STUDIES ON THE FOLATE POLYGLUTAMYL COMPONENTS OF HUMAN DIETS

Being a Thesis
submitted for
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by

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Abstract.

The folate in human diets has been determined by microbiological assay. The various folate derivatives in the diet were separated by column chromatography and the quantitative data on the total amount, and type, of folates present obtained.

Yeast polyglutamyl folates in a form suitable for use in human absorption studies were prepared. The absorption of these polyglutamates and of various synthetic folate compounds which may be present in a normal diet was studied in volunteers, and the results are discussed in the context of the relevant literature.

The possible role of antagonists to the enzyme splitting polyglutamyl folates in human diets was explored.

Finally an estimate was made of the amount of polyglutamyl folates which may be utilized by normal subjects.

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Glossary of terms and abbreviations.

Folate: non-specific term for pteroylglutamic acid and its derivatives.

Mono-,di- or triglutamyl folate; mono-,di- or triglutamate: terms used for folate derivatives with one, two or three glutamic acid residues.

Polyglutamyl folate; polyglutamate: folate derivatives with more than three (usually seven) glutamic acid residues.

"Free" folate;unconjugated folate: mono-,di- or triglutamyl folates. These folates are available for microbiological assay without the use of conjugase enzymes..

"Total" folate: all those folate compounds, including "free" folate as well as polyglutamyl derivatives, available for microbiological assay following incubation with conjugase enzymes.

Conjugase; carboxypeptidase (pteroylglutamyl hydrolase): the enzyme hydrolysing polyglutamyl folates to mono- or diglutamates. The source of the enzyme is stated in the text i.e. plasma conjugase, chick pancreatic conjugase etc.

Ascorbate: solution of L- ascorbic acid in either buffer or distilled water. The diluent, concentration and pH of the solution used is stated in the relevant section of the text.

PteGlu: pteroylglutamic acid, folic acid.

$PteGlu_3$: pteroyltriglutamic acid, (Teropterin).

H_2PteGlu: dihydropteroylglutamic acid; dihydrofolate.

H_4PteGlu: tetra hydro pteroylglutamic acid; tetrahydrofolate.

10 CHO PteGlu: 10 formyl pteroylglutamic acid.

10 CHO H_4PteGlu : 10 formyltetrahydropteroylglutamic acid.

5 CH_3 H_4PteGlu: 5 methyltetrahydropteroylglutamic acid.

5,10 CH H_4PteGlu: 5,10 methenyltetrahydropteroylglutamic acid.

5 CHNH H_4PteGlu: 5 formiminotetrahydropteroylglutamic acid.

5,10 CH_2 H_4PteGlu: 5,10 methylenetetrahydropteroylglutamic acid.

Section 1.

Introduction.

1. Historical Background

The elucidation and characterization of the many compounds in the folic acid group was preceded by early work based on the nutritional requirement of bacteria, animals, and man. In 1926 Minot and Murphy reported dramatic responses in 45 patients with "pernicious anaemia" given a diet of raw liver. In the following years crude liver extracts were widely employed in the treatment of macrocytic anaemias of varied aetiology, most of which were considered at that time to be due to a deficiency of Castle's extrinsic factor and hence to make up a homogeneous group. However Lucy Wills in 1931 in Bombay obtained haematological responses in patients with "tropical macrocytic anaemia" and in "pernicious anaemia of pregnancy" not only with crude liver extracts but also with autolyzed yeast preparations (marmite). Patients with pernicious anaemia generally did not respond to yeast preparations and this suggested that more than one haematinic substance might be involved.

In an attempt to establish the nature of the missing dietary factor Wills and Stewart (1935) produced "tropical anaemia" in rhesus monkeys, fed a diet similar to that taken by poor Hindus in Bombay and found it could be cured by crude

liver extract and marmite. In 1938 Wills and Evans showed that their essential factor was missing from refined liver extracts, which although fully active in pernicious anaemia, had no effect on the experimental anaemia in the monkey. These results could be paralleled in man. Therefore a new haemopoietic factor must be present in the crude extracts of liver, and in autolysed yeast.

A separate series of nutritional studies with monkeys was reported in 1935 by Day and co-workers, who proposed the name "vitamin M" for the material in brewers yeast which prevented "nutritional cytopenia" in this species (Day, Langston and Darby, 1938), while an investigation into the vitamin requirement of chicks showed a similar growth factor, again in yeast, termed "factor U" by Stokstad and Manning (1938), and vitamin Bc by Hogan and Parrott (1939). In 1940 a growth factor for Lactobacillus casei was reported in liver and yeast extracts by Snell and Peterson, which, as it could be absorbed and eluted from charcoal, they termed "norit eluate factor". In the following year Mitchell, Snell and Williams described a substance obtained from spinach which was a growth factor for both Streptococcus faecalis R and L. casei. This material

they called "folic acid" while a substance which was also active for L.casei was isolated from liver in 1943 by Stokstad and given the name "Lactobacillus casei" factor.

Initially it was believed that these isolated factors were not only identical but also comprised a single biologically effective molecule. However, the field was further complicated by the observation of Hutchings et al that a material isolated from a culture of a Corynebacterium was much more active for L.casei than for S.faecalis (Hutchings, Stokstad, Bohonos and Slobodkin, 1944). This they termed "fermentation L.casei factor".

At the same time, another group of workers remarked that a substance isolated from yeast, although fully active in chick anaemia, was completely inactive in microbiological assay systems using S.faecalis and L.casei as the test organisms. They concluded that the chick antianaemia activity in yeast was "due to the presence of vitamin Bc held almost entirely in the form of a relatively simple "conjugate". The factor became highly active in microbiological assay after enzymatic digestion (Binkley, Bird, Bloom, Brown, Calkins, Campbell, Emmett and Pfiffner, 1944).

The enzymes producing the active form were termed "conjugases" (Bird, Binkley, Bloom, Emmett and Pfiffner, 1945).

The vitamin Bc conjugate isolated from yeast was subsequently shown to be a heptaglutamate (Pfiffner, Calkins, Bloom and O'Dell, 1945) that is, to have 7 glutamic acid residues, and the fermentation L.casei factor a triglutamate (Hutchings, Stokstad, Bohonos, Sloane and Subba Row, 1948)

The isolation of pure folic acid and its conjugates was immediately followed by an elucidation of their structures (Angier, Boothe, Hutchings, Mowat, Semb, Stokstad, Subba Row, Waller, Cosulich, Fahrenbach, Hultquist, Kuh, Northey, Seeger, Sickels and Smith 1945). The parent molecule was shown to be pteroylglutamic acid and the conjugated forms consisted of derivatives containing 3 or 7 glutamic acid residues linked by γ glutamyl peptide bonds (Hutchings, Stokstad, Bohonos, Sloane and Subba Row, 1948; Pfiffner, Calkins, Bloom and O'Dell 1946).

With the availability of synthetic L.casei factor, interest was stimulated in its action on human disease states. It was reported to be effective in producing a reticulocyte response and regeneration of red blood cells in pernicious anaemia (Moore,

Bierbaum, Welch and Wright, 1945; Spies, Vilter, Koch and Caldwell, 1945), sprue (Darby and Jones, 1945; Moore, Bierbaum, Welch and Wright, 1945), macrocytic anaemia of pregnancy (Moore, Bierbaum, Welch and Wright, 1945), and other forms of macrocytic nutritional anaemia (Spies, Vilter, Koch and Caldwell, 1945).

Recognition of the clinical efficacy of this new trace material in diet in macrocytic anaemias and even at least for a time in pernicious anaemia led to attempts to estimate the folate content of a vast number of foodstuffs, attempts to appraise nutritional value of diets and to estimate probable human requirements. Failure to appreciate that some form of folate in diet were highly labile however resulted in many early estimates of folate content being much too low, (Stokstad and Koch, 1967), as are many earlier assumptions as to the availability and daily intake of food folate.

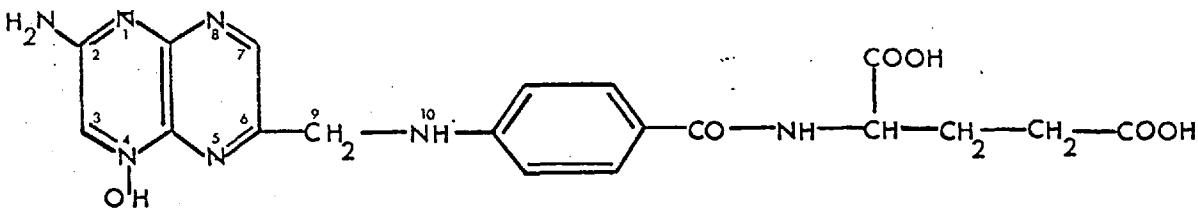

Figure I - Pteroylglutamic acid, showing the numbering of the atoms.

1.2 Physical and chemical properties of PteGlu, its derivatives and conjugates.

A. Pteroylglutamic acid.

(Vitamin U, Vitamin M, norit eluate factor, Vitamin Bc, liver L.casei factor).

The structure of the parent molecule was defined in 1946, and was shown to consist of 2 amino-4-hydroxy-6 pteridine, p-aminobenzoic acid and L glutamic acid (Angier, Booth, Hutchings, Mowat, Kuh, Northey, Seeger, Sickels and Smith), the name pteroylglutamic acid being assigned because of the presence of a pteridine moiety and glutamic acid in the molecule (figure 1).

When crystallized from water PteGlu forms yellow spear shaped leaflets, insoluble in most organic solvents but slightly soluble in acetic acid (Pfiffner, Binkley, Bloom and O'Dell, 1947). In solution it is decomposed by ultraviolet light and sunlight (Bloom, Vandenbelt, Binkley, O'Dell and Pfiffner, 1944) and enzymatic degradation by bacterial cells was reported by Stokes and Larsen (1945) and by tissue homogenates by Wright, Skeggs and Welch (1945). In both cases p-amino

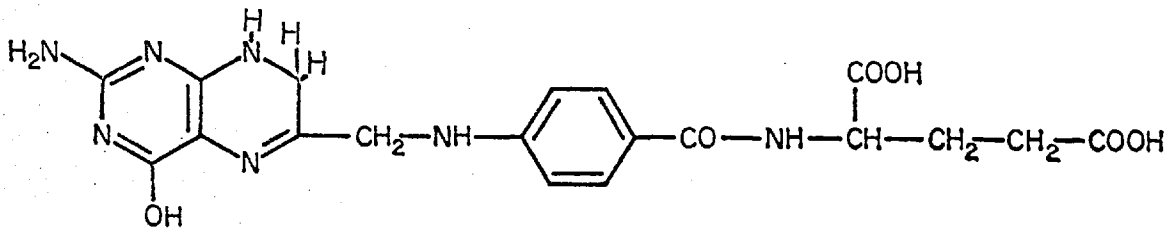

Figure II. Dihydropteroylglutamic acid

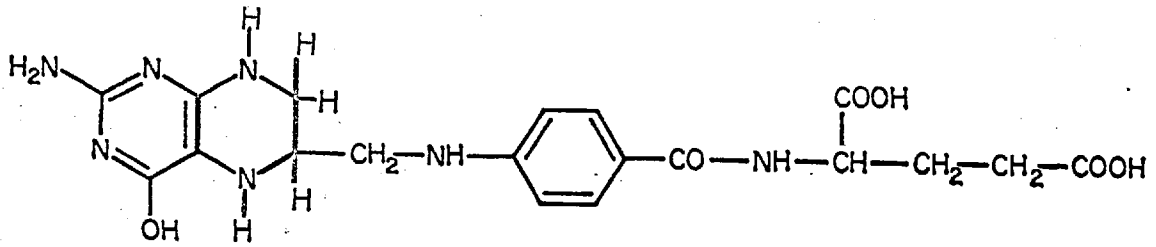

Figure III. Tetrahydropteroylglutamic acid

benzoic acid and glutamic acid was among the degradation products formed (Stokstad, Fordham and de Grunigen, 1947; Keresztesy and Silverman, 1953; Futterman and Silverman, 1957). The aerobic alkaline hydrolysis of PteGlu results in the formation of a fluorescent pigment and a diazotizable aromatic amine (Stokstad, Hutchings, Mowat, Boothe, Walker, Angier, Semb, and Subba Row, 1948) while reduction with zinc in acid solution yields an unstable form of PteGlu which rapidly splits to give the free aromatic amine (Hutchings, Stokstad, Mowat, Boothe, Waller, Angier, Semb and Subba Row 1948).

B. Reduction of PteGlu.

An important chemical feature of PteGlu is the reducibility of the pteridine ring, by either enzymatic or chemical means, to given either dihydro or tetrahydro-derivatives. Catalytic reduction in alkaline solution gives 7,8 H_2PteGlu (figure II) and in neutral solution 5,6,7,8, H_4PteGlu (figure III) (O'Dell, Vandenbelt, Bloom and Pfiffner, 1947; Blakley, 1957; Hatefi, Talbert, Osborn and Huennekens, 1959). H_2PteGlu can also be formed by reduction of PteGlu at room temperature with sodium dithionite in the presence of ascorbate (Futterman, 1957; Blakley, 1960), a similar

procedure at 75° yielding H_4 PteGlu (Silverman and Noronha, 1961). Chemically prepared H_4 PteGlu is the dl-L diastereoisomer and is only 50% active in enzyme systems, whereas the product of enzymatic reduction of H_2 PteGlu by dihydrofolate reductase (Osborn and Huennekens; 1958) being the l, L. form is 100% active (Matthews and Huennekens, 1960).

Both H_2 & H_4 PteGlu are unstable compounds, H_2 PteGlu in alkaline solutions being rapidly oxidised to folic acid and degradation products, a reaction which is slower in acid solutions (O'Dell, Vandenbelt, Bloom and Pfiffner, 1947; Futterman, 1957). H_4 PteGlu is also readily oxidised by atmospheric oxygen in neutral solution, being quickly converted to the dihydro derivative and degradation products, including xanthopterin, and more slowly to folic acid. In acid solutions as much as 40% of the molecule may be split to yield p-amino-benzoic acid (Blakeley, 1957).

C. Principal metabolically active forms of reduced PteGlu.

i. N^5 formyl tetrahydrofolic acid (N^5CHO H_4 PteGlu, folinic acid, citrovorum factor, CF, leucovorin). The discovery of

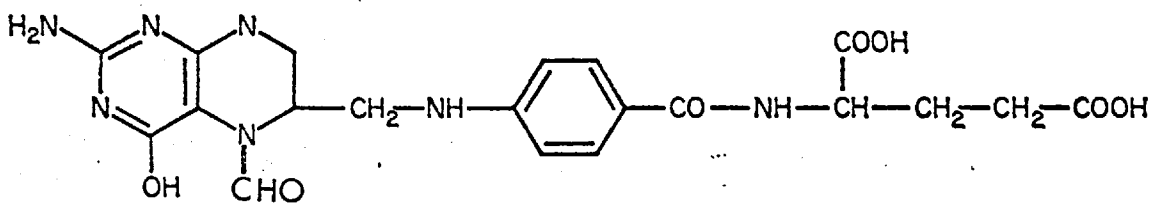

Figure IV. 5 formyltetrahydropteroylglutamic acid

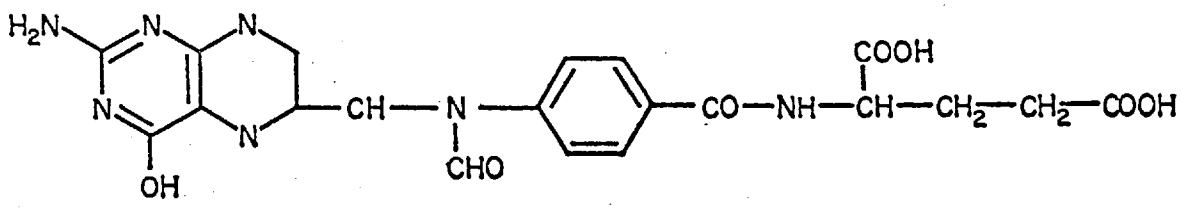

Figure V. 10 formyltetrahydropteroylglutamic acid

this growth factor, similar to but not identical with folic acid, followed soon after the synthesis of folic acid and was the first metabolically active tetrahydro derivative to be recognized.(figure IV) (Sauberlich, and Bauman 1948; Broquist, Stokstad, Hoffman, Belt, and Jukes 1949). Sauberlich and Baumann reported that an unidentified substance present in liver and yeast was required for growth by Leuconostoc citrovorum (now known as Pediococcus cerevisiae). Its relationship to PteGlu was confirmed by the observation that the level of 5 CHO H_4 PteGlu was low in the urine of rats on a folate free diet, but increased when normal feeding was resumed (Sauberlich, 1949) and also that it was more effective than folic acid in reversing Aminopterin toxicity in rats (Sauberlich, 1949a, Broquist, Stokstad and Jukes, 1950; Bond, Bardos, Silby and Shive, 1949). The suggestion that 5 CHO H_4 PteGlu was a formyl derivative of PteGlu resulted in the chemical synthesis of the compound by treating 10-CHO- H_4 PteGlu anaerobically in 0.1 N NaOH (Roth, Hultquist, Fahrenbach, Cosulich, Broquist, Brockman, Smith, Parker, Stokstad, and Jukes, 1952) or by autoclaving at pH 7.0 in the presence of ascorbate (Shive, Bardos, Bond and Rogers, 1950). Like

H_4PteGlu it contains an asymetric 6-carbon, and the synthetically prepared material is a mixture of two diastereoisomers, only the l, L-form being biologically active (Cosulich, Smith and Broquist, 1952). The natural material is fully active for P.cerevisiae (Keresztesy and Silverman, 1951, Sauberlich, 1952). 5 CHO H_4PteGlu is stable to oxidation, in contrast with other H_4 derivatives, and is stable in neutral and alkaline solutions. However in acid (pH 1-2), it is converted by the elimination of water to anhydroleucovorin (5-10 CH = H_4PteGlu) (May, Bardos, Berger, Lansford, Ravel, Sutherland and Shive, 1951).

ii. N^{10}formyltetrahydrofolic acid (10-CHO-H_4PteGlu. This derivative (figure V) which like H_4PteGlu was first discovered as an intermediate in the chemical synthesis of folinic fromfolic acid (May, Bardos, Berger, Lansford, Ravel, Sutherland and Shive 1957) can be chemically prepared by formylation of H_4PteGlu in formic acid (Roth, Hultquist, Fahrenbach, Cosulich, Broquist, Brockman, Smith Parker, Stokstad, and Jukes 1952) or by catalytic dehydrogenation of PteGlu in formic acid (Brockman, Roth, Broquist, Hultquist, Smith, Fahrenbach, Cosulich, Parker, Stokstad, and Jukes 1950, Shive, Bardos, Bond, and Rogers, 1950).

