Serum folates in man

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SUMMARY   In an aseptic microbiological assay of folate compounds and their breakdown compounds,
using *Lactobacillus casei*, *Streptococcus faecalis*, and *Pediococcus cerevisiae*, 4a-hydroxy-5-methyl-
4,5,6,7-tetrahydrofolate and 5-methyl-5,8-dihydrofolate were inactive under all conditions to all
three organisms and 5-methyl-5,6-dihydrofolate was inactive unless ascorbate was present in the
incubation medium, and then only to *L. casei*. 5-Methyltetrahydrofolate was active only for *L. casei*,
and activity in purified samples to *S. faecalis* was due to trace amounts of folic acid. Analysis of
S. faecalis values in the serum in normal subjects and in patients with various disorders showed
that levels of 10-formyltetrahydrofolate are raised in coeliac disease, leukaemia, rheumatoid
arthritis, and schizophrenia.

5-Methyltetrahydrofolate is readily absorbed by normal human subjects and by patients with
pernicious anaemia but poorly absorbed by patients with coeliac disease or leukaemia. 5-Methyl-
5,6-dihydrofolate was quickly absorbed by normal human subjects, being reflected by a considerably
raised level of 5-methyltetrahydrofolate in serum when sodium bicarbonate was given by mouth
before the 5-methyl-5,6-dihydrofolate. These higher levels were comparable to those in patients
with pernicious anaemia after oral administration of 5-methyl-5,6-dihydrofolate. Oral 5-methyl-
5,8-dihydrofolate and 4a-hydroxy-5-methyl-tetrahydrofolate did not appear as microbiologically
active folates in the serum.

The findings of this study suggest that the availability for biological utilisation of the major
dietary folate compounds will depend on the amount of gastric acidity and of ascorbate in the
intestinal chyme. Many may be unavailable for metabolic utilization in the body.

The folates present in foodstuffs before degradation
are 5-methyltetrahydrofolate and 10-formyltetra-
hydrofolate and their derivatives. Both compounds
are readily oxidised, the rates varying directly with
oxygen concentration, temperature, alkalinity, ex-
posure to light, and concentration of cupric and
ferric ions (Pearson, 1974; Blair and Pearson, 1974a,
1974b, Blair *et al.*, 1975). 5-Methyltetrahydrofolate
is oxidised to 5-methyl-5,6-dihydrofolate, which can
then rearrange to 5-methyl-5, 8-dihydrofolate at pH
4·0 or is oxidised to 4a-hydroxy-5-methyl-4a,5,6,7-
tetrahydrofolate (Fig. 1) (Larrabee *et al.*, 1961;
Donaldson and Keresztesy, 1962; Gupta and
Huennekens, 1967; Blair *et al.*, 1975). 10-Formyl-
tetrahydrofolate is oxidised to 10-formylfolate
(Robinson, 1971). Consequently there will be a
number of folate compounds present in the diet,

some of which may not be available for biological
utilization in the body but may be microbiologically
active for *L. casei*, *S. faecalis*, or *P. cerevisiae*.

In this paper we report the availability of the
folates derived from 5-methyltetrahydrofolate and
their metabolism in normal humans and in some
diseases. A further analysis of the *S. faecalis* values
in the serum of normal and diseased subjects is
reported.

Material and methods

5-Methyltetrahydrofolate was prepared by the method
of Blair and Saunders (1970), *4a-hydroxy-5-methyl-
4a,5,6,7-tetrahydrofolate* as given by Gapski *et al.*
(1971), and *7,8-dihydrofolate acid* as described
by Futterman (1963).

    5-Methyl-5,6-dihydrofolate was prepared by dis-
solving 400 mg of 5-methyltetrahydrofolate in 200 ml

