J. clin Path. (1964), 17, 165

Folic acid and vitamin B_{12} levels in pregnancy and their relation to megaloblastic anaemia

E. W. BALL AND C. GILES

From the City General Hospital, Stoke-on-Trent

SYNOPSIS There is a significant fall in the serum folic acid level during pregnancy, reaching its lowest level at term. This is most pronounced in twin pregnancies. A similar but less spectacular fall occurs in the vitamin B_{12} concentration.

In megaloblastic anaemia both folic acid and vitamin B_{12} levels are lower than in other pregnant women. The degree of megaloblastic change in the bone marrow, as measured by the type and number of megaloblasts, is reflected in the vitamin levels, cases with florid megaloblastosis showing the most marked depression of vitamin B_{12} and folic acid activity.

Although there is a significant difference in the mean folic acid levels between megaloblastic and normoblastic pregnant women, a considerable overlap exists between individual values in the two groups. When the labile folic-acid factor is determined separately the test becomes much more specific. In the present series, all cases of megaloblastic anaemia yielded labile-factor levels below 1·0 mμg. per ml., while a similar value was encountered in only one of 35 normal pregnancies.

In five women with megaloblastic anaemia the vitamin B_{12} concentration was less than 100 $\mu\mu$g. per ml. but rose to normal levels on folic acid therapy alone.

One of the main manifestations of both vitamin B_{12} and folic acid deficiency is megaloblastic anaemia. Osler (1919) was the first to describe megaloblastic anaemia in pregnancy, and until recently the disease was considered uncommon. During the last nine years, however, there have been several reports which suggest that megaloblastic anaemia is a frequent complication of late pregnancy responding promptly to the administration of folic acid (Scott, 1954; Cowan, 1957; Forshaw, Jones, Chisholm, and McGinley, 1957; Giles and Shuttleworth, 1958; Ainley, 1961).

Chanarin, MacGibbon, O'Sullivan, and Mollin (1959), using folic-acid clearance and absorption tests, showed that folic acid depletion in pregnancy is by no means confined to patients with megaloblastic anaemia, for abnormally rapid folic acid clearance was obtained even in women without anaemia, especially in cases of twin pregnancy. On the other hand, women who receive folic acid as routine prophylaxis in late pregnancy have significantly higher haemoglobin levels at term than comparable controls (Giles and Burton, 1960; Dawson, More, and Aird, 1962).

Much less is known about vitamin B_{12} metabolism in pregnancy. There can be little doubt that true Addisonian anaemia is an exceedingly rare complication of pregnancy. Since 1957 well over 18,000 antenatal patients, including 247 cases of megaloblastic anaemia, have been investigated in this hospital, but during this period not a single case of Addisonian anaemia in pregnancy or the puerperium has come to light. Nevertheless, a fall in the vitamin B_{12} serum level has been reported in a proportion of pregnant women both with and without anaemia (Heinrich, 1954; Spray and Witts, 1958; Dawson, 1962; Baker, Jacob, Rajan, Swaminathan, 1962).

In this hospital, estimation of the serum vitamin B_{12} concentration has been a routine procedure in the investigation of anaemia of pregnancy since 1960. Folic-acid clearance tests (Chanarin *et al.*, 1959) were introduced about the same time, but for various reasons this method was found unsatisfactory in our hands. In 1961, therefore, assay of the serum folic acid activity, using *Lactobacillus casei* (Baker, Herbert, Frank, Pasher, Hutner, Wasserman, and Sobotka, 1959; Waters and Mollin, 1961) was substituted. The term 'serum folic acid', as used in this paper, refers to this activity.

Although both tests were carried out in the same department, studies of vitamin B_{12} and folic acid

Received for publication 6 August 1963.

serum levels in pregnancy remained separate investigations and, as they were begun at different times, they do not necessarily cover the same case material. For this reason the results of the two vitamin assays have been recorded separately, and the analysis of the data differs in the two sections.

HAEMATOLOGICAL CLASSIFICATION OF MEGALOBLASTIC ANAEMIA

In Addisonian and other types of megaloblastic anaemia the bone marrow contains not only classical megaloblasts but also cells which in size and in texture of nuclear chromatin occupy an intermediate position between classical megaloblasts and normoblasts (Dacie and White, 1949). Israels (1951) named these cells 'transitional megaloblasts' and associated their presence with less severe forms of megaloblastic anaemia. Experience in this and other centres has shown that this concept also applies to megaloblastic anaemia in pregnancy (Giles, 1960; Dawson, 1962). In the florid form of this disease most erythroblasts are classical megaloblasts, but some transitional megaloblasts are also present. In the present paper such marrows have been classed as grade 3. In patients with anaemia of intermediate severity transitional megaloblasts predominate (grade 2), and in the mildest cases the marrow is in the main normoblastic but also contains a certain number of transitional megaloblasts (grade 1). When the marrow is predominantly megaloblastic, as in grades 2 and 3, a diagnosis can be established on the basis of the marrow film alone. In cases classified as grade 1, however, haematological diagnosis can be very difficult. Cases of this type have been included in the present series only if megaloblasts constituted at least 10% of all erythroblasts, if the initial serum iron concentration was raised, and if folic acid therapy produced a convincing clinical and haematological response.

An analysis of 247 cases diagnosed in this laboratory since 1957 (Table I) shows that the degree of megaloblastosis runs parallel to the severity of the other abnormalities. Classical megaloblasts are associated with the lowest mean haemoglobin, the highest mean serum iron levels, and the highest reticulocyte response to folic acid therapy. Anorexia and stomatitis, both very characteristic findings in megaloblastic anaemia of pregnancy, were likewise found most frequently in patients with a grade 3 type of marrow. Patients in whom the marrow was classified as grade 1 had the mildest form of the disease, while those in grade 2 exhibited an anaemia intermediate in severity between the other two groups.