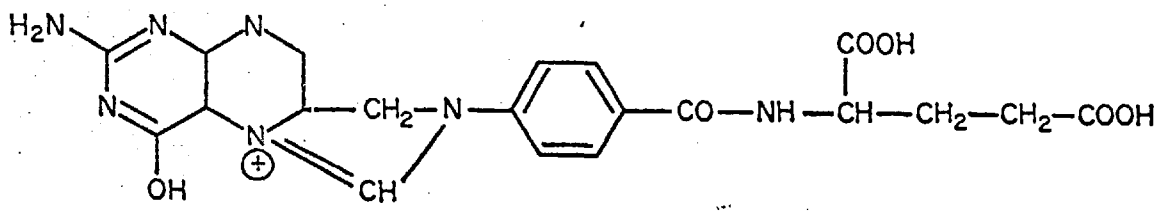

Figure VI. 5, 10 methenyltetrahydropteroylglutamic acid

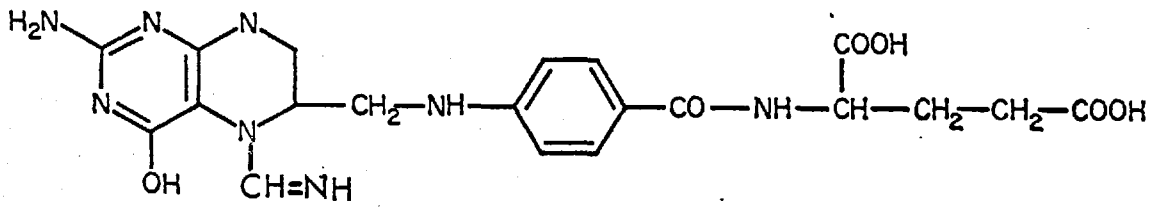

Figure VII. 5, formiminotetrahydropteroylglutamic acid

Solutions of 10-CHO-H_4PteGlu may be protected against atmospheric oxygen by ascorbate (Bakerman, 1961) or mercaptoethanol (Blakley, 1960). It is an intermediate in the enzymic synthesis of 5-CHO-H_4PteGlu (Nichol, Anton and Zarkrzewski, 1955).

iii. N^5 - N^{10} methenyltetrahydrofolic acid (5-10 CH= H_4 PteGlu, anhydroleucovorin)

This compound (figure VI) was discovered during studies on the structure of folinic acid (May, Bardos, Barger, Lansford, Ravel, Sutherland and Shive, 1951; Cosulich, Roth, Smith, Hultquist, and Parker 1951; Ibid, 1952). It can be formed from either 5 or 10 CHO H_4PteGlu in acid solution at pH 1-2, and though stable to oxygen in acid, in neutral or weakly alkaline solutions it is hydrolysed to the unstable 10 CHO H_4PteGlu form.

iiii. N^5 formiminotetrahydrofolic acid (5 CHNH-H_4PteGlu)

During studies on the enzymic breakdown of purines (Rabinowitz and Pricer, 1956) and histidine (Tabor and Rabinowitz, 1956; Miller and Waelsch, 1957) a new intermediate was discovered, the N^5 formimino derivative of H_4PteGlu (Figure VII). 5 CHNH-H_4PteGlu can be formed from the enzymatic reaction of H_4PteGlu with

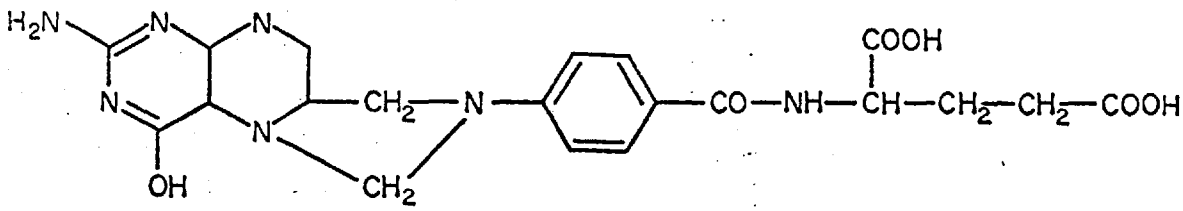

Figure VIII. 5,10, methylenetetrahydropteroylglutamic acid

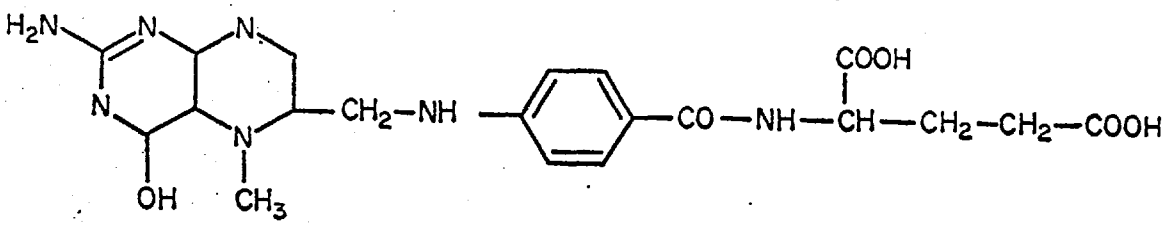

Figure IX. 5 methyltetrahydropteroylglutamic acid

formiminoglycine or formiminoglutamic acid, and can be converted into 5, 10 CH = H_4PteGlu by either 0.1 N HCl or the enzyme cydodeaminase (Takeyama, Hatch and Buchanan, 1961). 5, CHNH-H_4PteGlu is stable to air oxygen.

v. N^5, N^{10} methylenetetrahydrofolic acid (5, 10 CH_2 = H_4PteGlu) This derivative (Figure VIII) was also identified during enzyme studies with H_4PteGlu, in this case with serine (Deodhar, Sakami and Stevens, 1955; Jaenicke, 1956). It is also formed by the non-enzymatic reaction of H_4PteGlu and formaldehyde (Blakley, 1963; Jaenicke, 1956; Kisliuk, and Sakami 1954). 5, 10 CH_2 H_4PteGlu is more stable to atmospheric oxygen than H_4PteGlu, but less stable than 5 CHO-H_4PteGlu (Blakley, 1957).

vi. N^5 methyltetrahydrofolic acid (5 CH_3-H_4PteGlu). In 1959 Donaldson and Keresztesy isolated from fresh horse liver a compound which was active for L.casei but not for S.faecalis, and which, because prolonged autolysis of liver converted it to PteGlu, they called "prefolic A". In 1961, a new methylated H_4PteGlu derivative which functioned as an intermediate in methionine synthesis was reported (Larrabee and Buchanan, 1961).

"Prefolic A" was shown to be identical to this compound (Donaldson and Keresztesy, 1961) and was characterized as N^5 CH_3H_4PteGlu (figure IX). The compound is stable to air oxygen (Hatch, Larrabee, Cathou and Buchanan 1961) and like 5 CHO H_4PteGlu the chemically synthesized form comprises 2 diastereoisomers, so being only 50% active in biological systems. It can be prepared by reduction of a mixture of H_4PteGlu and formate by borohydride (Kisliuk and Woods, 1960; Jaenicke, 1961; Keresztesy and Donaldson, 1961; Sakami and Ukstins, 1961; Gupta and Huennekens, 1967) and from 5 CHO H_4PteGlu by conversion to 5,10 CH H_4PteGlu with subsequent borohydride reduction (Chanarin and Perry, 1967). Enzymatically , 5 CH_3 H_4PteGlu is formed from $N^{5,10}$ CH_2 H_4PteGlu by 5,10 CH_2 H_4PteGlu reductase which is found in the liver of many vertebrates (Donaldson and Keresztesy, 1961; Jaenicke, 1961) and in certain baderia (Hatch, Larrabee, Cathou and Buchanan, 1961; Cathou and Buchanan, 1963; Kisliuk, 1963; Guest, Foster and Woods, 1964). 5 CH_3H_4PteGlu is readily oxidised at

alkaline pH to dihydromethylfolate (Keresztesy and Donaldson, 1961; Gupta and Huennekens, 1967; Donaldson and Keresztesy 1963; Larrabee, Rosenthal, Cathou and Buchanan 1961) which, though relatively stable to oxygen, may be reduced back to 5 CH_3 H_4 PteGlu by ascorbic acid (Donaldson and Keresztesy 1962) by hydrogen in the presence of platinum (Larrabee, Rosenthal, Cathou and Buchanan, 1961) and by borohydride (Gupta and Huennekens, 1967).

D. Polyglutamyl forms of folate.

Even before the isolation of PteGlu itself it was recognised that natural materials contained substances which, although fully available as an essential growth factor to the chick, had only 2-5% activity when assessed by its microbiological growth effect on either L.casei or S.faecalis. (Stokstad, 1943; Binkley, Bird, Bloom, Brown, Calkins, Campbell, Emmett and Pfiffner, 1944). The existence of enzymes which were necessary to convert the naturally occurring forms of the vitamin to forms which were active in microbiological assay was remarked by Wright and Welch (1943, 1943[a]) who reported that incubation of

grasses and liver with fresh rat liver slices led to increased activity with L.casei or S.faecalis. The isolation of the "inert" or chick anti anaemic factor form from yeast followed, and it was reported as being non-protein in nature, and to have the same growth stimulating activity, absorbtion spectrum, colour, crystalline appearance and solubility after enzymic digestion as crystalline vitamin B_c, and so was considered to be a relatively simple conjugate (Pfiffner, Calkins, O'Dell, Bloom, Campbell and Bird, 1945).

The nature of the conjugate was reported by the same group of workers, and was shown by spectrophotometric studies to consist of one molecule of pteroylglutamic acid with 6 glutamic acid residues, probably joined in peptide linkage (Pfiffner, Calkins, Bloom, and O'Dell, 1946). Its molecular weight, based on PteGlu at 441, was approximately 1200.

Subsequently a form of PteGlu was isolated from the culture liquid of certain Corynebacteria (Hutchings, Stokstad, Bohonos, Sloane, and SubbaRow, 1948) which was more active for L.casei than S.faecalis and which yielded, on anaerobic alkaline hydrolysis, pteroylbenzoate glutamate and 2 moles of glutamic acid, thus establishing the polypeptide nature of the glutamate residues.

Subsequent isolation of folate derivatives has shown that all the biologically active forms, i.e. the formyl and methyl groups, their reduced forms, and the reduced form of PteGlu itself, exist in nature in the form of polyglutamates with either three or more glutamic acid residues. While there is direct evidence of the presence of heptaglutamates in yeast, the

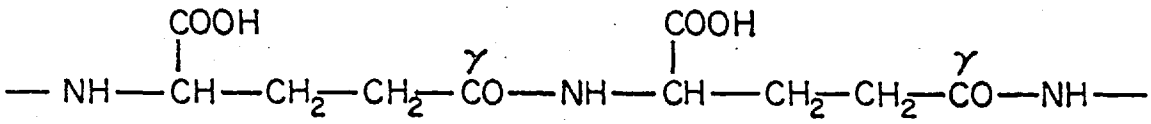

Figure X - The manner of linking glutamic acid residues in polyglutamyl forms of folate.

occurence of even higher conjugates, at least in yeast, is suggested by the isolation by Ratner, Blanchard and Green (1946) of a p-aminobenzoylpolyglutamic acid containing ten to eleven glutamic acid residues.

The manner of linking glutamic acid residues is shown in Figure X.

1.3 Microbiological assay of folate derivatives

The discovery of the various folate derivatives was due mainly to their growth - promoting effect on certain bacteria, which show different specificity in their response to the state of oxidation of the compound, its one-carbon substituent and the number of glutamic acid residues in the molecule. The three organisms most commonly employed in microbiological assay are Lactobacillus casei, Streptococcus faecalis (previously called S.lactis R) and Pedicoccus cerevisiae (previously called Leuconostoc citrovorum). The protozoon Tetrahymena geleii also has a requirement for . PteGlu, and is, unlike the other bacteria, able to utilize the higher polyglutamyl folates, its response to heptaglutamate being similar to that with PteGlu (Kidder and Dewey, 1949). Its use has not found universal acceptance however.

Of the 3 routinely used organisms P.cerevisiae is unique in its requirement for a reduced form of folate, probably due to inability to assimilate physiological concentrations of PteGlu (Wood and Hutchings, 1960). The original assumption was that only 5 CHO H_4 PteGlu would promote growth of this organism, with H_4 PteGlu being only 2.5% as active (Broquist, Fahrenbach, Brockman, Stokstad and Jukes, 1951).

Table 1. Growth response of assay organisms to naturally occurring folate derivatives*.

Compound	L.casei	S.faecalis	P.cerevisiae	Tetrahymena geleii W.
PteGlu	+	+	-	+
H_2PteGlu	+	+	-	
H_4PteGlu	+	+	+	
$PteGlu_2$	+	+	-	
$PteGlu_3$	+	-	-	+
$PteGlu_7$	-	-	-	+
5 CHO H_4PteGlu	+	+	+	
5 CHO $H_4PteGlu_2$	+	+	+	
5 CHO $H_4PteGlu_3$	+	-	+	
10 CHO PteGlu	+	+	-	
10 CHO $PteGlu_3$	+	-	-	
10 CHO H_2PteGlu	+	+	-	
10 CHO H_4PteGlu	+	+	+	
5 CH_3 H_4PteGlu	+	-	-	

* + = a response of at least 50% of the maximum

\- = a response of less than 5% of the maximum

However, this was shown to be a function of the oxidative instability of H_4 PteGlu, for when reducing conditions are maintained, for example with ascorbate, the response of P.cerevisiae is 40-80% of that obtained with 5 CHO H_4PteGlu (Silverman, Keresztesy, Koval and Gardiner, 1957; Silverman, Law and Kaufman, 1961; Bakerman, 1961). Under these same conditions 10 CHO H_4 PteGlu also supports the growth of P.cerevisiae.

S.faecalis and L.casei both respond to a number of folate derivatives, but differ in that only L.casei responds to triglutamate forms (Hutchings, Stokstad, Bohonos, Sloane & Subba Row, 1948). A second important difference is that L.casei alone responds tomethylated forms (Donaldson & Keresztesy, 1959; Keresztesy & Donaldson, 1960; Silverman, Law and Kaufman, 1961; Larrabee, Rosenthal , Cathou, and Buchanan, 1961; Herbert, Larrabee & Buchanan 1963). Reduced forms of folate (eg. H_4 folate and its formyl derivatives) also support the growth of both organisms, but precaution must be taken to

prevent oxidative decomposition of these compounds (Bakerman, 1961; Silverman, Law and Kaufman, 1961).

For some years the difference in response of S.faecalis and L.casei to polyglutamyl folate was thought to be a measure of the polyglutamyl forms present in a natural product (i.e. those containing three or more glutamic acid residues) but it is now known that L.casei does not respond to compounds above the level of triglutamate, the linkage of further glutamic acid residues producing only slight response by the organism.

The techniques of microbiological assay are discussed in Section II.

1.4 Folate conjugases.

A number of studies indicated that natural materials contained enzymes concerned with the removal or addition of glutamic acid residues from or to simpler folate compounds. The usual device that was used to demonstrate such enzymes was that after appropriate treatment folate became available to the three common microbiological assay organisms, L. casei, S.faecalis and P.cerevisiae, whereas the material did not support the growth of these organisms prior to treatment with the enzyme. Thus autolysis was often required to produce PteGlu and related compounds from natural materials which suggested they were being released by enzyme activity from other derivatives, and the presence of enzymes was demonstrated in various organs of the pig, dog, rabbit, rat, chick and turkey, ox and man (Bird, Binkley, Bloom, Emmett and Laskowski, 1945; Bird, Robbins, Vandenbelt & Pfiffner 1946; Simpson and Schweigert, 1949;).Termed "conjugase" the enzyme was shown, by its action in converting polyglutamyl folates to mono or di glutamates, to be a peptidase, being further classified as a γ glutamyl carboxypeptidase as it

does not attack the methyl ester of folylheptaglutamic acid (Pfiffner, Calkins, O'Dell, Bloom, Borwn, Campbell and Bird, 1945) or folyl-glutamyl glutamic acid (Dabrowska, Kazenko and Laskowski, 1949; Kazenko and Laskowski, (1948).

Conjugases, besides being present in high levels in animal tissue such as pancreas, liver, intestinal mucosa, kidney, spleen and brain, are also found in plasma (Wolff, Drouet and Karlin, 1949; Toennies and Phillips, 1959) leucocytes (Swendseid, Bethell and Bird, 1951) almonds, potatoes, (Bird, Binkley, Bloom, Emmett and Pfiffner, 1945; Bird, Robbins, Vandenbelt, and Pfiffner, 1946) other higher plants (Iwai and Nagakawa, 1958) and the gas glands of Physalia physalis (Wittenberg, Noronha and Silverman, 1962). These enzymes fall into two main classes, one active at pH 4.5, the other active at pH 7.5. The most widely employed of these conjugases are considered below.

A. Chick Pancreas Conjugase.

In comparing the activity as conjugase of various animal tissues Laskowsi, Mims and Day (1945) selected chick pancreas as being a potent source of enzyme and proceded to

purify it almost 3000-fold. Although unable to produce the conjugase in crystalline form they were able to show however that it was activated by Ca ions, had a pH optimum at 7.8 and a temperature optimum at about 32 C. The end product of the action of the purified enzyme on triglamyl folate was claimed to be the diglutamyl form (Kazenko and Laskowski, 1948). As it has been shown that chick pancreas releases reduced folates from non active, presumably polyglutamyl forms, in a number of natural materials, it can be concluded that it acts on polyglutamyl forms of H_4 PteGlu, 5 CHO H_4 PteGlu 10 CHO H_4 PteGlu and 5 CH_3 H_4 PteGlu (Hill and Scott 1952; Doctor and Couch, 1953; Hendlin, Koditschek and Soars 1953; Noronha and Aboobaker, 1963; Schertel, Boehne & Libby 1965). Consequently, due to its wide specificity and the comparative ease with which it may be purified (Mims and Laskowski, 1945; Laskowski, Mims and Day 1945; Iwai, Luttner and Toennies, 1964) chick pancreas conjugase has been widely used in the preparation of deconjugated forms of folate.

B. Hog kidney conjugase.

γ glutamyl carboxypeptidase from different sources can differ in both its pH and temperature optima, and in the end products of digestion (Bird, Robbins, Vandenbelt and Pfiffner, 1946). They showed that hog kidney conjugase, unlike chick pancreas conjugase, had a pH optimum at 4-4.5 a temperature optimum of 45-48 C, was not activated by Ca^{++} and appeared to produce PteGlu as its end product. Attempts to purify the enzyme were unsuccessful because of losses during dialysis and fractionation. Hog kidney conjugase appears to have the same braod specificity for polyglutamyl folates as chick pancreas carboxypeptidase, but it has been postulated (Sreenivasan, Harper, and Elvejhelm, (1949) that complete hydrolysis of polyglutamates is only achieved by the stepwise addition of both chick pancreas andhog kidney conjugases to natural folates. Iwai, Luttner and Toennies, (1964)using red blood cells as a substrate, state that the 2 conjugases used together are as effective as plasma conjugase in liberating assayable folates from the red cell.

