Metabolism of Folate Coenzymes in the Developing Chick Embryo (36738)

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Several authors have determined the content of many water-soluble vitamins, like vitamin B_{12} (1), choline (2, 3), nicotinic acid (4, 5), riboflavin (6), thiamin (6), and ascorbic acid (6) in the developing avian embryo. The only available data on folic acid (7) are defective as they concern only the egg *in toto* and were obtained by a method incapable of evaluating all forms of folic acid. These compounds, taking part in the metabolism of purine (8, 9) and pyrimidine (10) nucleotides, and of some amino acids (11, 12), play an important role in the biosynthesis of nucleic acids and proteins and, therefore, in the organogenesis processes.

For this reason we have studied the distribution of naturally occurring folates in the chick embryo and in the nonembryonic portions of the egg at various stages of development by means of a system of differential microbiological assays.

Materials and Methods. Preparation of tissue. White Leghorn × New Hampshire fertilized eggs, obtained from a commercial source, were incubated at 39° with relative humidity of 65% and forced air circulation. The eggs were placed on racks and turned twice daily. After 9, 12, 15 and 18 days of incubation the embryos, freed from extraembryonic membranes and yolk sac, as well as the nonembryonic portions of the egg (consisting of the egg content except the embryo) were pooled and immediately cooled. Part of the tissues was homogenized in a Virtis "45" homogenizer with 9 vol of ice-cold water; another part was homogenized with 9 vol of 1% (w/v) potassium ascorbate, pH 6.0. On days 15 and 18 the livers were excised from some embryos, pooled and homogenized in the same way.

Determination of folate coenzymes.[1] The homogenates were placed in a water bath at 95° for 5 min, quickly cooled in iced water and centrifuged. To the extracts were added hog kidney conjugase, prepared according to Eigen and Shockman (13), 0.1 *M* phosphate buffer (pH 4.7) and 1% potassium ascorbate (pH 4.7); potassium ascorbate was omitted in the enzyme treatment of the water extracts. The samples were incubated 4 hr at 37° and the folate derivatives were assayed by the method described by Bird, Mims McGlohon and Waitkus (14), which employs three test microorganisms in "autoclaved" and "aseptic" assays. Samples in potassium ascorbate were used for aseptic assays with the *L. casei* ATCC 7469 and *P. cerevisiae* ATCC 8081. The data obtained by aseptic *L. casei* assay indicate the sum of all folate forms, from which the percentages of other folate derivatives were calculated, while aseptic *P. cerevisiae* assay represents the value of all tetrahydro forms except 5-CH_3–H_4folate. Samples in water were used for autoclaved assays with *P. cerevisiae* and *S. faecalis* ATCC 8043. *P. cerevisiae* assay provides data on the amount of 5-HCO–H_4folate since it is the only compound which survives autoclaving in water and is active for this microorganism. The autoclaved *S. faecalis* assay of the water extracts represents the value of combined 5- and 10-HCO–H_4folate. In water solution 10-HCO–H_4folate is oxidized during autoclaving to a stable compound active for *S. faecalis* but inactive for *P. cerevisiae*,

[1] The following abbreviations are used: H_2folate = dihydrofolate; H_4folate = tetrahydrofolate; 5(10)-HCO–H_4folate = 5(10)-formyltetrahydrofolate; 5-CH_3–H_4folate = 5-methyltetrahydrofolate; 5,10-CH_2–H_4folate = 5,10-methylenetetrahydrofolate; 5, 10-CH=H_4folate = methylidyne-tetrahydrofolate.

