, and consistently gave an approximately 10-fold increase in folic acid content. Chick liver, in general, was less effective than rat liver. Increases of this order of magnitude still, however, did not adequately account for all the biological activity of factor R. Hence, resort was made to the use of dried kidney suspension, as described by Bird and associates.[9] This procedure resulted in an approximately 15-fold increase in folic acid content.

*Chick Experiments.* Two experiments were conducted with day-old White Leghorn chicks. Not less than 15 chicks were started in each lot. They were housed in batteries of individually heated, well lighted pens with raised wire mesh bottoms.

The basal diet used was purified diet 653 described by Hill, Norris and Heuser.[4] Factor S, prepared according to the method of Schumacher, Heuser, and Norris,[2] was supplied at a level equivalent to 5% of dried brewers' yeast, and 100 μg of 2-methyl-3-

[6] Pfiffner, J. J., Calkins, D. G., O'Dell, B. L., Bloom, E. S., Brown, R. A., Campbell, C. J., and Bird, O. D., *Science*, 1945, **102**, 228.

[7] Luckey, T. D., Briggs, G. M., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

[8] Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., SubbaRow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, 1945, **102**, 227.

‡ We are indebted to Lederle Laboratories, Pearl River, N.Y., for synthetic folic acid, to Dr. R. J. Williams, University of Texas, Austin, Tex., for folic acid concentrate, to Merck & Co., Inc., Rahway, N.J., for β-pyracin, and to Anheuser-Busch, Inc., St. Louis, Mo., for strain S dried brewers' yeast.

[9] Bird, O. D., Bressler, B., Brown, R. A., Campbell, C. J., and Emmett, A. D., *J. Biol. Chem.*, 1945, **159**, 631.

hydroxy-4-carboxy-5 - hydroxymethylpyridine ($\beta$-pyracin) were added per 100 g of diet.

The chicks were weighed individually at weekly intervals. Hemoglobin determinations were made at the 3rd, 4th, and 6th weeks in Experiment 1 by the colorimetric method of Sahli, using 0.1 N HCl to develop the acid hematin color, and in Experiment 2 by the oxyhemoglobin method developed in this laboratory by Robertson and Fiala,[10] using 0.1% $Na_2CO_3$, and measuring the color produced with a Coleman spectrophotometer.

*Experiment 1.* The *L. casei* factor of Hutchings and associates,[11] was used as a standard. The sample used was approximately 80% pure and was added at levels of 50, 100 and 150 $\mu$g per 100 g of diet. Factor R was included at levels equivalent to 5, 10, and 15% of original yeast. The results of the experiment indicated that 50 to 100 $\mu$g of *L. casei* factor were needed for optimum growth response in the chick. Using the same experimental conditions, Robertson, Daniel, Farmer, Norris, and Heuser[12] have reported that 45 to 55 $\mu$g of synthetic folic acid per 100 g of diet were required for optimum growth. Normal growth was obtained with factor R preparations that supplied approximately 25 $\mu$g of folic acid per 100 g of diet, as determined by *S. faecalis* assay after rat liver incubation.

*Experiment 2.* When synthetic folic acid became available, an experiment was conducted using 10, 20, 30, and 40 $\mu$g of folic acid per 100 g of diet. Factor R was included at levels equivalent to 3, 6, and 12% of original yeast. One group of chicks was supplied the various supplements alone, and a second group the supplements plus 2% succinylsulfathiazole. When the sulfonamide was added, *p*-aminobenzoic acid was omitted from the diet. The results of Experiment 2 are presented in Table I.

The amount of folic acid found in factor R by this chick experiment was approximately 3 $\mu$g per g of original yeast. This was obtained by comparing both the growth and hemoglobin responses at the various levels of factor R to the corresponding responses caused by synthetic folic acid. Comparing the biological and microbiological responses to factor R demonstrated that the microörganism was accounting for all of the chick activity after the sample had been incubated with hog kidney, but not when incubated with chick or rat liver.

No significant differences in the chick responses to factor R between the nonsulfonamide and sulfonamide diets were obtained. This indicated that potential folic acid in factor R was not liberated by intestinal bacteria and that under the conditions of the experiment no folic acid was synthesized by the intestinal flora. It appeared, therefore, that either the chick is able to use the folic acid of factor R directly, or metabolic processes make it available.

*Discussion.* Several explanations for the differences in microbiological results obtained by incubating factor R with chick or rat liver and hog kidney are possible. More than one conjugate of folic acid may be present in factor R. If this is the case, the hog kidney enzyme(s) is able to break down both conjugates, whereas rat and chick liver enzymes can split only one.

The presence of more than one conjugate of folic acid in factor R is supported by the recent findings of Ratner, Blanchard, and Green.[13] These investigators isolated a peptide of *p*-aminobenzoic acid from yeast having 10 or 11 glutamic acid residues. In one of the steps in the isolation of this peptide trichloracetic acid was used. This treatment may have split the peptide from the pterin nucleus of the folic acid conjugate, since Angier and associates[14] brought about the

10 Robertson, E. I., and Fiala, G. F., unpublished data, Cornell University, Ithaca, New York.

11 Hutchings, B. L., Stokstad, E. L. R., Bohonos, N., and Slobodkin, N. H., *Science*, 1944, **99**, 371.

12 Robertson, E. I., Daniel, L. J., Farmer, F. A., Norris, L. C., and Heuser, G. F., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 97.

13 Ratner, S., Blanchard, M., and Green, D. E., *J. Biol. Chem.*, 1946, **164**, 691.

14 Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., SubbaRow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, 1946, **103**, 667.