C. Plasma conjugases.

The presence of γ glutamyl carboxypeptidases in the blood of several species, including man, was reported by Simpson and Schweigert (1949), who noted a good release of free folate from its conjugate following incubation with turkey and rabbit blood at pH 6-8. In the same year Wolff, Drouet and Karlin showed that assayable folates could be released from a yeast preparation by a thermolabile enzyme in human plasma, active at pH 4.5. Toennies, Usdin and Philips (1956) demonstrated that this plasma factor was important in releasing polyglutamyl folate from red cells, and assigned it an optimal activity in vitro at pH 6.0. However, Hoffbrand, Newcombe, and Mollin (1966) reported that red cell folate activity was rapidly released when blood was lysed in 1% aqueous ascorbate, the final haemolysate pH being in the region of 3.6-4. Omer (1969) stated that a fall in pH alone, in the absence of plasma, was not associated with the release of assayable folate from erythrocytes, confirming that human plasma conjugase was activated at a low pH, although slow release of folate activity was

attained at pH values between 5-6.

The possibility of more than one plasma conjugase, as suggested by Toennies and Phillips (1969) cannot be excluded. However the physiological p H range of human plasma (7.38 - 7.5) would preclude the in vivo action of plasma conjugase, maximally activated at pH 4.5

D. Intestinal Conjugases. The presence of conjugase in the intestinal mucosa of the rat was noted by Laskowski, Mims and Day (1945), and this was followed in 1946 by a report, based on duodenal and gastric aspirates in 3 subjects, that intestinal conjugase was absent in the human being (Welch, Heinle, Nelson and Nelson, 1946 Buyze and Engel, 1948). However, Santini, Berger, Berdasco, Sheehy, Aviles and Davila (1962) reported conjugase activety in duodenal aspirates from two patients with tropical sprue, while Klipstein (1967) found the enzyme in the succus entericus in 5 out of 10 patients with tropical sprue and 7 out of 15 other subjects. The mucosa of human intestine was found to be a rich source of conjugase (Rosenberg, Godwin, Streiff & Castle, 1968, Hoffbrand &

Necheles, 1968; Rosenberg, Streiff, Godwin & Castle (1969) Hoffbrand, Necheles, Maldonado, Horta and Santini, (1969) and was reported to have a pH optimum of 4.6, the optimal temperature being 37 C. In the rat intestinal conjugase was stated to be 8 to 10 times more active than that found in chick pancreas (Rosenberg and Streiff, 1967).

E. Conjugase inhibitors. The presence in crude natural materials of substances which could inhibit the action of conjugases was noted by Bird, Robbins, Vandenbelt, and Pfiffner (1946) who found that, although hog kidney conjugase liberated "vitamin Bc" from its conjugate in yeast, the amount of "vitamin Bc" obtained depended on the purity of the yeast substrate. Using a series of yeast extracts ranging from a water extract of brewers yeast to crystalline vitamin Bc conjugate they demonstrated that over 100 times more conjugase was needed to release 1 µg of vitamin Bc from the yeast extract than from crystalline vitamin Bc conjugate. In a later study Mims, Swendseid and Bird (1947) implicated thymus and yeast nucleic acids in the inhibition of hog kidney conjugase activity, at the same time noting that in vivo experiments did not always parallel in vitro

studies. Sims and Totter (1947) showed that a glutamic acid polypeptide of p-aminobenzoic acid obtained from yeast had an inhibitory action on both rat liver and chick pancreas conjugases, when either yeast extract or crystalline vitamin Bc conjugate was used as substrate. Inhibition of intestinal conjugase by crude yeast extracts was assumed by Bethell, Meyers, Andrews, Swendseid, Bird and Brown (1947) and Swendseid, Bird, Brown and Bethell (1947) who in studies on patients with macrocytic anaemia, showed little response to the administration of a yeast extract with high inhibitor content to 3 patients with pernicious anaemia and one with post-gastrectomy anaemia, but a suboptimal response to the same material in a patient with probable nutritional macrocytic anaemia. In normal subjects tested the excretion of free PteGlu in the urine was greater in those given an inhibitor-free yeast preparation than in those who took a concentrate with high conjugase inhibition.

It has also been suggested (Druskin, Wallen and Bonagura, 1962; Hoffbrand and Necheles, 1968, Rosenberg, Streiff, Goodwin and Castle, 1968) that the anticonvulsant drug phenytoin induces folate dificiency by inhibiting intestinal conjugases, a view not supported by Baugh and Krumdieck (1969).

1.5 Natural occurance of folate derivatives.

A. Dietary materials.

The folic acid content of a number of natural foods was estimated in 1943 by Cheldelin, Wood and Williams, using S.faecalis as the assay organism and takadiastase as the source of conjugase. Unfortunately takadiastase does not liberate folate from its conjugates, nor was the need for the presence of a reducing agent to prevent oxidation of H_4 derivatives recognised, so neither this study, nor those of Ives, Pollard, Elvehjem and Strong (1946) and Schweigert, Pollard, and Elvehjem (1946) gave a true estimate of folates present in foodstuffs. The most extensive study on the folate composition of foods is that of Toepfer, Zook, Orr and Richardson (1951) but again this was carried out without the use of ascorbate, although hog kidney and chick pancreas conjugases were employed, and numbers of the foods were assayed with L.casei as well as S.faecalis. Among the richest sources of folate these workers reported were yeast, liver, asparagus, endive, broccoli, lettuce and spinach, all of which contained more than 1 mgm of folate

activity/100 gm of dry weight. Most other leafy greens were in the range 0.4-1 mgm PteGlu/100 gms dry weight, as were liver, dried beans, blackeye peas and soy flour. In the range 0.03 to 1 mgm folate were root vegetables, most fresh fruit, grains, nuts and lean beef, while in the lowest range, 0.03 mgms or less, were included eggs, milk and meats other than beef and poultry. The type of folate derivative in various foods and diets has been investigated by Butterworth and his colleagues. Assay by S.faecalis of 17 American diets gave a mean value of 52 µg, and after conjugase digestion 184 µg, for a 24 hour period. Fractionation of the digested material on DEAE cellulose showed the presence of N^{10} CHO PteGlu, N^{5} CHO H_4 PteGlu and PteGlu representing 55% 34% and 11% respectively of the total activity (Butterworth, Santini and Frommeyer, 1963). Assay of 4 diets by L.casei gave mean values of 156 µg and 689 µg pre and post conjugase treatment so that most of the folate would appear to be present as $5CH_3$ H_4 PteGlu and its polyglutamates.

In a report on the folate compounds in various individual foods (Santini, Brewster and Butterworth, 1964) formyl folates were again found to be the main constituents of the selected vegetables, but in contrast a higher L.casei than S.faecalis activity with yeast, chicken, liver, calf liver, navy beans, red beans and black-eyed peas indicated the presence of appreciable quantitites of 5 CH_3 H_4 PteGlu. In neither study was a reducing agent employed in the initial preparation of the foodstuffs to protect oxygen-labile derivatives of PteGlu, which could explain the discrepancy between this report and that of Iwai and Nagakawa (1958) who found most of the folate present in green leaves was a labile reduced derivative, presumably 10 CHO H_4 PteGlu, and its polyglutamates.

The folate content of cow's milk has received considerable attention and varying values have been reported, mean values being 38 µg/litre of L.casei active material and 12.6 µg of P.cerevisiae active material (Matoth, Pinkas and Sroka, 1965) 42 µg/litre (Sullivan, Luhby and Streiff, 1966) 62-100 µg/litre (Naiman & Oski, 1964) 100 µg/quart (Luhby and Cooperman, 1963)

55 µg/litre (Ghitis, 1966) and 85 µg/litre (Hurdle, 1967). The latter worker reported a value of 145 µg after the use of chick pancreas conjugase.

B. Yeast. Reference has been made to the isolation of heptaglutamate from yeast, Schertel, Boehne and Libby (1965) chromatographed on DEAE cellulose a yeast extract in ascorbic acid, and showed it to consist of approximately 98% polyglutamyl compounds. After conjugase digestion 50% of the material was inactive for S.faecalis and probably represents 5 CH_3H_4PteGlu, 8% was active for P.cerevisiae, representing 10 CHO H_4PteGlu, 5 CHO H_4PteGlu and H_4PteGlu, while the remainder is presumed to consist of PteGlu, 10 CHO PteGlu and their dihydro derivatives. There appears to be present at least 4 major types of 5 CH_3H_4PteGlu polyglutamates as shown by their elution pattern, while the S.faecalis activity of these same fractions indicates the presence of poluglutamates of other folate derivatives.

C. Folate derivatives in serum, plasma and whole blood.

A large number of studies have been carried out

on the forms of folate in these tissues, the primary objective being the detection of folate deficient anaemias. Using S.faecalis (Schweigert and Pearson 1947; Wolff, Drouet and Karlin 1949; Toennies and Gallant, 1949; Girdwood, 1953; Nieweg, Faber de Vries and Kroese 1954; Condit and Grob 1958; Cox, Meynell, Cooke and Gaddie, 1960) and/or L.casei (Schweigert, 1948; Simpson and Schweigert 1949; Spray 1952; Spray and Witts , 1952) as test organisms no consistent difference in serum or blood levels could be detected between normal subjects and those with megaloblastic anaemias until Toennies, Frank and Gallant (1953) and Toennies Usdin and Phillips (1956) showed that the folate material in blood was heat labile, but could be protected in assay systems by ascorbic acid. It could also be preserved for assay by low temperature dialysis. Using these techniques they showed that blood folate activity was 20 in 100 times higher than the values previously reported. Differentation of serum folate levels between normal subjects and those with folate deficiency was achieved by Baker, Herbert, Frank, Parker, Hutner,

Wasserman and Sobotka (1959) and the L.casei active material in human plasma was tentatively identified as 5 CH_3 H_4 PteGlu by Herbert, Larrabee and Buchanan (1962). These workers could not establish the presence in serum of peptide forms of folate, a view supported by Noronha and Aboobaker (1963), and Bird, McGlohon and Vaitkus (1965). However increased serum folate levels after chick pancreas digestion have been reported by Banerjea and Chatterjea (1966 and 1968).

The nature of the relatively large amounts of folate derivatives in red cells was studied by Noronha and Aboobaker (1963) who, using chromatographic separation in the presence of ascorbate, demonstrated 4 peptide derivatives of 5 CH_3 H_4 PteGlu, presumably of increasing chain length. 86-90% of the red cells folates were found to be peptide bound, and no significant amounts of 5 CHO H_4 derivatives were detected. This result is in contrast with those of Usdin (1959) and Iwai, Luttner and Toennies (1964), who found that about 18% of the poly glutamate folates in red cells were active for P.cerevisiae after the use of conjugase. It has also been shown that foetal blood contains a greater proportion of H_4 PteGlu

and its formyl derivaties than does the maternal blood or infant blood (Grossowicz, Aronovitch, Rachmilewitz Izak, Sadowsky and Bercovici, 1960; Matoth, Pinkas and Sroka (1965).

1.6 Folate content of daily diets.

Man appears to be totally dependent on extraneous or dietary sources for his folate supply, and estimation of the amount available from natural foodstuffs is complicated by the fact that some three quarters of the folate present are peptide bound. (The availability of these forms is considered later).

The folate content of whole diets were assayed by Butterworth, Santini and Frommeyer (1963), who reported average values of 4 American diets, using L.casei, of 157 µg "free" folate and 688 µg after enzyme treatment. Measuring free folate only Hurdle (1967) found a range of 161-297 µg daily in 8 young controls, with a mean of 223 µg, while sixteen elderly people at home consumed between 129 to 250 µg, with amean of 145µg. Other estimated values reported for daily dietary intake are 52-97 µg (Pace, Stier, Taylor and Goodman, 1960) and 43-86 µg (Denko, Grundy, Porter, Berryman, Friedmann and Youmans (1946). Distinguishing between "high cost", "low cost" and "poor" diets Chung, Pearson, Darby Miller and Goldsmith (1961)

reported folate values of 193, 157 and 47 µg respectively, while Santini, Berger, Berdasco, Sheehy, Aviles and Davila (1962) found a variation between 380 µg in a rural Puerto Rican diet and 650 µg in an urban one. Other estimates of daily food folate intake have been 400 µg (Jukes, 1961) and 1000-1500 µg (Jandl and Lear, 1956).

1.7 Availability of dietary folate.

Although there have been a number of reports on the absorption and utilization of PteGlu in man there have been few concerning polyglutamyl folates, which comprise the greater part of food folate material. PteGlu given orally is rapidly and efficiently absorbed (Denko, 1951; Spray and Witts, 1952, Clark, 1953; Chanarin, Anderson and Mollin 1958; Butterworth, Nadel, Perez-Santiago, Santini and Gardner 1957; Klipstein, 1963; Baker, Frank, Feingold, Ziffer, Gellene, Leevy and Sobotka 1965). Anderson, Belcher, Chanarin and Mollin 1960, reported an average of 79% of a 200 µg dose of H_3 labelled PteGlu absorbed by a group of 13 control subjects; and PteGlu itself is without doubt utilized by man as judged by clinical responses in folate deficiency anaemias.

The absorption and utilization of derivatives of PteGlu have been studied to a much lesser degree than that of the parent compound. However, comparing the absorption of PteGlu, 5 CHO H_4PteGlu, Pteroylglutamyl-a-glutamic acid (Diopterin) and $PteGlu_3$ (Teropterin) Baker, Frank, Feingold, Ziffer, Gellene, Leevy and Sobotka (1965) found increased

urinary excretion of folates after the administration of each analogue, thus indicating absorption of these substances. Oral 5 CHO H_4 PteGlu caused an optimum response, when given at a dosage of 50 µg daily, in a apatient with tropical sprue while 4 out of 9 patients responded to 100 µg synthetic 10 CHO PteGlu (Butterworth, Santini, Wheby and Perez-Santiago 1964).

Little of the above data on folate absorption can be applied to dietary material, where the main constituents consist of folate derivatives containing more than 3 glutamate residues, and few investigations of the availability of such folates have been made. However, Baumslag and Metz (1964) reported the effectiveness of feeding large quantities of lettuce, presumably containing some formyl polyglutamates, to patients with megaloblastic anaemia of pregnancy, similar observations being made on 2 patients only using soups made from lettuce, asparagus and spinach by Butterworth, Brewster, Perez-Santiago and Santini (unpublished data quoted by Butterworth, 1968).

Yeast preparations have been commonly used to test the absorption of polyglutamyl folates in the relatively small number of studies that have been reported. Although these forms appear to be equivalent to "free" PteGlu in the nutrition of the chick (Cropper and Scott, 1966) and rat (Kodicek and Carpenter, 1948) the evidence as regards man is conflicting. One of the first reports on yeast polyglutamate absorption was that of Bethell, Swendseid, Bird, Meyers, Andrews and Brown (1947) who treated 2 patients with pernicious anaemia in relapse and 1 with post gastrectomy anaemia with the compound, the reponse not being as effective as that to the free vitamin (a view supported by Welch, Heinle, Nelson and Nelson 1946) and indeed being very low when the yeast material contained an inhibitor of conjugase activity. Several groups have however reported haematological responses to conjugated yeast folate in patients with pernicious anaemia and sprue (Suarez, Welch, Heinle and Nelson 1946; Spies, Lopez, Milanes and Aramburu, 1947; Jandl and Lear 1956) although the amount of conjugate needed to produce a response appeared to be

greater, even on a molar basis than the "free" vitamin.

Jandl and Lear (1956) comparing urinary excretion values following equivalent amounts of oral PteGlu, yeast, and yeast pre-treated with conjugase suggested that approximately 25% of the yeast preparation was absorbed, compared to 60% after conjugase treatment and 95% with PteGlu. Spray (1952a) and Spray and Witts (1952) reported similar findings using doses of approximately 1 mgm of yeast polyglutamate or PteGlu.

Following the report by Schertel, Boehne, and Libby (1965) on the fractionation of yeast extract on DEAE columns, Streiff and Rosenberg (1967) gave 13 normal subjects oral doses of 10 µg of yeast folate obtained from those fractions free of monoglutamate and conjugase inhibitor, finding a plasma folate rise of 10-35% compared with a 20-50% rise with PteGlu. Hoffbrand, Necheles, Maldonado, Horta and Santini (1969) using a similar preparation in oral doses of 200 µg reported peak plasma folate increases over the fasting level of 86.6% and 105.4% for polyglutamate and PteGlu respectively. Without attempting to compare "free"

folate absorption with that of the polyglutamyl forms Rosenberg, Streiff, Godwin and Castle (1969) obtained peak plasma rises over baseline of between 6 and 17 µg/ml after 200 µg of DEAE fractionated material.

Recently the first study has been reported which utilizes synthetic C^{14}-labelled PteGlu polyglutamates (Butterworth, Baugh and Krumdieck, 1969) the conclusion being drawn from 2 cases that 50-70% of ingested polyheptaglutamate is available to man inasmuch as it is not lost in the faeces.

Whether polyglutamyl folates are absorbed unchanged or are broken down to more simple forms in the gut is uncertain. The presence of intestinal conjugases has been reported, and Streiff and Rosenberg (1967) and Rosenberg, Streiff, Godwin and Castle (1969) claim that everted sacs of rat intestine, and homogenates of intestinal mucosa of rat and man convert conjugated folate to free folate. Hoffbrand and Necheles, (1968) and Hoffbrand, Necheles, Maldonado, Horta and Santini (1969) report a similar effect using jejunal mucosal homogenates, while Hoffbrand and Peters (1969) localized the conjugase activity, using guinea pig mucosa, in the lysosomal fraction.

After ingestion of polyglutamyl folates the material appearing in the plasma and urine of the subject is usually an L.casei active form of folate, presumably 5 CH_3 H_4 PteGlu monoglutamate. However, Cooperman and Luhby (1965) reported the unchanged absorption of polyglutamate, stating that the peptide form became available for assay in the plasma only after chick pancreas conjugase treatment of the plasma. Plasma peptide forms have also been reported by Banerjea and Chatterjea (1966), who claimed a 2-11 fold increase in the L.casei values for normal subjects after treatment of plasma with fresh chick pancreas conjugase, and who also reported a 4-16 fold increase in conjugated plasma folate after the injection of 5 mgms PteGlu monoglutamate (Banerjea and Chatterjea, 1968).

1.8 Folate requirements in man.

The greater part of the work published in connection with folate requirement concerns the concentration of PteGlu itself, an unnatural form of folate, needed to produce haematological remission in patients with either nutritional megaloblastic anaemia or syndromes associated with folate malabsorption. These results were interpreted as being synonymous with normal daily requirements, and the values postulated vary considerably, from 5 µg daily in children (Velez, Ghitis, Pradilla and Vitale, 1963), to 25µg (Sheehy, Rubini, Perez-Santiago, Santini and Haddock, 1961; Druskin, Wallen and Bonagura, 1962) 50 µg (Zalusky and Herbert, 1961) 100 µg (Sheehy, 1961) 200 µg (Hansen and Weinfeld, 1962) and 200-300 µg(Jandl & Lear, 1956).