TABLE I. Total Folate Activity in the Developing Chick Embryo, in the Nonembryonic Portions of the Egg and in the Whole Egg at Different Days of Incubation.[a]

Incubation (days)	Embryo: Av dry wt (g)	Embryo: Folate activity[b] a	Embryo: Folate activity[b] b	Nonembryonic portions of the egg: Av dry wt (g)	Nonembryonic: Folate activity a	Nonembryonic: Folate activity b	Whole egg: Av dry wt (g)	Whole egg: Folate activity a	Whole egg: Folate activity b
0	—	—	—	—	—	—	16.65	53.30 ± 5.82	3.20 ± 0.28
9	0.15	1.80 ± 0.16	12.00 ± 1.23	16.01	51.30 ± 4.90	3.20 ± 0.23	16.16	53.10 ± 5.23	3.28 ± 0.31
12	0.68	9.30 ± 0.86	13.78 ± 1.26	14.64	50.00 ± 5.10	3.41 ± 0.31	15.32	59.30 ± 5.12	3.87 ± 0.40
15	4.11	18.80 ± 1.78	4.23 ± 0.45	10.63	32.90 ± 2.85	3.10 ± 0.29	14.74	51.65 ± 4.95	3.50 ± 0.49
18	8.21	30.00 ± 2.78	3.65 ± 0.30	6.75	9.50 ± 1.04	1.41 ± 0.13	14.96	39.50 ± 4.10	2.64 ± 0.11

[a] All values are the means of 6 pools of 3 embryos, 3 nonembryonic portions of the egg, 3 whole eggs, respectively, ± SEM.

[b] (a) μg/total wt of embryo, nonembryonic portions of the egg, whole egg, respectively; (b) μg/g dry wt of embryo, nonembryonic portions of the egg, whole egg, respectively.

while H_4folate is inactivated under these conditions.

Performing all these assays on the two extracts provides data for calculating the percentage of each of the main folate derivatives present originally in the different materials.

Calcium leucovorin was used as a reference standard and the concentration was adjusted to correct for the presence of the inactive D-isomer.

Enzyme assays. In assaying H_4folate dehydrogenase (EC 1.5.1.3), the livers were homogenized with 4 vol of 0.01 *M* Tris:HCl buffer (pH 7.0) and centrifuged at 20,000*g* for 10 min at 4°. The enzyme was determined in the supernatant by measuring the decrease in the absorbance at 340 mμ caused by the conversion of NADPH to $NADP^+$ and of H_2folate to H_4folate (15). To assay the other enzyme activities, the livers were homogenized with 9 vol of 0.05 *M* Tris:HCl buffer (pH 7.5) and centrifuged at 10,000*g* for 30 min at 4°. 5,10-CH_2–H_4Folate dehydrogenase (EC 1.5.1.5) was assayed determining spectrophotometrically at 355 mμ the 5,10-CH=H_4folate formed in the system (16). HCO–H_4Folate synthetase (EC 6.3.4.3) was assayed in the supernatant partially purified with protamine sulfate and solid ammonium sulfate, by measuring the 5, 10-CH=H_4folate formed in the reaction mixture (17). Protein was determined by the colorimetric method of Lowry *et al.* (18) with crystalline bovine plasma albumin as the standard.

Results. As shown in Table I the total folate activity of the embryo, referred to total embryo, increased constantly throughout development, while expressed per gram of dry weight, it was rather high until day 12 and then decreased considerably; the folate content of the nonembryonic portions of the egg, expressed per total material, remained constant until day 12 and decreased rapidly in the following days, while it decreased only on day 18 when expressed per gram of dry weight. As for the folate activity of the whole egg, it did not change markedly whichever expression was used: a slight increase can be seen at day 12 and a slight decrease in the last days of development.

From the data of Table II regarding the distribution of various coenzymic forms of folic acid in the chick embryo it can be seen that they tended to increase throughout the incubation in the same way as total folate activity did, expressed per total embryo; moreover ratios between the different coenzymes showed some variations.

In the nonembryonic portions of the egg (Table III) the amount of each coenzyme, expressed per total material, tended to decrease in parallel with total folate activity. Both in the embryo and in the nonembryonic portions of the egg ratios between various coenzymes were rather different according to the day of incubation.