TABLE I

CLINICAL AND LABORATORY FINDINGS IN PATIENTS WITH DIFFERENT GRADES OF MEGALOBLASTOSIS

	Grade 1 (71 Cases)	*Grade 2 (72 Cases)*	*Grade 3 (104 Cases)*
Mean haemoglobin concentration (g.%)	7·6	7·0	6·3
Mean serum iron concentration (μg.%)	139	187	242
Mean reticulocyte response to folic acid (%)	11	13	17
Incidence of stomatitis (% of cases)	23	27	40
Incidence of anorexia (% of cases)	14	30	35

FOLIC ACID DETERMINATIONS

SUBJECTS Blood was obtained from 58 normal subjects (laboratory staff, doctors, and nurses) and from 233 pregnant women who were not taking folic acid. The stage of pregnancy varied from nine weeks to full term. The pregnant women were referred to this laboratory for haematological examination from hospital and municipal ante-natal clinics, maternity wards, and general practitioners, and a few sera were received from other hospitals. The ante-natal clinic and ward patients were almost all anaemic (haemoglobin < 10 g.%) and iron-deficient (serum iron < 80 μg.% or M.C.H.C. < 31%) whilst only a third of the patients referred by general practitioners were anaemic.

SERA One portion of serum was mixed with ascorbic acid (5 mg. per ml.) to preserve labile folic acid (Waters and Mollin, 1961). Paired sera, with and without ascorbic acid, were stored at −20°C.

ASSAY OF FOLIC ACID The method of Waters and Mollin was modified in certain respects.

Double-strength medium was prepared as described by Jukes (1955), the stock solutions being kept no longer than recommended by Barton-Wright (1961). The complete medium was stored frozen for not longer than three weeks. Immediately before use, 100 mg. ascorbic acid was added to each 100 ml. of double-strength medium, as this enhanced the growth of both standards and tests and stabilized the relationship between the two. Certain batches of enzymic casein hydrolysate proved deficient in tryptophan, and sufficient of this was added to produce maximal growth. The complete medium is given in Table II.

Organism Lactobacillus casei (helveticus; A.T.C.C. no. 7469), received from Dr. Waters, was maintained by weekly subculture in Difco micro-inoculum agar, which after 18 hours at 37°C. was stored at 4°C. For use it was subcultured in Difco micro-inoculum broth and, after overnight incubation, was heavily seeded into a second bottle of broth which was incubated for a further seven hours. The organisms were then washed twice in sterile normal saline and suspended to a definite opalescence (about O.D. 0·4). One drop of this suspension was used to inoculate each tube.

TABLE II

COMPOSITION OF DOUBLE-STRENGTH MEDIUM

Reagent	*Concentration per Litre*
Enzymic casein hydrolysate (5% solution)[1]	200 ml.
Adenine sulphate	10 mg.
Guanine hydrochloride	10 mg.
Uracil	10 mg.
Xanthine	20 mg.
L-asparagine monohydrate	600 mg.
L-cysteine hydrochloride	500 mg.
Riboflavin	1 mg.
p-Amino-benzoic acid	2 mg.
Pyridoxine hydrochloride	4 mg.
Thiamine hydrochloride	0·4 mg.
Calcium pantothenate	0·8 mg.
Nicotinic acid	0·8 mg.
Biotin	0·02 mg.
Dextrose	40 g.
Tween 80 (0·5% solution)	20 ml.
Glutathione (reduced)	5 mg.
Salt solution[2]	10 ml.
Sodium acetate (hydrated)	66 g.
K_2HPO_4	1 g.
KH_2PO_4	1 g.
Adjust to *p*H 6·8 with saturated potassium hydroxide	
$MnSO_4 \cdot H_2O$	200 mg.
Immediately before use add:	
Ascorbic acid	1 g.

[1]Nutritional Biochemicals Corporation, Cleveland, Ohio.
[2]Of this solution, 10 ml. contains 400 mg. $MgSO_4 \cdot 7H_2O$, 20 mg. NaCl, 20 mg. $FeSO_4 \cdot 7H_2O$, 15 mg. $MnSO_4 \cdot H_2O$.

Standards A 0·4 mg. per ml. solution of pteroylglutamic acid (Halewood Chemicals Ltd. or Lederle) was prepared as described by Jukes (1955) and stored frozen. On the day of the test it was diluted in water to give final concentrations ranging from 0·003 to 0·4 mμg. per ml.

Procedure Sera were diluted 1 in 10 in M/10 phosphate buffer (*p*H 6·1) containing 100 mg. ascorbic acid in each 100 ml., and autoclaved at 15 lb./sq. in. for two and a half minutes to precipitate protein. The supernatant was further diluted 1 in 5 to give a final concentration of 1 in 100 after the addition of an equal volume of double-strength medium. The final volume in each 5 × $\frac{5}{8}$ in. tube was 10 ml. The tubes were covered by Oxoid caps, autoclaved at 10 lb./sq. in. for 10 minutes, inoculated and incubated at 37°C. in a water bath. Growth was measured in an E.E.L. nephelometer after about 20 hours' incubation, the exact time varying with the rapidity of growth.

ESTIMATION OF LABILE FOLIC ACID The specimen of serum stored without ascorbic acid was autoclaved in buffer containing no ascorbic acid to destroy labile folic acid. The residual stable folic acid was then assayed by the method described above, using a final dilution of 1 in 50. Ascorbic acid was added to the medium. Total and stable folic acids were always measured in the same assay to ensure identical conditions. The labile folic acid level was obtained by subtracting the stable from the total folic acid value.

All glassware was washed in Brylyanz, tap water, and de-ionized water and stored, for not longer than two weeks, in ultra-violet light.

RESULTS OF FOLIC ACID DETERMINATIONS

SERUM FOLIC ACID IN NORMAL SUBJECTS In 58 normal subjects the serum folic acid ranged from 3·4 to 11·6 mμg. per ml., with a mean of 6·57 mμg. per ml. The mean was somewhat higher in females than in males (Table III) but the difference was not statistically significant. The distribution in female subjects is shown in Figure 1.