TABLE I.
Effect of Factor R on Growth and Hemoglobin Formation in the Chick.

| Supplements | Weight | | Hemoglobin | | Folic acid added by supplement | | | |
|---|---|---|---|---|---|---|---|---|
| | 4 wk | 6 wk | 4 wk | 6 wk | Unincubated | Hog kidney | Rat liver | Chick liver |
| **Without succinylsulfathiazole.** | | | | | | | | |
| None* | 153 (11)† | 202 (5) | 3.9 | 2.4 | — | — | — | — |
| 10 μg folic acid‡ | 225 (15) | 345 (12) | 5.1 | 6.9 | 10 | — | — | — |
| 20 " " " | 249 (15) | 412 (15) | 8.0 | 9.5 | 20 | — | — | — |
| 30 " " " | 288 (15) | 476 (15) | 8.3 | 9.2 | 30 | — | — | — |
| 40 " " " | 274 (14) | 461 (14) | 8.7 | 9.3 | 40 | — | — | — |
| 3% factor R | 174 (12) | 266 (9) | 3.7 | 5.1 | 0.6 | 9.9 | 7.5 | 6.0 |
| 6" " " | 241 (12) | 445 (11) | 7.8 | 9.8 | 1.2 | 19.8 | 15.0 | 12.0 |
| 12" " " | 298 (12) | 492 (12) | 8.4 | 9.4 | 2.4 | 39.6 | 30.0 | 24.0 |
| **2% succinylsulfathiazole added.** | | | | | | | | |
| None | 153 (8) | 173 (2) | 3.2 | 1.6 | — | — | — | — |
| 10 μg folic acid | 206 (12) | 310 (11) | 4.8 | 6.6 | 10 | — | — | — |
| 20 " " " | 248 (14) | 431 (14) | 7.3 | 9.1 | 20 | — | — | — |
| 30 " " " | 282 (14) | 469 (14) | 8.2 | 9.5 | 30 | — | — | — |
| 40 " " " | 281 (14) | 470 (14) | 8.5 | 9.5 | 40 | — | — | — |
| 3% factor R | 186 (12) | 230 (8) | 4.4 | 3.5 | 0.6 | 9.9 | 7.5 | 6.0 |
| 6" " " | 239 (15) | 413 (11) | 6.2 | 9.1 | 1.2 | 19.8 | 15.0 | 12.0 |
| 12" " " | 301 (15) | 492 (15) | 8.0 | 9.2 | 2.4 | 39.6 | 30.0 | 24.0 |

* Basal diet contained 15 μg of folic acid per 100 g as determined by *S. faecalis* assay following chick liver incubation.
† Numbers in parentheses indicate number of surviving chicks of the original 15.
‡ Synthetic folic acid.

cleavage of the fermentation *L. casei* factor giving a pteridine fraction and an aromatic amine by using sulfurous acid, which is a milder treatment than that with trichloracetic acid.

The vitamin $B_c$ conjugate of Pfiffner and associates,[15] which is also obtained from yeast, has been shown to contain 7 molecules of glutamic acid. This evidence indicates that there may be at least 2 different conjugates in yeast. If there are 2 conjugates of folic acid present in factor R, it may be that there are 2 enzymes needed to split them, both of which are present in hog kidney and only one in rat and chick liver.

All conditions necessary for the optimum efficiency of the rat and chick liver enzyme systems may not have been met, so that complete liberation of folic acid from the conjugate was not obtained. Every effort was made to determine the optimum conditions for the enzyme systems involved. The proper concentrations of the enzymes and substrate were determined, as well as the optimum pH and temperature for the incubation.

Hog kidney may contain an enzyme system that can synthesize folic acid from simple precursors, whereas chick and rat liver may be devoid of such an enzyme. Many bacteria, yeasts, and molds are known to possess enzyme systems that are able to take the pyrimidine and thiazole fractions of thiamine and combine them into the vitamin.

*Summary.* The growth and hemoglobin responses obtained in chicks receiving a purified diet supplemented with factor R were found not to be due to the preformed folic acid present in factor R.

By means of chick liver, rat liver, and hog kidney incubation procedures the folic acid content of factor R was greatly increased. This indicated the presence of potential folic acid which did not stimulate the growth of *S. faecalis*, but which was available to the chick. The potential folic acid liberated by hog kidney enzymes was found to account for all of the responses obtained in the chick, whereas chick and rat liver enzymes were not as effective in liberating folic acid.

The most probable explanation for the discrepancy in the folic acid content of factor R obtained following hog kidney and rat or chick liver incubations is that factor R is a mixture of conjugates of folic acid rather than one conjugate.

The response to factor R was not affected by the presence of succinylsulfathiazole in the diet. Conjugated folic acid appeared, therefore, to be available as such to the chick, or was made available by metabolic processes.

[15] Pfiffner, J. J., Calkins, D. G., Bloom, E. S., and O'Dell, B. L., *J. Am. Chem. Soc.*, 1946, **68**, 1392.

15714

## Nerve Conduction After Inactivation of Choline Esterase.*

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Gilman reported in February, 1946[1] that di-isopropyl fluorophosphate (DFP) could block conduction in a stretch of frog nerve dipped in it; but that conduction returned on lifting out the nerve. Despite this "reversibility" of the block, the choline esterase (Ch.E.) was left inactivated. Objections were raised to the conditions of these experiments (atypical action potentials, manner of obtaining reversal, low frequency of stimula-

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[1] *The Physics and Chemistry of Nerve Conduction*, New York Academy of Sciences Symposium Monograph, in press.