The effect of periconceptional supplementation on blood vitamin concentrations in women at recurrence risk for neural tube defect

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1. We measured erythrocyte folic acid and riboflavin, serum folic acid and leucocyte vitamin C in women at high risk for neural tube defect (NTD) recurrence who were receiving periconceptional vitamin supplementation, before they received extra vitamins, after 28 d of supplementation and at the 8th week of pregnancy. Blood vitamin concentrations in unsupplemented high-risk women were also compared with the values found in unsupplemented low-risk women.

2. Vitamin supplementation with Pregnavite Forte F (Bencard®) raised the mean values for all vitamins measured by the 8th week of pregnancy. Mean erythrocyte folic acid rose from 250 to 478 ng/ml; plasma folic acid from 8·4 to 26·1 ng/ml; leucocyte vitamin C from 1·82 to 3·21 μg/ml blood; erythrocyte riboflavin (glutathione reductase (*EC* 1.6.4.2) activation ratio) from 1·08 to 1·04. All women receiving supplements had increased their serum and erythrocyte folic acid levels above the highest values found in women in an earlier study, who subsequently gave birth to children with NTD. Not all women, however, increased their leucocyte ascorbic acid or erythrocyte ribflavin levels above the highest values.

3. When vitamin concentrations in unsupplemented high-risk women were compared with levels in unsupplemented women at low risk for NTD, no significant differences were found in the mean values. However, a significantly higher proportion of high-risk compared with low-risk women had erythrocyte folic acid and leucocyte vitamin C values on or below the 5th percentile of the adult normal range.

4. The effectiveness of Pregnavite Forte F (Bencard®) for increasing maternal vitamin reserves is discussed with a view to preventing NTD and the possibility of identifying groups at risk for NTD because of low blood vitamin levels is considered.

There is increasing evidence that maternal vitamin deficiencies may be a factor in the causation of congenital defects of the neural tube (NTD). The geographical distribution of NTD in the UK and the relationship of NTD to social class (Fedrick, 1970; Leck, 1974) indicate that environmental factors could be involved. There is evidence for a nutritional component in the environmental contribution (Hibbard & Smithells, 1965). More recent work has shown that women who gave birth to an infant/foetus with NTD had lower average concentrations of some vitamins in their blood in early pregnancy than women who gave birth to children without NTD (Smithells *et al.* 1976).

Therefore, it was decided to test the hypothesis that vitamin deficiencies were a causal factor in NTD. Vitamin supplements were offered before and during early pregnancy to mothers who had already had one or more NTD-affected infant and who were planning a further pregnancy. The recurrence of NTD in the supplemented group was about eight times less than that in a comparable but unsupplemented group (Smithells *et al.* 1980; Smithells, *et al.* 1981*a*).

This paper describes the changes that occurred in the maternal blood vitamin concentrations during the study.

MATERIALS AND METHODS

Women were recruited who had had at least one child with NTD. Such a history carries a recurrence rate of approximately ten times the general population risk and hence such women were termed 'high-risk'. They were planning a further pregnancy but were not

Table 1. *Daily supplements in three tablets of Pregnavite Forte F (Bencard®)*

Retinol (μg)	1200
Vitamin D (μg)	10
Thiamin (mg)	1·5
Riboflavin (mg)	1·5
Pyridoxine (mg)	1·0
Nicotinamide (mg)	15
Folic acid (mg)	0·36
Ascorbic acid (mg)	40
Iron (as ferrous sulphate, mg)	75·6
Calcium phosphate (mg)	480

pregnant at the time of enrolling. Several centres were involved in the trial and details of recruitment are described elsewhere (Smithells, *et al.* 1981*a*). We now report on a subsection of the population who were recruited at Leeds and whose blood vitamin levels were measured. Volunteers were asked to take Pregnavite Forte F (Bencard®), an iron and multi-vitamin preparation (Table 1), 1 tablet 3 times/d, for not less than 28 d before conception. Those mothers who were taking oral contraceptives were asked to adopt an alternative form of contraception. Vitamin supplements were continued until the second missed menstrual period. Blood samples were taken before vitamin supplements were started, after 28 d of supplementation and at the end of the second month of pregnancy as measured from the last menstrual period. Because conception sometimes occurred very soon after the first 28 d of supplementation or could be delayed for several months, the duration of supplementation between the 2nd and 3rd blood samples varied between 52 and 168 d. Thirty-eight volunteers, believed to be fully supplemented and who subsequently became pregnant during the course of trial, were recruited at Leeds. Thirty-three of them provided blood samples on all three occasions. Because of inadequate samples, thirty-two had erythrocyte riboflavin and thirty serum folic acid assayed, and only twenty-one women had all three samples analysed for leucocyte vitamin C due to technical problems with the assay.

