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Microbiological assay of folic acid activity in human serum

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SYNOPSIS A method is described for the microbiological assay of folic acid activity in serum with *Lactobacillus casei* as test organism and a modified medium in which the organism gives a greater growth response than in media previously detailed. The results of experiments carried out to validate the use of this medium are shown.

In 94 control subjects levels of folic acid activity in the serum ranged from 2·1 to 28 mμg./ml. (mean 7·8). The values in nine out of 10 patients with megaloblastic anaemia due to deficiency of folic acid were 1·0 mμg./ml. or less and one result was 2·0 mμg./ml. In six patients with megaloblastic anaemia associated with pregnancy the results ranged from 0·7 to 4·0 mμg./ml., and in untreated pernicious anaemia 28 out of 31 results were within or above the control range and three values were just below the lower limit of normal.

Microbiological assay of the folic acid activity in serum has been used to help in the differential diagnosis of the megaloblastic anaemias (Herbert, Baker, Frank, Pasher, Sobotka, and Wasserman, 1960; Waters and Mollin, 1961). During an attempt to establish the method as described by Waters and Mollin difficulties were encountered which necessitated reappraisal of the method, and a better culture medium has therefore been prepared.

METHODS

REAGENTS AND GLASSWARE Chemicals of Analytical Reagent grade were used when possible, otherwise the purest commercial grade was used. Solutions were made up with water prepared by re-distilling de-ionized water through an all-glass still. Glassware was cleaned by soaking overnight in chromic acid-sulphuric acid cleaning mixture and washing six to eight times in hot tap water and twice in de-ionized water. Aluminium caps were rinsed in hot tap water and de-ionized water. The dispensing syringe was well washed with hot tap water and then with glass-distilled water just before use. With these precautions the only evidence of contamination by exogenous 'folic acid' was occasional tubes showing excessive growth. This occurred mainly in the early stages of the work and was confined to so few tubes that the aberrant readings were clearly distinguishable and could be eliminated.

BASAL MEDIUM Three stock solutions were maintained (Table I). To prepare solution A the casein hydrolysate, sodium acetate, KH_2PO_4, K_2HPO_4, and salt solution C were mixed and the *p*H adjusted to about 5·4 with 40% NaOH solution. The mixture was boiled for 30 minutes

TABLE I

STOCK SOLUTIONS FOR PREPARING THE BASAL MEDIUM FOR *LACTOBACILLUS CASEI*

Salt solution C

$MgSO_4 . 7H_2O$	40 g.
Ferrous ammonium sulphate, crystalline	2·8 g.
$MnSO_4 . 4H_2O$	8·0 g.
Concentrated HCl	4·0 ml.
Water to	1,000 ml.

Solution A

Vitamin-Free casein hydrolysate (enzymatic)[1]	1,000 ml.
Tryptophane	1·0 g.
Cysteine hydrochloride	1·0 g.
Asparagine	3·0 g.
Sodium acetate $3H_2O$	100 g.
KH_2PO_4	25 g.
K_2HPO_4	25 g.
Salt solution C	200 ml.
Adenine, guanine, uracil, xanthine	100 mg. each
Water to	5,000 ml.

Adjust *p*H to 5·4-5·6 with 40% NaOH solution, store in an ordinary refrigerator in plugged containers.

Solution B

Riboflavin	10 mg.
p-Aminobenzoic acid	20 mg.
Pyridoxine	40 mg.
Thiamine	4 mg.
Calcium pantothenate	8 mg.
Nicotinic acid	8 mg.
Biotin	200 μg.
Water to	100 ml.

Store under toluene in an ordinary refrigerator.

[1]Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

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or more until it gave a clear filtrate on filtering through Whatman no. 1 paper. Solid asparagine was dissolved in the hot filtrate. The cysteine HCl was dissolved in water; tryptophane was dissolved in dilute HCl; adenine, guanine, and uracil were dissolved together in hot dilute HCl; xanthine was dissolved in dilute NaOH solution. The solutions were added to the filtrate. The complete medium was prepared for each assay by making up a solution in the following proportions:

Solution A.....100 ml.
Solution B.....1 ml.

(The riboflavin does not dissolve completely and the solution was shaken to produce an even suspension before each removal.)

Dextrose.....4 g.
Ascorbic acid.....200 mg.
Water to 160 ml.