SERUM FOLIC ACID IN NORMOBLASTIC PREGNANT WOMEN After excluding cases of twin pregnancy and of megaloblastic anaemia, serum folic acid levels of 162 pregnant women were available for analysis. The levels ranged from 1·6 to 7·2 mμg. per ml., with a mean of 3·64 mμg. per ml. (Table III). This is significantly lower than the mean in non-pregnant

TABLE III

SERUM FOLIC ACID LEVELS IN NORMAL SUBJECTS AND IN PREGNANT WOMEN (NORMOBLASTIC AND MEGALOBLASTIC)

Type of Case	*No. of Cases*	*Folic Acid Levels (mμg./ml.)*			*Significance of Differences of Means*	
		Range	*Mean*	*Standard Deviation*	*(i) Between Adjoining Groups (P)*	*(ii) From Last Trimester of Normal Pregnancy (P)*
Normal subjects						
All cases	58	3·4-11·6	6·57	1·89		
Males	25	3·7-10·0	6·13	1·46	Not significant	
Females	33	3·4-11·6	6·90	2·12	< 0·01	
Pregnant women (normoblastic)						
All cases	162	1·6-7·2	3·64	1·30		
Last trimester	99	1·6-5·3	3·19	0·97	< 0·01	
Megaloblastic anaemia of pregnancy						
All cases	47	0·3-4·0	1·89	0·96		< 0·01
Marrow: grade 3	19	0·3-2·9	1·67	0·77		< 0·01
Marrow: grade 2	9	0·6-3·5	1·88	1·00	Not significant	< 0·01
Marrow: grade 1	10	0·7-4·0	2·76	1·05	< 0·01	Not significant
Marrow not examined	9	0·6-2·3	1·41	—		—
Twin pregnancies (normal)	12	1·1-5·3	2·60	—		—

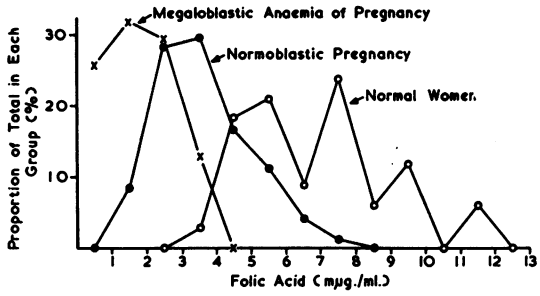

FIG. 1. *Distribution of serum folic acid levels in 33 normal women, 162 normoblastic pregnant women, and 49 women with megaloblastic anaemia of pregnancy.*

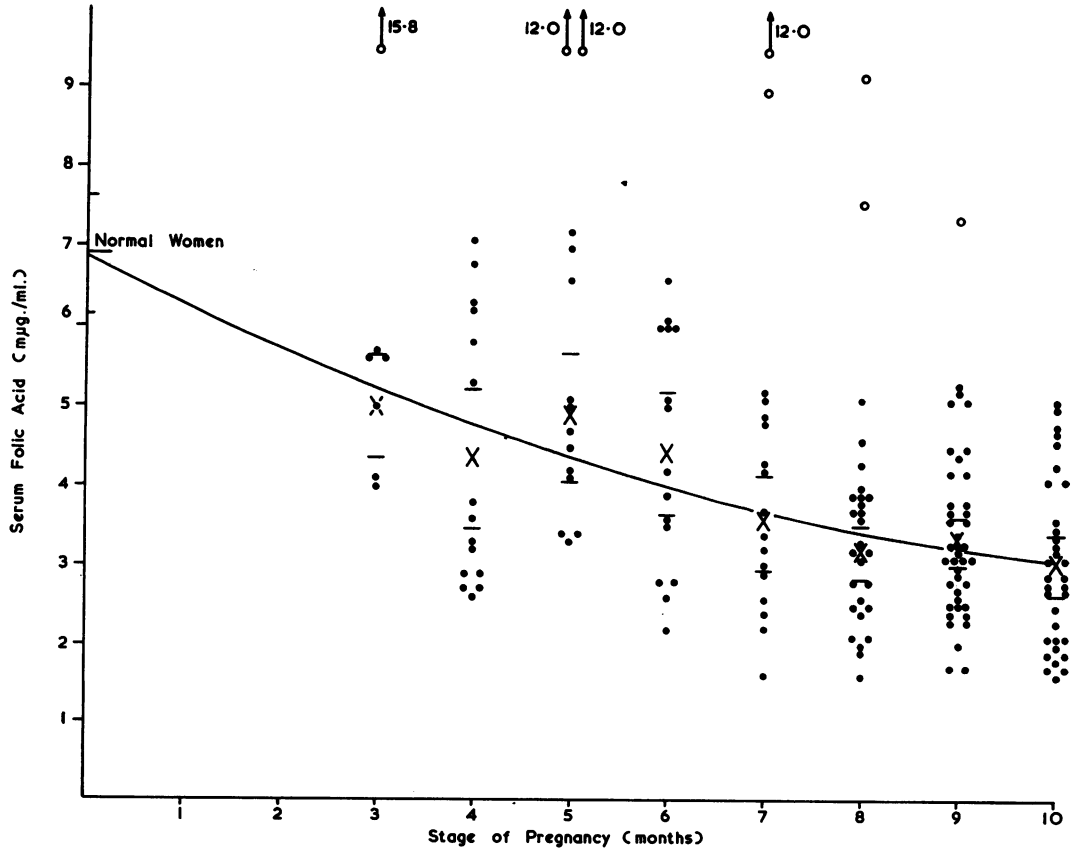

FIG. 2. *Serum folic acid levels in 170 normoblastic pregnant women, related to stage of pregnancy. Eight values (indicated by circles) lie outside the 95% probability limits for their monthly groups (David* et al., *1954) and have been excluded from the calculations: they are believed to be due to the administration of folic acid to the patients, although confirmation of this could not be obtained. The mean ± 2 S.E. for each month is indicated. The regression line is the best second degree polynomial through the monthly data and the levels in 33 normal women: its equation is* $y = 6{\cdot}870 - 0{\cdot}618x + 0{\cdot}024x^2$ *where y = serum folic acid in mμg./ml. and x = stage of pregnancy in months.*