Blood samples were taken into tubes containing acid-citrate-glucose for erythrocyte riboflavin, into EDTA anticoagulant for erythrocyte folic acid and into tubes without anticoagulant for serum vitamin C and serum folic acid determinations. The analytical methods are described elsewhere (Denson & Bowers, 1961; Smithells *et al.* 1976). Leucocyte vitamin C levels are expressed as vitamin C in leucocyte/ml blood to correct for the increase in leucocyte numbers occurring in pregnancy (Andrews & Bonsnes, 1951), which apparently decreases the concentration of vitamin C per 10^8 leucocytes whilst having little effect on the total white blood cell vitamin C. A possible explanation and the calculation involved are described elsewhere (Schorah *et al.* 1978).

In addition to the high-risk women in the supplementation study who became pregnant (study mothers), analyses of folic acid and vitamin C were performed on high-risk, unsupplemented women who were not planning a pregnancy. These results, together with the unsupplemented values obtained from the study mothers and volunteers who did not conceive, were compared with the vitamin concentrations found in a group of non-pregnant women whose infants had no NTD and were therefore termed low-risk, to determine whether there was a biochemical marker which would identify the high-risk group. The low-risk group were women from an earlier study of nutrition in early pregnancy (Smithells *et al.* 1976) who returned to give blood when not pregnant, plus a small group of blood donors of similar age. Fortuitously the overall social class structure of the low- and high-risk

Table 2. *The social class distribution of women in the high- and low-risk non-pregnant groups as a percentage of the total number of women in the groups*

Social class*	I+II+IIIN	IIIM	IV+V	Unclassified
High-risk	39	33	22	6
Low-risk	42	39	15	4

* Based on the Registrar General's classification (Office of Population Censuses and Surveys, 1970): I, professional; II, intermediate occupations; IIIN, skilled, non-manual; IIIM, skilled, manual; IV, partly skilled; V, unskilled. Unclassified includes Armed Forces, students and unemployed for > 6 months.

groups were similar (Table 2). Blood samples from both groups were analysed at the same time. For the purpose of this comparison only, we excluded women who were taking vitamin supplements and, for the folic acid analysis, women who were within 3 months of their previous pregnancy and therefore probably still affected by routine folate supplements taken up to the end of that pregnancy.

RESULTS

Table 3 shows the vitamin concentrations (mean and range) for study mothers who had blood samples taken at all three stages of the supplementation trial. The riboflavin values are expressed in terms of the activation ratio of erythrocyte glutathione reductase (*EC* 1.6.4.2) using FAD, the enzyme cofactor. A lower value indicates higher saturation of the enzyme with its cofactor and hence a more satisfactory riboflavin status. It should be noted that for all vitamins measured, particularly for folic acid, the initial pre-supplement values do not necessarily represent unsupplemented concentrations as several women were within 3 months of the end of their previous pregnancy during which they would almost certainly have been receiving folic acid supplements. This is illustrated in Table 4 which compares the folic acid concentrations in women whose initial blood sample was taken within 3 months of the end of their previous pregnancy with those who were more than 3 months from their last pregnancy.

Included in Table 3 are two thresholds below which vitamin concentrations were considered inappropriate for early pregnancy in high-risk women. These are the upper limits for the first-trimester blood vitamin levels in an earlier study of women who subsequently delivered an infant/foetus with NTD (Smithells *et al.* 1976). Two limits are shown; one for women whose infants had malformations involving neural tissue, and one which includes the rather higher values found in a woman whose child had a meningocele (no neural tissue involvement). It was hoped that all supplemented women (study mothers) would have raised their vitamin levels at least above the lower of the two limits by the 8th week of pregnancy (the appropriate comparison point with the first trimester values from the earlier study).