Herbert (1962) also studied the amount of folic acid needed to prevent a fall in serum folate values in 3 volunteers given a folate deficiency diet. Given folate supplements of 25-100 µg daily, all 3 had lower serum folates at the end of the study, showing that even 100 µg of folic

acid daily was insufficient to maintain the original serum folate value. Thus it would appear that man's daily requirement lies in the region of at least 100-200 µg of folate, in a readily absorbable form.

Section 11.

Materials, Methods and Subjects Studied.

1. General

Glassware: Glassware was washed in an automatic glass washing machine (Heinicke Instruments CO) using BHC Labrite 3357 detergent and a final distilled water rinse. Glassware washed in this way was satisfactory for microbiological assay work.

Pipettes were initially acid washed, subsequent cleaning being done in an automatic pipette washer using cold tap water followed by at least 3 rinses in distilled water before drying in a hot air oven.

Distilled Water: Glass-distilled water was used throughout.

Reagents: These were kept specifically for microbiological assay to reduce the chances of contamination with substances either stimulatory or inhibitory to the organisms.

L.ascorbic acid (BDH) was obtained as the dry powder and added to the buffer or aqueous solution immediately before use, on the day it was required, and the pH adjusted using a glass electrode pH meter.

SPECIMENS

A. Collection and storage.

i. Whole blood specimens for the estimation of red cell folate were collected into lithium heparin as anticoagulant, and one ml. of blood diluted and lysed in 9.0 mls of 1% aqueous ascorbate (Hoffbrand, Newcombe and Mollin, 1966). This was stored at -20° c until assayed.

ii. Serum and plasma samples were stored -20° c in plastic disposable containers after the addition of 3-5 mgms of dry ascorbic acid.

iii. Urine specimens were voided directly into a plastic wide mouthed container to which had been added approximately 1 gm of dry ascorbic acid. At the end of the collection period the volume of the specimen was measured and an aliquot stored at -20° c for assay.

iv. Food homogenates, prepared as described on page 100 were stored in 100 gm quantities in glass containers at -20° c.

v. Yeast preparations were kept in petri dishes in a vacuum desiccator, the desiccator being stored at room temperature in the dark.

B. Preparation of specimens for microbiological assay.

i. Whole blood. The initial 1/10 dilution in 1% aqueous ascorbate was diluted 1/10 again in the O.1.M. phosphate-ascorbate buffer pH 5.7, autoclaved, filtered, and the filtrate assayed.

ii. Serum.

L.casei assay: Unextracted sera were added directly to the assay tubes in the 'aseptic addition' technique. In the routine assay protein was precipitated and folate preserved with ascorbate by adding 0.2 mls serum to 9.8 mls O.1.M. phosphate buffer containing 150 mgms % ascorbate, pH 5.7, autoclaving for 10 mins at 116°, filtering and assaying the clear filtrate. (Chanarin and Berry 1964).

S.faecalis and P. cerevisiae assay: Whole serum was used. Any turbidity in the serum was allowed for by the inclusion in the assay of an uninoculated tube indentical with the triplicate inoculated assay tubes. The optical density value of the uninoculated tube was subtracted from the mean optical density of the 3 inoculated tubes.

iii. Urine. This was diluted appropriately either with 10% ascorbate for aseptic addition techniques and with water for routine assay.

iv. Food homogenates. Triplicate 4.0 gm aliquots of dietary homogenate (see page 100) were made up to 50 mls with O.1.M phosphate buffer containing 0.1% ascorbate pH 7.0 and autoclaved for 15 minutes at 121°C. When cool all samples were well mixed, and 25 mls (containing 2 gms homogenate) were removed, made up to 200 mls with distilled water, filtered and the clear filtrate retained for assay of "free folate".

The remaining 25 mls were treated with chick pancreas enzyme to release conjugate folate prior to assay. The method is described on page 73.

3. Preparation and use of conjugase enzymes.

Two main types of conjugase (carboxypeptidase; polyglutamyl hydrolase) were used in these studies.

1 Chick pancreatic conjugase, which has a pH optimum at approximately 7.0 (Mims and Laskowski, 1945).

2. Human plasma conjugase, active at pH 4.5 (Wolff, Drouet and Karlin, 1949; Toennies and Phillips, 1956; Omer, 1969)

A. Chick pancreatic conjugase:

This was chosen because of its wide specificity for polyglutamyl folate compounds, its ready availability and relatively high potency.

As some commerically available Difco chick pancreatic extracts proved to have very low 'conjugase activity', enzyme was prepared from fresh chick pancreas obtained from an abattoir and transported deep-frozen to the laboratory. The tissue from 10 chickens was used, the pancreas being cut into small pieces, ground in a mortar with two successive aliquots of acetone and filtered through gauze. The tissue was air dried and stored in the dark at 4°C. The enzyme was further concentrated and purified by the method of Iwai, Luttner and Toennies (1964) involving autolysis, Ca^{++} precipitation and re-suspension in O.1.M. phosphate buffer, pH 7.2. A large proportion of the folate compounds in the tissue were removed by adsorption with Amberlite resin IR 400.

This freshly prepared conjugase was fully active in deconjugating yeast polyglutamyl folate and its low folate activity made it more suitable for the assay of serum for polyglutamyl folate than the cruder preparation previously used.

1. Determination of quantities of chick pancreatic conjugase required per unit substrate. The concentration and pH values at which chick pancreas conjugase has been used by other workers are shown in the following table:

Author	Ratio of chick pancreas (mgms) to weight (gms) of homogenate assayed.	pH.
Toepfer et al (1951)	20:1/200:1	7.2
Butterworth et al (1963)	100:1	7.2
Herbert (1963)	2:1	6.1
Laboratory of the Govt. Chemist (1966)	25:1	6.1
Hurdle (1967)	3:1	6.0

To establish the optimal amount of conjugase required to hydrolyse polyglutamyl folate to an assable form, yeast extract (Difco) and a representative diet were both incubated at 37° with varying amounts of chick pancreatic conjugase.

The concentration of the conjugase was 25 mgms in 2 mls of O.1.M. phosphate buffer containing 25 mgms ascorbic acid,

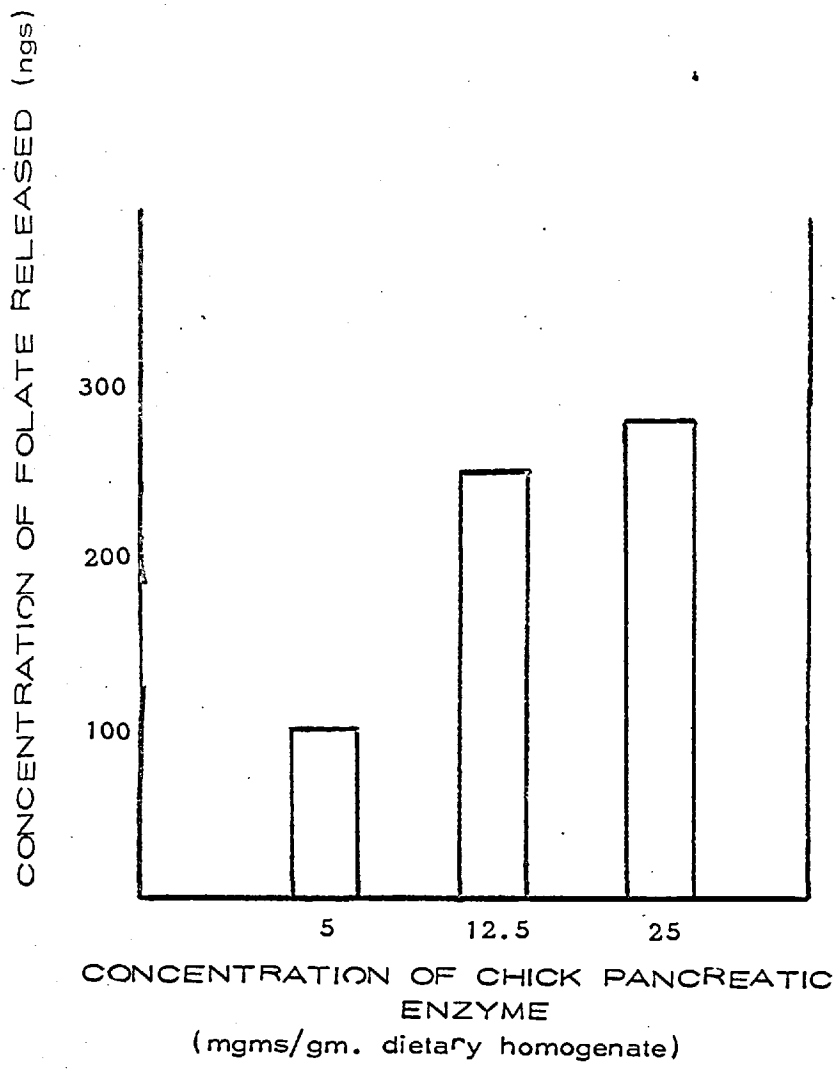

Figure XI - The release of assayable folates from a standard preparation of yeast polyglutamyl folate by varying concentrations of chick pancreatic enzyme.

pH 7.0. The time of incubation was left constant at 18 hours. The effect of varying concentrations of enzyme on the release of folate is shown in Fig XI., and from these values a concentration of 25 mgms of chick pancreatic suspension for every 2 gms of homogenate was used in the final assay procedure.

ii. Use of chick pancreatic enzyme:

a) With serum and urine. Specimens of serum or urine to be assayed were treated as follows:

0.1 mls of the sample in 9.8 mls of O.1.M. phosphate buffer pH 7.2 containing 50 mgms ascorbic acid was incubated for five hours at 37° C with 0.1 mls of purified chick pancreas conjugase. After incubation the specimens were brought to pH 5.7 with 3N HCl, heated for 10 minutes at 116° C, filtered, and the clear filtrate assayed.

Controls of the specimen without conjugase and of conjugase alone were treated in the same way, the folate value of the conjugase specimen being subtracted from the value of the serum or urine plus conjugase sample.

b) With dietary material. Prior to the collection of fresh pancreatic tissue as described on page 70, commercial dried chicken pancreas (Difco) was used at a concentration of 25 mgms to 2 gms of dietary homogenate in 25 mls O.1.M phosphate buffer, pH 7.0. The mixture was incubated for 18 hours at 37°, heated for 10 minutes at 121° C, filtered and the clear filtrate assayed.

Specimens of chick pancreas alone were treated and assayed in the same way, the folate value of the enzyme being

subtracted from the value of the food plus enzyme specimens.

B. Plasma conjugase:

Almost all human plasma or serum samples contain conjugase activity. Heparinized plasma was obtained from one or 2 donors and used either immediately or stored deep frozen until required. The substrates were either Difco yeast extract or a haemolysate prepared from washed human red cells, the systems being brought to the pH optimum for plasma conjugase by diluting the substrate with 1% aqueous ascorbate pH 4.5. Plasma conjugase was used in this study in a system to test for the presence of inhibitors of conjugase activity (Section V).

3. Microbiological assays.

These were done by two methods.

1. The entire assay was autoclaved prior to inoculation with the appropriate organism (routine assay). Stable folate compounds only (PteGlu, 5CHO H_4PteGlu, 10 CHO PteGlu, 5 CH_3 H_4PteGlu) are assayed by this method, but labile derivatives may be lost.

2. In order to protect samples from heat and oxidation an aseptic addition method (Bakerman, 1961) was used, whereby ascorbate and the test material were added to the cooled sterile basal medium immediately before inoculation. With this method H_2PteGlu and H_4PteGlu may be measured.

Labile folates are completely inactivated by autoclaving, as in the routine assay, but retain full microbiological activity in the aseptic addition assay. Thus the difference in the results obtained on a specimen assayed by both methods is a measure of the labile folate present.

Three assay organisms were used in these studies:

1. L.casei (NCIB 8010) which responds to all folate compounds up to the level of triglutamyl forms.

2. S.faecalis (NCIB 6459) which responds to folate excepting methyl compounds, tri- and polyglutamates.

3. P.cerevisiae (ATCC 8081) which has a requirement for tetrahydro folates only, excepting methyl forms, up to the level of triglutamate.

Assays with all 3 organisms were done using pyrex rimless tubes 150 x 19 mm. three tubes being set up for each specimen. Tubes were set up in stainless steel racks of 48-tube capacity, each rack of tubes being covered by a single aluminium lid.

A. L.casei assay.

The procedure followed was basically that described by Toepfer, Zook, Orr and Richardson (1951) which is in turn a modification of that of Teply and Elvehjem (1945).

i. Basal medium. This was a dehydrated preparation, Folic Acid casei medium (Difco) made into solution according to the makers' instructions but modified by the addition of 2 gms/ litre ascorbic acid and subsequent readjustment of the pH with 3 N. KOH to 6.6-6.8.

ii. Preparation of standard curve.

a) Stock solution. A commercial preparation of PteGlu (BDH) was dried in a hot air oven at 160° C for 2 hours, the dried product being stored in a desiccator. The standard solutions were made up from this by weighing accurately 100 mgms, adding 10 mls of distilled water and bringing the PteGlu into solution with the dropwise addition of N.NaOH. The solution was made up to 100 mls with distilled water, giving a concentration of 1 mgm/ml. The concentration of the solution was checked spectrophotometrically by diluting it 1/100 (0.01 mg/ml) in N/10 NaOH, which should give an optical density reading of 0.199 using a 1 cm light path at a wavelength of

Table 2.

L.casei Assay

Standard Growth Curve.

Routine assay

Water (ml)	Solution No.	Solution ml.	PteGlu content (ng)
2	-	0	0
1.75	E	.25	.05
1.5	E	.5	.1
1.0	E	1.0	.2
0.5	E	1.5	.3
0	E	2	.4
1.5	D	.5	.5
1.25	D	.75	.75
1.0	D	1.0	1.0

Aseptic addition assay

Solution No.	Solution ml	4% ascorbate (ml)	Water
	0	1	1
E	.25	"	0.75
E	.5	"	0.5
E	1.0	"	0
D	.3	"	0.7
D	.4	"	0.6
D	.5	"	0.5
D	.75	"	0.25
D	1.0	"	0

365 (Angier et al, 1945). Ten mls of solution containing 10 mgm PteGlu were then made up to 1,000 mls in 25% ethanol. This stock solution was stored in a dark bottle at 4°C for some months with no loss of activity.

b) Working standard solutions. For each assay the stock solution was diluted as follows:

Solution B : 25 mls of stock solution to 1000 ml water (250 ng/ml)

" C : 20 " " solution B to 500 mls water (10 ng/ml)

" D : 10 " " solution C to 100/mls (1 ng/ml)

" E : 20 " " " D " " " (0.2 ng/ml).

The assay range of the standard curve is from 0.05 - 1.0 ng PteGlu, triplicate tubes being diluted from the working standards as shown in table 2.

iii. Maintenance of organism. L.casei (NCIB 8010) was maintained in dehydrated culture media, namely B12 culture agar, USP (Difco) and B12 inoculum broth USP (Difco). The stock culture of the organism in the form of a "stab" in agar was kept at 4°C and was subcultured into broth at weekly intervals. As the assay requires a briskly growing organism the agar stab was subcultured into broth the morning prior to assay, put into fresh broth overnight, the 3rd broth culture made the following morning being the 6 hour culture used for inoculation of the assay.

iv. Preparation of the organism for use in the assay. At the end of the 6 hour incubation period the broth culture of L.casei is centrifuged to deposit the organism, and the folate rich supernatent discarded. To remove any traces of folate the organism was washed three times with 10 mls of sterile single strength basal medium by centrifuging and discarding each supernatent, the organism finally being suspended in 10 mls of sterile single strength medium.

v. The assay. Both standards and specimens were set up in tripilicate, the standard being set up as described above. The specimens were diluted in O.1.M. phosphate ascorbate buffer, pH 5.7, so that their folate content was about 0.1 - 0.4 μg, this being the optimal part of the growth curve. Two ml. volumes of the diluted specimens were used throughout, and 2 mls of basal medium added. A "zero" tube, consisting of water and basal medium was also included in the assay, this tube not being inoculated with

organism.

Each rack of tubes was covered with an aluminimum lid and all the racks autoclaved together at 10 lbs psi (116° C) for 10 minutes, cooled, and one drop of the washed culture added to each tube except the zero, using a sterile 50 dropper pipette. After shaking the tubes were incubated at 37° for 20-22 hours.

vi. Reading of assay results: At the end of the incubation period 5 mls of water was added to all the L.casei assay tubes to enable the optical densities of the tubes to fall within the sensitive range of the colorimeter. Each tube was well mixed to suspend the organism evenly, and finally the optical density read in a Unicam SP 300 colorimeter using a red orange filter (Ilford 204), the zero setting of the instrument being made with the "zero" tube. The optical densities of the standard tubes were then plotted on arithmetic graph paper with the concentration of PteGlu against the optical densities. The concentration of folate in the specimens is calculated from the graph using the formula:

$$\frac{\text{optical density} \times \text{dilution}}{\text{volume of filtrate.}}$$

vii. L.casei assay - "aseptic addition". This was done as described above for the routine assays but with the following important difference:

The basal medium (2 ml) and the appropriate quantity of diluent (table 2) were autoclaved together, cooled, and thereafter 1 ml of 4% aqueous ascorbate solution, pH 6.0 was

Table 3.

S. faecalis assay

Standard Growth Curve.

Routine assay				Aseptic addition assay			
water	Solution		PteGlu content	Solution		10% ascorbate	water
(ml)	No.	ml.	(ng)	No.	ml.	1	4
5	D	0	0	D	0	"	3.5
4.5	"	.5	.5	"	.5	"	3
4	"	1	1.0	"	1.0	"	2.5
3.5	"	1.5	1.5	"	1.5	"	2
3	"	2	2	"	2	"	1.5
2.5	"	2.5	2.5	"	2.5	"	1
2	"	3	3	"	3	"	0.5
1.5	"	3.5	3.5	"	3.5	"	0
1	"	4	4	"	4	"	3.5
0	"	5	5	"	0.5	"	

added aseptically, followed by one ml of test sample previously diluted in aqueous ascorbate (if necessary) to the appropriate concentration. Inoculation, incubation and reading of results were as described for the routine assay.

B. S.faecalis assay.

The same glassware, assay racks, method of autoclaving, inoculation and incubation were used for this assay as for L.casei. Points of difference are noted below.

i. Assay medium - Folic AOAC medium (Difco) was made up according to the maker's instruction. No extra ascorbate was added.

ii. Standard curve. The same solutions of folic acid were used as for the L.casei assay, but as the optimal part of the growth curve with S.faecalis is in the range of 2-3 ng the standard tubes were prepared from solution D (1 ng/ml) (table 3).

iii. Organism. This was stored in the form of dried gelatin discs in a vacuum desiccator. A fresh disc was used for each assay, being put into broth on the morning of the assay and incubated for 6-7 hrs at 37°C.

iv. The assay. 5 ml quantities of standards and tests were used, and 5 ml quantities of basal medium added. Any turbidity in the test material was compensated for by including in the assay a "blank" tube consisting of test and basal medium. This was not inoculated, and its density reading

Table 4.