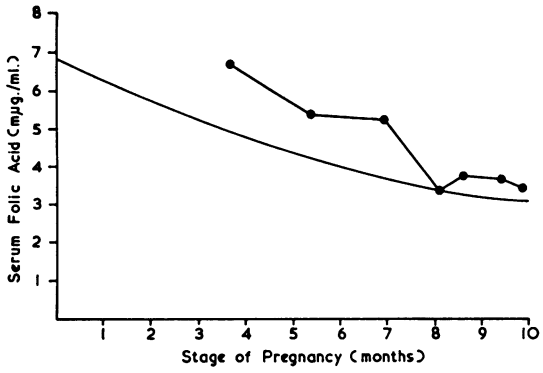

FIG. 3. *Serum folic acid levels of one woman throughout pregnancy (●—●) superimposed on regression line of all normoblastic pregnant women in the present series.*

women, but there was considerable overlapping between the two groups (Fig. 2).

There was a significant, progressive fall in the folic acid level during pregnancy, from 5·00 mμg. per ml. in the third month to 3·04 mμg. per ml. at term (Fig. 2). The monthly mean values showed no significant deviation from a linear regression line, but when this line was extrapolated back to the beginning of pregnancy, the mean for non-pregnant women deviated significantly from it. A second degree polynomial (a parabola) fitted all the points without significant deviation and is shown in Figure 2.

The relevance of this statistical pattern to the folic acid levels in individual women was confirmed by the examination of serial blood samples from one woman throughout pregnancy. The levels showed a progressive fall to full term, similar to that described above (Fig. 3.)

The patients who formed the basis of this investigation were not a random sample of the general population of pregnant women, but they showed no significant departure from a single population when their serum folic acid levels were grouped according to the source from which the patients were derived, those from general practitioners being subdivided into anaemic and non-anaemic (Table IV). It was concluded that, in this series, neither of these factors had any influence on the serum folic acid level. Since the anaemia was almost invariably associated with iron deficiency, it may be inferred that iron deficiency was also without influence.

SERUM FOLIC ACID IN MEGALOBLASTIC ANAEMIA OF PREGNANCY In 49 women with megaloblastic anaemia, diagnosed between the thirty-first week of pregnancy and 11 weeks after delivery, the range of serum folic acid levels was 0·3 to 4·0 mμg. per ml. (Table III). Excluding two women with twin pregnancies (folic acid levels 1·1 and 2·0 mμg. per ml.), the mean of the remaining 47 was 1·89 mμg. per ml. This is significantly lower than the mean value during the last trimester of normal pregnancy.

In 38 cases it was possible to group the serum folic acid levels according to the marrow picture, and the results of this analysis are shown in Table III. The mean values in patients with florid megaloblastosis and in cases of intermediate severity were significantly lower than the mean for the last trimester of normal pregnancy, but even in these groups there was no clear separation from the lower end of the normal range.

SERUM FOLIC ACID IN TWIN PREGNANCIES Fourteen patients with twin pregnancies were investigated, including the two cases of megaloblastic anaemia

TABLE IV

COMPARISON OF LINEAR REGRESSION OF SERUM FOLIC ACID ON STAGES OF PREGNANCY IN FOUR GROUPS OF NORMOBLASTIC PREGNANT WOMEN

Group	*No. of Cases*	*Regression Equation*[1] *y =*	*Deviation from Regression*		
			Sum of Squares	*Degrees of Freedom*	*Variance*
Analysis of variance					
From general practitioners (non-anaemic)	60	5·95 - 0·337x	89·6	58	
From general practitioners (anaemic[2])	44	7·44 - 0·475x	56·6	42	
From ante-natal clinics	32	5·13 - 0·176x	36·9	30	
From wards	26	5·43 - 0·242x	22·5	24	
Total deviation from four regression lines	162		205·6	154	1·334
Deviation of all data from one line	162	5·77 - 0·281x	216·0	160	
Difference			10·4	6	1·733

$F = \frac{1{\cdot}733}{1{\cdot}334} = 1{\cdot}30$ Difference between group regression lines is not significant.

[1]y = serum folic-acid levels in mμg./ml. x = stage of pregnancy in months.
[2]Haemoglobin below 10 g.%.

TABLE V

SERUM FOLIC ACID LEVELS IN TWIN PREGNANCY, COMPARED WITH THE EXPECTED LEVELS IN NORMAL PREGNANCY, TAKEN FROM FIGURE 2

Case No.	Age (yr.)	Stage of Pregnancy (wk.)	Hb (g.%)	M.C.H.C. (%)	Serum Folic Acid (mμg./ml.)	Expected Serum Folic Acid (mμg./ml.)
Megaloblastic						
1	22	38	9·3	33	1·1	3·04
2	33	Delivered 3 days	8·1	30	2·0	3·04
Normoblastic						
3	17	38	7·0	30	3·2	3·04
4	34	35	11·0	32	1·1	3·22
5	20	36	9·3	—	4·5	3·22
6	31	36	10·2	—	3·0	3·22
7	19	34	11·1	—	1·8	3·22
8	22	25	9·9	31	1·3	3·70
9	30	20	11·0	—	5·3	4·37
10	24	39	13·6	—	1·7	3·04
11	32	36	11·7	31	2·3	3·22
12	24	30	10·2	31	3·3	3·44
13	40	38	9·8	30	1·3	3·04
14	32	28	10·0	—	2·4	3·70
				Total	31·2	40·43
				Mean	2·60	3·37

