Biochemical responses of broiler chicks to folate deficiency

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The effects of folate sufficiency and deficiency in three pathways of folate metabolism were studied in 2- and 3-week-old broiler chicks. Erythrocyte phosphoribosylpyrophosphate concentrations and dihydrofolate reductase (*EC* 1.5.1.3) activity were significantly elevated in folate deficiency. Percentage incorporation of deoxyuridine into bone marrow DNA was reduced in folate deficiency. There was a trend towards reduced liver dihydrofolate reductase activity in deficient chicks. These studies identify further biochemical criteria that can be used to assess folate status of chicks.

Folic acid: Dihydrofolate reductase: Deoxyuridine suppression: Phosphoribosylpyrophosphate: Chicken

Little is known about the requirement for folic acid of modern strains of rapidly-growing poultry. Requirements have been calculated from experiments carried out several years ago using slower growing birds fed on purified or semi-purified diets. Since then, both genetic potential and management techniques have improved, leading to increased growth. Wong *et al.* (1977) estimated the requirement to be between 0·27 and 0·44 mg/kg, depending on the source of protein in the feed. National Research Council (1984) requirements for folate in broilers are set at 0·55 mg/kg. Subsequent biochemical studies involving the measurement of histidine oxidation suggested that 1·5–2·0 mg folate/kg was needed for maximal activity of this metabolic pathway (Whitehead & Rennie, 1989). Biochemical criteria can be useful for determining nutritional status or requirements for a vitamin. However, such criteria in poultry are largely lacking for folic acid. In order to develop such criteria and gain insight into folate requirements for metabolic function in broilers, a series of studies was undertaken on two areas of folate metabolism that have been used to assess folate status in mammals.

The deoxyuridine suppression test was developed by Metz *et al.* (1968) as an aid to diagnosing folate deficiency in man by assessing the level of function of the vitamin in a biochemical system. In bone marrow the preferred pathway for thymidine synthesis is by a *de novo* route, the starter substrate being deoxyuridine (dU). dU is converted to deoxyuridine monophosphate (dUMP) which is used to synthesize deoxythymidine monophosphate (dTMP). This second step requires the addition of a C_1 unit in the form of 5,10-methylene-tetrahydrofolate (5,10-methylene THF). A 'salvage pathway' independent of folate also exists for the synthesis of TMP from thymidine, but, in man, only approximately 10% of dTMP in bone marrow DNA is derived from this source. However, if folate is deficient the *de novo* route cannot function properly and up to 50% of dTMP is derived from the salvage pathway (Wickramasinghe & Longland, 1974).

Phosphoribosylpyrophosphate (PRPP) is the required substrate for those reactions in cells in which adenine, hypoxanthine and guanine are directly converted to ribonucleotides. It is also a required substrate in the ten-step biosynthetic pathway for the *de novo* synthesis

of inosine monophosphate which is used to synthesize purines. Folate is required at two stages in this pathway in the form of 10-formyltetrahydrofolate. It has been shown that folate-deficient, actively purine-synthesizing cells have elevated levels of PRPP as a result of both precursor accumulation and decreased negative feedback from purine nucleotides (Ghitis *et al.* 1987). PRPP concentrations have been found to be inversely related to the intracellular concentrations of folate. Folate-deficient cells pre-incubated with phosphate show even higher levels of PRPP, presumably resulting from stimulation of PRPP synthetase (*EC* 2.7.6.1) (Hershko *et al.* 1969).