PREPARATION OF SERUM EXTRACTS Blood was taken by venepuncture using stainless steel needles and all-glass syringes, and was allowed to clot for one to three hours in glass containers. The clots were stirred with glass rods, taking care to avoid haemolysis, and the samples were centrifuged. Solid ascorbic acid (5 mg./ml.) was dissolved in the serum which was stored at —15°C. until assayed.

Serum (0·5 ml.) was mixed with 0·1 ml. freshly prepared ascorbic acid solution in water (22·5 mg./ml.) and 2·2 ml. 0·2M sodium phosphate buffer *p*H 6·1 (750 ml. 0·2M NaH_2PO_4 solution + 250 ml. 0·2M Na_2HPO_4 solution). The volume was made up to 5 ml. with water and the solution was autoclaved at 15 lb. pressure for two and a half minutes, cooled, stirred with a glass rod, centrifuged, and the clear extract was decanted. Larger quantities of serum and other ingredients were used when more extract was needed for samples expected to have low folic acid activity.

STOCK STANDARD FOLIC ACID SOLUTION Pteroylglutamic acid was dried over anhydrous $CaCl_2$ and 10 mg. was dissolved in 100 ml. 0·001N NaOH solution and stored in a stoppered bottle at 0°C. Comparisons of the growth of the test organism with known amounts of folic acid from fresh and stored solutions showed that there was no appreciable deterioration in the solutions for up to 17 weeks. Nevertheless a fresh solution was prepared every four to six weeks.

METHOD OF ASSAY Assays were carried out in 5 in. × $\frac{5}{8}$ in. Pyrex test tubes, using four tubes containing identical samples for each standard and each dilution of each unknown. The standards, prepared from dilutions in water of the stock standard solution, contained 0, 0·05, 0·1, 0·2, 0·4, 0·6, 0·8, and 1·0 mμg. folic acid per tube respectively. Serum extracts (0·25 and 0·5 ml. per tube, or 0·5 ml. and 1 ml. per tube for samples expected to have low folic acid activity) were pipetted into other tubes and the volume of aqueous solution in every tube was made up to 1 ml. with water. Medium (4 ml.) was added to each tube with a dispensing syringe (Arnold R. Horwell Ltd.), the tubes were covered with aluminium caps, autoclaved at 10 lb. pressure for six minutes and allowed to cool.

MAINTENANCE OF CULTURES AND INOCULATING AND READING THE ASSAYS The test organism was *Lactobacillus casei* var. *rhamnosus* (National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, catalogue no. NCIB 6375). The inoculum for assays was prepared using a liquid medium prepared by a modification of the recipe for the complete medium. Ascorbic acid was omitted, 1 μg. pteroylglutamic acid was added, and the volume was made up to 200 ml. with water. The solution (10 ml.) was dispensed into 6 in. × $\frac{3}{4}$ in. tubes which were plugged with cotton wool and autoclaved at 10 lb. pressure for six minutes.

After recovering organisms from the freeze-dried culture using this medium, stab cultures were maintained in agar (Nymon and Gortner, 1946), transferred fortnightly and stored at 0°C. On the evening before setting up an assay, a tube of liquid medium was inoculated from a stab and incubated overnight at 37°C. Next morning 1 ml. of the suspension was added to a tube of sterile medium at 37°C. This culture was incubated for four to six hours, centrifuged, the medium was decanted and the pad of organisms was washed four times by decantation with sterile water and re-suspended in sterile water. The suspension was diluted with sterile water until opacity was only just visible, one drop of the diluted suspension was added to each assay tube, and all tubes were incubated for 40 to 44 hours at 37°C.

After incubation the contents of the tubes were shaken and the optical densities were read on a Hilger Spekker photoelectric absorptiometer with neutral grey filters, against a blank of uninoculated medium. The mean density from each set of four tubes was used to calculate the results. The folic acid activity of each dilution of each unknown was computed from a standard curve (Fig. 1) relating the amounts of folic acid to the densities for the standards. The final result was the mean value, expressed to two significant figures, from the two dilutions of each sample.

RESULTS

GROWTH RESPONSE Figure 1 shows standard curves obtained after 40 hours' incubation in different media. These curves were not obtained in the same experiment, but they are typical of curves obtained in several different experiments using different media.

EFFECT OF ASCORBIC ACID AND PHOSPHATE BUFFER ON GROWTH Waters and Mollin (1961) found that autoclaved mixtures of ascorbic acid and phosphate buffer did not affect the growth response. Using my medium without added ascorbic acid, growth was stimulated by such mixtures, but there was no stimulation when the medium contained ascorbic acid.

