

Vitamin deficiencies and neural tube defects

R. W. SMITHELLS, S. SHEPPARD, and C. J. SCHORAH

From the Department of Paediatrics and Child Health, University of Leeds

Smithells, R. W., Sheppard, S., and Schorah, C. J. (1976). *Archives of Disease in Childhood*, **51**, 944. **Vitamin deficiencies and neural tube defects.** Serum folate, red cell folate, white blood cell vitamin C, riboflavin saturation index, and serum vitamin A were determined during the first trimester of pregnancy in over 900 cases. For each of these there was a social class gradient. Social classes I + II showed the highest levels which differed significantly from other classes, except for serum folate.

In 6 mothers who gave birth to infants with neural tube defects, first trimester serum folate, red cell folate, white blood cell vitamin C, and riboflavin values were lower than in controls. In spite of small numbers the differences were significant for red cell folate ($P < 0.001$) and white blood cell vitamin C ($P < 0.05$).

These findings are compatible with the hypothesis that nutritional deficiencies are significant in the causation of congenital defects of the neural tube in man.

Congenital defects of the central nervous system account for a high proportion of all major birth defects, amounting in some places to as much as one-third of the total. In Liverpool, for example, from 1960–1966 inclusive, the incidence of neural tube defects was 7/1000 total births, and of all defects 22/1000 (Smithells, 1968). The population incidence varies widely in different parts of the world and in different ethnic groups. In parts of Ireland and South Wales the combined incidence of anencephaly and spina bifida exceeds 1% of all births (Elwood, 1972; Laurence, Carter, and David, 1968).

Although important progress is being made in the prenatal diagnosis of these conditions, and with it the potential for selective abortion, this is only acceptable as a 'second best' until true primary prevention is possible. Our ultimate aim must be the birth of a healthy baby rather than the abortion of an abnormal fetus.

Primary prevention demands knowledge of causes. It is widely accepted that neural tube defects are unlikely to be attributable to any single factor, either genetic or environmental. Nevertheless, knowledge of all contributory factors is not essential to prevention. If one relevant factor can be identified and eliminated, this might be

sufficient, by a threshold effect, to lead to a reduction in incidence.

Epidemiological studies have shown a number of fairly consistent features in the population patterns of neural tube defects which apply less strikingly or not at all to other birth defects (Leck, 1974). A social gradient, showing a higher incidence in social classes IV and V than in I and II, is one of the most consistent. In the UK social class is determined by the occupation of the father, but the cultural differences between the classes are not wholly determined by economic factors. Indeed the process of economic levelling continually erodes such income-determined differences as existed in the past.

These social class differences have been incorporated in a variety of theories of causation of neural tube defects. The theory of infection (Record, 1961) is consistent with greater overcrowding in poorer families; the theory of soft water (Stocks, 1970) is consistent with the concentrations of unskilled workers in the UK in soft water areas; the potato blight story (Renwick, 1972) is consistent with the consumption of cheap food by the poor.

The possibility that nutritional differences between the social classes might be a factor in the causation of neural tube defects deserves further examination. If significant differences exist, it

seems probable that in the UK at this time they would relate to the quality and balance of the diet rather than to the quantity of food.

The Leeds Pregnancy Nutrition Study was set up in 1969 to examine prospectively the nutrition of women in the first trimester of pregnancy and to correlate the findings with the outcome of the pregnancy. Several biochemical and haematological variables were determined on the mothers. This report is concerned only with the vitamins assayed—red blood cell folate, serum folate, white blood cell (WBC) vitamin C, riboflavin, and serum vitamin A. Comparisons are made between the levels of these parameters in the study population (control group), the social class subgroups, and those women who produced infants with defects of the central nervous system (CNS).

