Folate State of Premature Infants

P. M. ROBERTS, D. E. ARROWSMITH, S. M. RAU, and M. E. MONK-JONES

From the Department of Pathology, Elizabeth Garrett Anderson Hospital; the Department of Paediatrics, West Middlesex Hospital; and the Department of Medical Physics, Royal Free Hospital, London

The recent widespread application of the microbiological assay of folate in serum, whole blood, and red cells has led to many reports on the folate status of infants (Matoth *et al.*, 1964a, b; Matoth, Pinkas, and Sroka, 1965; Vanier and Tyas, 1966), including some on the folate status of premature infants (Shojania and Gross 1964; Strelling *et al.*, 1966; Vanier and Tyas, 1967).

This study was designed to investigate the serum and red cell folate levels in premature infants from birth to 6 months. The changes in folate levels of these premature infants were related to the folate status of the mothers, to the incidence of anaemia in the infants, and to the infants' dietary folate.

Patients and Methods

Investigations were made on 70 premature babies and a control group of 50 full-term babies. One blood sample was collected from the full-term infants within 8 days of birth. In the case of the premature babies repeated samples were often collected from within 8 days of birth up to the age of 6 months. The blood samples were taken by heel-prick into heparin and plain tubes.

The premature babies were divided into 3 groups according to birthweight, large (2000–2500 g.), intermediate (1500–2000 g.), and small (under 1500 g.).

Investigations were made on 20 of the mothers of the premature infants, one heparinized and one clotted venous blood sample being taken within 10 days of delivery. These mothers had all received at least 3.4 mg. folic acid daily by mouth since their first antenatal attendances.

On all the blood samples both from the infants and from the mothers, the Hb concentration and the packed cell volume were determined by cyanmethaemoglobin and microhaematocrit methods. Whole blood was diluted 1 in 20 in freshly prepared 10 mg./ml. ascorbic acid solution, and the plasma separated and stored frozen along with the 1/20 lysate until just before the assay.

Serum and red cell assay. The plasma and lysates were assayed in duplicate by the direct addition method for serum 'folic acid' activity (Harper, 1965) using

Bacto Folic Acid Casei media with additional L-tryptophan and ascorbic acid, but without added buffer. Sample blanks were included with each assay.

Variability of assay. 25 normal blood samples were assayed in duplicate on two successive occasions for red cell folate: the batch means of 240.0 and 236.9 ng./ml., respectively, giving a difference of 3.1 between the means, were not statistically significant. The standard deviation of the method was 11.2 and the coefficient of variation was 4.7%.

The serum was likewise assayed, the batch means for serum folate were 4.78 and 4.67 ng./ml., respectively: the difference of 0.11 between the means was not statistically significant. The standard deviation of the method* was 0.43 and the coefficient of variation was 9.1%.

Milk assay. The majority of the babies were fed on boiled pooled expressed breast milk (EBM). The free folate content of many samples of the EBM, both fresh and boiled, was estimated; so too was that of National Dried Milk, and of six commercial preparations, diluted according to the manufacturers' instructions. The majority of samples of fresh EBM were assayed on the day of collection; this was necessary due to the bacterial synthesis of metabolic products which support the growth of *L. casei* (Naiman and Oski, 1964). The milks were not subjected to conjugase treatment before assay, as only 'free folate' and not the polyglutamates was to be measured.

Results

The mean birthweight of the 50 normal full-term infants was 3.38 kg. (range 2.55–4.25 kg.) and the mean haemoglobin was 18.2 g./100 ml. (range 14.6–22.4 g./100 ml.). The mean serum folate for this group was 24.1 ng./ml. (range 6.2–30 ng./ml.), with a mean red cell folate of 460 ng./ml. (range 93–1100 ng./ml.).

The serum and red cell folate values in premature infants are shown in Table I and Table II. At birth the mean serum folate was very high; then

*Standard deviation of the method = $\sqrt{\frac{\sum d^2}{2n}}$, where d is the difference between duplicate assays of 1 sample and n is the number of samples.