AMOUNT OF ASCORBIC ACID NEEDED TO PROTECT THE FOLIC ACID ACTIVITY IN SERUM DURING AUTOCLAVING Ten samples of serum were extracted without ascorbic acid and with increasing amounts of ascorbic

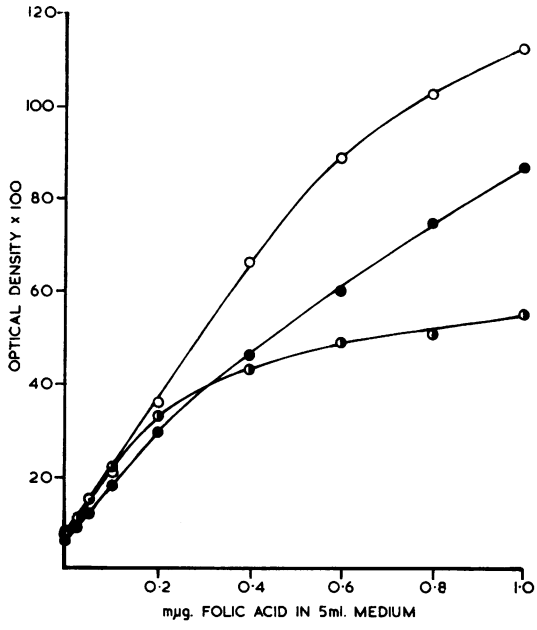

FIG. 1. *The growth response of* Lactobacillus casei *to pteroylglutamic acid in different basal media, all with 40 hours' incubation.*
◑ *represents the medium described by Waters and Mollin (1961);* ○ *represents the medium described in this paper;* ● *represents the medium described in this paper without ascorbic acid. These curves were not obtained in the same experiment, but are representative of results from several experiments under the above conditions.*

acid. The levels of ascorbic acid were equivalent to the 50, 100, 150, and 200 mg. ascorbic acid per 100 ml. buffer used by Waters and Mollin (1961). Full protection was obtained with 4·5 mg. ascorbic acid per ml. serum (Table II).

APPARENT FOLIC ACID ACTIVITY FROM DIFFERENT QUANTITIES OF SERUM EXTRACT The folic acid activity in serum is not due to pteroylglutamic acid which is used for the standards, so that different amounts of serum extract might give different results. The values from different quantities of the extracts from 11 samples of serum, using different amounts of ascorbic acid in the extractions, were similar (Table III).

TABLE II

YIELD OF FOLIC ACID ACTIVITY FROM SERUM USING DIFFERENT AMOUNTS OF ASCORBIC ACID IN THE EXTRACTION

Experiment No.	*Serum*	*Ascorbic Acid Added (mg./ml. serum)* 0	4·5	9	13·5	18
		Folic Acid Activity (mμg./ml. serum)				
1	Ho	1·6	8·4	8·4	8·0	—
	Maz	0·7	5·6	5·6	5·8	—
2	Al	1·0	8·3	7·9	8·1	7·4
	Mal	0·4	7·4	6·7	7·3	7·0
	Pit	0·6	6·3	6·1	5·9	6·1
	Sp	0·6	4·0	4·2	4·0	3·8
3	Br	1·2	5·6	5·4	5·3	5·6
	Ch	0·6	5·3	5·8	5·3	5·4
	Wr	0·8	4·8	4·5	4·6	4·5
4	Bo	0·7	8·0	8·0	7·6	8·0

EFFECT OF AUTOCLAVING SERUM AT HIGHER DILUTIONS Chanarin and Berry (1964) reported that when serum was diluted more than 1 in 10 with phosphate buffer containing ascorbic acid before autoclaving, the apparent folic acid activity increased. Samples of serum from six patients were therefore autoclaved at dilutions of 1 in 10, 1 in 20, 1 in 40, and 1 in 80, using the same concentration of ascorbic acid and buffer in each dilution. Under the present conditions the dilution of the serum had no consistent effect on the results (Table IV).