All women receiving vitamin supplements had moved out of the inappropriate zone for erythrocyte and serum folic acid by the 8th week of pregnancy. The rather slow initial rise in erythrocyte folic acid is to be expected, as the erythrocyte appears to receive its folic acid complement before it is released from the bone marrow. Hence, increases in erthrocyte folate will be seen gradually as a new cell population replaces the old. Rises in vitamin C and riboflavin levels were less impressive, 23 and 18% of the women respectively remaining below the lowest threshold at the 8th week of pregnancy. If deficiences of these vitamins are subsequently shown to be implicated in the causation of NTD, higher intakes may be indicated in an attempt to take all women above these inappropriate zones. However, the measurement of maternal cellular concentrations of vitamins may not be the best way of

Table 3. *Blood vitamin concentrations in high-risk women (the study mothers) before and during supplementation on all women who had vitamin levels measured at all three stages*

(Mean values plus ranges in parentheses)

	Before supplementation			After 28 d supplementation		8 weeks pregnant		Thresholds*	
	n	Mean	Range	Mean	Range	Mean	Range	A	B
Erythrocyte riboflavin (activation ratio)	32	1·08	(0·89–1·30)	1·03	(0·90–1·18)	1·04	(0·92–1·20)	1·12	1·12
Erythrocyte folic acid (ng/ml)	33	250	(49–698)	290	(91–600)	482	(263–900)	163	170
Serum folic acid (ng/ml)	30	8·3	(0·0–28)	17·0	(6·2–28)	26·5	(13–36)	5·3	7·6
Leucocyte vitamin C (μg/ml blood)	21	1·83	(0·96–3·5)	2·58	(1·44–6·5)	3·28	(1·15–6·14)	2·1	3·6

* Threshold levels represent: A, the upper limit of the first trimester vitamin concentration in women with a neural tube defect-affected foetus with neural tissue involvement; B, the upper limit including one woman who had a foetus with meningocele (no neural tissue involved) (Smithells *et al.* 1976).

Table 4. *A comparison of folic acid concentrations in non-pregnant women (according to period of time since end of previous pregnancy)*

(Mean values with their standard deviations)

Period since last pregnancy (months)	Serum folate (ng/ml)			Erythrocyte folate (ng/ml)		
	Mean	SD	*n*	Mean	SD	*n*
$\leqslant 3$	10·8*	7·6	13	362**	193	13
> 3	5·9	3·5	64	200	125	68

Values for serum and erythrocyte folic acid at $\leqslant 3$ months significantly different from that at > 3 months (Student's *t* test): * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$ according to variance equivalence and calculated from $\log_{10}$ transformed data, the distribution of which was closer to Gaussian.

Table 5. *Serum vitamin C (mg/100 ml) in high-risk women (the study mothers) before and during supplementation*

(Mean values plus ranges in parentheses)

Before supplementation			After 28 d supplementation			8 weeks pregnant			Thresholds*	
Mean	Range	*n*	Mean	Range	*n*	Mean	Range	*n*	A	B
0·83	(0·20–1·32)	8	1·45	(1·16–2·18)	8	1·43	(0·92–1·94)	7	0·80	1·10

* Threshold levels represent: A, the estimated upper limit of the first trimester vitamin concentration in women with a neural tube defect-affected foetus with neural tissue involvement; B, the upper limit including one woman who had a foetus with meningocele (no neural tissue involved) (Smithells *et al.* 1976).

assessing foetal vitamin status, as the foetus probably derives its vitamin supply from the maternal plasma and not from the cells. Vitamin levels in the serum rise more rapidly than in the cells (Table 3), and on reflection it would have been more appropriate to measure plasma or serum concentrations of vitamin C and riboflavin. Serum vitamin C was measured in a few women and the findings are shown in Table 5. We have no information on serum vitamin C from our earlier study (Smithells *et al.* 1976), but it is possible to estimate thresholds for the inappropriate zones from the maternal leucocyte vitamin C values in NTD-affected pregnancies in this earlier study, using the relationship between leucocyte and plasma vitamin C recorded in our laboratories on more than 400 individuals. These derived upper limits to the inappropriate zones are shown in Table 5. It is clear that the thresholds were exceeded by all high-risk mothers whose serum levels were assessed within 1 month of supplementation, although one mother still remained near the higher threshold at the 8th week of pregnancy.

