Nature of the Blood Folates in Young Chicks and Poults Fed Pteroylmonoglutamic Acid or Pteroylheptaglutamates. (31259)

W. J. CROPPER* AND M. L. SCOTT
Department of Poultry Science and Graduate School of Nutrition, Cornell University, Ithaca, N. Y.

Recent work has helped to elucidate the nature of the folate compounds existing in human blood(1,2,3). The human blood folates which supported growth of *Lactobacillus casei,* but not of *Pediococcus cerevisiae* and *Streptococcus faecalis,* originally were thought to be folylpolyglutamates(4,5). Later work suggested that serum folate is present as the monoglutamate, N^5-methyl-tetrahydrofolate (6) which also supports growth of *L. casei* alone(7,8), and that blood cell folate is a mixture of N^5-methyl-tetrahydrofolate derivatives of increasing complexity(1).

The present work was undertaken to determine the nature of the blood folate activity in young chicks and poults and to determine if this can be altered by feeding (a) pteroylmonoglutamic acid (PGA), or (b) brewer's dried yeast (containing conjugated forms of folic acid, largely pteroylheptaglutamates, including reduced forms) as the source of dietary folic acid(9,12). It was considered important to determine if the conjugated folic acid in brewer's dried yeast is deglutamated in the intestine or in the liver, since both tissues contain conjugase activity(13,14). Hence blood from the portal veins and hearts of chicks and poults given the two dietary folate treatments was analyzed microbiologically, using *S. faecalis, P. cerevisiae,* and *L. casei* with and without chicken pancreas conjugase treatment.

Materials and methods. In all microbiological assays, glassware was thoroughly cleaned by steeping 12 hours in chromic acid solution and washing several times with charcoal-treated distilled water. All the distilled water was either charcoal-treated or redistilled immediately before use.

Microbiological analysis of brewer's dried yeast. The "conjugated" or "bound" folic acid was determined by subtracting the value of the "free" folic acid as measured by the 1960 A. O. A. C. method(15) from that of the "total" folic acid, as measured by the 1955 A. O. A. C. method(15) using *S. faecalis*† and chicken pancreas as the source of conjugase. The assay medium used was Folic Acid Assay Medium‡ and the assay procedures were those of the 1955 A. O. A. C. method. The tubes were incubated at 30°C for 18 hours, stirred with a Vortex Junior mixer, and the turbidity measured spectrophotometrically at 610 mμ.

Microbiological analysis of blood. Preparation of the blood samples for microbiological assay was carried out according to the method of Grosswicz *et al*(16).

(a) *S. faecalis* assay was conducted for measurement of pteroic acid, N^{10}-formyl folic acid, all reduced forms of folic acid. The supernatant solution obtained from the blood sample was diluted 10 times to make the concentration of folic acid approximately 1.0 mμg/ml and then used directly in the assay. The samples were assayed before and after treatment with chicken conjugase.

(b) *P. cerevisiae*§ assay was used for measurement of all reduced forms of folic acid except N^5-methyl THFA. The method was similar to that described for *S. faecalis* except that Difco C. F. Assay Medium was used rather than Difco Folic Acid Assay Medium, and Calcium Leucovorin¶ rather than pteroylmonoglutamic acid was used as standard. The incubation period was 48 hours at 30°C.

(c) *L. casei*‖ assay was conducted for measurement of petroyl-di-, tri-, and mono-

* Present address: Dept. Food Science, Borough Polytechnic, London, England.

† *Streptococcus faecalis* A.T.C.C. 8043 was obtained from American Type Culture Collection, Washington, D.C.

‡ Obtained from Difco Laboratories, Detroit, Mich.

§ *Pediococcus cerevisiae* A.T.C.C. 8081 was obtained from American Type Culture Collection, Washington, D.C.

¶ Obtained from Lederle Laboratories, Pearl River, N. Y.

‖ *Lactobacillus casei* A.T.C.C. 7469 was obtained from American Type Culture Collection, Washington, D.C.

glutamic acids, N^{10}-formyl folic acid, and all reduced forms of folic acid including N^5-methyl THFA. The method was similar to that described for *S. faecalis*, except that a medium of the following composition, which was a modification of Scott, Norris and Heuser(17), was used: Casein hydrolysate (acid) 5 g, isoelectric casein (enzymatically hydrolyzed) 5 g, L-cystine (dissolved in HCl) 400 mg, L-tryptophan 400 mg, dextrose 40 g, sodium acetate trihydrate 40 g, K_2HPO_4 5 g, adenine 20 mg, guanine 20 mg, uracil 20 mg, xanthine 20 mg, salt solution B 10 ml, glutamine 200 mg, asparagine 200 mg. Adjust to pH 6.6-6.8, steam, filter through Celite. Add pyridoxine 2400 μg, thiamin 400 μg, riboflavin 400 μg, nicotinic acid 1200 μg, calcium pantothenate 800 μg, biotin 800 μg, p-aminobenzoic acid 20 μg, pyridoxal** 40 μg. Make to 1000 ml.