RECOVERY OF ADDED FOLIC ACID FROM SERUM When pteroylglutamic acid was added to 11 samples of serum, recoveries of between 64% and 116% were obtained (Table V). The mean recovery with 5

TABLE III

APPARENT FOLIC ACID ACTIVITY OF SERUM USING DIFFERENT AMOUNTS OF SERUM EXTRACTS

Experiment No.	*Ascorbic Acid (mg. per ml.) Serum in Extraction*	*Serum*	*Serum Extract (ml.) in 5 ml. Medium* 0·25	0·5	1·0
			Apparent Folic Acid Activity (mμg./ml.)		
1	9	Al	7·6	7·9	8·3
		Mal	6·6	6·7	7·1
		Pit	6·0	6·1	6·2
		Sp	3·8	4·2	4·2
2	4·5	Br	5·5	5·6	5·9
		Ch	5·8	5·3	5·7
		Wr	5·2	4·8	5·4
3	4·5	Bo	8·9	8·0	8·4
	9	Bo	8·9	8·0	8·2
4	4·5	Bun	9·6	8·4	7·7
5	4·5	Wr	2·8	3·2	3·6
6	4·5	Fa	9·6	9·8	9·0

TABLE IV

FOLIC ACID ACTIVITY OF SERUM AUTOCLAVED AT DIFFERENT DILUTIONS

Experiment No.	*Serum*	*Dilution of Serum before Extraction* 1:10	1:20	1:40	1:80
		Apparent Folic Acid Activity (mμg./ml.)			
1	Da	2·4	2·4	1·7	—
	McD	16	17	18	—
2	Gr	12	13	14	14
	How	8·0	8·2	8·7	—
	McD	17	19	20	21
3	Wi	12	11	11	10
4	Cha	9·2	8·7	8·4	7·7

mμg./ml. added was 88%, and with 10 mμg./ml. added, 98%.

REPRODUCIBILITY OF RESULTS IN DIFFERENT ASSAYS Sixty samples of serum were assayed on successive days. The ratio

$$\frac{\text{Difference between the two results}}{\text{Mean of the two results}} \times 100$$

varied from 0·2% to 39% (mean 12%).

TABLE V

RECOVERY OF ADDED PTEROYLGLUTAMIC ACID FROM SERUM

Experiment No.	*Serum*	*Pteroylglutamic Acid Added (mμg./ml. serum)* 5	10
		Folic Acid Activity Recovered (mμg./ml. serum)	
1	Bo	4·9	9·5
2	Ban	4·8	—
	Bun	5·1	10
	Mas	4·0	11
3	Pin	5·8	9·9
4	McN	3·8	10
	Wr	3·2	8·4
5	Hy	4·4	9·3
	Sta	4·3	8·8
6	Fl	4·4	10
	Pr	3·6	11
Means		4·38	9·79

STORAGE OF SERUM Sixteen sera were assayed when fresh and during storage at −15°C. without ascorbic acid, using 4·5 mg. ascorbic acid per ml. serum in the extractions. The folic acid activity decreased at rates which varied from sample to sample (Table

TABLE VI

EFFECT OF STORAGE AT −15°C. ON THE FOLIC ACID ACTIVITY OF SERUM

Serum	*Folic Acid Activity (mμg) per ml. serum. at the Following Times (weeks) after Taking* 0	2	4	8	12
(a) Samples stored without added ascorbic acid					
Hy	5·3	—	4·0	3·2	1·4 (1·6)[1]
Sta	7·1	—	5·9	6·1	2·2
Bai	7·6	—	5·9	1·6	—
Tr	8·6	—	7·1	2·9	—
Bal	12	—	12	3·3	—
Es	6·0	—	5·3	1·7	—
Fl	5·8	—	3·2	1·6 (1·9)[1]	—
Har	11	—	9·9	3·3 (4·0)[1]	—
Hi	11	—	10	3·2 (5·0)[1]	—
Pr	6·7	—	5·7	1·9	—
Ca	7·2	—	2·7	2·0 (2·2)[1]	—
Hai	12	—	8·4	3·0	—
Ho	11	—	5·4	3·3 (4·2)[1]	—
Le	16	—	9·1	4·6 (6·4)[1]	—
Sa	4·9	—	2·5	1·4	—
Ve	9·8	—	5·0	2·6	—
(b) Samples stored with ascorbic acid (5 mg./ml. serum) added					
He	10	7·9	8·7	12	9·0
Qu	5·0	4·5	4·8	7·1	5·5
Ro	6·2	5·1	6·0	8·1	6·4
Han	10	9·3	11	16	12
Bur	5·7	5·6	6·4	—	5·5
Be	6·4	7·2 (7·2)[2]	8·1 (7·7)[2]	7·0 (4·3)[2]	6·5 (4·0)[2]
Wo	3·8	4·2 (4·5)[2]	4·9 (3·2)[2]	4·3 (2·7)[2]	3·9 (2·5)[2]
Fi	9·5	9·9	12	9·4	8·6
La	4·4	4·6 (3·7)[2]	— (5·4)[2]	3·1 (3·2)[2]	3·7 (2·2)[2]
Fo	5·7	5·7 (3·3)[2]	6·8 (6·8)[2]	5·8 (3·6)[2]	3·9 (2·5)[2]
Ri	4·5	4·9 (3·0)[2]	5·8 (3·7)[2]	4·9 (2·9)[2]	3·7 (2·1)[2]
Sti	9·2	11 (11)[2]	—	8·3 (5·2)[2]	9·5 (6·5)[2]
Stn	21	19 (19)[2]	28 (19)[2]	21 (11)[2]	19 (10)[2]
Gr	5·9	5·5	8·6	7·2	5·4
No	22	22 (20)[2]	21 (18)[2]	21 (14)[2]	23 (17)[2]
Wy	9·4	10 (8·2)[2]	11 (5·6)[2]	12 (5·6)[2]	9·9 (5·4)[2]