5 - methyltetrahydrofolate

oxidation

5 - methyl - 5, 6 - dihydrofolate

4a - hydroxy - 5 - methyl - 4a, 5, 6, 7 - tetrahydrofolate

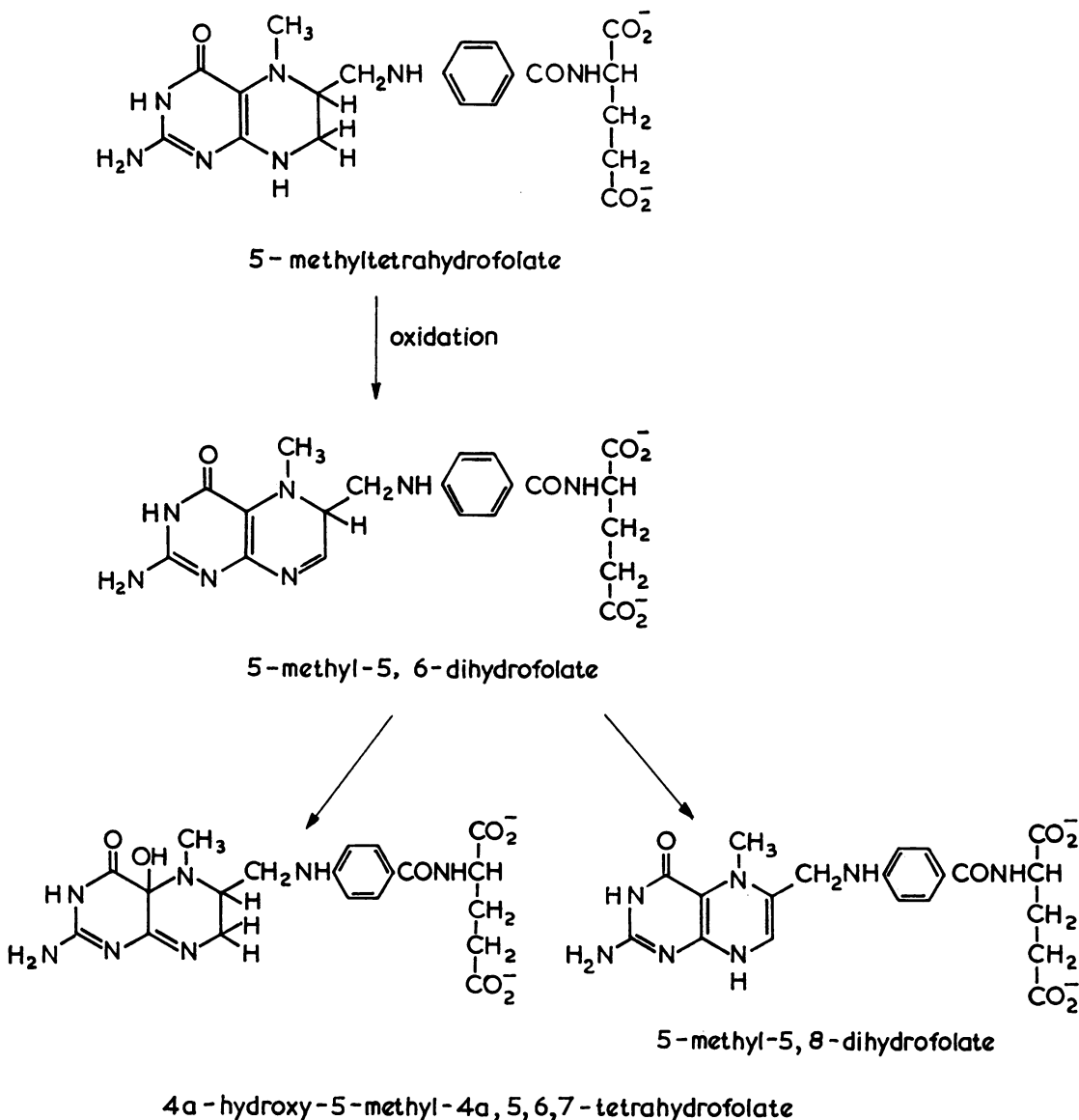5 - methyl - 5, 8 - dihydrofolate

Fig. 1   *5-Methyltetrahydrofolate and its derivatives.*

of water containing 5 mg of copper sulphate. The mixture was stirred under a slow stream of oxygen for 60-100 minutes and the reaction progress followed by the appearance of the characteristic ultraviolet spectrum of 5-methyl-5,6-dihydrofolate with maxima at 248 and 290 nm in 0·1 M phosphate buffer pH 7·0. The solution of 5-methyl-5,6-dihydrofolate was freeze-dried to yield a puffy yellow solid product of

90% purity as judged by ultraviolet spectroscopy. The crude product was then further purified as described by Gapski *et al.* (1971).

5-Methyl-5,8-dihydrofolate was prepared by acidic rearrangement of 5-methyl-5,6-dihydrofolate. A 5-mg sample of microbiologically active material of 5-methyl-5,6-dihydrofolate was dissolved in 0·1 M HCl 30 minutes before administration to normal

human volunteers. The resultant acidified samples of 5-methyl-5,8-dihydrofolate were neutralised with sodium hydroxide solution just before use.

MICROBIOLOGICAL ASSAY

Microbiological assays of 5-methyl-5,6-dihydrofolate; 5-methyl-5,8-dihydrofolate; 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydrofolate; and of the serum samples were performed aseptically using *L. casei*, *S. faecalis*, and *P. cerevisiae* (Herbert, 1966).

Since 5-methyl-5,6-dihydrofolate is microbiologically active for *L. casei* in the presence of ascorbate but inactive when ascorbate is omitted its presence cannot be detected in serum or plasma with added ascorbate, but it can be determined after its rapid conversion in acid to the microbiologically inactive 5-methyl-5,8-dihydrofolate by the difference in *L. casei* values in their two situations.

Blood samples from normal human volunteers and patients were separated quickly (30 minutes clotting time) to avoid oxidation and the sera divided into two equal portions. The first portion was adjusted to a pH of 3·0 with concentrated HCl and allowed to stand in the dark at room temperature for 20 minutes. At the end of the 20-minutes period a small amount of 20% (w/v) sodium hydroxide solution was added to bring the pH of the sample to the starting pH of around 7·4 and ascorbate (5 mg/ml) added. To the second portion an amount of distilled water was added equal to the volume of added acid and base and ascorbate (5 mg/ml). These samples were kept at −20°C until assayed microbiologically, which in all cases was within a week.

SERUM VALUES

Single blood samples were taken after a normal lunch from 52 normal persons and 353 patients with serum folate levels >3·0 μg/l. The patients were suffering from the following: rheumatoid arthritis (25); leukaemia (79); coeliac disease, treated and untreated (74); schizophrenia (7); treated pernicious anaemia (18); psoriasis (6); and regional enteritis (144). Likewise blood samples were taken from 19 normal persons and 190 patients with serum folate levels <3·0 μg/l. The patients were suffering from the following: coeliac disease (30); psoriasis (5); leukaemia (31); untreated pernicious anaemia (2); rheumatoid arthritis (22); regional enteritis (90); and schizophrenia (10).

Fasting blood samples were taken from eight patients with folic acid deficient megaloblastic anaemia.

Fasting blood samples were taken from five normal persons at hourly intervals for 10 hours.

ABSORPTION TESTS

After an overnight fast the serum levels of folate compounds after oral administration of 5-methyltetrahydrofolate were measured in 15 normal volunteers, two patients with untreated pernicious anaemia, six patients with untreated coeliac disease, and eight patients with chronic granulocytic leukaemia. Also the serum levels of 5-methyl-5,6-dihydrofolate were measured in five normal volunteers and five patients with untreated pernicious anaemia. The first venous blood samples were taken aseptically just before giving the test material. Further samples were taken while still fasting at 30, 60, 90, 120, and 180 minutes. Blood was allowed to clot, centrifuged, and ascorbate was added (2-5 mg/ml) to the separated serum and kept at −20°C until assayed.

The serum levels after oral administration of 10 mg 4a-hydroxy-5-methyltetrahydrofolate, 5-methyl-5,8-dihydrofolate, and dihydrofolate were similarly studied.

To study the effect of altering the gastric pH a dose of 3 or 5 g sodium bicarbonate was administered before additional tests, and to prevent oxidation 1 g ascorbic acid was similarly given in other experiments.

Results

MICROBIOLOGICAL ACTIVITY OF FOLATE COMPOUNDS

Aseptic microbiological assay of 5-methyl-5,6-dihydrofolate; 5-methyl-5,8-dihydrofolate; and 4a-hydroxy-5-methyl-4,5,6,7-tetrahydrofolate with all three micro-organisms in the presence of ascorbate showed that only the first compound was microbiologically active, and then only for *L. casei*, with equivalent activity to that of 5-methyltetrahydrofolate. The other two compounds were inactive for all three micro-organisms. When ascorbate was omitted from the incubation medium 5-methyl-5,6-dihydrofolate was also inactive for the three micro-organisms.

Bioautography of 1-μg samples of 5-methyltetrahydrofolate in 3% aqueous ammonium chloride using *L. casei* and *S. faecalis* as developing organisms (Leeming *et al.*, 1970; Brown *et al.*, 1973) showed a little *L. casei* activity (3-10%) at R_F 0·0 (folic acid) and much activity at R_F 0·65 (5-methyltetrahydrofolate) but *S. faecalis* activity only at R_F 0·0 and none elsewhere—that is, *S. faecalis* activity associated with highly purified samples of 5-methyltetrahydrofolate was due to trace amounts of folic acid.