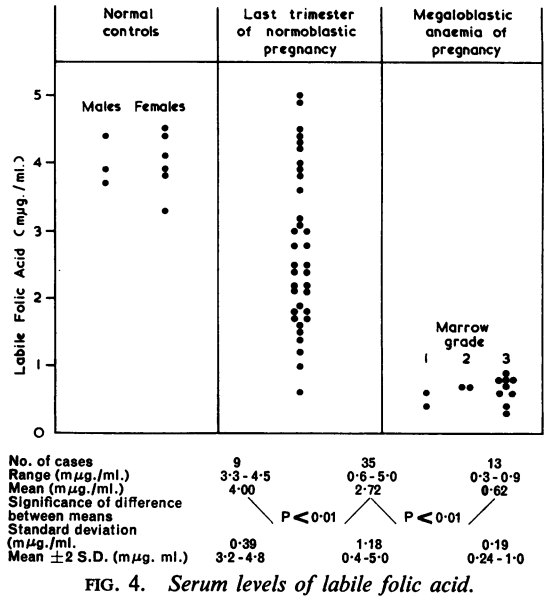

FIG. 4. *Serum levels of labile folic acid.*

already mentioned. In the remaining 12 the serum folic acid level ranged from 1·1 to 5·3 mμg. per ml., with a mean of 2·60 mμg. per ml. (Tables III and V). This is significantly lower than the expected mean of 3·37 mμg. per ml., calculated from the levels in normal pregnant women at the same stages of gestation (Fig. 2), and the range overlaps that found in megaloblastic anaemia.

LABILE FOLIC ACID ACTIVITY IN SERUM

Waters and Mollin (1961) have shown that the *L. casei* assay measures at least two separate components of the folic acid complex. One factor is stable to heating and storage, but its blood level bears no relationship to the degree of folic acid deficiency. The other factor is destroyed by heating and storage, but can be preserved by adding ascorbic acid to the serum. This second, labile, factor has recently been identified as N_5-methyl-tetra-hydrofolic-acid (Herbert, Larrabee, and Buchanan, 1962) and appears to be closely related to folinic acid.

An investigation of serum levels of this labile factor in relation to megaloblastic anaemia in pregnancy appeared to be of interest, especially since our results had shown that the total folic acid activity was not a reliable criterion in the diagnosis of this disease. Labile factor was estimated as the difference between simultaneous assays on two specimens of serum, one autoclaved with and the other without ascorbic acid.

RESULTS In nine normal subjects the labile factor level ranged from 3·3 to 4·5 mμg. per ml. (mean 4·0 mμg. per ml.). In 35 normal women during the last three months of pregnancy the range was from 0·6 to 5·0 mμg. per ml. (mean 2·72 mμg. per ml.), whilst in 13 patients with megaloblastic anaemia the labile factor levels ranged from 0·3 to 0·9 mμg. per ml., with a mean of 0·62 mμg. per ml., which is significantly lower than in normal pregnancy. The distribution of labile factor levels is illustrated in Fig. 4, which shows that, by taking 1·0 mμg. per ml. as the lower limit of normal, only one case would have been wrongly diagnosed as a result of this test.

MATERIAL AND METHODS FOR VITAMIN B_{12} DETERMINATIONS

Sera from pregnant women were derived from the same categories of patients as the specimens for folic acid assay. The vitamin B_{12} concentration was estimated using *Euglena gracilis* (z strain) as the test organism (Hutner, Bach, and Ross, 1956). Tubes containing both sera and standards were incubated for four days at 28 to 30°C. in a perspex tank illuminated from below by fluorescent light strips. Tests were read in a photoelectric absorptiometer.

RESULTS OF VITAMIN B_{12} DETERMINATIONS

VITAMIN B_{12} IN PREGNANCY Vitamin B_{12} serum levels in 320 pregnant women, both with and without anaemia, were compared with 564 controls, consisting of healthy non-pregnant subjects and of general

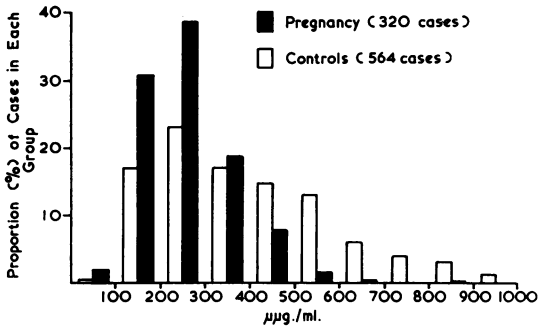

FIG. 5. *Distribution of vitamin B_{12} levels in pregnant women and non-pregnant controls.*

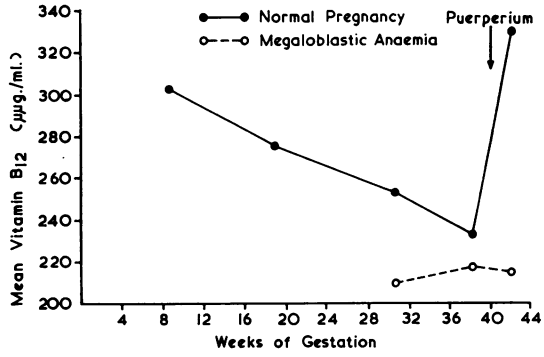

FIG. 6. *Mean vitamin B_{12} levels at different stages of pregnancy.*

hospital patients. Cases of relapsed or treated Addisonian anaemia, of leukaemia, liver disease, and intestinal malabsorption were excluded from this control group.

The mean vitamin B_{12} concentration in all pregnant women was 248 $\mu\mu$g. per ml. (S.E. 6·72) and that of the control group 356 $\mu\mu$g. per ml. (S.E. 7·00). The difference is statistically significant ($P < 0.001$). The distribution of vitamin B_{12} levels in the two categories (Fig. 5) shows that 33% of pregnant women had values below 200 $\mu\mu$g. per ml. compared with only 17% of controls.

Of the 320 pregnant women in this series, 61 had normal haemoglobin levels; the remainder presented with anaemia. The majority of the anaemic patients (159 cases) suffered from simple iron deficiency, sometimes complicated by urinary infection, whilst 100 patients had megaloblastic anaemia. The mean vitamin B_{12} concentration in the three groups is shown in Table VI.