[1]Results from portions of the same sample extracted with 18 mg. ascorbic acid per ml. serum.
[2]Results from batches of the original samples stored without added ascorbic acid.

VIa). Seven duplicate samples were extracted using 18 mg. ascorbic acid per ml. serum. The results were higher than those using 4·5 mg. ascorbic acid per ml. but the lost activity was not fully restored.

Sixteen more samples were assayed when fresh and during storage at −15°C. with 5 mg. ascorbic acid added per ml. serum. Portions of nine of these samples were stored without ascorbic acid. No loss of activity occurred for up to 12 weeks in the samples stored with ascorbic acid, but there was some loss in all the samples stored without ascorbic acid (Table VIb). For reasons which are not understood the variation between the values on the samples stored with ascorbic acid was often greater than that between the results from samples assayed on successive days.

RESULTS FROM CONTROL SUBJECTS AND PATIENTS WITH MEGALOBLASTIC ANAEMIA These results are summarized in Table VII.

Control subjects Sera from 94 healthy control subjects were examined, 46 men and 48 women aged between 17 and 83, who were members of staff, medical students, or visitors to patients on medical wards. Since blood may be taken from patients at any time, no consideration was given to the times at which the control samples were taken in relation to meals or similar factors. The results varied from 2·1 to 28 mμg./ml. (mean 7·8); except for the value of 28 mμg./ml., the highest figure was 17 mμg./ml. Sixty-four results are the means of two values from independent assays on the same sample of serum and the remainder are from one assay only.

Patients with clinical evidence of folic acid deficiency Only 10 patients with megaloblastic anaemia thought clinically to be due to folic acid deficiency have been available for study. The results from nine patients were 1·0 mμg./ml. or less. The other patient, a man aged 70 who had had a gastrectomy and had polycythaemia vera, gave a value of 2·0 mμg./ml., which is just below the control range but is equivocal because of the inaccuracy of the method. Serum vitamin B_{12} values were obtained on eight of these patients and only one result was below the control range.

Megaloblastic anaemia of pregnancy and the puerperium Four of the six results (0·7, 1·1, 1·1, and 1·5 mμg./ml.) were below the control range, and two values (2·8 and 4·0 mμg./ml.) were in the lower control range.

Untreated pernicious anaemia In 31 patients the results varied from 1·8 to >40 mμg./ml. The result which was above 40 mμg./ml. was not repeated because the serum was stored without ascorbic acid and the activity had decreased when the sample was re-assayed. The mean from the other 30 patients was 12·6 mμg./ml. Three patients gave values of 1·8 mμg./ml., just below the control range.

DISCUSSION

Under the conditions described *Lactobacillus casei* shows a greater growth response to pteroylglutamic acid than was obtained using the medium described by Waters and Mollin (1961). Compared with these authors' medium, the present medium includes ascorbic acid and added tryptophane but contains no Tween 80 or glutathione; it contains twice as much adenine, guanine, and uracil but less cysteine. Over three times less sodium acetate but five times more KH_2PO_4 and K_2HPO_4 are added, and it contains four times more Mg and Fe and a little more Mn, though some metals may be precipitated during the preparation of solution A. The amounts of vitamins are identical in both media. The increased growth response is advantageous in increasing the sensitivity of the estimations.