The difference between the *S. faecalis* and *P. cerevisiae* values was nil at short clotting times (Table 1). The *S. faecalis* value remained constant over six hours while that of *P. cerevisiae* steadily declined from the same as the *S. faecalis* value to

60% of it after eight hours (Table 1). Since the only known *P. cerevisiae*-active folate which would rapidly decompose to yield an equivalent and stable *S. faecalis* activity is 10-formyltetrahydrofolate, this provides further evidence that 10-formyltetrahydrofolate is a folate present in serum. The *S. faecalis* value would therefore seem to be the best measure of 10-formyltetrahydrofolate in the serum of normal subjects. However, when oral doses of other folates are given, folic acid may be present as an impurity, so that levels of 10-formyltetrahydrofolate are best represented in such experiments by the *P. cerevisiae* values.

SERUM VALUES

Table 2 compares the serum *S. faecalis* values of normal and diseased subjects whose *L. casei*-active

folates ranged from 3·0 μg/l to 20 μg/l in the non-fasting state. Table 3 is a similar comparison of those with serum folates below 3·0 μg/l. In many cases the *S. faecalis* level is significantly increased in the diseased state. The diurnal variation experiment showed a varying level of serum folates in man (Fig. 2), and though the differences in levels are not significant the existence of a diurnal variation is established by the smooth curve obtained.

Measurement of the 5-methyltetrahydrofolate component in normal persons in this study showed it to contain a small amount of 5-methyl-5,6-dihydrofolate (Table 4) in some subjects but not in others, suggesting a possible analytical artefact. In pernicious anaemia the increase in the level of serum 5-methyl-5,6-dihydrofolate was significant and too large to be an artefact.

Table 1 *Effect of clotting time* (T) *on serum folates (mean ± SEM of three samples)*

Test organism	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	$T_{2\frac{1}{2}}$	T_3	T_4	T_6	T_8
L. casei	7·0	7·0	6·8	7·0	7·2	7·0	6·7	6·7	6·8
	±0·7	±0·7	±0·7	±0·7	±0·8	±0·8	±0·8	±0·8	±0·8
S. faecalis	0·8	0·8	0·8	0·8	0·8	0·8	0·8	0·8	0·8
	±0·1	±0·1	±0·1	±0·1	±0·1	±0·1	±0·1	±0·1	±0·1
P. cerevisiae	0·8	0·7	0·65	0·6	0·65	0·6	0·6	0·6	0·6
	±0·1	±0·1	±0·1	±0·1	±0·1	±0·1	±0·1	±0·1	±0·1

T = time in hours.

Table 2 *Serum folate levels as microbiologically assayed with* L. casei *and* S. faecalis *of normal subjects and patients with* L. casei *values of 3·0-20 μg/l. All samples taken in early afternoon after usual diet*

Diagnosis	No. of subjects	L. casei (μg/l ± SEM)	Student's t test (P)	S. faecalis (μg/l ± SEM)	Student's t test (P)
Normal	52	5·57 ± 0·28	—	0·78 ± 0·03	—
Adult coeliac disease	74	6·10 ± 0·40	NS	1·00 ± 0·06	0·005
Psoriasis	6	4·73 ± 0·50	NS	0·95 ± 0·14	0·100
Leukaemia	79	5·56 ± 0·28	NS	1·65 ± 0·10	0·001
Pernicious anaemia*	18	6·50 ± 0·70	NS	0·89 ± 0·10	NS
Rheumatoid arthritis	25	4·72 ± 0·36	0·10	1·85 ± 0·30	0·001
Regional enteritis	144	5·29 ± 0.22	NS	0·84 ± 0·03	NS
Schizophrenia	7	3·94 ± 0.40	0·005	0·94 ± 0·10	0·05

NS = not significant.
*Receiving vitamin B_{12}.

Table 3 *Comparison of serum folate levels of normal subjects and patients with* L. casei *values <3·0 μg/l. All samples taken in early afternoon after usual diet*

Diagnosis	No. of subjects	L. casei (μg/l ± SEM)	Student's t test (P)	S. faecalis (μg/l ± SEM)	Student's t test (P)
Normal	19	2·00 ± 0·20	—	0·40 ± 0·06	—
Adult coeliac disease	30	1·85 ± 0·15	NS	0·90 ± 0·08	0·001
Psoriasis	5	1·72 ± 0·18	NS	1·14 ± 0·18	0·001
Leukaemia	31	1·67 ± 0·14	0·10	1·03 ± 0·10	0·001
Pernicious anaemia*	2	1·50	NS	0·70	0·05
Rheumatoid arthritis	22	2·30 ± 0·10	NS	1·29 ± 0·15	0·001
Regional enteritis	90	1·50 ± 0·07	0·05	0·38 ± 0·03	NS
Schizophrenia	10	1·65 ± 0·18	0·10	0·84 ± 0·10	0·001

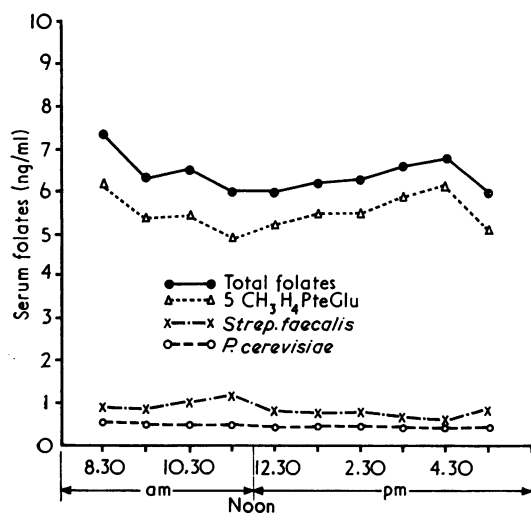*Receiving vitamin B_{12}.
NS = not significant.

Fig. 2 *Diurnal variation of serum folates of fasting normal persons during working day.*

Eight patients with megaloblastic anaemia had a mean cell volume of 108 ± 1.5 μm^3 and serum 10-formyltetrahydrofolate of 0.16 ± 0.04 $\mu g/l$, significantly less than that in normal subjects (Tables 2 and 3). Patients with coeliac disease, leukaemia, rheumatoid arthritis, and schizophrenia had significantly increased levels of 10-formyltetrahydrofolate, but those with regional enteritis and pernicious anaemia had levels not significantly different from normal. Patients with psoriasis had significantly raised levels of 10-formyltetrahydrofolate when the total serum folate was <3.0 $\mu g/l$, but when the serum folate was >3.0 $\mu g/l$ the level of 10-formyltetrahydrofolate when the total serum folate was tetrahydrofolate, although raised, was not significantly different from normal.