TABLE VI

SERUM VITAMIN B_{12} IN NORMAL AND ANAEMIC PREGNANCIES

Group	*No. of Cases*	*Mean Vitamin B_{12} ($\mu\mu$g. per ml.)*
Normal pregnancies	61	270
Iron-deficiency anaemias	159	262
Megaloblastic anaemias	100	216

STAGE OF GESTATION AND VITAMIN B_{12} CONCENTRATION When vitamin B_{12} levels in the sera of normal or iron-deficient pregnant women were classified according to the stage of pregnancy at which specimens had been taken a slight but steady fall in the vitamin B_{12} concentration became apparent, reaching the lowest level at term, to be followed by a rapid rise during the first four weeks of the puerperium (Fig. 6).

OTHER FACTORS IN RELATION TO THE VITAMIN B_{12} CONCENTRATION A relationship could not be established between vitamin B_{12} serum levels and the haemoglobin concentration when the two values were compared in 233 pregnant women.

Likewise there was no evidence to suggest that the fall in vitamin B_{12} concentration during pregnancy was in any way related to iron deficiency. Of 209 cases in which both the serum iron and vitamin B_{12} had been determined, only 12 patients (5·7%) showed a dual deficiency (Fig. 7). This was not an unexpected finding since the mean vitamin B_{12} level of iron-deficient pregnant women was virtually the same as that in normal pregnancy. More surprising perhaps was the lack of a significant degree of correlation between vitamin B_{12} and folic acid serum levels in 59 women during the latter half of pregnancy.

In 12 cases of twin pregnancy the vitamin B_{12} concentration did not differ significantly from that in other pregnant women. Finally, no significant fall in vitamin B_{12} levels could be demonstrated with advancing age of the patient, nor with an increasing number of previous pregnancies.

VITAMIN B_{12} LEVELS IN MEGALOBLASTIC ANAEMIA The mean vitamin B_{12} concentration in 100 cases of megaloblastic anaemia of pregnancy was 216 $\mu\mu$g. per ml. (S.E. 11·17). This compares with the mean of 266 $\mu\mu$g. per ml. for all other pregnancies (S.E. 7·69), a difference which is statistically significant ($P < 0.01$). Figure 8 shows the distribution of vitamin B_{12} levels plotted against those in other pregnancies and reflects the difference in the mean values.

When the mean vitamin B_{12} levels in cases of megaloblastic anaemia were classified according to the stage of gestation at which the disease was diagnosed, *i.e.*, from the 25th week of pregnancy to the fourth week in the puerperium, quite a different pattern emerged from that observed in other pregnant women (Fig. 6), for the mean vitamin B_{12} concentration remained at a constant level between 212

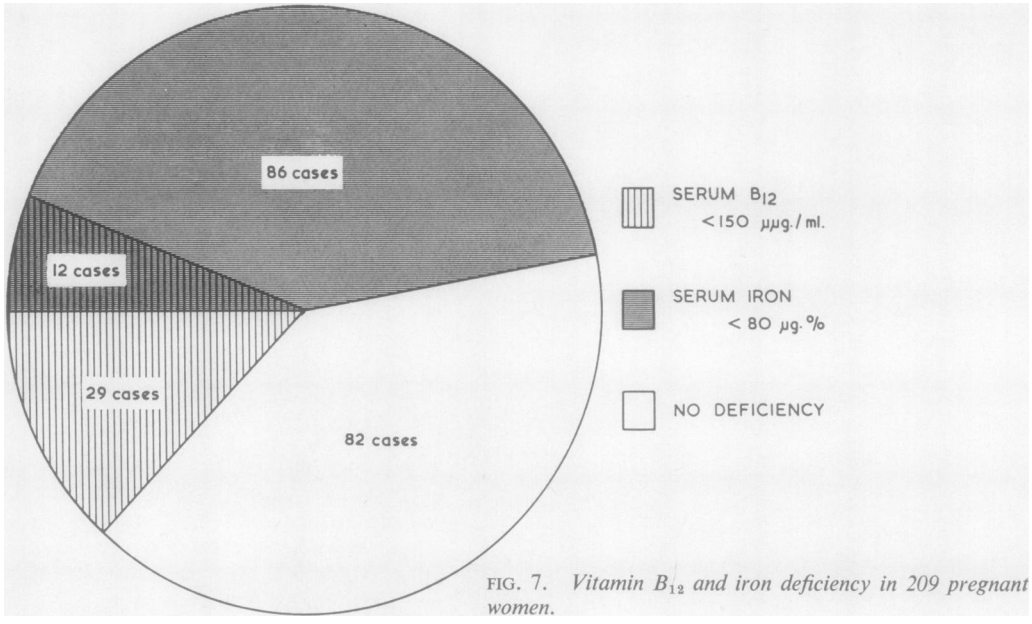

FIG. 7. *Vitamin B_{12} and iron deficiency in 209 pregnant women.*

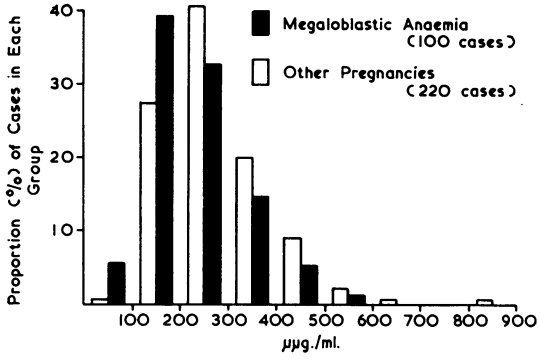

FIG. 8. *Distribution of vitamin B_{12} levels in megaloblastic anaemias and in other pregnancies.*

and 218 $\mu\mu$g. per ml., irrespective of the stage of gestation.