Further studies were also carried out on dihydrofolate reductase (DHFR; *EC* 1.5.1.3). DHFR is a key enzyme in folate metabolism, catalysing the reduction of dihydrofolic acid to tetrahydrofolic acid (THF) with NADPH providing reducing power. THF then participates in a wide range of biochemical reactions involving C_1 group transfer, e.g. histidine oxidation, the conversion of phenylalanine to tyrosine, the inter-conversion of serine and glycine, dTMP and inositate synthesis. DHFR is located in the cell cytosol, and enzyme activity has been detected in a wide variety of tissues such as liver, thymus and bone marrow, although levels are low in non-proliferating tissues such as skeletal muscle, heart, lung, brain and erythrocytes. Burns & Jackson (1976) reported that hepatic DHFR activity fell in folate-deficient chickens. However, Whitehead & Rennie (1989) found that erythrocyte DHFR activity rose in deficient chicks. The responsiveness of the enzyme in both tissues to dietary folate was therefore reassessed to confirm and extend the earlier observations.

MATERIALS AND METHODS

Birds and husbandry

Three experiments were carried out using batches of 1-d-old male broiler chicks (DB Marshall, Newbridge, Midlothian) randomly divided into groups of ten birds housed in separate compartments of an electrically-heated, wire-floored brooder unit. The chicks were fed on a purified diet (Table 1) based on wheat-starch and vitamin-free casein, supplemented with various amounts of a commercial preparation of 800 g folic acid/kg, Rovimix 80 SD (Roche Products Ltd, Welwyn Garden City, Herts.) as described previously (Whitehead & Rennie, 1989). Food and water were available *ad lib.* At 2 and 3 weeks of age, blood samples were taken into heparinized tubes for measurement of erythrocyte PRPP and DHFR activity. Plasma from the chicks in the different groups was pooled for use in the dU suppression test. Birds were then killed by cervical dislocation and liver and bone marrow removed to measure DHFR activity and percentage dU suppression respectively.

Reagents

All reagents were of analytical grade and supplied by Sigma (Poole, Dorset) or BDH (Glasgow), unless otherwise stated.

Biochemical assays

DHFR. Erythrocyte DHFR activity was measured as previously described (Whitehead & Rennie, 1989). Liver DHFR activity was measured by the following procedure. After killing, the liver was immediately removed, rinsed in ice-cold NaCl solution (9 g/l), blotted dry on tissue, and quickly frozen to $-20°$ until the assay could be performed. For the assay, a 1 g portion of tissue from the left lobe was homogenized with 4 ml ice-cold 0·05 M-sodium citrate, pH 7·2, using a Polytron homogenizer. The homogenate was centrifuged at 20000 ***g*** for 30 min. The supernatant fraction was removed for the assay, a small portion being taken for protein assay using a dye-binding assay (Bio-Rad, Hemel Hempstead, Herts.)

Table 1. *Composition of experimental diets (g/kg)*

Ingredient	Control diet	Imbalanced diet
Wheat	559	563
Soya-bean meal	350	350
Vegetable oil	45	50
Limestone	13	—
Dicalcium phosphate	21	25
Salt	4	4
Methionine	2	2
Lysine	2	2
Vitamin and mineral supplement*	4	4
Calculated analyses (g/kg)		
Ca	12	7·5
P	6	7·6

* Provided (mg/kg diet): retinol 1376 μg, cholecalciferol 25 μg, α-tocopherol 10, menadione 1·3, thiamine 1·3, riboflavin 3·3, pyridoxine 1·7, cyanocobalamin 0·6 μg, folic acid 0·7, biotin 267 μg, pantothenic acid 5·3, nicotinic acid 23·3, zinc 40, copper 2·9, iodine 320 μg, iron 64, manganese 80, selenium 120 μg.

adapted for use with a Titertek microplate reader. The DHFR assay was performed as described previously, except that the assay mixture contained 150 μl citrate–mercaptoethanol buffer, and the reaction was initiated by the addition of 10 μl liver preparation.

PRPP. Erythrocyte PRPP was measured by a modification of the method of Ghitis & Waxman (1987). [Carboxyl-^{14}C]orotic acid was supplied by New England Nuclear Research, Du Pont (UK Ltd) (Stevenage, Herts.) and had a specific activity of 50–60 mCi/mmol. Polystyrene flat-bottomed tubes (16·5 × 95 mm) with polythene caps (Sarstedt Ltd, Leicester) were used for the PRPP assay.