ABSORPTION TESTS

Microbiologically active 5-methyl-5,6-dihydrofolate 5 mg by mouth quickly appears in the serum (Table 5) and at significantly higher levels when given with sodium bicarbonate (Table 6). At the same time 5-methyl-5,6-dihydrofolate is converted at some stage after administration to 5-methyltetrahydrofolate. The resultant serum levels of this latter compound are still further enhanced by the simultaneous administration of sodium bicarbonate, the differences being significant by Student's t test at 60, 90, 120, and 180 minutes after ingestion (Table 6) (P < 0.01).

Serum levels after the oral administration of 5-methyltetrahydrofolate are shown in Table 7 and also with the simultaneous ingestion of ascorbate (Table 8) and of bicarbonate (Tables 9 and 10), when significantly higher serum levels were found. Oral administration of 5-methyl-5,8-dihydrofolate (Table 11) and 4a-hydroxy-5-methyl-4a,5,6,7-tetrahydrofolate (Table 12) showed little rise in folate serum levels. Administration of 7,8-dihydrofolate gave a marked increase in total serum folates but not serum 10-formyltetrahydrofolate (Table 13). Studies in patients with pernicious anaemia after oral 5-methyltetrahydrofolate (Table 14) and after 5-methyl-5,6-dihydrofolate (Table 15) showed that significant levels of the relevant folates were readily attained. Oral administration of 5-methyl-5,6-dihydrofolate to patients either with coeliac disease (Table 16) or with leukaemia (Table 17) showed significantly reduced serum levels compared with similar experiments in normal subjects.

Discussion

Although published information on the serum levels of folate in normal and diseased subjects after oral administration of folic acid is considerable, little is known about the levels after the administration of 5-methyltetrahydrofolate and its decomposition products 5-methyl-5,6-dihydrofolate, 4a-hydroxy-5-

Table 4 *Fasting serum 5-methyl-5,6-dihydrofolate levels in normal subjects and those with various diseases*

Diagnosis	No. of subjects	L. casei ($\mu g/l \pm SEM$)		5-CH$_3$-5,6-H$_2$-PteGlu ($\mu g/l \pm SEM$)	5-CH$_3$H$_4$-PteGlu* ($\mu g/l \pm SEM$)
		Before acid treatment	After acid treatment		
Normal	30	5.75 ± 0.48	5.00 ± 0.63	0.75 ± 0.15	4.95 ± 0.40
Leukaemia	5	5.18 ± 0.50	4.50 ± 0.93	0.68 ± 0.43	4.00 ± 0.45
Regional enteritis	8	4.78 ± 0.38	4.47 ± 0.63	0.31 ± 0.25	4.00 ± 0.35
Pernicious anaemia†	4	9.50 ± 4.30	5.50 ± 2.90	4.00 ± 1.41	8.70 ± 4.20
Rheumatoid arthritis	30	3.3 ± 0.28	2.42 ± 0.44	0.88 ± 0.16	1.80 ± 0.20
Adult coeliac disease	4	6.4 ± 1.00	5.55 ± 0.50	0.85 ± 0.15	5.40 ± 0.90

*Summation of 5-CH$_3$H$_4$PteGlu and 5-CH$_3$-5,6-H$_2$PteGlu activities.
†Receiving vitamin B$_{12}$.

Table 5   Serum folate levels in five normal persons after oral administration of 10 mg of 5-methyl-5,6-dihydrofolate

Serum folate	Serum folate levels ($\mu g/l \pm SEM$)							
	T_0	$T_\frac{1}{2}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3	T_4*	T_6*
Total folates	$6\cdot2 \pm 1\cdot3$	$37\cdot6 \pm 5\cdot9$	$32\cdot8 \pm 4\cdot5$	$27\cdot8 \pm 2\cdot0$	$23\cdot6 \pm 0\cdot9$	$21\cdot8 \pm 0\cdot8$	$21\cdot0$	$20\cdot0$
5-CH_3H_4PteGlu†	$5\cdot5 \pm 1\cdot3$	$36\cdot6 \pm 5\cdot9$	$31\cdot6 \pm 4\cdot6$	$26\cdot8 \pm 2\cdot0$	$22\cdot7 \pm 0\cdot9$	$21\cdot1 \pm 0\cdot2$	$20\cdot0$	$19\cdot0$
5-CH_3-5,6-H_2PteGlu*	$0\cdot0$	$2\cdot0$	$1\cdot0$	$1\cdot0$	$1\cdot0$	$1\cdot0$	$0\cdot5$	$0\cdot5$
10-CHO H_4PteGlu‡	$0\cdot6 \pm 0\cdot2$	$0\cdot9 \pm 0\cdot2$	$1\cdot0 \pm 0\cdot2$	$0\cdot9 \pm 0\cdot2$	$0\cdot8 \pm 0\cdot2$	$0\cdot6 \pm 0\cdot1$	$1\cdot0$	$1\cdot0$
S. faecalis	$0\cdot7 \pm 0\cdot25$	$1\cdot0 \pm 0\cdot25$	$1\cdot2 \pm 0\cdot28$	$1\cdot0 \pm 0\cdot25$	$0\cdot9 \pm 0\cdot25$	$0\cdot7 \pm 0\cdot1$	$1\cdot0$	$1\cdot0$

*Results from only one subject
†Summation of 5-CH_3H_4PteGlu and 5-CH_3-5,6-H_2PteGlu activities.
‡*P. cerevisiae* values.
T = time in hours.