Experimental procedures used with chicks and poults. Chicks. Twenty one-day-old White Plymouth Rock male chicks per treatment were reared for 4 weeks on the following dietary treatments: (1) pteroylmonoglutamic acid (30 μg/100 g diet); (2) brewer's dried yeast supplying 6 μg/100 g diet as "free" folic acid and 24 μg/100 g diet as "conjugated" folic acid. The basal diet (Table I) was a purified, casein-glucose diet adequate in all known nutrients except folic acid. Brewer's yeast was substituted for equal quantities of casein and sucrose in the treatments in which it was used.

Poults. Twenty one-day-old Wilford Medium White male poults per treatment were reared for 4 weeks on the following dietary treatments: (1) pteroylmonoglutamic acid (50 μg/100 g diet); (2) brewer's dried yeast, supplying 10 μg/100 g diet as "free" folic acid and 40 μg/100 g diet as "conjugated" folic acid.

The basal diet (Table II) was a casein-gelatin-glucose diet, adequate in all known nutrients with the exception of folic acid. The energy:protein ratio in this diet was lower than in the chick diet, to satisfy the higher protein requirements of poults. Higher levels of brewer's yeast and folic acid were used

** Pyridoxal was added aseptically after the tubes were autoclaved using a Swinney apparatus.

TABLE I. Basal Diet for Chick Experiments.

Ingredients	%
Casein	33.4
Glucose (Cerelose)	59.071
Cellulose (Solka-Floc)	3.0
Stripped lard*	4.0
L-Arginine HCl	1.2
Glycine	.6
DL-Methionine	.34
Choline chloride (70% solution)	.214
Antioxidant†	.025
Vitamin mixture‡	1.0
Mineral mixture§	6.15
	100.000
Protein, %	30.53
Energy, Cal/lb ME	1628.4
ME/protein ratio	53.3

* Distillation Products Industries, Rochester, N. Y.

† Santoquin, Monsanto Chemical Co., St. Louis, Mo.

‡ Vitamin A 2400 IU/kg, vitamin D 180 IU/kg; in mg/kg—vitamin K 0.5, thiamin 1.7, riboflavin 2.7, pantothenic acid 8.5, nicotinic acid 25, choline 1250, vitamin B_{12} 0.01, pyridoxine 2.7, biotin 0.1.

§ g/kg diet: $KHCO_3$ 3.0, $CaHPO_4 \cdot 2H_2O$ 23.5, $CaCO_3$ 15.0, KH_2PO_4 9.0, NaCl 8.0, $FeSO_4 \cdot 7H_2O$ 0.35, $MgSO_4$ 2.5, $CuSO_4 \cdot 5H_2O$ 0.017, ZnO 0.1, $CoCl_2 \cdot 5H_2O$ 0.001, $NaMoO_4 \cdot 5H_2O$ 0.008, Na_2SeO_4 0.0002, $Ca(IO_3)_2$ 0.004.

TABLE II. Basal Diet for Poult Experiments.

Ingredients	%
Casein	30.0
Gelatin	10.0
Glucose (Cerelose)	35.3
Cellulose (Solka-Floc)	10.0
Stripped lard*	4.0
L-Arginine	.3
DL-Methionine	.25
Choline chloride (70% solution)	.82
Antioxidant†	.025
Mineral mixture‡	8.305
Vitamin mixture§	1.000
	100.000
Protein, %	35.55
Energy, Cal/lb ME	1442.2
ME/protein ratio	40.6

* Distillation Products Industries, Rochester, N. Y.

† Santoquin, Monsanto Chemical Co., St. Louis, Mo.

‡ g/kg diet: $CaHPO_4 \cdot 2H_2O$ 40, $CaCO_3$ 15, KH_2PO_4 15, NaCl 6.0, $FeSO_4 \cdot 7H_2O$ 0.3, $MgSO_4 \cdot 7H_2O$ 6.5, KI 0.0015, $CuSO_4 \cdot 5H_2O$ 0.1, $ZnCl_2$ 0.15, $NaMoO_4 \cdot 5H_2O$ 0.005, Na_2SeO_4 0.002.