Phosphate–Krebs–Ringer (PKR) buffer was freshly prepared before each assay by mixing stock solutions of (g/l): NaCl 9, KCl 11·5, $CaCl_2$ 12·2, KH_2PO_4 21·1, $MgSO_4 . 7H_2O$ 38·0 and 0·1 M-Na_2HPO_4 buffer, pH 7·4, in the ratio of 100:4:3:1:1:21 and readjusting the pH to 7·4. Bovine serum albumin (BSA; 20 g/l) was added with gentle stirring until fully dissolved.

Erythrocytes were separated by centrifugation and washed twice with ice-cold PKR buffer, and then re-suspended in 1 ml of the same buffer. A 50 μl portion of suspension was taken at this stage for cell-counting using an automated Coulter counter. The cell suspension was incubated for 1 h at 38° and then centrifuged. The supernatant fraction was drawn off, and the cells mixed with 1 ml cold 10 mM-glycylglycine–1 mM-EDTA buffer, pH 8·0 at 38°. The mixture was quickly frozen to −20°, thawed and quickly refrozen to rupture the erythrocytes. Samples were centrifuged at 2500 *g* for 10 min to remove cell membranes. The supernatant fraction was drawn off and kept frozen for no longer than 4 d at −20°, or no longer than 8 d at −80°, before being assayed.

A 1 mM stock solution of PRPP was prepared and frozen at −80° as 0·5 ml portions. Each portion was thawed only once for use, any excess being disposed of. A working solution of PRPP was made by diluting the stock solution 1000 times with glycylglycine–EDTA buffer to give a solution containing 1000 pmol/ml.

[^{14}C]orotic acid was diluted with deionized water to give a solution containing 10 μCi/ml and stored at −20° until required. 'Cold' 600 μM-orotic acid was divided into 0·2 ml portions and stored at −20°. Working orotic acid solution was freshly prepared before each assay by mixing equal volumes of labelled and unlabelled solutions, which were then further diluted twice with deionized water. The enzyme solution was prepared by dissolving

5 mg orotate phosphoribosyl transferase (*EC* 2.4.2.10)–orotidylate decarboxylase (*EC* 4.1.1.230) mixed enzymes (0·2–0·3 U/mg) in 1 ml 100 mM-$MgCl_2$ solution.

Reaction tube caps were prepared by soaking filter papers (Whatman no. 1, 10 × 100 mm) in hyamine hydroxide, then quickly rolling them up and inserting them into the tube caps. The caps were stored under N_2 in a sealed glass vessel until required.

Standard solutions of 0–1200 pmol PRPP were pipetted into tubes and made up to 1·2 ml with glycylglycine–EDTA buffer. Thawed cell lysates were diluted by mixing 250 μl with 750 μl glycylglycine–EDTA and pipetted into tubes. All tubes were placed in a boiling water bath for 45 s before immediate assay. Working orotic acid solution (15 μl; equivalent to 35 nCi) was added to each tube, followed by 25 μl mixed enzyme solution. The tubes were sealed with the prepared caps, and then incubated at 38° in a shaking water-bath for 45 min. 2 M-HCl (200 μl) was added to all tubes using a hypodermic needle, in order to stop the enzymic reactions and to release the CO_2 from solution. The hole made by the needle was quickly sealed with typewriter correction fluid, and the tubes returned to the water-bath for a further hour. At the end of this equilibration each filter paper was carefully removed from its cap and placed in a mini scintillation vial with 4·5 ml Optiphase X (FSA Laboratory Supplies, Leicester). The $^{14}CO_2$ was estimated using an LKB Rackbeta liquid scintillation counter. All assays were performed in duplicate. Results were expressed as pmol PRPP/10^6 erythrocytes.