P.cerevisiae assay

Standard Growth Curve

Routine assay				Aseptic addition assay			
water	solution		5 CHO H_4 PteGlu content	Solution		10% ascorbate	water
(ml)	No.	ml.	(ng)	No.	ml.		
5	C	0	0	C	0	1 ml	4
4.8	"	0.2	0.1	"	0.2	"	3.8
4.6	"	0.4	0.2	"	0.4	"	3.6
4.4	"	0.6	0.3	"	0.6	"	3.4
4.2	"	0.8	0.4	"	0.8	"	3.2
4	"	1.0	0.5	"	1.0	"	3
3.5	"	1.5	0.75	"	1.5	"	2.5
3	"	2	1.0	"	2	"	2

subtracted from that of the inoculated tubes.

v. "Aseptic addition" assay. Preparation of the standard curve is shown in table 4. The assay protocol is that described above for L.casei i.e. with the addition after autoclaving of the medium of 1 ml 10% ascorbate followed by the test material.

C.P.cerevisiae assay. Both routine andaseptic addition assays were done as described for S.faecalis, using 5 ml quantities of test and basal medium.

i. Medium. CF assay medium (Difco) was used.

ii. Standard curve. As P.cerevisiae has a requirement for pre-formed tetrahydrofolate derivatives the standard used was 5 CHO H_4PteGlu. The synthetic compound used here, which may be obtained commercially (Calcium leucovorin, Lederle) is a mixture of two diastereoisomers only one of which, the L. form, is microbiologically active. Allowance was made when making the standard dilutions for the inactive (D) isomer present.

Working solutions. Solution A: 1 ampoule Leucovorin (= 1.5 mgms L.form) diluted to 150 mls in 30% ethanol containing 0.5 mls NH_4OH (stock solution).

Solution B - 1 ml solution A to 1000 mls (10 ng/ml)

" C - 5 ml " B to 100 " (0.5 ng/ml).

Standard growth curve dilutions are shown in table 4.

iii. Organism. The stock culture was in the form of a stab in agar, three successive subcultures into broth being made before using the organism in the assay.

D. L.casei and chloramphenicol assay.

In addition to these assay techniques a few L.casei assays were performed by the method of Millbank, Davis, Rawlins, and Waters (1970). This method utilizes a chloramphenicol resistant strain of L.casei and chloramphenicol may be added to the basal medium thus making heat sterilization unecessary. As experience with this method was limited it was used only as a screening assay on fractions collected after column chromatography of yeast and diet specimens.

E. Control of assays.

Each assay performed was controlled by the addition of a known standard, treated identically with the test material. For serum assays this comprised a serum of known folate content and a further sample of the same serum to which was added synthetic PteGlu, where L.casei or S.faecalis were the test organism, and 5 CHO H_4PteGlu for P.cerevisiae assays. Where recoveries of added material were lower than 80% or greater than 120% the assay was discarded.

Where food or yeast folate were being assayed, the standard used was Difco dried yeast extract. This had previously been assayed with L.casei in triplicate on 10 separate occasions and gave a mean value per gram of 0.6 µg "Free" folate and 44 µg "total" folate. In the food folate assay, 0.5 gms of dried

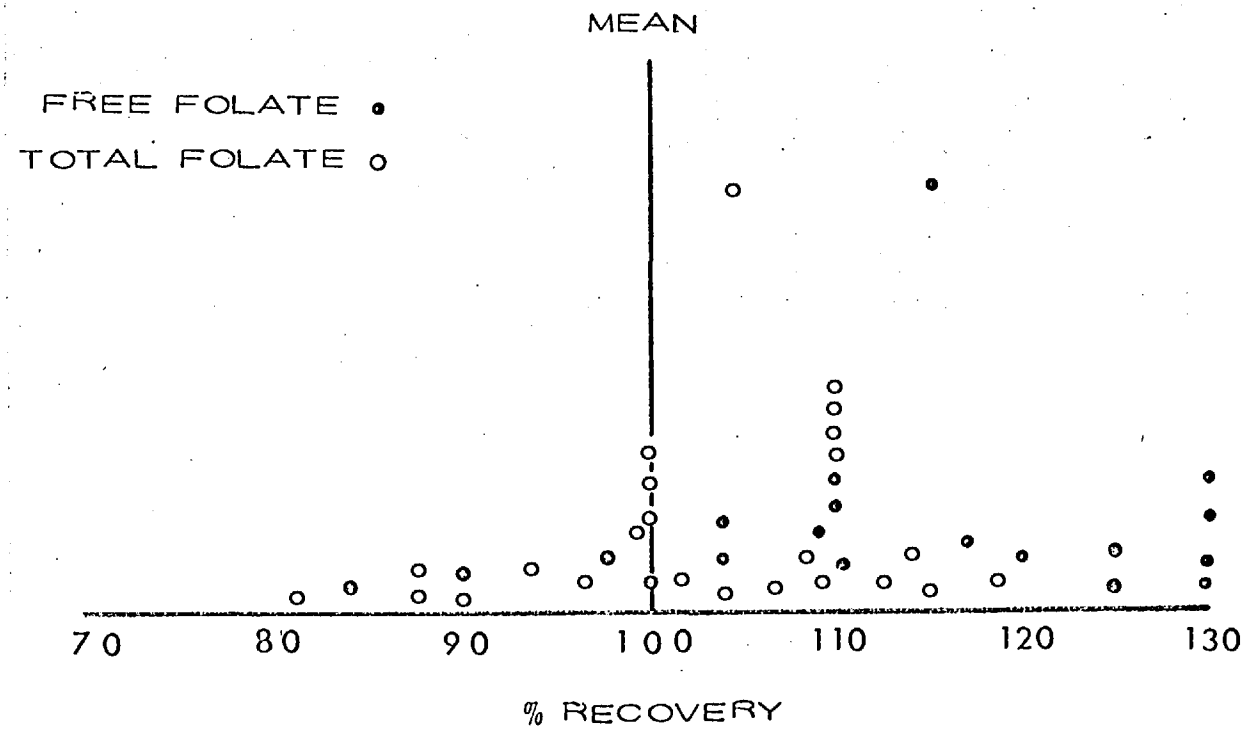

Figure XII - Percentage recovery of folate in the form of yeast added to a homogenate of a 24-hour food collection.

yeast extract was added to 4 gms of dietary homogenate and the whole treated as previously described. The recoveries in 22 assays are shown in fig XII, the mean recovery being 113% for free folate and 104% for polyglutamyl folate.

The same yeast extract was also used as a control when specimens of serum or urine were assayed for polyglutamyl folate content, but was diluted such that 1 ml. contained 4.4 ng polyglutamyl folate. 0.1 ml of this added to serum and assayed gave a mean recovery of polyglutamyl folate of 99% with a range from 78-121% in 18 assays.

II. 5. Column chromatography.

Chromatographic fractionation of folates in yeast preparations and diets was performed by the method of Schertel, Boehne and Libby (1965).

Preparative separation of large amounts of material was done in a 40 x 2.4 cm glass column using DEAE cellulose (Whatmans). Gradient elution of folates from the column was achieved by allowing a solution of 0.2% 2,- mercaptoethanol in 0.5 M phosphate buffer pH 7.0 to drip into a mixing chamber attached to the column containing 0.2% 2,- mercaptoethanol.

The presence of 2,- mercaptoethanol in the system preserved oxygen-labile folates. The eluate leaving the column was collected in 100 ml amounts in an LKB Ultrorac fraction collector.

Analytical fractionation of microgram quantities of folates was done in 20 x 1.5 cm. columns using Whatman's DE 23 cellulose, an improved form allowing better separation of folate analogues. Gradient elution was carried out as described above, and five ml fractions were collected for assay.

A. Preparation of natural materials for chromatography.

a) Yeast. The yeast sample was extracted by stirring for 30 minutes in 1% aqueous ascorbate pH 6.0 at 70 °C.

b) Food. Five gram aliquots of dietary homogenate were extracted as described for yeast. The material, which contained some insoluble substances, was then centrifuged at 10,000 r.p.m. for 15 minutes and the resulting supernatent was applied to the column.

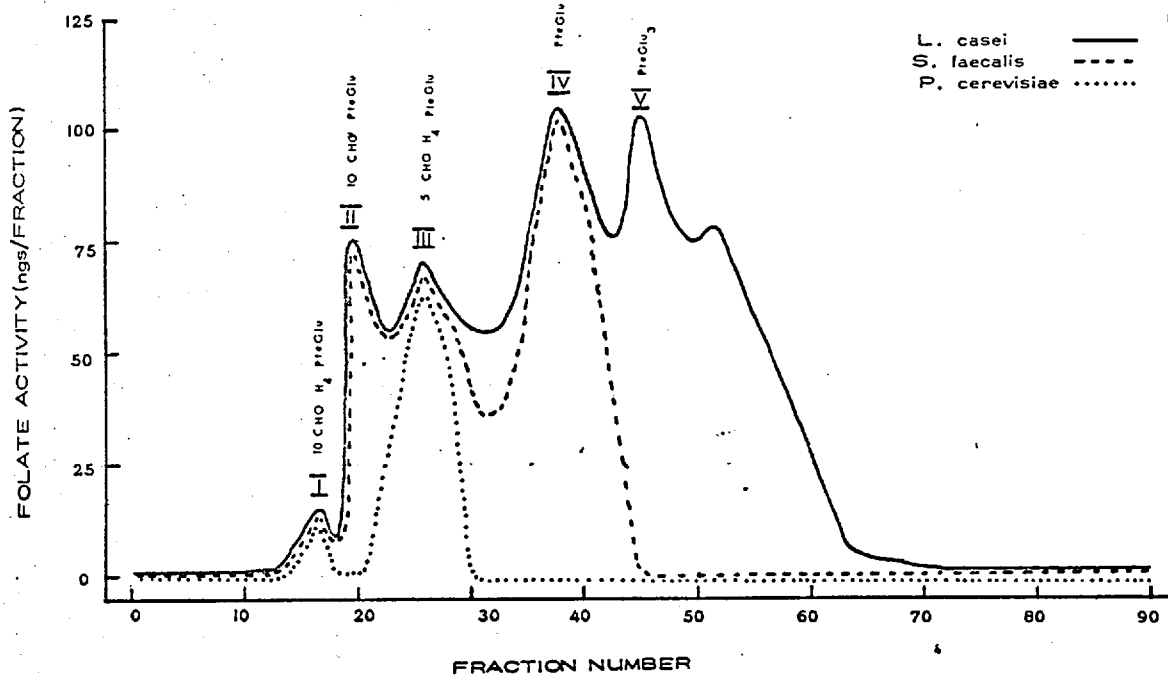

Figure XIII. Chromatogram representing the microbiological activity of successive fractions eluted from an analytical column of DEAE cellulose. The material applied to the column was a mixture of chemically prepared monoglutamyl folates.

Identification of the peaks was as follows:

I. 10 CHO H_4 PteGlu
II. 10 CHO PteGlu
III. 5 CHO H_4 PteGlu
IV. PteGlu
V. $PteGlu_3$

2. Chemically prepared folate derivatives.

PteGlu, 5 CHO H_4PteGlu, and $PteGlu_3$ (Teropterin) were all obtained commercially. 10 CHO PteGlu was prepared by the method of Silverman, Law and Kaufman (1961).

B. Chromatographic fractionation of synthetic folate derivatives.

Although the order of elution of folate derivatives from DEAE cellulose has been described (Silverman, Law and Kaufman, 1961; Wittenberg, Noronha and Silverman, 1962; Noronha and Silverman, 1962; Schertel, Boehne and Libby, 1965) the order of elution of PteGlu, $PteGlu_3$ 5 CHO H_4PteGlu and 10 CHO PteGlu was determined for the chromatographic system used in these studies. Microgram quantities of each compound were applied to the column and eluted in 5 ml fractions.

Results: Microbiological assay using L.casei, S.faecalis and P.cerevisiae showed 4 major peaks of folate activity, and one minor one (figure XIII). The peaks were identified by their support for the growth of the 3 test organisms as follows:

I 10 CHO H_4PteGlu*
II 10 CHO PteGlu
III 5 CHO H_4PteGlu
IV PteGlu
V $PteGlu_3$

* possibly formed as a by-product of the chemical preparation of 10 CHO PteGlu.

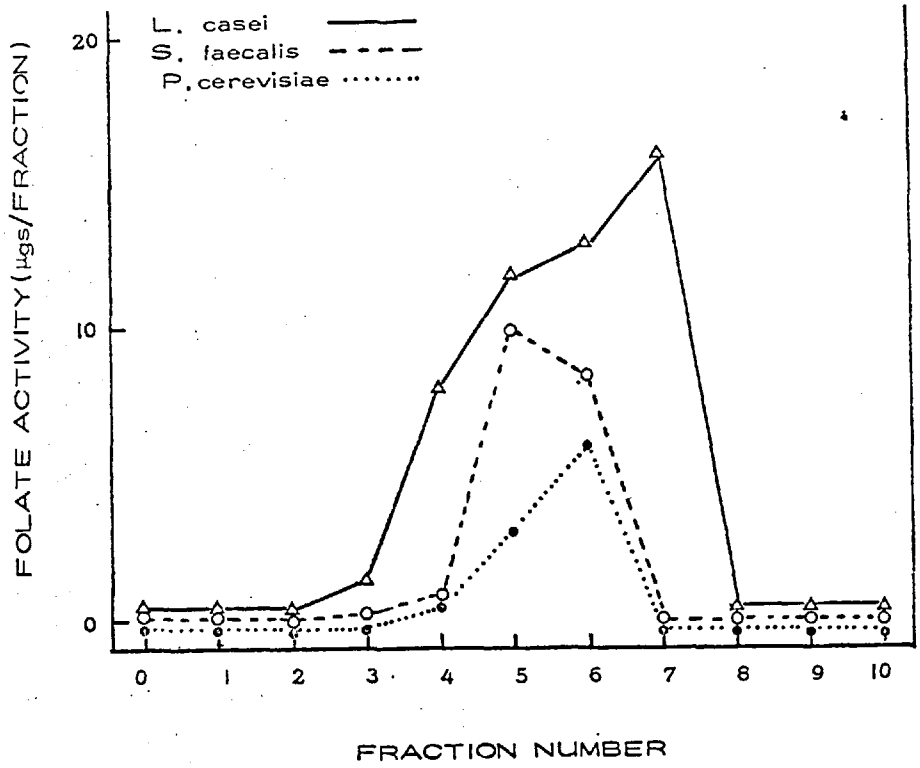

Figure XIV. Chromatogram representing the folate components of yeast (50 gms) eluted from a preparative column of DEAE cellulose.

The microbiological activity shown is that obtained prior to incubation of the fractions with chick pancreatic conjugase, and corresponds to mono-, di-, and triglutamyl folates.

C. Chromatographic fractionation of yeast.

Difco dried yeast extract was fractionated both by the large scale technique described above, and also on an analytical column.

i. Large scale fractionation. Fifty grams of yeast powder solution was applied to the column and 100 ml fractions were collected and assayed microbiologically using L.casei, S. faecalis and P.cerevisiae as assay organisms. The fractions were assayed both with and without chick pancreatic conjugase.

Results. Differential microbiological assay results for fractions 1 to 10 prior to the use of chick pancreatic enzyme are shown in figure XIV. There was no significant folate activity in the subsequent fractions. The collection of 100 ml fractions did not allow complete separation of each folate derivative present but the assay response of the three organisms used appeared to show the presence of mono, di or triglutamyl 10 CHO PteGlu (fractions 4 and 5) 5 CHO H_4PteGlu (fraction 6) and 5 CH_3 H_4PteGlu (fractions 7 and 8).

Incubation of an aliquot of each fraction with chick pancreatic conjugase resulted in the appearance of further peaks of folate activity (figure XV). These were identified as polyglutamyl derivatives of the following:

A - 10 CHO H_2PteGlu

B - 10 CHO PteGlu

C - 5 CHO H_4PteGlu

D - 5 CH_3 H_4 PteGlu

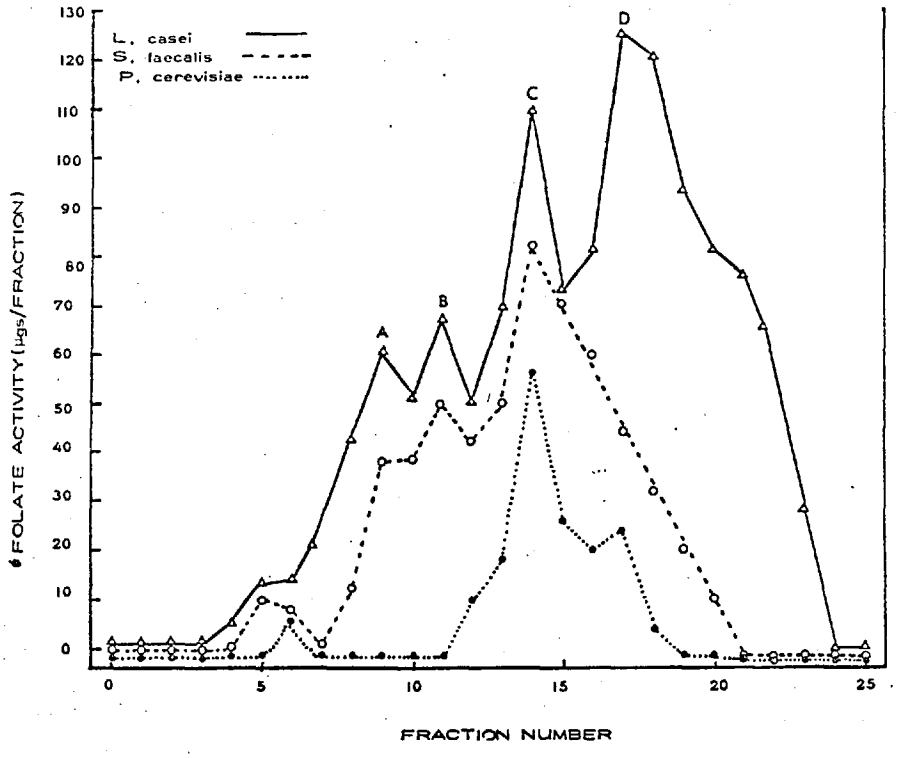

Figure XV. Chromatogram representing the folate components of yeast (50 gms) eluted from a column of DEAE cellulose.

The microbiological activity shown is that obtained following incubation of the fractions with chick pancreatic conjugase (cf Fig. XIV), the additional peaks of folate activity being those due to polyglutamyl folates.