Five women did not have blood taken on all three occasions. However, the changes in blood vitamin concentrations, where available in these women, were within the range found in women who had all three measurements taken.

Blood vitamin C and folic acid concentrations in unsupplemented, non-pregnant high-risk women were compared with those of social-class-matched mothers with no previous history of NTD (low-risk). Table 6 shows that there were no significant differences in the mean values for leucocyte vitamin C and erythrocyte and serum folic acid between the two groups, although the values for the high-risk mothers tended to be lower for each vitamin except serum folic acid. There was, however, a noticeably wider distribution of the values in the

Table 6. *Blood vitamin concentrations in unsupplemented non-pregnant high-risk and low-risk women*

(Mean values with their standard deviations)

	High-risk			Low-risk			Significance of difference
	Mean	SD	*n*	Mean	SD	*n*	
Erythrocyte folic acid (ng/ml)	200	125	68	222	100	100	NS
Serum folic acid (ng/ml)	5·9	3·5	64	5·7	2·5	91	NS
Leucocyte vitamin C (μg/ml blood)	2·08	0·85	67	2·19	0·75	70	NS

NS, not significantly different.

Table 7. *Number and percentage of unsupplemented, non-pregnant high-risk and low-risk women with blood vitamin concentrations on or below the 5th percentile of the normal range for healthy adults*

	5th percentile	High-risk			Low-risk		
		no.	%	*n*	no.	%	*n*
Erythrocyte folic acid (ng/ml)	100	16**	24	68	7**	7	100
Serum folic acid (ng/ml)	2·2	2	3	64	2	2	91
Leucocyte vitamin C (μg/ml blood)	1·35	14**	20	67	3**	4	70

$2 \times 2\chi^2$ analysis shows significant differences between high- and low-risk values: $^{**}P < 0{\cdot}01$.

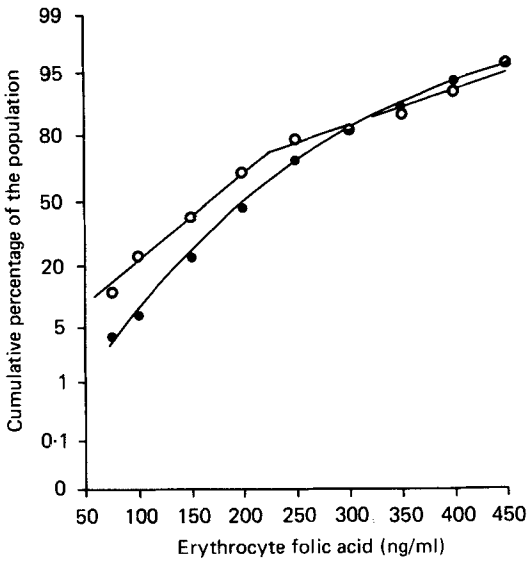

Fig. 1. The frequency distribution of erythrocyte folic acid values in the high-risk (○) and low-risk (●) groups.

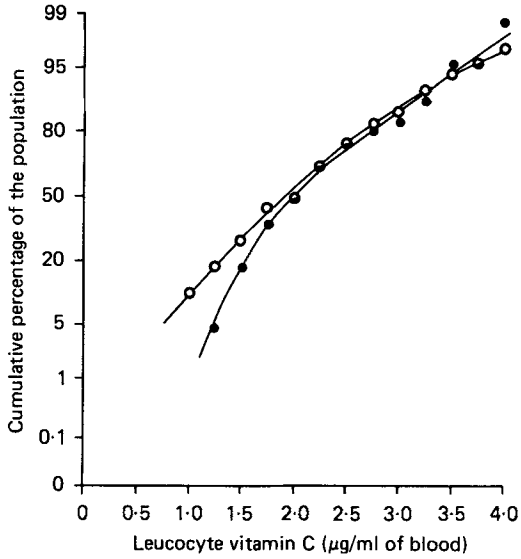

Fig. 2. The frequency distribution of leucocyte vitamin C values in the high-risk (○) and low-risk (●) groups.