Deoxyuridine suppression test. Percentage deoxyuridine suppression was measured by the method of Deacon *et al.* (1981) with minor modifications. [6-^{3}H]Thymidine was supplied by Amersham International plc (Aylesbury, Bucks.) and had a specific activity of 25 Ci/mmol. Chicks were killed by cervical dislocation and bone marrow quickly extracted from both the right and left femurs into 10 ml ice-cold Hanks' buffer, pH 7·4, containing a small amount of heparin. The marrow samples were all pooled according to diet because of the very small quantities available from young chicks and, therefore, each pooled sample contained marrow from about five chicks. The cell clumps were broken up by forcing through a wide bore hypodermic needle four or five times. The cells were then washed twice with ice-cold Hanks' solution before being resuspended in 5 ml buffer. A portion of suspension was removed for cell counting to check that all suspensions contained approximately 0·1 × 10^6 cells/ml.

The assay was set up in duplicate in 1·5 ml polypropylene Eppendorf tubes. 'Test' tubes contained 1 ml marrow suspension, 10 μl of 33 μM-dU in Hanks' buffer and 100 μl pooled plasma. 'Blank' tubes contained 1 ml marrow suspension, 10 μl Hanks' buffer and 100 μl plasma. The tubes were incubated at 37° for 15 min in a shaking water-bath. ^{3}H-Thymidine (1 μCi; equivalent to 40 pmoles) in Hanks' buffer was added to all tubes, which were mixed and then incubated for a further hour. They were then placed on ice to stop any further reactions from taking place. The cells were washed twice in ice-cold NaCl solution (9 g/l) and centrifuged at 6500 ***g*** for 2 min in a microcentrifuge. DNA was extracted by the following procedure: 1·5 ml ice-cold 0·5 M-perchloric acid (PCA) was added to all tubes which were then thoroughly mixed and left on ice for 10 min. The tubes were centrifuged at 6500 ***g*** for 2 min and the supernatant fraction drawn off with a syringe. 0·5 M-PCA (0·5 ml) was added to all tubes followed by thorough mixing. The tubes were placed in an 80° oven for 15 min, before centrifuging at 13000 ***g*** for 10 min. Two 100 μl portions of supernatant fraction were drawn off and added to 4·5 ml Optiphase X, and ^{3}H counted using liquid scintillation counting. The ability of the bone marrow cells to synthesize thymidine from deoxyuridine or their need to make use of the salvage pathway was then calculated from the equation: (mean test counts/min (cpm)/mean control cpm) × 100, and was expressed as 'percentage suppression'.

Table 2. *Erythrocyte phosphoribosylpyrophosphate (PRPP; pmol/10^6 erythrocytes) in chicks fed on diets containing different levels of folic acid**

(Mean values with their standard errors; no. of replicates in parentheses)

Dietary supplement of folic acid (mg/kg)	Expt 1† 3 weeks		Expt 2			
			2 weeks		3 weeks	
	Mean	SE	Mean	se	Mean	SE
0	2074[a]	417 (4)	6805[a]	1124 (3)	8825[a]	898 (5)
0·5	‡		611[b]	86 (3)	4450[b]	386 (5)
1·0	1242[b]	174 (4)	3522[c]	851 (5)	3319[b]	721 (5)
1·5	1332[b]	190 (5)	2708[c]	233 (4)	2812[b]	429 (5)
2·0	1226[b]	184 (5)	3221[c]	787 (3)	3405[b]	791 (4)
4·0	1695[b]	215 (5)	2224[c]	217 (2)	3720[b]	119 (4)

[a, b, c] Within a column, values with different superscript letters were significantly different ($P < 0·05$).

* For details of dietary treatments, see Table 1 and p. 802.

† No phosphate pre-incubation.

‡ The 0·5 mg folic acid/kg diet was not introduced until Expt 2.

Statistical analysis

Statistical analysis of results was carried out by analysis of variance using Genstat statistical package.