The peaks were identified as:

A. 10 CHO H_2 polyglutamate

B. 10 CHO H_o polyglutamate

C. 5 CHO H_4 polyglutamate

D. 5 CH_3 H_4 polyglutamate

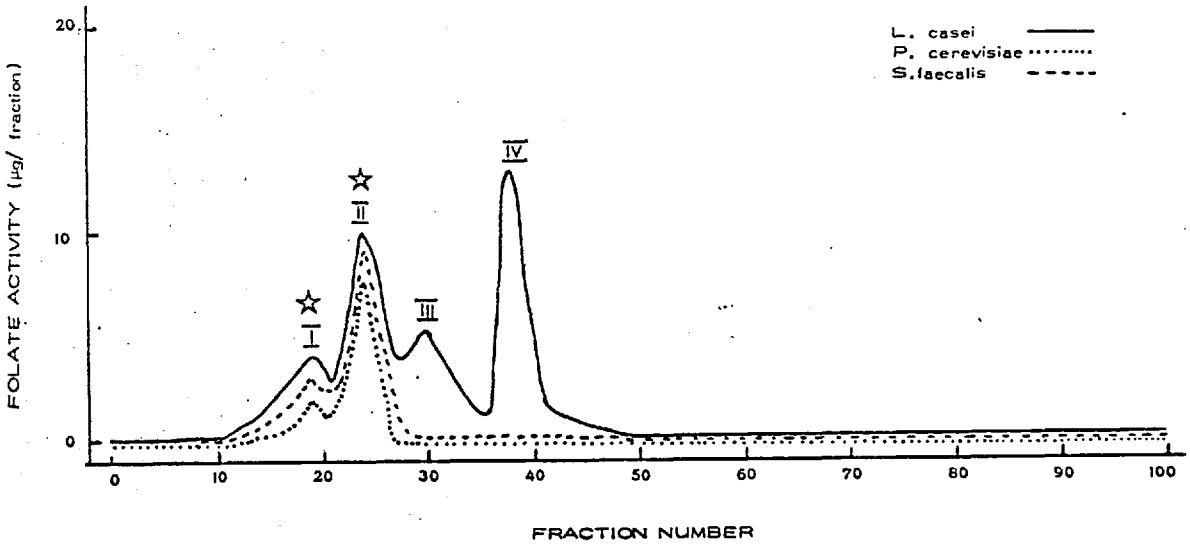

Figure XVI. Chromatogram representing the elution of yeast folates from an analytical column of DEAE cellulose.

The microbiological activity shown is that obtained prior to the use of chick pancreatic conjugase ("free folate").

The nature of the folate compound in the peaks marked ☆ have been identified by their elution characteristics (cf. Fig. XIII) as well as by microbiological assay.

The peaks were identified as:

I. 10 CHO H_0 PteGlu
II. 5 CHO H_4 PteGlu
III. 5 CH_3 H_4 PteGlu
IV. 5 CH_3 H_4 $PteGlu_3$

ii. Analytical fractionation. 0.5 gms of concentrated yeast (p_{104}) was applied to a column and 5 ml fractions were collected and assayed, with and without chick pancreatic conjugase treatment.

Analytical fractionation allowed better differentiation of folate compounds, and more peaks of activity were detected than by large scale chromatography.

Results. Direct assay of the eluates prior to conjugase digestion showed 4 small peaks of folate activity (fig XVI). These were identified as:

I 10 CHO PteGlu*

II 5 CHO H_4PteGlu

III 5 CH_3 H_4PteGlu

IV 5 CH_3 H_4 $PteGlu_3$

* plus a trace of fully reduced (i.e. H_4) formyl folate (P.cerevisiae assay).

Following chick pancreatic conjugase treatment (figure XVII) 6 further peaks were detected; As these peaks of folate activity were not available to the assay organism prior to deconjugation, they represented folate derivatives linked to more than three glutamic acid residues (polyglutamates). The peaks were identified as follows:

V 10 CHO polyglutamate

VI 10 CHO H_2 "

VII 5 CHO H_4 "

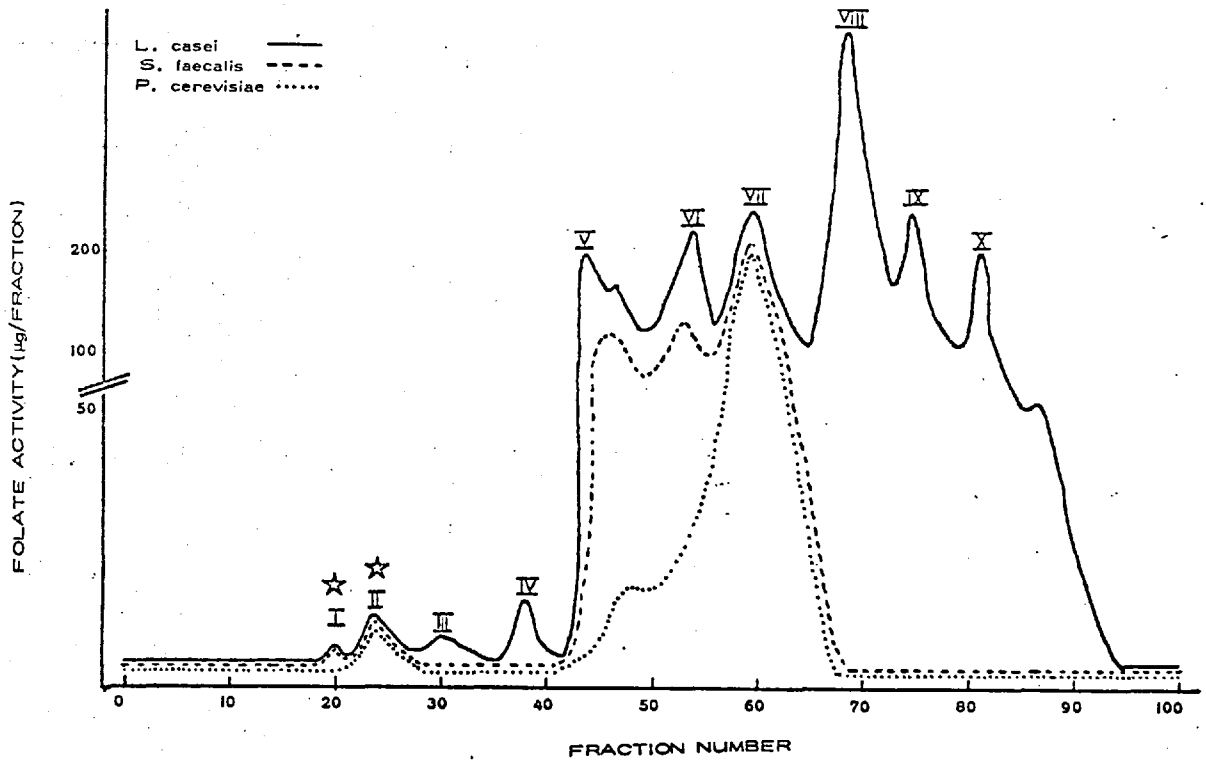

Figure XVII. Chromatogram representing the elution of yeast folates from an analytical column of DEAE cellulose.

The microbiological activity shown is that obtained following incubation of the fractions with chick pancreatic conjugase (cf Fig. XVI).

The polyglutamyl fractions were identified as:

V.	10 CHO H_0	polyglutamate
VI.	10 CHO H_2	polyglutamate
VII.	5 CHO H_4	polyglutamate
VIII.	5 CH_3 H_4	polyglutamate *
IX.	5 CH_3 H_4	polyglutamate *
X.	5 CH_3H_4	polyglutamate *

* polyglutamates of increasing chain length.

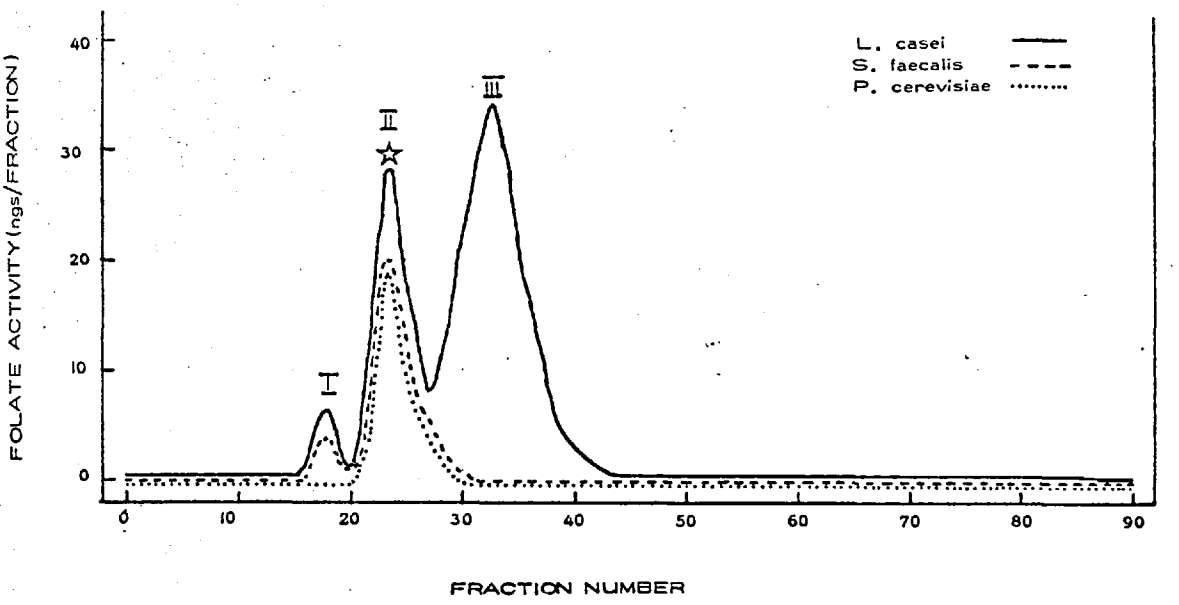

Figure XVIII. Chromatogram representing the elution of pooled yeast folates from DEAE cellulose (fractions 50-80, Fig. XVII).

The yeast preparation had been incubated with chick pancreatic conjugase before being placed on the column.

The peak marked ☆ was identified by its elution characteristics (cf Fig XIII) as well as by microbiological assay.

The peaks were identified as:

I. 10 CHO H_4 or H_2PteGlu

II. 5 CHO H_4 PteGlu

III. 5 CH_3 H_4 PteGlu

VIII	5 CH_3 H_4	polyglutamate
IX	5 CH_3 H_4	"
X	5 CH_3 H_4	"

Peaks VIII, IX and X probably represent reduced methyl folates of increasing chain lengths.

iii. Chromatography of deconjugated yeast polyglutamyl folate.

The polyglutamyl folates contained in fractions VII and VIII and IX (eluates nos. 50-70, figure XVII) were pooled, incubated with chick pancreatic enzyme (0.5 mls) and an aliquot re-fractionated.

Results. Two major peaks of activity, together with a 3rd minor one, could be detected by mi crobiological assay of the eluates, representing the polyglutamyl folates derivatives 10 CHO H_o or H_2 PteGlu, 5 CHO H_4Pt Glu and 5 CH_3 H_4PteGlu deconjugated to mono, di and triglutamyl folate (fig XVIII). Further treatment of the column eluates with deconjugating enzyme did however result in the appearance of further folate activity (fig XIX), the conclusion being that not all of the polyglutamyl folate present had been deconjugated to the level of a triglutamate, or less, by the enzyme preparation.

The chromatographic fractionation of human diets is presented in Section III.

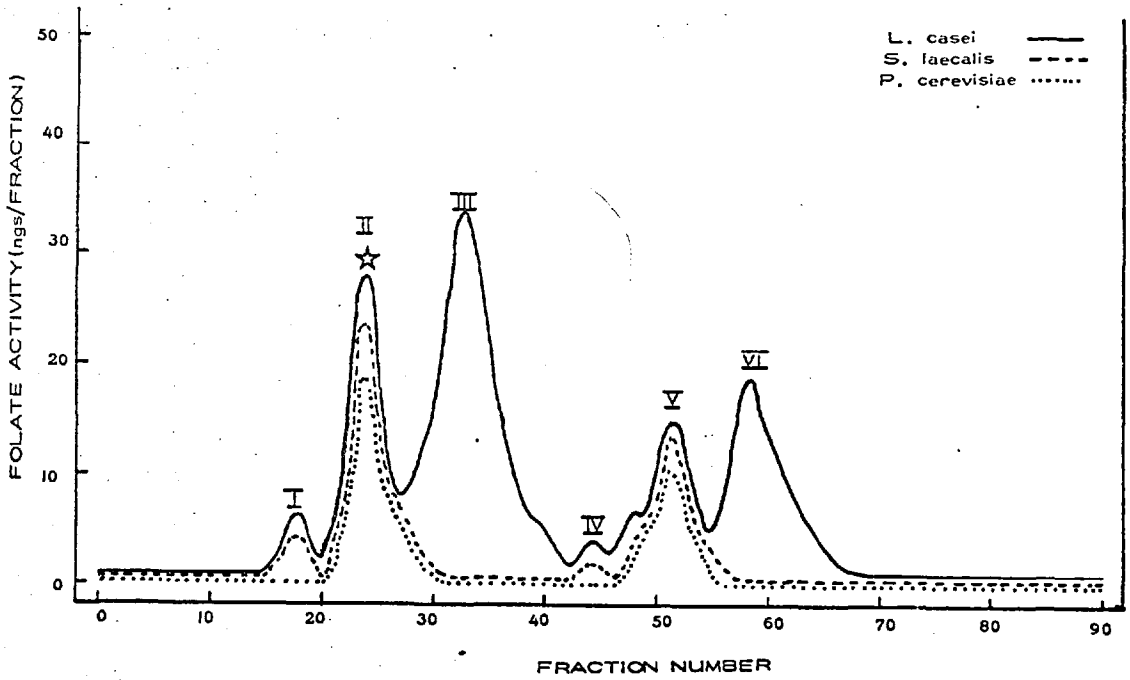

Figure XIX. Chromatogram representing the elution of pooled yeast folates from DEAE cellulose (fractions 50 - 80, Fig. XVIII).

The eluted fractions have been incubated with chick pancreatic conjugase (cf Fig. XVIII).

Three new peaks of folate activity appeared and were identified as.

IV. 10 CHO H_0/H_2 polyglutamate

V. 5 CHO H_4 polyglutamate

VI. 5 CH_3 H_4 polyglutamate

6. Preparation of natural substrates and synthetic folate analogues.

A. Natural folates.

i 24-hour food collections.

Sixteen subjects attending the antenatal clinic at St. Mary's Hospital agreed to prepare their own meals in duplicate for a period of seven days. Each volunteer was given £5 to cover additional expense and was visited daily by a health visitor. Each subject was supplied with a weighed 5 litre capacity wide mouthed polyethylene contained. To preserve labile reduced folates to each container was added 500 mls of freshly made O.1.M. phosphate buffer with 3 gms % ascorbic-acid added, the pH being brought to 7.0 with 10 N. NaOH. This pH value was chosen so that both acid (5 CHO H_4PteGlu) and alkaline (5 CH_3 H_4PteGlu, H_2PteGlu) sensitive folate analogues might be preserved, and the pH still be optimal for the activity of chick pancreatic conjugase.

During the 24 hour collection period the container was refrigerated where possible or alternatively kept in a cool dark place. The food collection was returned to the laboratory daily and a fresh one supplied. On arrival each collection was weighed, homogenised in an Ato-mix homogeniser, and two 100 ml aliquots stored deep frozen.

In addition to the diet collections 10 24 hour samples and 4 complete midday meals were obtained from one of the wards of St. Mary's Hospital, as well as one home prepared meal. These were all collected and treated as described above.

Table 5.

Recovery of yeast folates after charcoal adsorption*

	Standard yeast solution.		after charcoal		water wash of charcoal		Eluate		% recovery of polyglutamates
	free	total	free	total	free	total	free	total	
1. Activated charcoal	3	220	0	0	0	0	0	9.8	4.6
2. Stearic acid treated charcoal.	3	20	.034	22.7	.018	5	2.9	12.5	57

* results are expressed as µg folate (L.casei assay)

ii. Yeast.

As yeast is a rich source of folate compounds, approximately 98% of which is polyglutamate, it was selected for use in studies on the absorption of polyglutamyl folates. Three different preparations were used:

1. Brewer's yeast (Obtained by courtesy of the Guinness Breweries)
2. Difco yeast extract
3. Yeast tablets (B.P.) containing 300 mgms of dried yeast per tablet.

a) Concentration of yeast polyglutamyl folates.

Average assay values of brewer's yeast were 0.4 µg/g "free" and 5.5 µg/g "total" folate and for Difco yeast extract 0.6 µg/gm "free" and 44 µg/gm "total" (L.casei). As these values would necessitate large doses of the crude material in order to give sufficient folate to produce plasma rises in the subjects studied, the yeast polyglutamates were concentrated as follows by a simple method of charcoal adsorption, with subsequent elution and evaporation of the eluate.

(1) Adsorption and elution from charcoal. It was not found possible to recover more than about 5% of folate which had been taken up by activated charcoal using simple elution methods. Therefore the modification of Asatoor and Dalgleish (1956) was used, whereby binding sites on the charcoal were blocked by preliminary addition of stearic acid to the charcoal. In this way, using the same procedure and concentration of solutions as before, 57% of the yeast polyglutamate was recovered from the deactivated charcoal (table 5). Although the decreased

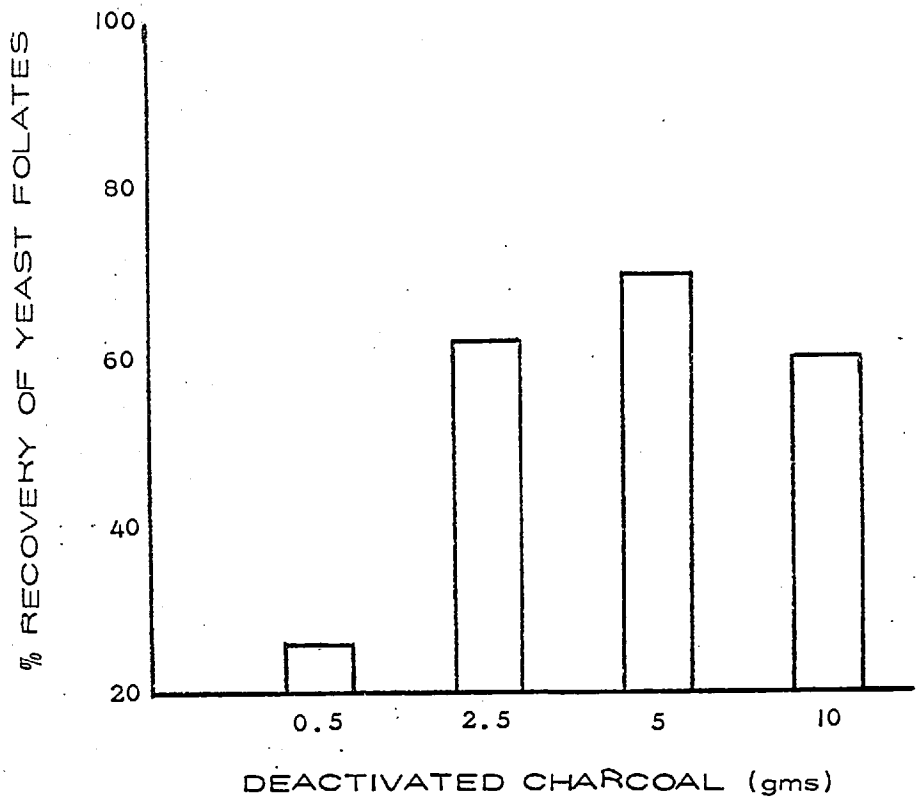

Figure XX - The recovery (%) of yeast folates from varying amounts of deactivated charcoal.

binding capacity of deactivated charcoal prevented 100% uptake of the original folates, and also allowed some loss after water washing of the charcoal "cake", folate recovery after elution varied between 50-70% in 22 preparations.