§ mg/kg: Menadione sodium bisulfite 10, biotin 0.15, vitamin B_{12} 0.02, calcium pantothenate 16.0, nicotinic acid 80.0, pyridoxine HCl 5.0, riboflavin 6.0, thiamin hydrochloride 3.5. Units/kg: vitamin A 6,000, vitamin D_3 1,500.

TABLE III. Folic Acid Content of Blood Collected from the Heart and Portal Vein of Chicks and Poults Fed Pteroylmono- or Heptaglutamates.

Form of folic acid	Source of blood	Folic acid activity of blood (mγ/ml)					
		S. faecalis		*P. cerevisiae*		*L. casei*	
		Incub.	Un-incub.	Incub.	Un-incub.	Incub.	Un-incub.
CHICK:							
Pteroylmonoglutamic acid	Heart	98	7.2	72	8.0	798	5.2
	Portal vein	117	6.4	81	7.0	733	11.6
Pteroylheptaglutamate (brewer's dried yeast)	Heart	92	6.0	76	5.0	813	11.8
	Portal vein	86	8.0	65	6.1	726	8.4
POULT:							
Pteroylmonoglutamic acid	Heart	180	7.2	81	8.0	571	4.7
	Portal vein	349	9.0	89	6.2	598	14.0
Pteroylheptaglutamate (brewer's dried yeast)	Heart	95	6.7	85	7.1	423	16.8
	Portal vein	111	9.1	79	7.0	546	12.7

since the folic acid requirement of the poult is higher than that of the chick. Brewer's yeast was substituted for equal quantities of casein and sucrose in the treatments in which it was used.

Feed was withdrawn for 12 hours from the chicks and poults at 4 weeks of age and then *ad libitum* feeding was allowed for a period of 8 hours prior to the collection of blood. This was done to ensure that the folic acid would be in process of absorption at time of collection of the blood.

Blood was collected from the hepatic portal veins and hearts of anesthetized birds; samples from several birds on each treatment were composited.

Results. Both portal and systemic blood of chicks and poults were analyzed for "free" and "total" forms of folic acid active for each of the 3 microorganisms used (Table III).

Incubation of all blood samples with chick pancreas enzyme indicated higher total folic acid levels in the blood of chicks as compared with poults receiving both dietary treatments. This may have been due to the level of folic acid in the poult diet which was considerably below the requirement, while that in the chick diet was barely deficient.

The large differences obtained with all 3 bacteria in values after incubation with conjugase enzyme, as compared with the unincubated blood samples, indicated a predominance of folic acid form(s) in both the chick and poult blood containing more than 2 glutamic acid residues per molecule(18).

In the *L. casei* assay, the large differences obtained between incubation and non-incubation with the conjugase indicate that the main form of folic acid conjugate in blood of both chicks and poults is N^5-methyl THFA, since this form is active for *L. casei* but not for *S. faecalis* or *P. cerevisiae*. This form has been shown to occur in human blood(1). Although N^5-methyl THFA is difficult to distinguish microbiologically from pteroyltriglutamate, the activity of the conjugase-treated blood for *L. casei* which was much higher than that for *S. faecalis* argues against the presence of pteroyltriglutamate.

Discussion. The results showing that the predominant folic acid in the hepatic portal vein is conjugated and apparently present largely as the N^5-methyl form irrespective of whether brewer's dried yeast or pteroylmonoglutamic acid was the dietary source of folic acid indicate that glutamation and substitutions on the pteridine ring may occur in the intestinal wall. Although systemic blood is recirculated through the portal vein in chicks and poults fed pteroylmonoglutamic acid, somewhat higher levels should have been present in portal than in systemic blood if the pteroylmonoglutamate were reduced, methylated and glutamated in the liver only and not in the intestinal mucosa.

The values obtained by microbiological analysis of the chick and poult blood using *L. casei, S. faecalis* and *P. cerevisiae* before

conjugase treatment were similar to values obtained by Noronha and Aboobaker(1), who analyzed human blood from normal adult males. However, conjugase treatment increased *L. casei* activity in chick and poult blood to a much greater extent than in human blood (chick 733-813 mγ/ml; poult 423-598 mγ/ml; human 66-116 mγ/ml).

Higher values were obtained in the *P. cerevisiae* and *S. faecalis* assays of chick and poult blood after conjugase treatment, whereas no increases were reported for human blood (chick 65-81 and 86-117 mγ/ml; poult 79-89 and 95-349 mγ/ml, respectively).