Table 6   Serum folate levels in five normal persons after oral administration of 3 g sodium bicarbonate and 10 mg dl-5-methyl-5,6-dihydrofolate

Serum folate	Serum folate levels ($\mu g/l \pm SEM$)							
	T_0	$T_\frac{1}{2}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3	T_4*	T_6*
Total folates	$8\cdot8 \pm 3\cdot0$	$19\cdot0 \pm 1\cdot0$	$51\cdot6 \pm 3\cdot4$	$112\cdot3 \pm 10\cdot0$	$99\cdot5 \pm 12\cdot0$	$51\cdot3 \pm 6\cdot0$	45	32
5-CH_3H_4PreGlu†	$8\cdot0 \pm 3\cdot0$	$18\cdot2 \pm 1\cdot0$	$50\cdot3 \pm 4\cdot0$	$109\cdot9 \pm 10\cdot2$	$96\cdot4 \pm 12\cdot4$	$48\cdot8 \pm 6\cdot4$	$43\cdot5$	$30\cdot8$
5-CH_3-5,6-H_2 PteGlu*	$0\cdot5$	$11\cdot0$	$18\cdot0$	$33\cdot0$	$26\cdot0$	$10\cdot0$	—	—
10-CHO H_4PteGlu‡	$0\cdot7 \pm 0\cdot1$	$0\cdot8 \pm 0\cdot1$	$1\cdot0 \pm 0\cdot1$	$1\cdot3 \pm 0\cdot2$	$1\cdot5 \pm 0\cdot4$	$1\cdot2 \pm 0\cdot5$	$1\cdot0$	$0\cdot9$
S. faecalis	$0\cdot8 \pm 0\cdot1$	$0\cdot8 \pm 0\cdot1$	$1\cdot4 \pm 0\cdot4$	$2\cdot4 \pm 0\cdot2$	$2\cdot9 \pm 0\cdot3$	$2\cdot5 \pm 0\cdot4$	$1\cdot5$	$1\cdot2$

*Results from only one subject.
†Summation of 5-CH_3H_4PteGlu and 5-CH_3-5,6-H_2PteGlu activities.
‡*P. cerevisiae* values.
T = time in hours.

Table 7   Serum folate levels in six normal persons after oral administration of 10 mg 5-methyltetrahydrofolate

Serum folates	Serum folate levels ($\mu g/l \pm SEM$)								
	T_0	$T_\frac{1}{2}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3	T_4	T_5	T_6*
Total folates	$9\cdot1 \pm 2\cdot1$	$106\cdot0 \pm 15\cdot2$	$166\cdot0 \pm 21\cdot0$	$188\cdot0 \pm 19\cdot0$	$180\cdot0 \pm 14\cdot7$	$110\cdot0 \pm 9\cdot4$	$81\cdot0 \pm 9\cdot8$	$55\cdot8 \pm 5\cdot2$	$41\cdot0$
5-CH_3H_4PteGlu†	$8\cdot3 \pm 2\cdot3$	$103\cdot1 \pm 16\cdot0$	$162\cdot0 \pm 21\cdot6$	$184\cdot3 \pm 21\cdot0$	$175\cdot4 \pm 14\cdot7$	$106\cdot3 \pm 9\cdot4$	$77\cdot5 \pm 10\cdot6$	$52\cdot4 \pm 5\cdot8$	$37\cdot8$
10-$CHOH_4$ PteGlu‡	$0\cdot6 \pm 0\cdot1$	$0\cdot5 \pm 0\cdot1$	$0\cdot7 \pm 0\cdot1$	$0\cdot6 \pm 0\cdot1$	$0\cdot8 \pm 0\cdot09$	$0\cdot7 \pm 0\cdot09$	$0\cdot7 \pm 0\cdot1$	$0\cdot7 \pm 0\cdot1$	$0\cdot7$
S. faecalis	$0\cdot8 \pm 0\cdot15$	$2\cdot9 \pm 0\cdot6$	$3\cdot8 \pm 0\cdot6$	$3\cdot7 \pm 0\cdot6$	$4\cdot6 \pm 0\cdot6$	$3\cdot7 \pm 0\cdot5$	$3\cdot5 \pm 0\cdot5$	$3\cdot4 \pm 0\cdot6$	$3\cdot2$

*Results from only one subject.
†Summation of 5-CH_3H_4PteGlu and 5-CH_3-5,6-H_2PteGlu activities.
‡*P. cerevisiae* values.
T = time in hours.

Table 8   Serum folate levels in three normal persons after oral administration of 20 mg 5-methyltetrahydrofolate in 200 ml water containing 1 g ascorbic acid

Serum folate	Serum folate levels ($\mu g/l \pm SEM$)					
	T_0	$T_\frac{1}{2}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
Total folates	$9\cdot75 \pm 0\cdot8$	$251\cdot2 \pm 43\cdot0$	$450\cdot0 \pm 23\cdot8$	$620\cdot0 \pm 47\cdot8$	$530\cdot0 \pm 66\cdot6$	$430\cdot0 \pm 47\cdot2$
5-CH_3H_4PteGlu*	$9\cdot05 \pm 0\cdot8$	$243\cdot0 \pm 41\cdot0$	$436\cdot4 \pm 17\cdot0$	$602\cdot2 \pm 42\cdot0$	$511\cdot2 \pm 60\cdot0$	$413\cdot4 \pm 42\cdot0$
5-CH_3-5,6-H_2PteGlu	$0\cdot0 \pm 0\cdot0$	$25\cdot0 \pm 10\cdot4$	$60\cdot0 \pm 17\cdot3$	$56\cdot7 \pm 17\cdot6$	$56\cdot7 \pm 17\cdot6$	$40\cdot0 \pm 15\cdot3$
10-CHO H_4PteGlu†	$0\cdot53 \pm 0\cdot05$	$1\cdot68 \pm 0\cdot45$	$3\cdot25 \pm 0\cdot52$	$3\cdot32 \pm 0\cdot58$	$4\cdot35 \pm 0\cdot30$	$4\cdot0 \pm 0\cdot4$
S. faecalis	$0\cdot7 \pm 0\cdot0$	$8\cdot2 \pm 2\cdot5$	$13\cdot6 \pm 5\cdot2$	$17\cdot8 \pm 6\cdot2$	$18\cdot8 \pm 7\cdot0$	$16\cdot6 \pm 6\cdot1$

*Summation of 5-CH_3H_4PteGlu and 5-CH_3-5,6-H_2PteGlu activities.
†*P. cerevisiae* values.
T = time in hours.

Table 9 *Serum folate levels in four normal persons after oral administration of 3 g of sodium bicarbonate and 10 mg of 5-methyltetrahydrofolate*

Serum folate	Serum folate levels (μg/l $\pm$ SEM)					
	T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
Total folates	7·5 $\pm$ 2·2	75·0 $\pm$ 7·6	155·0 $\pm$ 21·8	176·7 $\pm$ 24·8	147·5 $\pm$ 28·7	106·0 $\pm$ 29·0
5-CH$_3$H$_4$PteGlu*	7·0 $\pm$ 2·0	72·2 $\pm$ 7·9	149·6 $\pm$ 22·0	170·0 $\pm$ 25·0	141·0 $\pm$ 29·0	101·5 $\pm$ 29·9
5-CH$_3$-5,6-H$_2$PteGlu	3·0 $\pm$ 2·1	17·0 $\pm$ 2·7	48·0 $\pm$ 17·3	74·0 $\pm$ 40·0	65·0 $\pm$ 31·5	50·0 $\pm$ 16·7
10-CHO H$_4$PteGlu†	0·5 $\pm$ 0·1	1·0 $\pm$ 0·3	1·5 $\pm$ 0·3	1·7 $\pm$ 0·4	1·5 $\pm$ 0·5	1·4 $\pm$ 0·5
S. faecalis	0·5 $\pm$ 0·1	2·8 $\pm$ 1·9	5·4 $\pm$ 2·3	6·7 $\pm$ 2·5	6·5 $\pm$ 3·5	4·5 $\pm$ 2·0

*Summation of 5-CH$_3$H$_4$PteGlu and 5-CH$_3$-5,6-H$_2$PteGlu activities.
†P. cerevisiae values.
T = time in hours.