Much more evident are the percentage variations (Table IV) between different components of the total folate activity in the whole egg compared with those in the embryo and in the nonembryonic portions of the egg, when separately considered. In particular, a higher percentage of reduced forms, especially of H_4folate, was observed between days 12 and 15 of incubation. In the same period a lower amount of 5-CH_3–H_4folate occurred.

The liver content of various coenzymes (Table V) increased from day 15 up to day 18, when the whole organ was considered, while it tended to decrease when the amount was considered per weight unit; a higher percentage of 5-CH_3–H_4folate and a lower one of H_4folate was observed in this organ with respect to the distribution of coenzymes in the whole embryo.

Lastly, the data of Table VI regarding enzymic activities involved in the metabolism of folate coenzymes, determined at various stages of development, show that 5, 10-CH_2–H_4folate dehydrogenase and 10-HCO–H_4folate synthetase increased between days 10 and 12; on the contrary from day 14 onward these activities tended to decrease rapidly. H_4Folate dehydrogenase activity remained constant until day 14 and then it, too, decreased rapidly.

Discussion. The data obtained in the present research demonstrate first of all that the folate activity of the egg does not vary during incubation, as had been partially seen

TABLE II. Distribution of Various Folate Derivatives in the Developing Chick Embryo.[a]

Compounds	Days: 9[b] a	9[b] b	12 a	12 b	15 a	15 b	18 a	18 b
All forms	1.80 ± 0.16	12.00 ± 1.13	9.30 ± 0.86	13.78 ± 1.86	18.80 ± 1.86	4.25 ± 0.51	30.00 ± 3.10	3.65 ± 0.40
All tetrahydro forms except 5-CH_3–H_4 folate	1.40 ± 0.15	9.30 ± 0.77	5.60 ± 0.53	8.29 ± 0.92	14.10 ± 1.32	3.18 ± 0.27	21.97 ± 1.40	2.68 ± 0.15
5-CH_3–H_4folate	0.40 ± 0.03	2.70 ± 0.20	3.70 ± 0.32	5.48 ± 0.61	4.70 ± 0.46	1.06 ± 0.12	8.03 ± 0.69	0.97 ± 0.11
5- and 10-CHO–H_4 folate	0.48 ± 0.06	3.20 ± 0.21	3.02 ± 0.23	4.46 ± 0.32	8.90 ± 0.79	2.00 ± 0.21	10.59 ± 1.02	1.29 ± 0.17
H_4folate	0.92 ± 0.10	6.10 ± 0.52	2.72 ± 0.29	4.02 ± 0.44	5.20 ± 0.68	1.17 ± 0.13	11.38 ± 1.18	1.38 ± 0.12

[a] All values are the means of 6 pools of 3 embryos, ± SEM.
[b] (a) μg/total embryo; (b) μg/g dry wt of embryo.

TABLE III. Distribution of Various Folate Derivatives in the Nonembryonic Portions of the Egg at Different Days of Incubation.[a]

Compounds	Days: 0[b] a	b	9 a	b	12 a	b	15 a	b	18 a	b
All forms	53.30 ± 5.00	3.20 ± 0.25	51.30 ± 4.85	3.20 ± 0.41	50.00 ± 4.92	3.41 ± 0.46	32.90 ± 2.75	3.10 ± 0.39	9.50 ± 0.88	1.41 ± 0.15
All tetrahydro-forms except $5\text{-}CH_3\text{-}H_4$folate	40.80 ± 3.90	2.50 ± 0.20	38.00 ± 3.50	2.40 ± 0.19	43.10 ± 3.62	2.94 ± 0.12	27.60 ± 2.01	2.60 ± 0.18	8.27 ± 0.71	1.22 ± 0.13
$5\text{-}CH_3\text{-}H_4$folate	12.40 ± 0.87	0.75 ± 0.09	13.30 ± 1.03	0.80 ± 0.12	7.20 ± 0.63	0.49 ± 0.05	5.20 ± 0.43	0.50 ± 0.03	1.23 ± 0.16	0.19 ± 0.02
5- and 10-CHO-H_4folate	16.10 ± 1.04	1.00 ± 0.13	17.10 ± 1.25	1.10 ± 0.17	15.68 ± 1.32	1.07 ± 0.12	6.80 ± 0.53	0.65 ± 0.80	4.71 ± 0.34	0.70 ± 0.06
H_4folate	24.80 ± 2.03	1.50 ± 0.17	21.00 ± 2.36	1.30 ± 0.09	27.28 ± 1.92	1.86 ± 0.13	20.90 ± 1.67	2.00 ± 0.23	3.56 ± 0.28	0.52 ± 0.06