Material and methods

Mothers were recruited on a voluntary basis through general practitioners and hospital antenatal clinics and were therefore not a representative population sample. All were resident in Leeds. No restrictions were made on age or parity. The duration of pregnancy at the time of study was not more than 13 completed weeks from the start of the last menstrual period. Mothers were interviewed at home when a comprehensive questionnaire was completed which incorporated a section detailing all drugs, including vitamin supplements, taken since the beginning of pregnancy. Mothers were asked to attend for blood sampling in the mornings after eating only tea and toast. On the day of venepuncture a record was made of any pharmaceutical preparations being taken and of the food eaten that day. Samples were taken into sterilized acid-citrate-dextrose anticoagulant for WBC vitamin C estimation and assessment of riboflavin status. They were prepared for analysis within 3 hours. Subsequent storage of the leucocytes was for not more than 2 weeks in 5% trichloroacetic acid at -18°C . Riboflavin levels were usually assessed on the day of collection and always within 3 days. Red cell folate was analysed using 0.5 ml of whole blood sequestrene samples diluted with 4.5 ml of 1% ascorbic acid and stored, usually for no longer than one week, at -18°C . Serum samples were used for the analysis of vitamin A (Wild, Schorah, and Smithells, 1974) and for serum folate, the latter being stored in approximately 0.5% ascorbic acid at -18°C .

Vitamin C in the buffy layer was measured by the technique of Denson and Bowers (1961) modified to give a more satisfactory separation of leucocytes (details to be published). Riboflavin status was assessed by the glutathione reductase technique of Glatzle *et al.* (1970). Details of sample preparation, minor modifications and chemicals used are recorded elsewhere (Schorah and Messenger, 1975). Folic acid was measured microbiologically using chloramphenicol-resistant *Lactobacillus casei*. The method is a manual adaptation of the technique of Davis, Nicol, and Kelly (1970) and is

similar to that described by Chanarin, Kyle, and Stacey (1972). Vitamin A was measured by the technique of Hansen and Warwick (1969). Results are expressed as free retinol.

All methods were controlled throughout the survey by using pooled control samples run with each analysis batch or, in the case of the riboflavin estimation, by the differences between results for individual samples estimated in consecutive batches.

The average between-batch coefficient of variation of the control samples throughout the survey period was WBC vitamin C $\pm 11.4\%$; riboflavin $\pm 4.4\%$; red cell folate $\pm 9.6\%$; serum folate $\pm 9.2\%$; vitamin A $\pm 7\%$.

Results

In the following analysis the control group is defined as all mothers tested. However, folate and vitamin C values have been excluded from this group and from the social class subgroups for mothers who were known to be taking these supplements. Folate values have also been disregarded in all mothers taking chemotherapeutic substances because of the inhibiting effect of these drugs on the growth of *L. casei in vitro*.

In this report all means are calculated directly from the raw data. However, examination of the results in the control group suggests that the values are not always normally distributed but that in some cases the log or square-root values more nearly approach normal distribution (Wild *et al.*, 1974). Details of data distribution not yet published will be reported elsewhere. The numbers in the CNS group are too small to indicate a distribution but they give a variance which differs significantly from that of the control population for folate and vitamin C. Differences in variance, therefore, invalidate the use of the ordinary 2-sample t-test in comparing the means of the control and the CNS populations. However, as the control group is large (approximately 1000) the standard errors in the estimates of the control means are very small. For each vitamin, therefore, we can consider the control means as fixed points and perform 1-sample t-tests for the CNS mean against a fixed (known) alternative. The social class means are compared by 2-sample t-tests using unadjusted distributions as numbers are large and variances of the populations similar.

Table I shows the mean values and 95% ranges for the five assays in the control group and the means in the social class subgroups. Classes I+II and IV+V are grouped together. Classes III manual and III nonmanual are treated separately. The mean for the CNS group is also shown. The P values of all significant differences between social classes, and between CNS defect infants and controls, are shown in the final column.

TABLE I

Vitamin assays: mean values for control population, social class subdivisions, and the CNS defect infants (numbers in each group shown in brackets)

	Controls Mean 95% range	Social class				CNS defect	Significant differences
		I + II	III Nonmanual	III Manual	IV + V		
Red cell folate (ng/ml)	228 (959) 86-460	249 (245)	218 (148)	221 (420)	220 (146)	141 (6)	I + II v III NM P < 0.005 I + II v IIIM P < 0.001 I + II v IV + V P < 0.01 CNS defect v controls P < 0.001
Serum folate (ng/ml)	6.3 (953) 1.9-15.8	6.7 (245)	5.8 (148)	6.3 (412)	6.0 (148)	4.9 (5)	(No significant differences)
Vitamin C ($\mu\text{g}/10^8$ WBC)	34.5 (1098) 15-66	36.9 (229)	35.3 (177)	33.4 (441)	32.5 (181)	23.9 (4)	I + II v III M P < 0.001 I + II v IV + V P < 0.001 III NM v IV + V P < 0.05 CNS defect v controls P < 0.05
Riboflavin (saturation index)	1.23 (1284) 1.03-1.53	1.20 (357)	1.24 (204)	1.24 (519)	1.26 (204)	1.28 (6)	I + II v III NM P < 0.001 I + II v III M P < 0.001 I + II v IV + V P < 0.001
Serum vitamin A ($\mu\text{g}/100$ ml)	68.2 (971) 43-108	71.7 (288)	67.3 (150)	66.4 (377)	66.6 (156)	75.7 (3)	I + II v III NM P < 0.05 I + II v III M P < 0.001 I + II v IV + V P < 0.01