Table 10 *Serum folate levels in five normal persons after oral administration of 5 g sodium bicarbonate and 10 mg of 5-methyltetrahydrofolate*

Serum folate	Serum folate levels (μg/l $\pm$ SEM)					
	T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
Total folates	8·0 $\pm$ 3·0	172·0 $\pm$ 18·8	246·0 $\pm$ 19·4	259·0 $\pm$ 18·5	220·0 $\pm$ 22·6	145·0 $\pm$ 23·2
5-CH$_3$H$_4$PteGlu*	7·3 $\pm$ 3·1	170·1 $\pm$ 19·0	242·8 $\pm$ 19·7	255·3 $\pm$ 18·8	217·3 $\pm$ 22·9	143·4 $\pm$ 23·5
5-CH$_3$-5,6-H$_2$PteGlu	0·9 $\pm$ 0·3	70·0 $\pm$ 28·8	86·0 $\pm$ 40·7	115·4 $\pm$ 43·0	75·0 $\pm$ 31·7	54·0 $\pm$ 28·2
10-CHO H$_4$PteGlu†	0·65 $\pm$ 0·05	1·46 $\pm$ 0·46	2·20 $\pm$ 0·53	1·90 $\pm$ 0·54	1·56 $\pm$ 0·36	1·10 $\pm$ 0·22

*Summation of 5-CH$_3$H$_4$PteGlu and 5-CH$_3$-5,6-H$_2$PteGlu activities.
†P. cerevisiae values.
T = time in hours.

Table 11 *Serum folate levels in three normal persons after oral administration of 10 mg 5-methyl-5,8- dihydrofolate*

Serum folate	Serum folate levels (μg/l $\pm$ SEM)					
	T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
Total folates	7·3 $\pm$ 3·0	7·7 $\pm$ 2·7	8·2 $\pm$ 2·7	8·3 $\pm$ 2·8	7·9 $\pm$ 3·1	7·2 $\pm$ 3·6
5-CH$_3$H$_4$PteGlu*	6·8 $\pm$ 3·0	7·0 $\pm$ 2·8	7·3 $\pm$ 2·8	7·5 $\pm$ 2·7	7·3 $\pm$ 3·0	6·6 $\pm$ 3·5
10-CHO H$_4$PteGlu†	0·5 $\pm$ 0·1	0·6 $\pm$ 0·1	0·6 $\pm$ 0·1	0·5 $\pm$ 0·1	0·5 $\pm$ 0·1	0·5 $\pm$ 0·1
S. faecalis	0·5 $\pm$ 0·1	0·7 $\pm$ 0·1	0·9 $\pm$ 0·1	0·8 $\pm$ 0·2	0·6 $\pm$ 0·2	0·6 $\pm$ 0·2

*Summation of 5-CH$_3$H$_4$PteGlu and 5-CH$_3$-5,6-H$_2$PteGlu activities.
†P. cerevisiae values.
T = time in hours.

Table 12 *Serum folate levels in three normal persons after oral administration of 10 mg 4a-hydroxy-5-methyl-4a,5,6,7-tetra-hydrofolate*

Serum folate	Serum folate levels (μg/l $\pm$ SEM)					
	T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
Total folates	11·8 $\pm$ 6·7	11·5 $\pm$ 6·6	10·8 $\pm$ 6·0	10·6 $\pm$ 7·0	10·8 $\pm$ 7·1	11·8 $\pm$ 7·3
5-CH$_3$H$_4$PteGlu*	11·2 $\pm$ 6·7	10·8 $\pm$ 6·7	10·1 $\pm$ 6·0	10·0 $\pm$ 7·0	10·2 $\pm$ 7·1	11·2 $\pm$ 7·3
10-CHO H$_4$PteGlu†	0·6 $\pm$ 0·0	0·6 $\pm$ 0·0	0·6 $\pm$ 0·0	0·6 $\pm$ 0·0	0·6 $\pm$ 0·0	0·6 $\pm$ 0·0
S. faecalis	0·6 $\pm$ 0·0	0·7 $\pm$ 0·1	0·7 $\pm$ 0·1	0·6 $\pm$ 0·0	0·6 $\pm$ 0·0	0·6 $\pm$ 0·0

*Summation of 5-CH$_3$H$_4$PteGlu and 5-CH$_3$-5,6-H$_2$PteGlu activities.
†P. cerevisiae values.
T = time in hours.

methyl-4a,5,6,7-tetrahydrofolate, and 5-methyl-5,8-dihydrofolate.

The results of oral administration of 5-methyl-tetrahydrofolate vary. When exposed to the acid contents of the normal fasting human stomach a substantial proportion will be converted to 5-methyl-5,8-dihydrofolate by oxidation and acid-catalysed rearrangement. Not surprisingly, therefore, the serum levels of 5-methyltetrahydrofolate in normal persons are much lower than when the administration

Table 13   *Serum folate levels in four normal persons after oral administration of 5 mg of 7,8-dihydrofolate*

Serum folate	Serum folate levels (μg/l $\pm$ SEM)					
	T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
5-CH$_3$H$_4$PteGlu	5·9 $\pm$ 0·7	132·0 $\pm$ 18·8	200·0 $\pm$ 28·6	260·0 $\pm$ 27·4	210·0 $\pm$ 22·7	122·5 $\pm$ 20·9
5-CH$_3$-5,6-H$_2$PteGlu*	0	0	0	0	0	0
10-CHO H$_4$PteGlu†	0·60 $\pm$ 0·03	1·50 $\pm$ 0·09	1·80 $\pm$ 0·21	1·80 $\pm$ 0·15	1·50 $\pm$ 0·23	1·10 $\pm$ 0·19
10-CHOPteGlu‡	0·10 $\pm$ 0·03	1·10 $\pm$ 0·10	2·60 $\pm$ 0·30	1·20 $\pm$ 0·10	1·50 $\pm$ 0·10	0·10 $\pm$ 0·10

*Mean of only two subjects.
†*P. cerevisiae* activity.
‡Difference between *S. faecalis* and *P. cerevisiae* activities.
T = time in hours.