Subnormal vitamin B_{12} levels (below 150 $\mu\mu$g. per ml.) were found in 30·6% of patients with megaloblastic anaemias but in only 14·1% of other pregnant women, a significant difference ($P < 0.01$). Five patients, all below the age of 40, who presented with megaloblastic anaemia had serum vitamin B_{12} concentrations below 100 $\mu\mu$g. per ml. In all these cases the vitamin B_{12} rose to normal levels after one to three weeks' administration of folic acid alone (Table VII).

As in the case of folic acid, the mean vitamin B_{12} concentration was lowest in cases with a florid megaloblastic bone marrow (grade 3). In patients whose marrow was classified as grade 2 the vitamin B_{12} concentration was rather higher, and in the mildest cases (grade 1) the vitamin B_{12} level approached that of normal women during late pregnancy (Table VIII).

TABLE VII

EFFECT OF FOLIC-ACID THERAPY IN FIVE PATIENTS WITH SUBNORMAL VITAMIN B_{12} LEVELS

Case	*Age (yr.)*	*Haemoglobin (g.%)*	*Marrow*	*Serum Vitamin B_{12} (μμg./ml.) Level*	
				Before Folic Acid	*After Folic Acid*
1	28	7·8	Megaloblastic	60	250
2	22	5·2	Megaloblastic	90	380
3	39	7·4	Megaloblastic	60	240
4	36	6·9	Megaloblastic	56	152
5	20	6·5	Megaloblastic	60	200

DISCUSSION

The two studies reported in this paper have demonstrated that both the serum folic acid and

TABLE VIII

VITAMIN B_{12} LEVELS CLASSIFIED ACCORDING TO DEGREE OF MEGALOBLASTOSIS

Marrow	*No. of Cases*	*Mean Vitamin B_{12} (μμg./ml.)*
Megaloblastic, grade 3	46	178
Megaloblastic, grade 2	24	195
Megaloblastic, grade 1	30	226
Normoblastic (25-40 weeks)	110	247

vitamin B_{12} concentration steadily diminish throughout pregnancy, reaching their lowest levels at term. The fall is much more pronounced in the case of folic acid and confirms that in pregnancy the demand for this vitamin is greatly increased, a fact which has been recognized for some time (Chanarin *et al.*, 1959; Girdwood and Delamore, 1961; Scott, 1962) and which is also reflected in the relatively high folic acid levels in the blood of the foetus (Baker, Ziffer, Pasher, and Sobotka, 1958; Solomons, Lee, Wasserman, and Malkin, 1962).

The significance of the corresponding fall in the vitamin B_{12} concentration is open to conjecture. Baker *et al.* (1958) found that vitamin B_{12} levels in specimens of cord blood were higher than in the corresponding maternal blood samples. This suggests that the maternal stores of vitamin B_{12} undergo depletion along with those of folic acid. Although haemodilution may play a part in lowering vitamin levels towards the end of pregnancy, this is unlikely to be the whole explanation.

In patients with megaloblastic anaemia the serum levels of both vitamins are lower than in normal pregnancy. Moreover, the mean serum concentrations reflect the degree of megaloblastic change in the bone marrow and thus lend support to the concept that there are varying degrees of megaloblastic erythropoiesis characterized by the number and types of megaloblasts in the marrow (Giles, 1960; Dawson, 1962). Some authorities have questioned the diagnostic significance of transitional megaloblasts (Witts, 1962; Pinkerton, 1962). On the other hand, these cells occur in undoubted cases of Addisonian anaemia, in which they can be the predominant erythroblasts. The results of the vitamin assays recorded in this paper suggest that transitional megaloblasts merely indicate a milder degree of the disease process which culminates in florid megaloblastosis.

It is difficult to explain why subnormal vitamin B_{12} levels are commoner in megaloblastic anaemia than in normal pregnancy, for previous workers (Badenoch, Callender, Evans, Turnbull, and Witts, 1955; Metz, Lewis, Keeley, and Hart, 1960) have found no evidence of impaired absorption of vitamin B_{12} in this disease. A possible explanation was suggested by Cox and his co-workers (1959), who attributed the low vitamin B_{12} concentration to excessive utilization of this vitamin in an effort to compensate for the lack of folic acid. Only in five cases of the present series were the vitamin B_{12} levels low enough to raise a suspicion of Addisonian anaemia, and in all these patients the vitamin B_{12} level returned to normal on folic-acid therapy alone. Cox, Meynell, Gaddie, and Cooke (1959) reported similar findings in megaloblastic anaemia of pregnancy and Narayanan *et al.* (1956) in nutritional megaloblastic anaemia. Estimation of the serum vitamin B_{12} is, therefore, of little help in the diagnosis of megaloblastic anaemia of pregnancy.

Assay of folic acid activity, however, has considerable practical application as a diagnostic test in anaemia of pregnancy. Cytological interpretation of blood and marrow films can at times be very difficult and a diagnosis in such cases rests on individual opinion. By contrast, the serum folic acid level is an objective measurement. In the present series, folic acid levels in megaloblastic anaemia were invariably low and readily distinguished from those in normal non-pregnant subjects. There was, however, a considerable overlap between folic acid levels in patients with megaloblastic anaemia and those of other pregnant women, especially in cases of twin pregnancy. This confirms the findings of Chanarin and his co-workers (1959) who established by different methods that folic acid deficiency in pregnancy can exist without any detectable megaloblastosis.

At first sight it is not easy to understand why of two pregnant women with identical serum folic acid levels one may present with megaloblastic anaemia while the other has maintained normal erythropoiesis. In order to explain this observation one might assume the existence of an additional factor necessary for the production of megaloblastic changes in the bone marrow (Scott, 1962). This might be a constitutional predisposition to the disease, which is suggested by the tendency for the same patient to develop megaloblastic anaemia in successive pregnancies and also by an apparently abnormal blood group distribution (Giles, 1960; Ainley, 1961).