TABLE I
Serum Folate Levels in Premature Infants

	Age									
	1-9 dy.	10-19 dy.	20-29 dy.	30-39 dy.	40-49 dy.	50-59 dy.	2-3 mth.	3-4 mth.	4-5 mth.	5-6 mth.
Mean (ng./ml.)	18.7	12.3	12.4	6.8	4.2	2.7	1.9	2.6	2.3	3.1
Range (ng./ml.)	5.5-30.0	2.5-30.0	3.1-30.0	2.4-12.0	1.4-6.6	2.0-3.2	1.0-3.6	0.6-9.0	0.6-6.0	0.3-10.5
No. of cases	18	27	15	15	13	6	10	11	11	12

TABLE II
Red Cell Folate

	Age									
	1-9 dy.	10-19 dy.	20-29 dy.	30-39 dy.	40-49 dy.	50-59 dy.	2-3 mth.	3-4 mth.	4-5 mth.	5-6 mth.
Mean (ng./ml.)	563	503	314	260	208	185	150	140	106	103
Range (ng./ml.)	230-1250	146-1250	55-780	45-615	55-355	68-305	28-360	68-207	45-185	41-185
No. of cases	29	34	20	20	17	10	16	12	10	9

there was a marked fall in the mean value of serum folate in each 10-day period during the first 2 months of life; it then remained well below 6 ng./ml. till 6 months of age. The same pattern was seen in the red cell folate levels which were very high neonatally, continuing to fall till 6 months of age. The differences in the mean values in each 10-day period are statistically significant ($p < 0.001$) for red cell folate. In Fig. 1 the changes in red cell folate with age are shown, the very high neonatal levels fall rapidly, and frequently reach subnormal levels by adult standards at 3 months of age. Fig. 2 shows a similar fall in serum folate levels, all but one falling below 6 ng./ml. by 7 weeks of age. The serum levels fell to subnormal values before that of the red cells.

When the babies were divided into three groups according to birthweight, the results of the red cell folate estimations for the first 10 days and for the second 10 days after birth are shown in Table III. Though there was a fall in the mean values for red cell folate from the large to the small babies during both these 10-day periods, the differences are not statistically significant.

It can be seen in Fig. 3 that the serum folate and the red cell folate both fell exponentially with age for the first 90 days of life. This followed because there was a highly significant ($p < 0.001$) linear regression between the logarithm of the serum

folate and the age of the infants, as well as between the logarithm of the red cell folate and the age of the infant.

In Fig. 4 the linear relation between the Hb level and the red cell folate level in each infant is shown ($p < 0.001$).

Fig. 5 shows the linear relation between the red cell folate level in each mother and that in her infant ($p < 0.001$).

Table IV shows the result of *L. casei* assay of 13 samples of human milk and of 7 artificial feeds; the values are for free folate. It is seen that the fresh breast milk had a low level of free folate, ranging from 0.3-3.9 $\mu\text{g./l.}$, and the commercial preparations varied considerably in their content of free folate, from 5.2-22.1 $\mu\text{g./l.}$

Discussion

The normal adult levels of red cell folate were considered by Hoffbrand, Newcombe, and Mollin (1966) to lie between 160 and 640 ng./ml. of packed red cells. Vanier and Tyas (1966) found a range of 43-837 ng./ml., one normal adult having a persistently low value, but the remainder being above 100 ng./ml. We found all except one of 25 normal adults to have a red cell folate level above 130 ng./ml., the one exception having a value of 99 ng./ml. We found the upper normal value to be 530 ng./ml.

FIG. 1.—Infants' red cell folate values related to age.

FIG. 2.—Infants' serum folate values related to age.