Table 14   *Serum folate levels in two patients with pernicious anaemia after oral administration of 10 mg of 5-methyltetrahydrofolate*

Serum folate	Serum folate levels (μg/l $\pm$ SEM)					
	T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
Total folates	3·8	92·0	190·0	240·0	220·0	160·0
5-CH$_3$H$_4$PteGlu*	3·4	91·5	188·6	238·7	218·7	158·7
5-CH$_3$-5,6-H$_2$PteGlu†	1·7	20·0	70·0	120·0	70·0	20·0
10-CHO H$_4$PteGlu‡	0·3	0·4	0·6	0·5	0·9	0·8
S. faecalis	0·4	0·5	1·4	1·3	1·3	1·3

*Summation of 5-CH$_3$H$_4$PteGlu and 5-CH$_3$-5,6-H$_2$PteGlu activities.
†Results from only one subject.
‡*P. cerevisiae* values.
T = time in hours.

Table 15   *Serum folate levels in five patients with pernicious anaemia after oral administration of 10 mg of 5-methyl-5,6-dihydrofolate*

Serum folate	Serum folate levels (μg/l $\pm$ SEM)					
	T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
Total folates	8·6 $\pm$ 2·8	47·0 $\pm$ 6·8	72·4 $\pm$ 10·2	96·8 $\pm$ 14·1	97·4 $\pm$ 15·0	84·8 $\pm$ 15·9
5-CH$_3$H$_4$PteGlu*	7·9 $\pm$ 2·8	45·8 $\pm$ 6·8	70·8 $\pm$ 10·1	95·1 $\pm$ 13·9	95·7 $\pm$ 14·7	83·6 $\pm$ 15·9
5-CH$_3$-5,6-H$_2$PteGlu†	4·6 $\pm$ 1·8	32·0 $\pm$ 17·5	65·0 $\pm$ 22·0	79·0 $\pm$ 34·0	70·0 $\pm$ 26·2	47·0 $\pm$ 16·1
10-CHO H$_4$PteGlu‡	0·56 $\pm$ 0·08	0·73 $\pm$ 0·14	0·95 $\pm$ 0·14	0·86 $\pm$ 0·12	0·85 $\pm$ 0·06	0·73 $\pm$ 0·09
S. faecalis	0·7 $\pm$ 0·1	1·2 $\pm$ 0·2	1·6 $\pm$ 0·2	1·7 $\pm$ 0·25	1·7 $\pm$ 0·3	1·2 $\pm$ 0·2

*Summation of 5-CH$_3$H$_4$PteGlu and 5-CH$_3$-5,6-H$_2$PteGlu activities.
†Results from three subjects.
‡*P. cerevisiae* values.
T = time in hours.

Table 16   *Serum folate levels in six adults with untreated coeliac disease after oral administration of 10 mg of 5-methyltetrahydrofolate*

Serum folate	Serum folate levels (μg/l $\pm$ SEM)					
	T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2	T_3
Total folates	4·0 $\pm$ 0·8	65·4 $\pm$ 23·9	96·9 $\pm$ 24·3	138·0 $\pm$ 25·4	132·0 $\pm$ 15·7	100·0 $\pm$ 20·0
5-CH$_3$H$_4$PteGlu*	3·2 $\pm$ 0·7	61·4 $\pm$ 22·1	89·6 $\pm$ 21·2	128·2 $\pm$ 21·0	121·8 $\pm$ 10·5	92·6 $\pm$ 16·5
5-CH$_3$-5,6-H$_2$PteGlu†	0·85 $\pm$ 0·5	16·6 $\pm$ 16·0	20·0 $\pm$ 15·0	20·0 $\pm$ 15·0	33·7 $\pm$ 24·0	36·7 $\pm$ 20·0
10-CHO H$_4$PteGlu‡	0·6 $\pm$ 0·13	1·0 $\pm$ 0·24	1·4 $\pm$ 0·51	1·8 $\pm$ 0·88	2·2 $\pm$ 1·19	1·2 $\pm$ 0·78
S. faecalis	0·8 $\pm$ 0·1	4·0 $\pm$ 1·75	7·0 $\pm$ 3·1	9·8 $\pm$ 4·4	10·2 $\pm$ 5·3	7·4 $\pm$ 3·48

*Summation of 5-CH$_3$H$_4$PteGlu and 5-CH$_3$-5,6-H$_2$PteGlu activities.
†Results from three subjects.
‡*P. cerevisiae* values.
T = time in hours.

Table 17   *Serum folate levels in eight patients with leukaemia after oral administration of 10 mg of 5-methyltetrahydrofolate*

Serum folate	Serum folate levels ($\mu g/l \pm SEM$)				
	T_0	$T_{\frac{1}{2}}$	T_1	$T_{1\frac{1}{2}}$	T_2
Total folates	5.7 ± 1.1	65.6 ± 12.6	117.9 ± 19.7	105.3 ± 13.8	94.8 ± 12.7
5-CH₃H₄PteGlu*	4.8 ± 1.0	64.6 ± 12.6	116.4 ± 19.5	103.8 ± 13.8	93.0 ± 12.7
5-CH₃-5,6-H₂PteGlu†	2.5	35.0	23.0	27.0	45.0
10-CHO H₄PteGlu‡	0.8 ± 0.05	0.8 ± 0.07	1.0 ± 0.17	1.0 ± 0.13	1.0 ± 0.16
S. faecalis	0.9 ± 0.08	1.0 ± 0.09	1.5 ± 0.29	1.5 ± 0.39	1.8 ± 0.54

*Summation of 5-CH₃H₄PteGlu and 5-CH₃-5,6-H₂PteGlu activities.
†Results from two subjects.
‡P. cerevisiae values.
T = time in hours.

is accompanied by either ascorbate or sodium bicarbonate (Tables 7, 8, 9, 10). Thus these findings reflect chemical changes within the stomach and not necessarily differences in absorption. They contrast with the findings with folic acid, a synthetic compound, where the processes of absorption are depressed (Benn *et al.*, 1971).

Administration of 5-methyltetrahydrofolate to patients with pernicious anaemia resulted in total serum folate levels greater than those in normal subjects as might be deduced from the absence of gastric acidity (Table 14). Weir *et al.* (1973) found that the absorption of 5-methyltetrahydrofolate was reduced in coeliac disease, and the serum levels in our absorptive experiments in coeliac disease (Table 16) were significantly less than in normal subjects. Similarly, significantly lower serum values were found in the patients with leukaemia (Table 17).