high-risk group and this was most apparent at the lower levels for erythrocyte folic acid and leucocyte vitamin C. Table 7 shows that there were significantly more high-risk than low-risk women with erythrocyte folic acid and leucocyte vitamin C concentrations below the 5th percentile of the normal ranges for these assays (established on healthy adults). The 5th was chosen as the lowest percentile to include sufficient numbers for analysis. Below the 2·5 percentile, the lower limit for most biochemical reference ranges, the differences between the high-and low-risk groups for erythrocyte folic acid and leucocyte vitamin C were maintained, but were only significant for vitamin C. Cumulative percentage plots (Figs 1 and 2) for the high- and low-risk groups confirmed these differences and indicated that the distribution of the erythrocyte folate values represented two populations within the high-risk group. Because these distribution differences were small, the findings must be interpreted with caution. However, the results suggest that within the high-risk group there is a sub-populaton of mothers who, for some reason, maintain lower erythrocyte folic acid and leucocyte vitamin C reserves than do the low-risk comparison group.

DISCUSSION

One of the concerns of any therapeutic trial is whether or not the treatment achieves effective concentrations of the agent at the site of its action in the body. With regard to prevention of NTD, it is impossible to assess vitamin concentrations in the neural tissue of the foetus. The closest one can come to such an assessment is a measurement of the vitamin concentrations in maternal blood. This will give an indication of the mother's compliance, assuming there is no serious problem affecting either absorption or metabolism leading to low maternal concentrations despite high intakes. The analysis of maternal vitamin concentrations following supplementation (Table 3) shows no evidence of any of the study mothers either failing to comply with the instructions or of having any defect in vitamin absorption or metabolism that could not be overcome by the dose of vitamins given in the present study. All women increased their serum folic acid levels during the period of supplementation with the exception of one who had high levels initially and who had been

receiving folate supplements a few weeks prior to the trial. Three women failed to increase their erythrocyte folic acid, but all three had pre-supplement concentrations higher than the 90th percentile of the normal range for erythrocyte folate (340 ng/ml). All study mothers examined achieved both serum and erythrocyte folic acid concentrations at the 8th week of pregnancy well above the thresholds found in the first trimester in NTD-affected pregnancies but erythrocyte and leucocyte measurements suggested that intakes of riboflavin and vitamin C respectively were insufficient to raise all the maternal reserves to appropriate levels in the time available. However, we have noted that plasma vitamin C levels suggest that the intake is probably sufficient for the foetus. One other centre in the trial also analysed serum and erythrocyte folic acid routinely on the pre-supplement sample and after 1 month of supplementation. Their analytical technique produced rather higher values for erythrocyte folic acid (*n* 31, mean values: first sample 303, second sample 387) than those reported here, a finding which was also supported by the normal ranges, which in Leeds were lower for erythrocyte folate. However, the mean and range of values for serum folic acid for the pre-supplement and 1 month supplement samples (6·5, 1·2–21, *n* 41; 15·4, 3·3–37, *n* 41 respectively) were very similar to the Leeds values with all 41 women showing an increase in their serum folic acid concentrations, suggesting compliance, adequate absorption and metabolism. Our experience differs from that of Laurence and colleagues (Laurence *et al.* 1981) who reported 27% of their supplemented patients failing to achieve serum folic acid concentrations above 10 ng/ml, although prescribed doses over ten times greater than that used in this study. In contrast, all Leeds study mothers whose vitamin concentrations were measured at the 8th week of pregnancy, a similar time to that used by Laurence *et al.* (1981), had levels $\geqslant$ 13 ng/ml. If compliance was the problem in Laurence's study, the difference between the two studies probably reflects how closely the patients were supervised. Patients in the current trial were highly motivated and were contacted at least twice during the trial. Supervision is of crucial importance to the success of further research in this field and to the wider application of vitamin supplementation if its effectiveness is confirmed.