RESULTS

Erythrocyte PRPP values are given in Table 2. Expt 1 was carried out without phosphate pre-incubation of cells and without chicks fed on a diet containing 0·5 mg added folic acid/kg. Expt 2 was carried out with phosphate pre-incubation of cells. Significantly raised ($P < 0·05$) erythrocyte PRPP concentrations were seen in chicks fed on the diet containing no added folic acid in both experiments, but PRPP levels were greater when cells from both the 2- and 3-week-old birds were pre-incubated with phosphate buffer. Supplemental folate of between 1 and 4 mg/kg gave PRPP concentrations that did not differ significantly ($P > 0·05$) at both ages. When the diet contained 0·5 mg added folate/kg, intermediate concentrations of PRPP were obtained. Excessively low results obtained from some 2-week-old chicks were thought to have been caused by destruction of PRPP in the original cell lysates due to inadequate water-bath treatment failing to inactivate naturally occurring PRPP-destroying enzymes in the samples.

Values for percentage dU suppression are given in Table 3. Statistical analysis showed that, in both experiments, percentage suppression was significantly higher ($P < 0·05$) in birds fed on the diet containing no added folic acid, compared with the other groups of birds. There was no significant difference in percentage suppression in birds fed on the supplemented diets. In the second experiment percentage suppression was significantly lower ($P < 0·05$) in 3-week-old chicks compared with 2-week-old chicks from all six dietary treatments.

Three experiments were carried out investigating erythrocyte and liver DHFR activity in response to various dietary additions of folic acid. The results from all three experiments are summarized in Table 4. In both Expts 1 and 3, erythrocyte DHFR activity was significantly increased ($P < 0·05$) when the diet contained no added folic acid. There was no significant difference ($P > 0·05$) in erythrocyte DHFR activity in birds consuming diets containing different amounts of supplemental folic acid. In both Expts 2 and 3 there was

Table 3. *Deoxyuridine suppression test results obtained from chicks fed on diets containing different levels of folic acid (% suppression)**

(Mean values of duplicate observations. Pooled SE of the mean was 6·2 over both experiments)

Dietary supplement of folic acid (mg/kg)	Expt 1	Expt 2	
	3 weeks	2 weeks	3 weeks
0	73[a]	80[a]	61[a]
0·5	†	31[b]	19[b]
1·0	12[b]	23[b]	13[b]
1·5	34[b]	40[b]	12[b]
2·0	36[b]	24[b]	14[b]
4·0	32[b]	22[b]	13[b]

[a, b] Within a column, values with different superscript letters were significantly different ($P < 0·05$).
* For details of dietary treatments, see Table 1 and p. 802.
† The 0·5 mg folic acid/kg diet was not introduced until Expt 2.

Table 4. *Liver and erythrocyte (RBC) dihydrofolate reductase* (EC *1.5.1.3*) *activity** *in chicks fed on diets containing different levels of folic acid*†

(Mean values with their standard errors; no. of replicates in parentheses)

Dietary supplement of folic acid (mg/kg)	Expt 1 RBC		Expt 2 Liver		Expt 3			
					RBC		Liver	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0	0·51[a]	0·06 (9)	2·0[a]	0·46 (9)	2·5[a]	0·19 (10)	3·9[a]	0·27 (8)
0·5		‡	2·2[a]	0·48 (7)		—	2·6[a]	0·16 (8)
1·0	0·28[b]	0·03 (10)	2·5[a]	0·42 (7)	1·5[b]	0·10 (10)	5·6[a]	1·04 (11)
1·5	0·32[b]	0·01 (5)	2·7[a]	0·53 (9)		—		—
2·0	0·34[b]	0·07 (6)	2·4[a]	0·31 (8)	1·5[b]	0·06 (10)	5·9[a]	0·73 (11)
4·0	0·35[b]	— (2)	2·4[a]	0·34 (10)	1·6[b]	0·12 (8)	4·6[a]	0·68 (8)

[a, b] Within a column, values with different superscript letters were significantly different ($P < 0·05$).
* RBC assay, pmol THF/10^6 red blood cells per 20 min; liver assay, pmol THF/mg protein per 20 min.
† For details of dietary treatments, see Table 1 and p. 802.
‡ The 0·5 mg folic acid/kg diet was not introduced until Expt 2.

no significant difference in liver DHFR activity between groups. However, variation within treatments was large and the results do suggest a trend towards lower activity of the enzyme in birds fed on the diet containing no added folic acid.