(2) Effect of variation in charcoal quantities. Figure XX shows the effect on polyglutamate recovery of adding 0.5, 2.5 5 and 10 gms of deactivated charcoal to the standard 5% yeast solution. The highest recovery was obtained when equal quantities of charcoal and yeast were used. As the difference between recoveries with 2.5 and 5 gms of charcoal was of the order of 8% only, it was decided for practical reasons to use a ratio of 1:2 charcoal/yeast when large quantities of yeast were being concentrated.

(3) Elution of charcoal adsorbed yeast folates. Ammonia-ethanol (ethanol 4 parts, ammonia 1 part, water 5 parts) as eluting agent gave approximately 70% recovery of yeast folates adsorbed onto stearic acid treated charcoal as compared to a 50% recovery with an acid phenol mixture (aqueous phenol pH 7.2, 5 parts, conc HCl 1 part) used by Asatoor and Dalgleish (1956).

(4) Final method adopted for concentration of yeast polyglutamyl folates.

Five hundred grams of yeast were made up to two litres with 1% aqueous ascorbate solution, heated for 5 minutes at 15 lbs pressure, cooled, and the pH of the solution brought to 4.0 with acetic acid.

Table 6.

Assay values for brewer's yeast concentrates.

(µg folate/gm)

	L.casei		S.faecalis	
	free	total	free	total
1	2.4	75	2	40
2	1.4	40	1	25
3	1.6	40	1.6	15
4	1.4	50	1.6	40
5	2	44	-	-
6	1	40	-	-
7	0.5	50	-	-
8	0.6	33	-	-
9	1.9	50	-	-
10	2	55	-	-

Charcoal, 250 gms(previously deactivated with 10 gms stearic acid in ethanol and air dried) was added and the solution allowed to stand, with occasional stirring, in the cold for one hour. The charcoal was filtered off in a Buchner funnel and the charcoal cake washed with 1 litre of distilled water. Folates were eluted by washing the charcoal with 2 litre of ammonical ethanol, and the eluate was evaporated to dryness in a rotary evaporator, the flask containing the eluant being suspended in a water bath at 80°C. The result of evaporation was light brown semi-crystalline substance which was then stored in a vacumm desiccator in the dark. The amount of dried material obtained from 500 gms yeast was between 10-20 gms.

Microbiological assay values for Brewer's yeast concentrate and Difco dried yeast concentrate are shown in tables 6 and 7.

B. Preparation of synthetic folate analogues.

i. Folic acid.

A solution of folic acid was prepared by weighing out 200 mgms of synthetic PteGlu (BDH) previously dried at 160°C for 2 hours, and dissolving this in 100 mls of 0.2 N. NaOH. The concentration of PteGlu was 2 mgms/ml, and the solution was stored at -20°C till required.

ii. Reduced folate derivatives.

a) Tetrahydrofolic acid. This was prepared by the method of Silverman and Noronha (1961) involving the reduction of PteGlu with sodium dithionite at 75°C in the presence of ascorbate. In on absorption study commercial H_4PteGlu (Sigma) was used.

Table 7.

Assay values for Difco yeast concentrates

(µg folates/gm)

Batch no:	L.casei		S.faecalis	
	free	total	free	total
1	2	150	0.85	102
2	2	190	0.62	102
3	1.6	150	0.6	100
4	2.2	160	0.6	91
5	1.2	120	0.3	90
6	1.5	150	0.7	60
7	1.2	120	0.5	70
8	1.3	80	-	-
9	2.3	160	-	-
10	1.5	150	-	-
11	1.7	220	-	-
12	1.3	140	-	-
13	1.2	120	0.6	77.5

Allowance was made when calculating the dosage for the biochemically inactive (D)-isomer formed when PteGlu is chemically reduced. Oxidative degradation of H_4PteGlu was prevented by making the material immediately prior to use. Microbiological assay of the product of reduction was carried out using S.faecalis and P.cerevisiae as assay organisms, both by routine and aseptic addition methods, the assay results ranging from 52-80% of the anticipated value. The actual H_4PteGlu value was almost certainly higher, as in P.cerevisiae assays H_4PteGlu has a lower response to the organism, especially in weak dilutions, than 5 CHO H_4PteGlu which was the standard used. This results in spuriously low assay values by as much as 30-50% (Bakerman, 1961). Another cause of lower than expected assay values is the rapid degradation of H_4PteGlu itself, as it was frequently found impossible to assay the material on the day of its preparation. The stable folate material detected by routine S.faecalis assay was probably due in some p art to re-oxidation of the H_4PteGlu to folic acid, and probably also to non-reduction of a small amount of the original material (table 8).

Table 8.

Assay of THF formed from reduction of PteGlu at 75°*

	Aseptic addition assay		routine assay	
	P.cerevisiae	S.faecalis	P.cerevisiae	S.faecalis
1	80	96	-	11
2.	70	92	1.2	16
3	65	88	1	15
4	52	64	1	12
5	70	98	-	-

* results expressed as a percentage of PteGlu concentration of starting material.

(b) Dihydrofolic acid. Crystalling H_2PteGlu was also prepared by dithionite reduction of folic acid, the reaction taking place at room temperature. It was isolated by precipitation at pH 2.8 and stored if necessary in a vacuum desiccator in the dark (Blakely, 1960a). Spectrophotometric measurement of the product in O.1.N. NaOH gave a characteristic peak at 284 mμ.

Differential microbiological assays, aseptic and routine, were of limited value as the material available to S.faecalis on routine assay was probably, to an unknown extent, due to folic acid formed from the oxidation of dihydrofolate at the neutral pH of the assay medium.

(c) 5 methyl tetrahydrofolic acid. This material was made by acidifying 5 formyl H_4PteGlu, forming 5,10 methenyl H_4PteGlu, which was then reduced to 5 CH_3 H_4PteGlu by borohydride (Chanarin and Perry, 1967). Both microbiological assay results and absorption spectra were those of 5 CH_3 H_4PteGlu. The product was used immediately after preparation.

(d) 5 formyl tetrahydrofolic acid. A commercial product was used (Lederle), allowance being made for the presemce of the inactive (D) isomer in the material.

iii. Tritium labelled folate compounds.

a) Folic acid, specificially labelled in the 3', 5' positions of the phenyl group, was obtained from the Radiochemical Centre, Amersham. The specific activity was approximately 60 μCi per μg.

(b) Reduced folate derivatives.

Tritium labelled H_2PteGlu and H_4PteGlu were prepared by adding 1-2 µg of H^3 folic acid to non-radio active folic acid, and reducing the mixtures as described for the appropriate compound.

7. Counting of tritium in plasma samples.

Plasma samples obtained after oral or intravenous administration of H^3 labelled folate analogues were treated by adding 1 ml of Hyamine hydroxide 10 X (Packard) to 1 ml of plasma, with mixing, followed by overnight incubation at 37° C. Hyamine 10 X has been reported as having a high quenching effect on tritium counting (Yoshino, 1968) but 1 ml was necessary to assimilate the plasma sample. After incubation the sample was added to 15 mls of a liquid scintillator solution consisting of:

Ethanol 50 mls

Tolune 46 g mls

* Liquaflor 2.1 mls (Halstead, Griggs, & Harris 1967)

(* Liquaflor - 50 gms PPO - 2,5 - diphenyloxazole

0.625 POPOP - P 4,bis-2(4 methyl-5-phenoloxazolyl) benzine

500 mls toluene)

Each specimen was counted for 50 minutes at -5° C in Packard Tri Carb liquid scintillation counter, counting efficiencies (channel ratio method) averaging 14%.

A standard was included in each series of plasmas counted, made by adding 0.1 mls of a 1/500 dilution of the test dose to 1 ml of the patient's resting plasma. Counts per litre of the patients plasma were expressed as a percentage of this standard.

8. Clinical methods.

Absorption tests.

These were carried out at a dose level of 20 µg/kg body weight except where specifically stated otherwise. The subjects were usually saturated with 15 mgms of folic acid orally 36 hours prior to the test dose being given, in order to achieve a more standard rate of clearance of absorbed folate from plasma. Zero blood specimens were taken at the start of the test, and then at 1,2,3 and where possible 4 hourly intervals thereafter. Where tritium-labelled folates were given specimens were taken at 10 minutes intervals for the first 40 minutes, then at 30 minute intervals after the first hour had elapsed until $2\frac{1}{2}$ hours after the oral dose.

Urines were collected over a 6 hour period.

B. Clearance tests.

The rate of clearance from plasma of intravenously injected tritium labelled folates was measured on samples taken by means of an indwelling needle, at 3 minute intervals up to 15 minutes, then at 5 minute intervals until 27 minutes after the dose followed by 12 minute intervals until 88 minutes had elapsed.

9. Subjects studied.

Absorption tests with non radioactive mono- or polyglutamyl folates were carried out on healthy young subjects, either members of the hospital and laboratory staff or medical students.

Tritium-labelled folate absorption and clearance tests were done on patients hospitalized for minor surgery or medical treatment. All patients had the procedure explained to them before the test, and all agreed to co-operate in the studies. In addition 5 absorption tests were done on 5 patients of St. Mary's Hospital with proven pernicious anaemia.

Section III.

Folate Compounds in Human Diets.

Introduction.

Values reported in the literature for normal daily dietary folate intake vary from 53 µg (Read, Gough, Pardoe and Nicholas, 1965) to 1500 µg (Jandl and Lear, 1956).

This section reports the microbiological assay results for free and total folate content of 111 home prepared 24 hour food collections from 16 subjects, and for 10 24 hour food collections from a ward of St. Mary's Hospital. In addition 4 main meals from the hospital, and one home prepared meal, were assayed.

To determine the type of folate derivatives in human diets chromatographic separation of a dietary homogenate was undertaken, and the eluted fractions assayed.

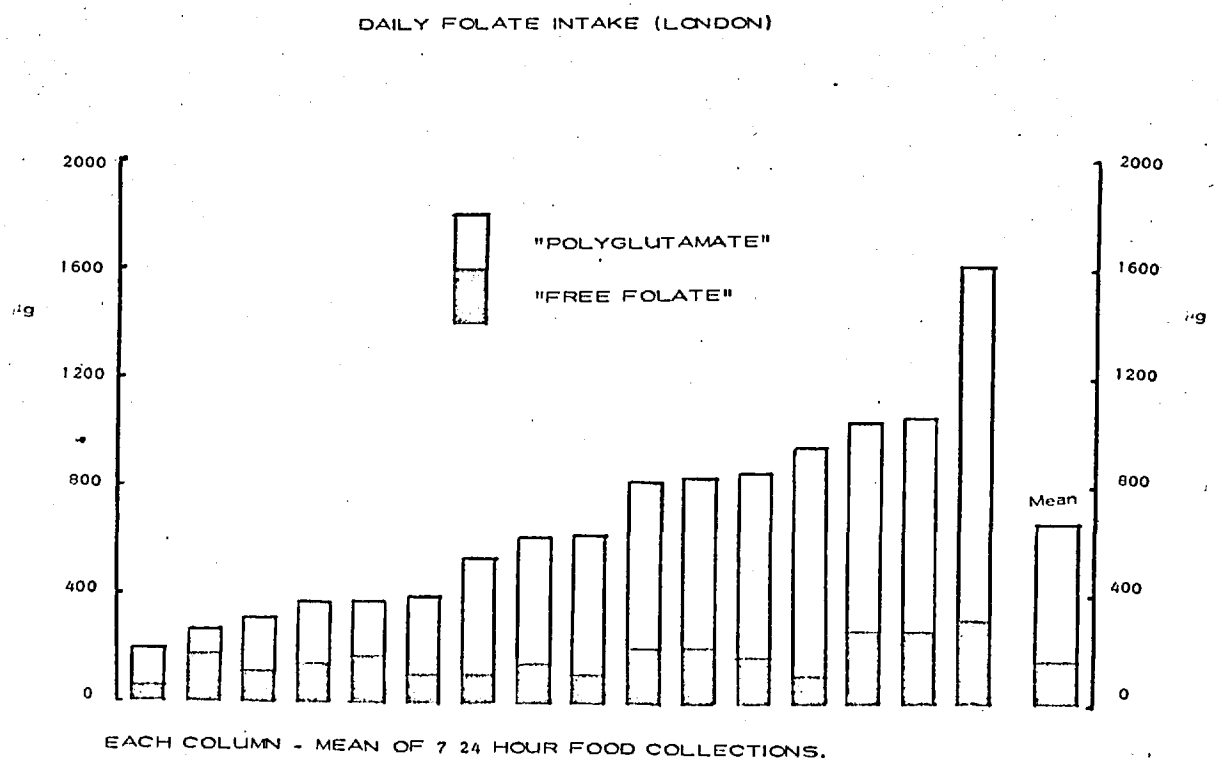

Figure XXI - The mean "free" and "total" folate in 16 weekly diets prepared at home.

2. Free and total folate intake in subjects living at home.

The mean daily intake of free folate in individual subjects ranged from 53-296µg when assayed with L. casei, with day to day variations ranging from 21-430 µg (table 9 and figure XXI). The mean free folate content of the 111 24 hour diets assayed was 160 µg.

After deconjugation of polyglutamyl folates with chick pancreatic enzyme the mean daily intake per subject was 198-1616 µg total folate, day to day variations being from 90-2300 µg. The mean total folate content of the 111 24 hour diets (fig XXI) was 676 µg (L.casei assay).

Table 9.

Folate content (L.casei assay) of home prepared diets (µg/24 hrs)

Subject	Free		Total	
	Mean	Range	Mean	Range
1	202	115–290	830	499–1650
2	101	24–270	945	270–1430
3	133	70–180	257	210–430
4	141	50–197	607	310–1150
5	53	38–78	198	109–282
6	271	65–350	1051	650–2070
7	162	114–226	361	260–455
8	172	44–430	265	180–465
9	96	27–422	387	167–1000
10	199	150–245	820	520–1150
11	161	21–400	849	240–1950
12	115	58–285	355	240–1950
13	265	105–340	1041	315–1420
14	97	47–132	529	90–1237
15	98	27–202	616	290–1140
16	296	190–400	1615	1090–2300
Mean	160		676	

S. faecalis assays were performed on 35 24 hour diet collections from 6 individuals, the mean daily range being 34-75 µg of free folate with individual variations of 11-125 µg. The mean free folate content of the 35 diets was 61 µg.

The mean daily range of total folates, using S. faecalis as assay organism was 104-492 µg, the individual variation 58-800 µg, and the mean total folate in 35 diets 245 µg (table 10).

Table 10.

Folate content (S.faecalis assay) of home prepared diets (µg/24 hrs)

Subject	Free		Total	
	Mean	Range	Mean	Range
1	57	22-91	104	52-178
2	52	19-80	492	130-800
4	70	40-92	216	154-316
9	34	11-77	184	122-246
12	61	31-96	106	72-132
13	75	25-125	322	84-446
Mean	61		245	

Table 11.

Folate content of hospital diets

(µg/24 hrs).

	Free folate		Total folate	
Day	L.casei	S.faecalis	L.casei	S.faecalis
1	120	53	472	187
2	35	29	280	427
3	165	73	294	188
4	1052	46	174	173
5	112	-	298	164
6	68	52	465	308
7	157	68	690	340
8	152	34	465	380
9	100	-	1000	-
10	156	-	780	-
Mean	117	51	487	271

3. Free and total folate in hospital diets.

Using L.casei as assay organism the range of the 10 24 hour hospital diets was from 35-165 µg of free folate with a mean of 117 µg, and 174-1000 µg of total folate, mean 487 µg.

S.faecalis assay values ranged from 29-68 µg as free folate, mean 51 µg, and 173-427 µg total folate, mean 271 µg (table 11)

Table 12.

Folate content of 5 individual diets.

	Free folate			Total folate		
Diet	L.casei	S.faecalis	P.cerevisiae	L.casei	S.faecalis	P.cerevisiae
1	68	12	6	628	52	32
2	32	18	8	269	83	55
3	20	14	8	372	86	79
4	40	6	3	262	43	24
5	104	18	9	954	118	69
Mean	53	13	7	497	76	52

4. Free and total folate in individual diets.

Individual diets.

In addition to the L.casei and S.faecalis assay reported above P.cerevisiae was employed to determine the proportion of reduced folate, other than methyl folates, in five representative main meals, 4 collected from a ward of St Mary's Hospital, and one home prepared. (table 12.)

5. Summary of findings:

Mean microbiological assay results of the 24 hour food collections studied are summarised in table 13.

Table 13.

	L.casei	S.faecalis	L.casei	S.faecalis
Home prepared diets	160	61	676	245
Hospital diets	117	51	487	271

The mean assay results on 5 individuals main meals are shown in Table 14 below.

Table 14.

Free folate			Total folate		
L.casei	S.faecalis	P.cerevisiae	L.casei	S.faecalis	P.cere.
53	13	7	497	76	52

Analysis of the home diets showed that only some 25% of food folate was available to L.casei and 10% to S.faecalis before the use of chick pancreatic enzyme. The S.faecalis activity presumably was due to the presence of mono and diglutamyl folates, with the exception of methyl compounds.

The additional L.casei activity presumably represented methyl and triglutamyl folates, which are inactive for S. faecalis. Three quarters of the dietary folate became available for assay only after the use of deconjugating enzyme, implying that some 75% of the folate was linked tomore than 3 glutamyl residues. L.casei activity was again higher than that for S.faecalis, and though the greater part was probably due to methyl polyglutamates, some could have been due to complex polypeptide forms being deconjugated only to the level of triglutamates, inactive for S.faecalis.

Hospital diets contained less folate than home meals, although the proportion of S.faecalis activity was slightly higher.

P.cerevisiae assays on the 5 individual diets analysed gave a mean value of 52 µg or 12% of total activity. This represents reduced folate derivatives other than methyl folates.