The outcome of our non-randomized trial showed an NTD recurrence in thirteen of 308 infants/foetuses examined in the unsupplemented high-risk group but only one recurrence in 196 in the high-risk group receiving full supplementation (Smithells *et al.* 1981*a*). The mother having a recurrence in the supplemented group had her blood vitamins analysed at the time of therapeutic abortion and both erythrocyte folic acid and leucocyte vitamin C were markedly elevated. Close questioning confirmed that she had taken her supplements as directed. Although there have been two recurrences in the supplemented mothers in our current trial, the results are still encouraging (Smithells *et al.* 1981*b*). There are three possible explanations for these recurrences. There could be an inappropriate maternal vitamin concentration in spite of supplementation or a defect in placental or foetal uptake of vitamins which would not be detected by the current study. More probably, vitamin deficiency may not be a major aetiological factor in all cases of NTD.

The greater frequency of low erythrocyte folic acid and leucocyte vitamin C values in the unsupplemented high-risk group than in the low-risk women (Table 7, Figs 1 and 2) is of interest and may indicate some dietary, absorption or metabolic problem within the high-risk populaton, which can be overcome by supplementation. The lack of any such finding for serum folic acid raises the possibility of early vitamin B_{12} deficiency which produces a low to normal erythrocyte folic acid concentration associated with rather higher serum folate values. Indeed, there is some evidence that women who produce anencephalic foetuses have, in the first trimester of pregnancy, low vitamin B_{12} concentrations (Schorah *et al.* 1980). However, the differences in the unsupplemented vitamin values between the high- and low-risk groups are small. It is possible that changes in demand during pregnancy

(Rothman, 1970) may accentuate these differences between blood vitamin concentrations in high- and low-risk women, especially if vitamin intakes are sub-optimal. In addition, a single estimate of blood vitamin concentration is only a crude assessment of vitamin metabolism. It follows that there is a need to look at the metabolism of vitamins, particularly folic acid, in greater detail, in both pregnant and non-pregnant high-risk women, if the proposition that vitamin deficiencies are a causal factor in NTD is upheld. Here there is some urgency, because it might be possible to identify women at primary risk before they embark on child-bearing.

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REFERENCES

Andrews, W. C. & Bonsnes, R. W. (1951). *Am. J. Obstet. Gynecol.* **61**, 1129.
Denson, K. W. & Bowers, E. F. (1961). *Clin. Sci.* **21**, 157.
Fedrick, J. (1970). *Ann. hum. Genet.* **34**, 31.
Hibbard, E. D. & Smithells, R. W. (1965). *Lancet* **i**, 1254.
Laurence, K. M., James, N., Miller, M. H., Tennant, G. B. & Campbell, H. (1981). *Br. med. J.* **282**, 1509.
Leck, I. (1974). *Br. med. Bull.* **30**, 158.
Office of Populaton Censuses and Surveys (1970). *Classification of Occupations.* London: HM Stationery Office.
Rothman, D. (1970). *Am. J. Obstet. Gynecol.* **108**, 149.
Schorah, C. J., Smithells, R. W. & Scott, J. (1980). *Lancet* **i**, 880.
Schorah, C. J., Zemroch, P. J., Sheppard, S. & Smithells, R. W. (1978). *Br. J. Nutr.* **39**, 139.
Smithells, R. W., Sheppard, S. & Schorah, C. J. (1976). *Archs Dis. Childh.* **51**, 944.
Smithells, R. W., Sheppard, S., Schorah, C. J., Seller, M. J., Nevin, N. C., Harris, R., Read, A. P. & Fielding, D. W. (1980). *Lancet* **i**, 339.
Smithells, R. W., Sheppard, S., Schorah, C. J., Seller, M. J., Nevin, N. C., Harris, R., Read, A. P. & Fielding, D. W. (1981*a*). *Archs Dis Childh.* **56**, 911.
Smithells, R. W., Sheppard, S., Schorah, C. J., Seller, M. J., Nevin, N. C., Harris, R., Read, A. P., Fielding, D. W. & Walker, S. (1981*b*). *Lancet* **ii**, 1425.

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