Table II gives details of the 6 cases with defects of the CNS. 3 were anencephalics, 1 of which aborted spontaneously at 13 weeks: 1 was a meningocele, a lesion which does not involve neural tissue but closely resembles failure of closure of the neural tube. 1 case of myelomeningocele and hydrocephalus died in the neonatal period. The sixth infant had microcephaly, a condition of mixed aetiology but for which no cause could be found in this instance.

Red cell folate. The mean RBC folate in 959 control mothers was 228 ng/ml. The mean for social classes I+II (249 ng/ml) was significantly higher than the other social class means. The mean RBC folate in the 6 mothers of infants with defects of the CNS was 141 ng/ml which is significantly lower than the control group ($P < 0.001$).

Serum folate. The mean serum folate in 953 control mothers was 6.3 ng/ml. The mean for

TABLE II
Details of 6 cases with defects

Case no.	Defect	Date of last menstrual period	Social class	Gestation (w)	Outcome
1	Anencephaly	12 Mar 70	IIIM	41	Stillbirth
2	Anencephaly	8 Apr 71	IIIM	13	Spontaneous abortion
3	Anencephaly	11 Mar 72	IIIM	32	Neonatal death
4	Meningocele	22 Apr 70	V	40	Live birth
4	Myelomeningocele + hydrocephalus	7 Dec 70	IIIM	39	Neonatal death
6	Microcephaly	25 May 70	IV	41	Live birth
Mean \pm SD					

*A higher riboflavin saturation index means a greater degree of riboflavin deficiency.

†This mother had been taking a folate supplement for one week. The serum folate

‡This mother was receiving a vitamin C supplement. NA, not available.

social classes I+II was higher than the other social class means but none of the social class differences is significant. The mean serum folate of 5 mothers of CNS defect infants was 4.9 ng/ml which is lower than the lowest social class mean but not significantly so.

Vitamin C. The mean WBC vitamin C in 1098 controls was 34.5 $\mu\text{g}/10^8$ WBCs. The mean for social classes I+II was significantly greater than the means for III manual and IV+V, and that for class III nonmanual was significantly greater than the mean for classes IV+V. The mean for 4 mothers of CNS defect infants was 23.9 $\mu\text{g}/10^8$ WBCs which is lower than all social class means, the difference from the overall mean being of borderline significance ($P < 0.05$).

Riboflavin. The mean saturation index in 1284 controls was 1.23. The mean for social classes I+II (1.20) is significantly lower (indicating a more satisfactory riboflavin status) than all other social class means ($P < 0.001$). The mean for social class III (manual and nonmanual) is lower than that for social classes IV+V, but not significantly so. The mean for 6 mothers of CNS defect babies was 1.28 which is higher than all social class means but not significantly different from the mean of all controls.

Vitamin A. The mean serum vitamin A in 971 control mothers was 68.2 $\mu\text{g}/100$ ml. The mean value for social classes I+II (71.7 $\mu\text{g}/100$ ml) is significantly higher than all other social class means. Values were available for only 3 mothers of CNS defect babies. The mean (75.7 $\mu\text{g}/100$

ml) is higher than all social class means, but is attributable to a single high value (see Table II).

These observations may be summarized as follows.

Social class differences. For all vitamin levels determined, the mean values were most satisfactory in social classes I+II, and this difference was significant except for serum folate. This statement assumes that higher values for folate and vitamin C and a lower saturation index for riboflavin are more satisfactory, and this is probably valid. In view of the possibility of adverse effects of high levels of vitamin A on the embryo there may be an optimum level, as yet unknown. However, in the light of all the information from this study it seems reasonable to accept that the vitamin A values found in healthy women from social classes I+II are satisfactory.