DISCUSSION

The experimental results indicated that erythrocyte PRPP concentrations are raised in folate deficiency in chicks, as in man, and that these levels are amplified following pre-incubation of the cells with phosphate buffer. The concentrations of PRPP (pmol/10^6 cells) when dietary folate is either sufficient or deficient are similar to values reported for humans (Ghitis & Waxman, 1987) and showed little change with age. A dietary folate supplement of 0·5–1·0 mg/kg is sufficient to normalize erythrocyte PRPP concentrations in the chick.

The experiments also showed that the dU suppression test can be used to measure the folate status of chicks, as well as that of man and rats (Deacon *et al.* 1981). However, the

actual percentage suppression values differ markedly between species. In folate-sufficient humans, values of less than 10% are found (Metz *et al.* 1968), whereas in the chick, values of 20–25% were found in the 2-week-old chick, and 10–15% in the 3-week-old chick (both Expt 2). It is possible that this normal value would be reduced even further in more mature birds, as work done previously in the setting up of this assay would imply (results not shown). In folate-deficient humans the percentage suppression ceiling is 40–50% (Metz, 1984), whereas values of 60–80% were obtained from folate-deficient chicks.

These differences in dU suppression values that are seen with age are perhaps due to changing enzyme activities in the growing chick related to its growth rate and requirement for DNA synthesis. The 2-week-old chick may be able to make greater use of the salvage pathway than the 3-week-old chick, resulting in more ^{3}H-thymidine being incorporated into the DNA, even with adequate dietary folate.

The results of the two experiments investigating DHFR activity in both chick erythrocytes and liver confirmed that the activity of the enzyme is affected by dietary deficiency of folic acid. They showed that the activity of the enzyme is significantly increased in erythrocytes (Whitehead & Rennie, 1989) and that it is reduced, although not significantly, in liver. Burns & Jackson (1976) found that dietary deficiency of folic acid significantly reduced the activity of liver DHFR in chicks, but did not measure enzyme activities in other tissues.

It is of interest to speculate on these different responses of the same enzyme. DHFR, as well as being inhibited by folate analogues such as methotrexate, is also inhibited by various naturally-occurring folate species such as 5,10-methylene-, 5,10-methenyl-, and 10-formyl-THF, but not by 5-methyl-THF (Brody *et al.* 1984). Therefore, it might be expected that in deficient chicks where blood cell stores of 5-methyl-THF, the main form of folate in red cells (Cossins, 1984), are lowered, that erythrocyte activity would not change, as it is not inhibited by 5-methyl-THF. The observed increase may be a localized attempt to overcome the metabolic lack of folate.

In folate-deficient birds, liver stores of THF polyglutamates and 5,10-methenyl-THF polyglutamates will fall. These folate species are known inhibitors of DHFR and, therefore, it might be expected that enzyme activity would rise following removal of these inhibitors. However, experimental results showed a trend towards a drop in activity, although the changes were not significant. This fall in activity could be the result of a reduction in synthesis of the enzyme. An alternative explanation is folate deficiency inducing changes in other areas of C_1 metabolism exerting a negative feedback effect on DHFR activity.

It is concluded that the measurement of erythrocyte PRPP and DHFR activity may be a useful criterion of folate status in broilers. Measurement of dU suppression also gave an indication of folate status in chicks but the variation with age means that this test would be less useful for field diagnosis. The results of these studies suggest that the functions of all the folate-dependent pathways tested were normalized by dietary folate supplements of 1 mg/kg.

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