There is, however, an alternative explanation which may be sought in the complexity of folic acid derivatives in the serum. If serum folic acid activity is assayed using *Streptococcus faecalis* as the test organism, or if the *Lactobacillus casei* assay is performed without prior addition of ascorbic acid, much lower values are obtained which do not in any way reflect the degree of folic acid depletion of the patient. These low values are merely a small stable fraction of the total serum folic acid. By adding ascorbic acid to the serum an additional labile factor can be preserved (Waters and Mollin, 1961), and the total folic acid activity corresponds very much better with the degree of folic acid deficiency of the patient, as measured by different methods. It is, therefore, possible that the presence of variable amounts of stable factor may render the *L. casei* assay inaccurate. Since stable factor activity is usually low it is unlikely to affect the result where total folic acid activity is normal or high. With low total values, however,

which are common in late pregnancy, the stable factor activity may materially affect the results and thus account for the overlap in serum levels between patients with megaloblastic anaemia and in normal women in late pregnancy.

By the simultaneous assay of total folic acid and stable factor activity in two samples of the same serum the labile folic acid was estimated in a series of 48 pregnant women, including 13 cases of megaloblastic anaemia. The results of this preliminary study are encouraging, for all patients with megaloblastic anaemia showed very low serum levels of labile factor and the overlap with other pregnancies has been virtually eliminated. If these findings can be confirmed in a larger series of cases it would appear that the serum assay of folic acid can now take its place as a practical laboratory test in the diagnosis of megaloblastic anaemia of pregnancy.

We wish to record our thanks to the staff of the Pathology Department of the City General Hospital, Stoke-on-Trent, especially to Mr. A. C. Key, F.I.M.L.T., Miss Wendy Cooke, and Miss Rosemary Thompstone, for their help with the vitamin assays; to Miss M. R. Haslock, B.Sc., for the serum iron results; to Mr. P. H. Johnson, F.I.M.L.T., and Mrs. Margaret Clowes, A.I.M.L.T. for their help with the haematological work; to Dr. A. J. McCall and Dr. J. A. H. Brown for their advice; to all the obstetricians, many general practitioners, and pathologists in many other hospitals for supplying case material; and to Mr. A. H. Iliffe and Dr. D. G. Beech for their help with the statistical analysis.

Our thanks are due to the Research Subcommittee of the Birmingham Regional Hospital Board for apparatus and technical assistance.

REFERENCES

Ainley, N. J. (1961). *J. Obstet. Gynaec. Brit. Emp.*, **68**, 254.
Badenoch, J., Callender, S. T., Evans, J. R., Turnbull, A. L., and Witts, L. J. (1955). *Brit. med. J.*, **1**, 1245.
Baker, H., Herbert, V., Frank, O., Pasher, I., Hutner, S. H., Wasserman, L. R., and Sobotka, H. (1959). *Clin. Chem.*, **5**, 275.
——, Ziffer H., Pasher, I., and Sobotka, H. (1958). *Brit. med. J.*, **1**, 978.
Baker, S. J., Jacob, E., Rajan, K. T., and Swaminathan, S. P. (1962). *Ibid.*, **1**, 1658.
Barton-Wright, E. C. (1961). *Lab. Pract.*, **10**, 715.
Chanarin, I., MacGibbon, B. M., O'Sullivan, W. J., and Mollin, D. L. (1959). *Lancet*, **2**, 634.
Cowan, B. (1957). *Scot. med. J.*, **2**, 433.
Cox, E. V. Meynell, M. J., Gaddie, R., and Cooke, W. T. (1959). *Lancet*, **2**, 998.
Dacie, J. V., and White, J. C. (1949). *J. clin. Path.*, **2**, 1.
David, H. A., Hartley, H. O., and Pearson, E. S. (1954). *Biometrika*, **41**, 482.
Dawson, D. W. (1962). *J. Obstet. Gynaec. Brit. Cwlth*, **69**, 38.
——, More, J. R. S., and Aird, D. C. (1962). *Lancet*, **2**, 1015.
Forshaw, J. W. B., Jones, A. T., Chisholm, W. N., and McGinley, W. K. (1957). *J. Obstet. Gynaec. Brit. Emp.*, **64**, 255.
Giles, C. (1960). *Lancet*, **2**, 1063.
——, and Burton, H. (1960). *Brit. med. J.*, **2**, 636.
——, and Shuttleworth, E. M. (1958). *Lancet*, **2**, 1341.
Girdwood, R. H., and Delamore, I. W. (1961). *Scot. med. J.*, **6**, 44.
Heinrich, H. C. (1954). *Klin. Wschr.*, **32**, 205 and 867.
Herbert, V., Larrabee, A. R., and Buchanan, J. M. (1962). *J. clin. Invest.*, **41**, 1134.
Hutner, S. H., Bach, M. K., and Ross, G. I. M. (1956). *J. Protozool.*, **3**, 101.
Israels, M. C. G. (1951). In *Recent Advances in Clinical Pathology*, edited by S. C. Dyke, p. 358. Churchill, London.
Jukes, T. H. (1955). In *Methods of Biochemical Analysis*, edited by D. Glick, vol. 2, p. 121. Interscience, New York.
Metz, J., Lewis, S. M., Keeley, K. J., and Hart, D. (1960). *J. clin. Path.*, **13**, 394.
Narayanan, M. S., Shenoy, K. G., Ramasarma, G. B. (1956). *Nature (Lond.)*, **178**, 1347.
Osler, Sir W. (1919). *Brit. Med. J.*, **1**, 1.
Pinkerton, J. H. M. (1962). *Proc. roy. Soc. Med.*, **55**, 766.
Scott, J. M. (1954). *J. Obstet. Gynaec. Brit. Emp.*, **61**, 646.
—— (1962). *Postgrad. med. J.*, **38**, 202.
Solomons, E., Lee, S. L., Wasserman, M., and Malkin, J. (1962). *J. Obstet. Gynaec. Brit. Cwlth*, **69**, 724.
Spray, G. H. and Witts, L. J. (1958). *Brit. med. J.*, **1**, 295.
Waters, A. H., and Mollin, D. L. (1961). *J. clin. Path.*, **14**, 335.
Witts, L. J. (1962). *Brit. med. J.*, **2**, 402.