Matoth *et al.* (1964a, 1965) made several studies of folic and folinic acid in infant and maternal bloods. They noted in 373 healthy infants that the levels of whole blood 'folic acid' were high at birth and dropped to a subnormal level by 8 weeks, and from then on to 1 year remained well below the normal adult mean. The breast-fed infants had considera-

bly higher values than those of the group as a whole, and the *L. casei* activity of the bloods in these breast-fed infants was 50% higher than those of artificially-fed infants. These authors discussed the possibilities that these results could be due to a higher level of folic acid in breast milk than in artificial foods, or that it could be related to differ-

TABLE III
Red Cell Folate

	Age (days)					
	1—9			10—19		
	Large BW 2000–2500 g.	Intermediate BW 1500–2000 g.	Small BW <1500 g.	Large BW 2000–2500 g.	Intermediate BW 1500–2000 g.	Small BW <1500 g.
Mean (ng./ml.)	647	570	464	581	562	374
Range (ng./ml.)	230–1250	328–820	288–695	146–1250	313–985	235–740
No. of cases	13	9	7	15	11	8

FIG. 3.—Infants' red cell folate and serum folate values related to age plotted on a semilogarithmic scale. ○ Mean red cell folate. ● Antilog of mean log RCF. △ Mean serum folate. In each period of 10 days.

ences in the infants' intestinal bacterial flora. Vanier and Tyas (1966, 1967) estimated the folate status of 24 normal full-term and 20 premature infants neonatally, at 3–6 months and at 6–8

FIG. 5.—Infants' red cell folate values related to mothers' red cell folate values within 10 days of birth of infant.

FIG. 4.—Infants' red cell folate values related to Hb levels.

months of age. They found very high neonatal levels with subnormal levels at 3–6 months, rising slowly at 6–8 months of age. Strelling *et al.* (1966) studied 54 premature and 22 full-term infants and found evidence of megaloblastic anaemia in 11 of the premature infants. These authors reported whole blood folate, the result of which varies with the haematocrit, making it a less reliable index of folate status than the red cell content.

Our findings for the levels of serum folate and red cell folate, in both full-term and premature

TABLE IV
Folate Content of Milks

Type of Milk	Mean ($\mu\text{g./l.}$)	Range ($\mu\text{g./l.}$)
Fresh expressed breast milk (13 samples)	2.7	0.3–3.9
Full Cream National Dried Commercial preparations (made up as directed)	10.3	—
1	8.7	—
2	5.2	—
3	13.4	—
4	22.1	—
5	6.7	—
6	10.3	—

infants neonatally, confirm the findings of Vanier and Tyas (1966, 1967). In the three groups of infants separated by birthweight into large, intermediate, and small babies, there appeared to be a correlation between the birthweight and their neonatal red cell folate levels; these differences, however, were not statistically significant. Shojania and Gross (1964) found that by dividing their premature babies into two groups, according to birthweight, above and below 1700 g., there was a correlation with their serum folate levels after the age of 1 month, 60% of their low birthweight infants having a serum folate under 5 ng./ml. at this age.

From the age of 2 months, the mean red cell folate and the mean serum folate levels in premature babies were subnormal by adult standards, most of these remaining subnormal up to 6 months of age. Many of the levels did not rise as they did in the infants studied by Shojania and Gross (1964), where there was a rise in serum folate after 2 months, but their infants were fed on solids including fruit and vegetables from 6–8 weeks of age. The infants we studied were nearly all on mixed feeds by 4 months of age, but this seemed to have little significant effect on either serum or red cell folate levels.

It was interesting to note that the same infant followed for a number of months showed constant values for folate levels, and some babies showed practically identical values on repeated assays over a period of several months.

The analysis of our results shows that the fall in both serum and red cell folate related to age is highly significant for the first 3 months of life. The exponential type of curve obtained is typical of that seen in biological decay, and this suggests that there was an initial high intracellular level of folate at birth, which was gradually utilized, with little or no replacement of folate, for at least the first 3 months of life.

There was a significant relation between each mother and her infant's red cell folate when both were estimated within 10 days of birth. The regression line showed that the infant's level was higher than the mother's, which is in agreement with other studies on maternal and cord blood, notably that of Grossowicz, Izak, and Rachmilewitz (1966).

There was also a significant relation both between the serum folate and Hb levels and between the red cell folate and Hb levels up to the age of 6 months. Shojania and Gross (1964) found no such correlation.