Mothers of CNS defect infants. The mean vitamin A value was higher than all social class means (reflecting a single high value among 3 observations), but not significantly so. The mean values for serum folate, RBC folate, and WBC vitamin C were lower, and the saturation index for riboflavin poorer than in all social classes. These differ significantly from controls for RBC folate ($P < 0.001$) and vitamin C ($P < 0.05$) in spite of small numbers.

Discussion

Nutrition is so fundamental to human health that its contribution to social class differences in health patterns in the past has been accepted as self-evident. Improved dietary standards in the UK since

II of the CNS in infant

Maternal

Red cell folate (ng/ml)	Serum folate (ng/ml)	Vitamin C ($\mu\text{g}/10^8$ WBCs)	Riboflavin* (saturation index)	Serum vitamin A ($\mu\text{g}/100$ ml)
157	4.5	21.8‡	1.41	NA
116	3.6	28.1	1.12	62.4
116	(23.4)†	NA	1.26	94.9
170	7.3	33.2	1.13	NA
165	5.8	12.5	1.19	69.9
123	3.2	NA	1.59	NA
141 ± 25.5	4.9 ± 1.7	23.9 ± 8.9	1.28 ± 0.18	75.7 ± 17.2

‡ therefore disregarded.

the 1939–45 war have tended to encourage the belief that nutritional factors are no longer of much significance. Deficiency of calories and protein in this country occurs only in those who are neglected by themselves or by others. It is certainly not a problem of expectant mothers. Similarly, classical vitamin deficiency diseases are no longer seen in the UK except among a few identifiable groups such as immigrant children. There is therefore an understandable reluctance to regard nutritional factors as likely to contribute to health problems in the UK today.

In the field of experimental teratology, a wide variety of developmental defects can be induced in most species of laboratory animals by feeding expectant mothers on diets deficient of one or more vitamins, by giving vitamin antagonists, or by a combination of both (Kalter and Warkany, 1959). The degrees of deficiency induced have usually been so extreme that the relevance of these studies to human 'spontaneous' malformations is obscure.

The value of dietary surveys, especially in early pregnancy when nausea and vomiting may be problems, is limited. The quantity of nutrients consumed is at best an indirect way of determining what is available to the embryo and fetus. Maternal blood levels of vitamins, or biochemical challenges (e.g. histidine loading), give more direct evidence of maternal vitamin status. The assessment is still being made on the wrong side of the placenta, but is the best available in early pregnancy.

Attempts to correlate human vitamin deficiency with congenital defects have concentrated particularly on folic acid. The susceptibility of animal embryos to folate deficiency and to folate antagonists is well documented (Nelson, 1960). There are also several reports of human malformations associated with maternal ingestion of folate antagonists in pregnancy (Thiersch, 1952; Meltzer, 1956; Warkany, Beaudry, and Hornstein, 1959; Emerson, 1962). Pregnancy itself may have profound effects on folate metabolism, which may be of more significance than changes in folate consumption (Rothman, 1970).

Investigations of the relation between maternal nutritional deficiency and fetal abnormalities give apparently conflicting results. The Vanderbilt Cooperative Study of Nutrition (McGanity *et al.*, 1954) examined nutrient intake and laboratory data in 2046 women, of whom 278 entered the study in the first trimester. No significant differences were found between the 55 mothers who gave birth to infants with major or minor defects and the remainder. Vitamin intake was recorded for vita-

mins A, C, & D, thiamine, niacin, and riboflavin. Laboratory data included serum vitamin A, vitamin C, and carotene, and urinary excretion of thiamine, riboflavin, and N'-methylnicotinamide. Folate intakes and blood levels were not estimated.

Hibbard and Hibbard (1963) reported a high incidence of folate deficiency in pregnant women with placental abruption and found an increased rate of birth defects in their offspring (4.5% compared with 1.3%, $P < 0.001$). The same group of workers found a smaller but still significant difference in a later study (Hibbard, Hibbard, and Jeffcoate, 1965) and a smaller and not significant difference in a third study (Hibbard, 1964). Hibbard and Smithells (1965) studied formimino-glutamic acid (FIGLU) excretion after histidine loading in late pregnancy and the immediate post-partum period and found abnormalities in 69% of 35 mothers of infants with CNS defects, compared with 17% of 35 controls matched for age and parity. Fraser and Watt (1964) reported 17 women with megaloblastic anaemia of pregnancy, 4 of whom gave birth to babies with major birth defects (3 involving the CNS).