[a] All values are the means of 6 pools of 3 nonembryonic portions of the egg, ± SEM.

[b] (a) μg/total nonembryonic portions of the egg; (b) μg/g dry wt of the nonembryonic portions of the egg.

by Hayes and Luckey (7). Therefore folic acid behaves like vitamin B_{12}, whose amount in the fertilized egg does not increase but, on the contrary, decreases towards the end of incubation (1). One may therefore reasonably think that a *de novo* synthesis of folic acid from its precursors does not take place, as on the contrary happens for nicotinic acid (4, 5). Thus, it must be thought that all folic acid necessary for the development of the embryo is already present in the yolk sac from the beginning and during incubation the vitamin passes to the embryo. A similar transfer was observed by Tsuji, Brin and Williams (3) for choline.

It is interesting to point out that the percentages of different coenzymes both in the embryo and in the nonembryonic portions of the egg vary continuously during development. This phenomenon is particularly evident in the whole egg. In fact, while the total activity remains unchanged it is possible to see, around day 12, a significant percentage increase of the reduced forms, particularly of H_4folate and, at the same time, a decrease of $5\text{-}CH_3\text{-}H_4$folate. It is well known that H_4folate and the other reduced derivatives represent the actual coenzymic forms of folic acid, while $5\text{-}CH_3\text{-}H_4$folate can be considered a form not directly available. In fact $5\text{-}CH_3\text{-}H_4$folate tends to accumulate at the expense of H_4folate when the metabolic processes utilizing 1-C fragments are slackening as in vitamin B_{12} deficiency. In this case the block of the $5\text{-}CH_3\text{-}H_4$folate: homocysteine transmethylase activity leads to a remarkable storage of the 5-methyl derivative and, consequently, to a drawing of H_4folate and of the other reduced forms (19). It could be thought that the greatest utilization of these coenzymes takes place at day 12 when the biosynthesis processes are more active in the developing embryo.

The higher levels of folate coenzymes in the 12-day-old embryo are surely dependent on the increase of enzymic activities involved in their synthesis. Thus H_4folate dehydrogenase, $5,10\text{-}CH_2\text{-}H_4$folate dehydrogenase and 10-HCO-synthetase are more active in this period. On the other hand an increase was also observed by Silber, Huennekens and Ga-

TABLE IV. Folate Derivatives in the Fertilized Whole Egg at Different Days of Incubation.[a]

Compounds Days:	0	9	10	15	18
All forms	53.30 ± 5.12	53.10 ± 5.23	59.30 ± 4.12	51.65 ± 4.95	39.50 ± 3.10
All H_4 forms except 5-CH_3–H_4folate	41.00 ± 3.90 77%[b]	39.40 ± 3.12 74%	48.70 ± 3.00 82%	41.75 ± 3.94 81%	30.24 ± 2.96 77%
5-CH_3–H_4folate	12.40 ± 1.07 23%	13.70 ± 1.24 26%	10.90 ± 1.12 18%	9.90 ± 0.87 19%	9.26 ± 1.04 23%
5- and 10-CHO–H_4folate	16.10 ± 1.14 30%	17.50 ± 1.07 33%	18.70 ± 1.98 31%	15.68 ± 1.08 30%	15.30 ± 1.39 39%
H_4folate	24.80 ± 2.23 40%	21.90 ± 1.88 41%	30.00 ± 2.50 51%	26.06 ± 2.45 50%	14.94 ± 1.20 38%

[a] Values are the means of 6 pools of 3 eggs, ± SEM, expressed as μg/whole egg.
[b] Percentages are referred to the "All forms" values.

brio (20) studying characteristics of these enzymes in the developing embryo compared with their counterpart in other tissues. Therefore it is possible to think that the increased demand may induce these enzymic activities to produce the suitable coenzymic forms.