Some authors have emphasized that folate deficiency is precipitated by infection (Gray and Butler, 1965; Matoth *et al.*, 1964b; May *et al.*, 1952).

The premature babies we investigated were clinically well, with no evidence of infection.

The serum and red cell folate results obtained on the premature infants suggest that the initial store of folate derived from the mothers is exhausted by about 3 months of age. After that time the infant's only source of folate is dietary. The values for boiled pooled EBM were low, ranging from 1.0–7.5 $\mu\text{g./l.}$, which would provide a 3 kg. baby with only 0.5–3.4 $\mu\text{g. folate per 24 hours}$, whereas artificial foods would provide the 3 kg. baby with 2.5–9.0 $\mu\text{g. folate per 24 hours}$.

It is well known that the folate content of breast-milk increases during the post-partum period. Ramasastry (1965) found that human colostrum contained a mean of 4.4 $\mu\text{g./l.}$ of folate; transitional milk, 5–15 days *post partum*, a mean of 8.4 $\mu\text{g./l.}$ of folate; and mature milk, more than 15 days *post partum*, a mean of 16.5 $\mu\text{g./l.}$ of folate. These samples were taken from Indian women of the lower social classes who may well have been taking a diet high in folate, being very largely vegetarians. The pooled EBM fed to the infants in our study was mainly obtained in the early post-partum period and would, therefore, have a low folate content.

The effect of boiling on the folate content of milk was studied by Ghitis (1966), who showed that boiling milk for even 5 seconds reduced the folate content to approximately 50% of the original value. Nicol and Davis (1967) showed similar differences between fresh and boiled cow's milk. The pooled boiled EBM in the present series had probably lost half the original folate content by boiling.

Naiman and Oski (1964) analysed various types of milk for their folate content, but not human milk; they found goat's milk to have the lowest folate content. Matoth *et al.* (1965) analysed fresh human breast-milk and found a mean folate content of 24 $\mu\text{g./l.}$, though they did not mention at what time *post partum* this level was found. Vanier and Tyas (1967) assayed half-cream Cow and Gate milk for *L. casei* activity, and calculated the average content to be 17 $\mu\text{g./l.}$, and our findings agree with this value.

The normal infant requirement of folate is not known with certainty. Sullivan, Luhby, and Streiff (1966) suggested that infants might need 4 to 10 times the adult requirement as calculated on body weight, and therefore require 20–50 $\mu\text{g. of folic acid per day}$. Ghitis (1966) concluded that infants required more than 5 $\mu\text{g./kg. body weight per day}$, and Matoth *et al.* (1965) suggested a daily requirement of 20 $\mu\text{g. folate per}$

day. The availability of 'free' and of 'polyglutamate' forms of folate is not known, and in the present series only 'free' folate was measured with the *L. casei* assay.

The folate intake of the premature babies we investigated was clearly below the generally accepted limits of normal requirements, as the boiled pooled EBM contained between 1.0 µg. and 7.5 µg./l. of folate. Had the babies been fed on artificial foods the folate intake would have been greater, but still well below the 50 µg. of folate per day which has been regarded as optimal. Though the older infants were on mixed feeding the folate content of their diet was probably low, particularly as canned foods are often used for infant feeding.

The low milk folate may explain the initial fall in both the red cell and the serum folate values of premature infants, and the continuing low levels up to the age of 6 months. In this context the red cell folate level is probably the most significant measure of the folate stores (Hoffbrand *et al.*, 1966) of these infants, and this measurement correlated with the dietary folate intake (Chanarin *et al.*, 1968). There may be a connexion between the folate levels and the anaemia of prematurity, as there is a significant correlation between the Hb values and red cell folate levels.

Summary

The normal values for serum and red cell folate in 70 premature infants followed for the first 6 months were investigated. The values found were related to dietary folate intake, to Hb levels, and in some cases to maternal levels. High levels of both serum and red cell folate were found in the infants at birth; the levels dropped rapidly until they reached low values at 3 months of age. These low levels were probably related to a low dietary folate intake, and the exhaustion of the high initial stores derived from the mother. There was good correlation between the red cell folate and Hb levels in the infant, and between the red cell folate levels of the mother and of her infant during the neonatal period.