Hibbard and Hibbard (1966) noted the recurrence of folate deficiency in successive pregnancies in the same women. An abnormal FIGLU test recurred in 73% of 200 women, including 18 of 27 retested in the first trimester. By contrast, Hall (1972) found no association between folate deficiency at first booking or at 30 weeks and placental abruption. Giles (1966) reported 335 cases of megaloblastic anaemia of pregnancy and the puerperium with no significant increase in fetal malformation rate. Pritchard *et al.* (1970) reported one birth defect among 82 live births to women with megaloblastic anaemia of pregnancy. The same group (Scott, Whalley, and Pritchard, 1970) compared the post-partum plasma folate levels of 29 mothers of defective infants with those of 82 controls and found no significant difference.

The apparent conflict is not very surprising. Folate status has been assessed by different methods, at different stages of pregnancy or the puerperium, and in groups of women from widely differing backgrounds and selected for study on different criteria. In relation to fetal malformation, only nutritional deficiencies operating before conception or during the embryonic period could be of aetiological significance.

The Leeds Pregnancy Nutrition Study has shown that there are significant social class differences in nutritional intake (unpublished observations). The present report shows that there are also social class differences in blood levels of all the

vitamins studied which run in the anticipated direction. For social class I+II mothers the levels of RBC folate, WBC vitamin C, riboflavin, and vitamin A are significantly better than in the other social classes. As correlations between dietary intake and blood levels, estimated at the same time, are very poor (unpublished observations), the lower blood levels in classes III, IV, and V may not directly reflect vitamin consumption.

The low mean values for folate, vitamin C, and riboflavin among the mothers of infants with CNS defects is consistent with the hypothesis that deficiency of one or more vitamins is a contributory factor in the genesis of these malformations. In view of the very small number of infants with CNS defects in this study it is hardly surprising that the differences between their mothers and controls is significant only for two vitamins (RBC folate and WBC vitamin C). It will be noted in Table II that the mother of the infant with meningocele (not involving the neural tube) has the best levels of the vitamins estimated. In contrast to these results a group of 7 mothers whose infants had malformations of the cardiovascular system had mean values close to the control means (RBC folate 239 ng/ml, serum folate 6.1 ng/ml, vitamin C 30.0 $\mu\text{g}/10^8$ WBC, riboflavin index 1.22, vitamin A 68.1 $\mu\text{g}/100$ ml).

These results must be interpreted with caution. If vitamin deficiency is a factor in the genesis of CNS defects, appropriate vitamin supplementation might make a contribution to primary prevention. As the neural tube closes before antenatal supervision normally begins, such supplementation would have to be started before conception. This would apply even more forcibly to women who were taking contraceptive pills before conception because of the known tendency for these pills to depress the levels of some important vitamins (Wynn, 1975).

The most direct way of testing this hypothesis is by preconceptional vitamin supplementation of mothers who have already had one or more infants with CNS defects. Such a study is now being undertaken.

We are grateful to the mothers who volunteered for this study; to the general practitioners and obstetricians who helped in their recruitment; to Mr. Peter Zemroch for statistical analysis; to the doctors who assisted in the study, in particular Drs. A. Meynell and M. E. Carver, and to Mrs. J. Wild for technical assistance. We gratefully acknowledge generous financial support from Action for the Crippled Child, Roche Products Ltd., and the West Riding Medical Research Trust.

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Correspondence to Prof. R. W. Smithells, Department of Paediatrics and Child Health, 27 Blundell Street, Leeds LS1 3ET.

Addendum

Since submitting this paper a patient in our current survey has had her pregnancy terminated because of raised amniotic fluid α -fetoprotein levels. The abortus was an anencephalic fetus. The first trimester (12th week) vitamin levels in the mother were: vitamin C 12 $\mu\text{g}/10^8$ WBC, red blood cell folate 82 ng/ml, serum folate 3.4 ng/ml, and riboflavin saturation index 1.41. The values for red blood cell folate and vitamin C are below the lower limit of the 95% range for the study population reported in this article and augment our findings of lower levels for these vitamins in mothers of CNS malformed children.