Using the serum levels and the appropriate logarithmic plot the half life of serum folate was 2·2 hours for normal subjects and 3·0 hours for coeliac disease, pernicious anaemia, and leukaemia, the rate of clearance of serum folate being of first order. Hence in the absorptive tests in this paper the serum levels will maintain a relationship to the rate of absorption and thus reflect absorptive ability.

When 5-methyl-5,6-dihydrofolate is given orally to normal subjects, there is a rapid rise in serum folate which reaches a maximum (Table 5) earlier and at much lower levels than that found with the equivalent dose of 5-methyltetrahydrofolate (Table 7), explained most likely by the acid decomposition in the stomach of 5-methyl-5,6-dihydrofolate. This suggestion receives support by the findings that when 5-methyl-5,6-dihydrofolate is administered with sodium bicarbonate to normal subjects or in patients with a histamine fast achlorhydria, such as pernicious anaemia, serum folate reaches much higher peak values at the normally expected time (Tables 6 and 15).

Since   4a-hydroxy-5-methyl-4a,5,6,7-tetrahydro-

folate is readily absorbed by the rat and excreted unchanged in the urine (Dransfield, 1973), it is likely to be absorbed in man even though the administration of this compound and of 5-methyl-5,8-dihydrofolate produced no change in serum folate (Tables 8 and 9). These two compounds probably do not enter the folate metabolic pool.

It has been suggested (Ratanasthien *et al*, 1974) that only two folates, 5-methyltetrahydrofolate and 10-formyl-tetrahydrofolate, are present in serum and that the difference observed between the *S. faecalis* and *P. cerevisiae* serum assays in normals is due to the oxidation of 10-formyltetrahydrofolate to 10-formylfolic acid. The results of the bioautographic analysis of purified 5-methyltetrahydrofolate clearly show that the weak activity of this preparation for *S. faecalis* is due to trace amounts of folic acid.

The results obtained measuring *S. faecalis* and *P. cerevisiae* values over a range of clotting times with the steady decline in the *P. cerevisiae* values (Table 1) can only indicate that the relevant folate is 10-formyltetrahydrofolate and the changes in values are due to oxidation to 10-formylfolic acid. Of the two other possible folates with identical *S. faecalis* and *P. cerevisiae* activity, one, 5-formyltetrahydrofolate is stable to oxidation and the other, tetrahydrofolate, is rapidly oxidised to inactive compounds (Blair and Pearson, 1974a; Pearson, 1974). Consequently, measurement of the levels of 10-formyltetrahydrofolate in the serum (in the absence of administered folic acid) is best determined by the *S. faecalis* value. Interestingly, DaCosta and Rothenberg (1971), using a radioimmune technique, showed that extract human serum contained small amounts of a folate ($1·4 \pm 0·1$ $\mu g/l$) whose properties suggested it was derived from 10-formyltetrahydrofolate.

A comparison of the serum total folate levels in normal subjects and patients with serum levels of < 3 $\mu g/l$ (Table 3) and in those with levels > 3 $\mu g/l$ (Table 2) shows that the serum levels of 10-formyl-tetrahydrofolate are significantly increased in many

diseases. The reason for this is not immediately apparent but clearly has some significance in the interpretation of total serum folate levels. It is also apparent that there is some clinical variation, though the changes are small, in these levels throughout the day.

There seems to be a homeostatic mechanism for keeping the serum levels of 10-formyltetrahydrofolate constant in health (Ratanasthien *et al.*, 1974), though clearly the levels must fall when the total serum folate diminishes to low levels. Since 10-formyltetrahydrofolate is the coenzyme necessary for purine biosynthesis (Blakely, 1969) a substantial fall in its level could be one cause of megaloblastic anaemia. As was noted earlier, values of 10-formyltetrahydrofolate in such patients were significantly less than normal, confirming the observations made by DaCosta and Rothenberg (1971). Low values of serum 10-formyltetrahydrofolate could also result from a lack of vitamin B_{12}, for this coenzyme controls the rate of formation of 10-formyltetra-hydrofolate from 5-methyltetrahydrofolate, and so bring about megaloblastic anaemia. The existence of a significant fall in 10-formyltetrahydrofolate can be derived from the data of DaCosta and Rothenberg (1971) in the serum of B_{12}-deficient subjects with megaloblastic anaemia.

There is now substantial evidence that there is considerably increased production of 10-formyltetra-hydrofolate in rapidly proliferating cells. The enzymes of the folate cycle show marked variation with the rate of cell multiplication (Rosenblatt and Erbe, 1973), while studies of urinary folates in rats show that when large implanted Walker 256 tumours are present the relative amount of 10-formyltetra-hydrofolate is increased while 5-methyltetrahydro-folate is decreased (Barford and Blair, 1975). This could be the reason for the increased levels found in psoriasis and leukaemia but the cause is not so clear in coeliac disease and rheumatoid arthritis.

A large proportion of the serum folate increase after oral administration of 5-methyl-5,6-dihydro-folate to pernicious anaemia subjects (Table 15) and the combined oral administration of it plus bicarbonate and of 5-methyltetrahydrofolate plus bicarbonate to normal subjects (Tables 6 and 9) is 5-methyl-5,6-dihydrofolate. This seems to persist without being metabolised and does not appear therefore to enter the folate metabolism pool in either normal subjects or in patients with pernicious anaemia. In the fasting state only patients with pernicious anaemia have a substantial portion of serum folate as this compound (Table 4). In all other cases the small proportion found could have been an analytical artefact.

Lastly, oral administration of 7,8-dihydrofolate (Table 13) showed the same pattern of rapid conversion to serum 5-methyltetrahydrofolate with little change in serum 10-formyltetrahydrofolate levels as had been previously noted with tetrahydrofolate (Ratanasthien *et al.*, 1974).

The major dietary folate compounds are 5-methyltetrahydrofolate and its derivatives. The data in this study suggest that their availability will depend on the amount of gastric acidity and of ascorbate in the intestinal chyme. In particular 5-methyl-5,6-dihydrofolate may not be available in the absence of ascorbate and in an acid stomach. Even when it does appear in the serum it seems unlikely that it will enter the folate metabolic pool since the serum levels tend to remain relatively constant for long periods.

These findings are particularly relevant, for total food folate is conventionally measured by *L. casei* assay. Since our own observations suggest that about 50% of total food folate is 5-methyl-5,6-dihydrofolate (unpublished) measurements of food folate by *L. casei* assay may greatly exaggerate the utilisation of folate in food by man. If 5-methyl-5,6-dihydrofolate constitutes a major portion of food folate it may explain the increased proportion of this compound in the serum of patients with pernicious anaemia.

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