Our thanks are due to Dr. Dynski-Klein, Dr. N.

O'Doherty, Dr. Fawzi, Dr. D'Sa, Sister Paterson, and to Dr. P. D. Roberts for assistance with specimens, to Mr. Philip Lock for enthusiastic technical assistance, and to the British Nutrition Foundation for financial assistance.

REFERENCES

- Chanarin, I., Rothman, D., Perry, J., and Stratfull, D. (1968). Normal dietary folate, iron and protein intake, with particular reference to pregnancy. *Brit. med. J.*, **2**, 394.
- Ghitis, J. (1966). The labile folate of milk. *Amer. J. clin. Nutr.*, **18**, 452.
- Gray, O. P., and Butler, E. B. (1965). Megaloblastic anaemia in premature infants. *Arch. Dis. Childh.*, **40**, 53.
- Grossowicz, N., Izak, G., and Rachmilewitz, M. (1966). The effect of anaemia on the concentration of folate derivatives in paired fetal-maternal blood. *Israel J. med. Sci.*, **2**, 510.
- Harper, T. A. (1965). A modified aseptic addition assay procedure for the measurement of serum folic-acid activity. *Nature (Lond.)*, **207**, 947.
- Hoffbrand, A. V., Newcombe, B. F. A., and Mollin, D. L. (1966). Method of assay of red cell folate activity and the value of the assay as a test for folate deficiency. *J. clin. Path.*, **19**, 17.
- Matoth, Y., Pinkas, A., and Sroka, C. (1965). Studies on folic acid in infancy. III. Foliates in breast fed infants and their mothers. *Amer. J. clin. Nutr.*, **16**, 356.
- , —, Zamir, R., Mooallem, F., and Grossowicz, N. (1964a). Studies on folic acid in infancy. I. Blood levels of folic and folinic acid in healthy infants. *Pediatrics*, **33**, 507.
- , Zamir, R., Bar-Shani, S., and Grossowicz, N. (1964b). Studies on folic acid in infancy. II. Folic and folinic acid blood levels in infants with diarrhoea, malnutrition, and infection. *ibid.*, **33**, 694.
- May, C. D., Stewart, C. T., Hamilton, A., and Salmon, R. J. (1952). Infection as a cause of folic acid deficiency and megaloblastic anemia. *Amer. J. Dis. Child.*, **84**, 718.
- Naiman, J. L., and Oski, F. A. (1964). The folic acid content of milk; revised figures based on an improved assay method. *Pediatrics*, **34**, 274.
- Nicol, D. J., and Davis, R. E. (1967). The folate and vitamin B₁₂ content of infant milk foods with particular reference to goats' milk. *Med. J. Aust.*, **2**, 212.
- Ramasastri, B. V. (1965). Folate activity in human milk. *Brit. J. Nutr.*, **19**, 581.
- Shojania, A. M., and Gross, S. (1964). Folic acid deficiency and prematurity. *J. Pediat.*, **64**, 323.
- Strelling, M. K., Blackledge, G. D., Goodall, H. B., and Walker, C. H. M. (1966). Megaloblastic anaemia and whole-blood folate levels in premature infants. *Lancet*, **1**, 898.
- Sullivan, L. W., Luhby, A. L., and Streiff, R. R. (1966). Studies of the daily requirement for folic acid in infants and the etiology of folate deficiency in goat's milk megaloblastic anemia. (Abstr.). *Amer. J. clin. Nutr.*, **18**, 311.
- Vanier, T. M., and Tyas, J. F. (1966). Folic acid status in normal infants during the first year of life. *Arch. Dis. Childh.*, **41**, 658.
- , and — (1967). Folic acid status in premature infants. *ibid.*, **42**, 57.

Correspondence to Dr. P. M. Roberts, Pathology Department, Elizabeth Garrett Anderson Hospital, Euston Road, London N.W.1.