Whether the higher levels of conjugated folic acid found in the chick and poult blood as compared with human blood represent a true species difference or are due to differences in methods of analysis is not known. Noronha and Aboobaker used a purified conjugase from chick liver; a chick pancreas homogenate was used in the present study. Hill and Scott(11,19) showed that the release of "Citrovorum factor" from its conjugate in brewer's yeast by chick liver enzyme was dependent upon the cysteine and ascorbic acid levels in the incubation mixture.

Summary. (1) Chick and poult blood folates were measured microbiologically, using *L. casei, S. faecalis* and *P. cerevisiae,* with and without incubation with chicken pancreas conjugase. Before incubation with conjugase, folate activity was low in blood of both chicks and poults. It increased greatly, particularly for *L. casei,* following incubation with chicken pancreas. (2) The use of pteroylmonoglutamic acid or pteroylheptaglutamates (in brewer's dried yeast) as different sources of dietary folic acid did not alter the form of folate activities found by *L. casei, S. faecalis,* or *P. cerevisiae* in chick or poult blood, either before or after incubation with chicken pancreas. (3) The major folate activity in both chick and poult blood was present as a form which was most active for *L. casei* following chicken pancreas conjugase treatment. Therefore, folate appears to be present in chick and poult blood mainly as a conjugate of N^5-methyl-tetra-hydropteroylglutamic acid. (4) Although the extent of dilution of portal blood with systemic blood was not measured, the very low level of pteroylmonoglutamic acid in portal blood coupled with the approximately equivalent levels of conjugated folate active for *L. casei* in both portal and systemic blood indicate that both pteroylmonoglutamic acid and the pteroyl polyglutamates of brewer's yeast may be transformed to a conjugate of N^5-methyl-tetra-hydropteroylglutamic acid in the intestinal mucosa.

The authors wish to acknowledge grants in support of this work by Agway, Inc., Syracuse, N. Y., and The Distillers Feed Research Council, Cincinnati, O.

1. Noronha, J. M., Aboobaker, V. S., Arch. Biochem. Biophys., 1963, v101, 445.
2. Iwai, K., Littner, P. M., Toennies, G., J. Biol. Chem., 1964, v239, 2365.
3. Bird, O. D., McGlohon, V. M., Vaitkus, J. W., Anal. Biochem., 1965, v12, 18.
4. Herbert, V., J. Clin. Invest., 1961, v40, 81.
5. Usdin, E., J. Biol. Chem., 1959, v234, 2373.
6. Herbert, V., Larrabee, A. R., Buchanan, J. M., J. Clin. Invest., 1962, v41, 1134.
7. Larrabee, A. R., Rosenthal, S., Cathon, R. E., Buchanan, J. M., J. Am. Chem. Soc., 1961, v83, 4094.
8. Keresztesy, J. C., Donaldson, K. O., Biochem. Biophys. Res. Comm., 1961, v5, 286.
9. Pfiffner, J. J., Calkins, D. G., O'Dell, B. L., Bloom, E. S., Brown, R. A., Campbell, C. J., Bird, O. D., Science, 1945, v102, 228.
10. Pfiffner, J. J., Calkins, D. G., Bloom, E. S., O'Dell, B. L., J. Am. Chem. Soc., 1946, v68, 1392.
11. Hill, C. H., Scott, M. L., J. Biol. Chem., 1952, v196, 189.
12. Schertel, M. E., Boehne, J. W., Libby, D. A., ibid., 1965, v240, 3154.
13. Charkey, L. D., Daniel, L. J., Farmer, F., Norris, L. C., Heuser, G. F., Proc. Soc. Exp. Biol. and Med., 1947, v64, 102.
14. Chitre, R. C., Agasha, S., J. Univ. Bombay, 1954, v36, 38.
15. Association of Official Agricultural Chemists, 9th Ed., 1960; 8th Ed., 1955.
16. Grosswicz, N., Mandelbaum-Shavit, F., Davidoff, R., Aronovitch, J., Blood, 1962, v20, 609.
17. Scott, M. L., Norris, L. C., Heuser, G. F., J. Biol. Chem., 1946, v166, 481.
18. Kazenko, A., Laskowski, M., ibid., 1948, v173, 217.
19. Hill, C. H., Scott, M. L., ibid., 1952, v196, 195.

Received March 21, 1966. P.S.E.B.M., 1966, v122.