Lastly, it is interesting to point out that also in the embryo the liver is the most interested organ in the metabolism of folic acid, since its coenzymic content is higher than that of other tissues, weights being equal.

Summary. Folate coenzymes were determined in the chick embryo, in the nonembryonic portions of the egg and in the whole egg at different stages of development by means of differential microbiological assays. Moreover, the enzymic activities involved in the metabolism of these structures were evaluated in the chick embryo.

During development the total folate content of the whole egg remains constant, only in the latest stages does a slight decrease occur. In the embryo, folate activity increases constantly during development, while it decreases in the nonembryonic portions of the egg, in particular from day 12 onward. Similar behavior was observed for each folate derivative.

Nevertheless, it is possible to observe in the whole egg as well as in the embryo, significant percentage variations among the coenzymes at various stages of development; particularly on day 12 the amount of H_4folate is higher, while that of CH_3–H_4folate is lower. As for the enzymes

TABLE V. Distribution of Various Folate Derivatives in the Livers of the 15- and 18-Day-Old Embryos.[a]

Compounds	15 Days		18 Days	
	a	b	a	b
All forms	4.10 ± 0.30	52.90 ± 4.10	5.65 ± 0.51	44.40 ± 4.32
All tetrahydro forms except 5-CH_3–H_4folate	2.65 ± 0.95	33.80 ± 3.04	3.20 ± 0.39	24.90 ± 3.24
5-CH_3–H_4folate	1.50 ± 0.10	19.10 ± 1.48	2.50 ± 0.20	19.50 ± 1.76
5- and 10-CHO–H_4 folate	1.65 ± 0.13	20.90 ± 1.92	2.10 ± 0.21	16.50 ± 1.82
H_4folate	1.00 ± 0.07	12.90 ± 1.00	1.00 ± 0.09	7.70 ± 6.95

[a] All values are the means of 3 pools of 5 livers, ± SEM.
[b] (a) μg/total liver; (b) μg/g dry wt of liver.

TABLE VI. Enzymic Activities Catalyzing the Synthesis of Folate Coenzymes in the Developing Chick Embryo.[a]

Incubation (days)	H_4folate dehydrogenase[b]	10-HCO–H_4folate synthetase[c]	5, 10-CH_2–H_4folate dehydrogenase[c]
4	2.20 ± 0.17 (40)	1237 ± 104	103 ± 12.3
6	2.13 ± 0.16 (20)	1490 ± 123	79 ± 7.5
8	1.85 ± 0.12 (10)	1580 ± 120	86 ± 6.7
10	2.12 ± 0.14 (5)	2140 ± 205	129 ± 9.0
12	2.08 ± 0.13 (5)	1830 ± 128	148 ± 8.7
14	1.62 ± 0.10 (3)	1343 ± 107	128 ± 9.3
16	1.28 ± 0.10 (3)	516 ± 56	77 ± 5.4
18	1.00 ± 0.08 (3)	450 ± 38	60 ± 7.2

[a] All values are the means of 3 pools, ± SEM; in parentheses the number of embryos per pool.
[b] nmoles of H_2folate reduced/min/mg protein.
[c] nmoles of 5, 10-CH = H_4folate formed/20 min/mg protein.

studied, an increase of activity between days 10 and 12 was observed. Therefore it is possible that an increase of the synthesis, followed by an increased storage of coenzymes occurs when the demand for the latter increases.

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