TABLE VII

FOLIC ACID ACTIVITY IN THE SERUM OF CONTROL SUBJECTS AND PATIENTS WITH MEGALOBLASTIC ANAEMIA

Group	*No. of Subjects*	*Folic Acid Activity (mμg./ml. serum)*		*Vitamin B_{12} Activity*[1] *(μμg./ml. serum)*
		Mean	*Observed Range*	
Control subjects	94	7·8	2·1-28	Not determined
Megaloblastic anaemia due to folic acid defficiency				
Malabsorption syndrome	5	0·6	0·4-1·0	160-580
Post-gastrectomy steatorrhoea	1	2·0		430
Anticonvulsant therapy	1	0·6		——
Nutritional deficiency	1	0·3		——
Uncertain origin, free acid in gastric juice	1	0·9		92
Uncertain origin, responding clinically to folic acid but not to vitamin B_{12}	1	0·9		170
Megaloblastic anaemia of pregnancy and the puerperium	6	1·9	0·7-4·0	160-360
Untreated pernicious anaemia	31	>13·4	1·8->40	<10-95

[1]Assayed with *Lactobacillus leichmannii* as test organism (Spray, 1955).

In addition to the effect of ascorbic acid in increasing growth response and eliminating stimulation by the ascorbic acid and phosphate buffer in the serum extracts, its inclusion in the medium seems logical to protect the folic acid activity in the serum extracts during autoclaving of the medium. This may explain why 4·5 mg. ascorbic acid per ml. serum gave full protection to the activity (Table II). Waters and Mollin (1961) recommended 9 mg./ml., and later (Waters and Mollin, 1963) used 18 mg./ml., and Herbert (1961) advocated 13·5 mg./ml. Under these authors' conditions protection of the activity during autoclaving of the medium would depend on the ascorbic acid carried over in the serum extracts.

The use of two dilutions for each sample of serum nearly doubles the work but gives greater confidence in the results and could help to detect inhibitors which might be present in an extract. The data on the recovery of added pteroylglutamic acid from serum, and on the reproducibility of the results in different assays, are comparable with those of Waters and Mollin (1961). Serum for this estimation should have 5 mg. ascorbic acid added per ml. if it is to be stored before assay, otherwise the folic acid activity will decrease at an unpredictable rate (Table VI). This agrees with the findings of Waters and Mollin (1961), but I could not confirm their claim, which was later withdrawn (Waters and Mollin, 1963), that the activity lost during storage was restored by autoclaving with large amounts of ascorbic acid.

The results from control subjects by this method are lower than those of Waters and Mollin (1961), whose range was 5·9—21 mμg./ml. (mean 9·9). Of the present control results, 38 (40%) were below 6·0 mμg./ml. and 25 (27%) were below 5·0 mμg./ml. The reasons for the differences are not clear, but normal ranges of 2·7—18·5 mμg./ml. (Davis and Kelly, 1962) and 3·2—15 mμg./ml. (Grossowicz, Mandelbaum-Shavit, Davidoff, and Aronovitch, 1962) have been reported by others. The values from patients with megaloblastic anaemia are comparable with those of other authors when considered in relation to the corresponding control ranges. Thus nine out of 10 patients with clinical evidence of folic acid deficiency gave unequivocally low results, and the results in megaloblastic anaemia of pregnancy were below or just within the normal range, as found by Waters and Mollin (1961). Most values in untreated pernicious anaemia were normal or high, with a higher mean than normal, but three patients out of 31 gave values just below the normal range. Cooper and Lowenstein (1961) found low values in two patients with untreated pernicious anaemia, and Waters and Mollin (1961) obtained one low value among 42 such patients.

This study was designed to obtain an independent assessment of the value of the estimation on isolated samples of serum as a diagnostic aid. The results so far obtained indicate that the method offers some advantages over previous methods and differentiates between folic acid deficiency and deficiency of vitamin B_{12} in most patients.

I am grateful to Professor L. J. Witts in whose Department this work was done; to Miss Susan Mason, Mrs. Vera Ilic, and Miss Beverley Symonds who have assisted in turn with the laboratory work; and to the physicians and haematologists in Oxford and elsewhere who have supplied serum and clinical details on the patients included in the study.

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