6. Column chromatography of dietary material:

In an attempt to characterize further the folate derivatives in foodstuffs chromatographic fractionation of an aliquot of a home diet consisting of pork sausages, spinach, pumpkin and potatoes, with salt, pepper and butter (no. 5. table 12) was undertaken. Preparation of the food and chromatography techniques used were those described in Section II. 110 5 ml fractions were collected, and each fraction was assayed using L.casei, S.faecalis & P.cerevisiae both before and after

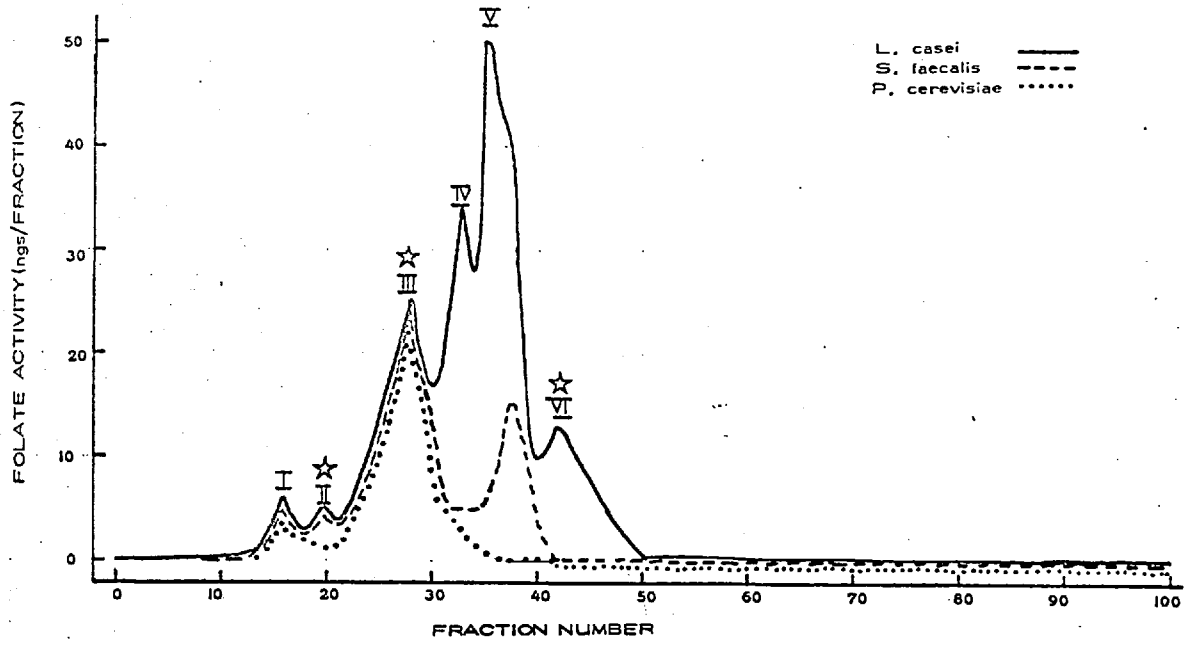

Figure XXII. Chromatogram representing the elution of dietary folates from a column of DEAE cellulose.

The microbiological activity shown is that obtained prior to the use of chick pancreatic conjugase.

The peaks ☆ were identified by their elution characteristics (cf Fig. XIII) as well as by microbiological assay.

The peaks were identified as follows:

I. 10 CHO H_4PteGlu

II. 10 CHO PteGlu

III. 5 CHO H_4PteGlu

IV. 5 CH_3 H_4PteGlu

V. 5 CH_3 H_4PteGlu + PteGlu

VI. $PteGlu_3$

incubation with chick pancreatic enzyme.

The results are shown in figures XXII and XXIII. Free or unconjugated folate in this diet appeared in two major peaks of activity and 2 minor ones. Judged by the elution order of folates from DEAE cellulose (Noronha and Silverman, 1962; Silverman, Law and Kaufman 1961; Wittenberg, Noronha & Silverman, 1962; Schertel, Boehne and Libby, 1965) the elution data presented on page 90 and microbiological assay results, these were as follows.

	L.casei	S.faec.	P.cere.
I - 10 CHO H_4 PteGlu	+	+	+
II - 10 CHO PteGlu	+	+	-
III - 5 CHO H_4 PteGlu	+	+	+
IV - 5 CH_3 H_4 PteGlu	+	-	-
V - 5 CH_3 H_4 PteGlu + PteGlu	+	+	-
VI - $PteGlu_3$	+	-	-

There was no activity for S.faecalis or P.cerevisiae after fraction 42, the residual L.casei activity in peak VII being presumably due to a triglutamate of PteGlu, as following chick pancreatic conjugase activity the material became active for S.faecalis, but not P.cerevisiae.

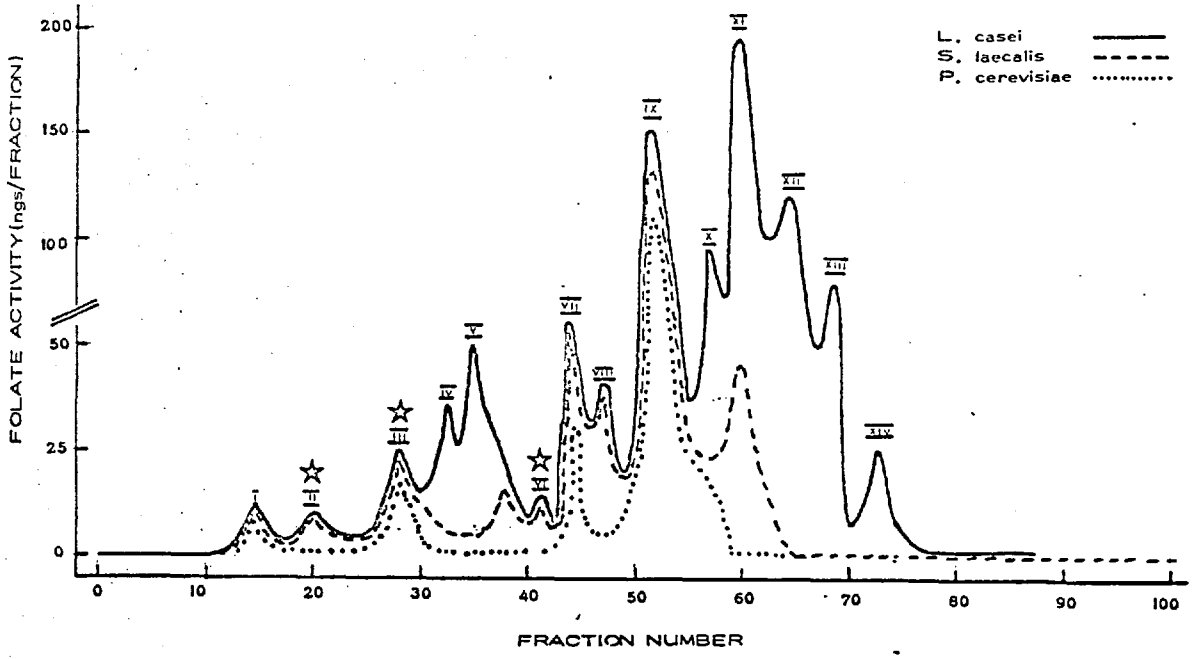

Figure XXIII. Chromatogram representing the elution of dietary folates from a column of DEAE cellulose.

The microbiological activity shown is that obtained following incubation of the fractions with chick pancreatic conjugase which resulted in the appearance of further peaks of folate activity (cf Fig. XXII).

The peaks were identified as:

VII.	10 CHO H_4PteGlu	polyglutamate
VIII.	10 CHO PteGlu	polyglutamate
IX.	5 CHO H_4PteGlu	polyglutamate
X.	5 CH_3 H_4PteGlu	polyglutamate
XI.	5 CH_3 H_4PteGlu + $PteGlu_3$	polyglutamate
XII, XIII, XIV.	5 CH_3 H_4PteGlu	polyglutamates, presumably of increasing chain lengths.

Incubation of the column eluates with chick pancreatic enzyme resulted in the appearance of several new major peaks identified as

	L.casei	S.faec.	P.cere.
VII - 10 CHO H_4PteGlu polyglutamate	+	+	+
VIII - 10 CHO PteGlu polyglutamate	+	+	-
IX - 5 CHO H_4PteGlu polyglutamate	+	+	+
X - 5 CH_3 H_4PteGlu polyglutamate	+	-	-
XI - 5 CH_3 H_4PteGlu + PteGlu polyglutamate	+	+	-
XII XIII XIV - 5 CH_3 H_4PteGlu presumably of increasing chain length	+	-	-

The approximate amount of each folate analogue present in the complete diet was calculated from the quantity of dietary homogenate placed on the column and the microbiological assay data obtained for each eluted fraction, and is shown in table 15.

Table 15.

Chromatographic peak	Folate derivative	Degree of conjugation	Amount in diet µg	Amount in diet %
I	10 formyl H_4	mono or di glutamate	3	0.3
II	10 formyl H_o	"	2	0.3
III	5 formyl H_4	"	23	2.6
IV, V	5 methyl H_4	mono, di and triglutamate	69	4.0
V	folic acid	mono or diglutamate	10	1.1
VI	folic acid	triglutamate	4	0.4
VII	10 formyl H_4	polyglutamate	36	4.0
VIII	10 formyl H_0	"	22	2.4
IX	5 formyl H_4	"	157	17.3
X	5 methyl H_4	"	454	50.4
XI	folic acid	"	68	7.6
XI	5 methyl $H_{4(a)}$*	"	30	3.3
XII	" (b)*	"	14	1.5
XIII	" (c)*	"	8	0.9
		Total	900	100

* polyglutamates of increasing chain length.

Discussion.

Microbiological estimates of the folate content of foods are few. The most extensive study is that of Toepfer, Zook, Orr and Richardson (1951) who assayed individual foodstuffs with L.casei or S.faecalis, and in addition employed conjugase to estimate total folates. Unfortunately this study, and those of Butterworth, Santini and Frommeyer (1963); and Santini, Brewster and Butterworth (1964) were carried out without the use of ascorbate preservative in the preliminary treatment of the samples, which may have led to loss of labile folate before assay.

Differences in treatment of the dietary material prior to assay, the frequent use of S.faecalis as the only assay organism, failure to render polyglutamyl folates available to assay by the use of conjugases, and failure to use a reducing agent as a preservative of labile folates has led to marked variations in the published reports on the folate content of a daily diet. Assay results range from 62 µg (Denko, Grundy, Porter, Berryman, Friedmann and Youmans, 1946) 47 µg for a poor diet, 157 µg for a low cost diet and 193 µg for a high cost diet (Chung, Pearson, Darby, Miller and Goldsmith, 1961) 101 µg for elderly hospital patients, 145 µg for the elderly at home, and 223 µg for young controls (Hurdle, 1967) 380 µg for a rural and 650 µg for an urban diet (Santini, Berger, Berdasco, Sheehy, Aviles and Davila, 1962) and 157 µg as free folate and 689 µg as total folate (Butterworth, Santini and Frommeyer, 1963).

Estimates of daily folate made from food tables range from 53µg (Read, Gough, Pardoe and Nicholas, 1965) to 1000-1500 µg (Jandl & Lear, 1956).

The mean values for daily dietary folate found in this study are in agreement with those found by Butterworth et al (1963) who assayed 4 complete daily diets with L.casei, and 17 with S.faecalis (table 16).

Table 16.

	Free folate (µg)		Total folate (µg)	
	L.casei	S.faecalis	L.casei	S.faecalis
Present study	160	61	676	245
Butterworth et al	157	52	689	184

They also are in accord with those of Hurdle (1967) who used L.casei assay values of individual foods to estimate daily folate intake.

The lower assay values obtained for hospital diets in the present study are probably due to methods of cooking and storage of the meal before it is served to the patients. Herbert (1963) produced a 'folate free' diet by boiling the foodstuffs in large quantities of water. Hurdle (1967) demonstrated that 80% of the folate in cabbage was lost by boiling for 8 minutes, the loss being due to destruction of folate and not leaching out into the water.

Broccoli, potatoes and eggs also lost 80-90% of folate on boiling. Frying too has a destructive effect on food folate, beef, porkand bacon losing 67-95% of their folate content (Cheldelin, Woods and Williams, 1943; Schweigert, Pollard and Elvehjem, 1946).

The type of folate compound in daily diets has been reported by Butterworth, Santini, Frommeyer (1963) who employed column chromatography to separate the folate analogues in 14 meals. This work was extended to 12 individual foods (Santini, Brewster and Butterworth, 1964) but in neither study were any reduced folates, other than the stable 5 CHO H_4PteGlu, detected, and no reference is made to 5 CH_3 H_4PteGlu or to the elution of polyglutamates. Three folate derivatives only were found, namely 1o CHO PteGlu (55% of the total activity) 5 CHO H_4PteGlu (34%) and PteGlu (11%). Iwai and Nagakawa however, investigating folate derivatives in green leaves using S.faecalis and P.cerevisiae as assay organisms,demonstrated that most of the folate was present as labile reduced derivatives, presumably predominently 10 CHO H_4PteGlu. As L.casei was not used for microbiological assay there is no information concerning the concentration of 5CH_3 H_4PteGlu. The discrepancies in these reports is again probably due to the presence or absence of reducing agents during the handling of the test materials.

In the chromatographic study reported here the greatest activity in the diet(which consisted of pork sausages, spinach, pumpkin and potatoes) was provided by methylated reduced folates, present not only as monoglutamyl but also polyglutamyl forms of differing chain lengths. 5 CHO H_4PteGlu and its polyglutamate comprised the next major peak of activity, with 10 CHO PteGlu and PteGlu, probably formed from the degradation of reduced formyl components (Stokstad, 1952) providing relatively minor fractions.

	Proportion in diet (%)	
Compound	Chromatographic fractionation	mean whole diet assay
10 CHO H_4	5.4	20
5 CHO H_4	20	
10 CHO PteGlu	2.8	10
	8.6	
5 CH_3	63	70
$PteGlu_3$	0.4	

The proportions of each derivative may vary from meal to meal depending on the dietary components (Santini, Brewster and Butterworth 1964). The amount of each folate compound obtained here after chromatography of a representative diet correlates well with the mean assay values obtained from the dietary homogenates studied. This suggests that on average most human diets comprise approximately 60-70% of reduced

methylated folates, 20% reduced and reduced formyl folates, and 10% non reduced, unsubstituted and formyl folate, apparently all in both mono and polyglutamyl forms.

Section IV.

The Intestinal Absorption of Monoglutamyl Folates.

This section describes absorption studies with chemically prepared analogues of those monoglutamyl folates present in human diets. Observations were also made with tritium labelled folate compounds, and the absorption data compared with that obtained from tritiated folate clearance studies on 3 subjects.

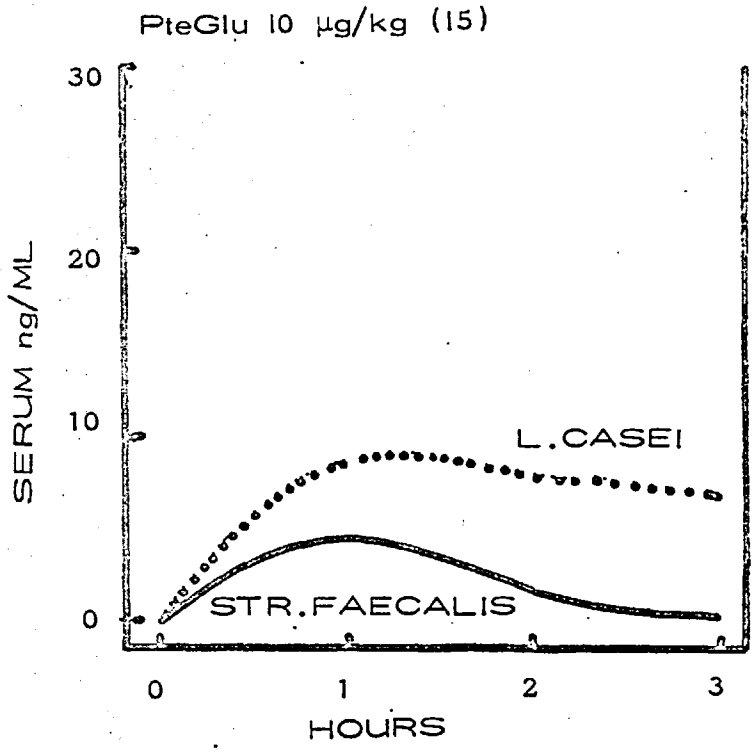

Figure XXIV. The mean serum folate levels following an oral dose of PteGlu 10µg/kg (13 subjects)

The zero serum folate values have been subtracted from the remaining values.

1. Pteroylglutamic acid:

Absorption studies were carried out at 2 dose levels, fifteen subjects being given 10 µg/kg body weight, and thirteen subjects, 20 µg/kg.

Serum folate values after both dose levels are shown in tables 17 and 18 and fig XXIV and XXV. The L.casei level in the fasting sample has been substracted from the folate levels in the later samples. There was no significant activity in the fasting sample with S.faecalis.

All subjects tested, at both dose levels, showed a rise in serum folate over the base line sample with both L.casei and S.faecalis as assay organisms. The values obtained with L.casei were higher than those obtained with S.faecalis, which suggests that a large proportion of serum folate was 5 CH_3 H_4PteGlu, (active for L.casei not S.faecalis) but that a significant amount was absorbed unchanged.

Table 17.

Serum folate level after 10 µg/kg PteGlu orally.*
(ng/ml)

Subject	Assay organism	Time (after oral dose) 1 hour	2 hours	3 hours
(1)	L.casei	13	8	4
	S.faecalis	8	2.5	0
(2)	L.casei	9	4	1
	S.faecalis	4	2.5	2.5
(3)	L.casei	6	4	6
	S.faecalis	4	2	0
(4)	L.casei	3	3	3
	S.faecalis	0	0	0
(5)	L.casei	6	6	6
	S.faecalis	4	4	0
(6)	L.casei	9	6	5
	S.faecalis	4	0	0
(7)	L.casei	8	3	3
	S.faecalis	2.5	0	0
(8)	L.casei	7	5	7
	S.faecalis	5	0	0
(9)	L.casei	8	9	6
	S.faecalis	0	0	0
(10)	L.casei	17	18	10
	S.faecalis	4	0	0
(11)	L.casei	13	2	4
	S.faecalis	2	0	0
(12)	L.casei	13	13	9
	S.faecalis	6	0	0
(13)	L.casei	2	9	25
	S faecalis	2.5	1.5	1.5
(14)	L.casei	21	25	7
	S.faecalis	0	4	0
(15)	L.casei	5	2	-
	S.faecalis	2	0	-
Mean	L.casei	8.7	7.8	7.0
	S.faecalis	4.8	1.1	0.3

* The L.casei serum folate zero value has been subtracted from the remaining values.

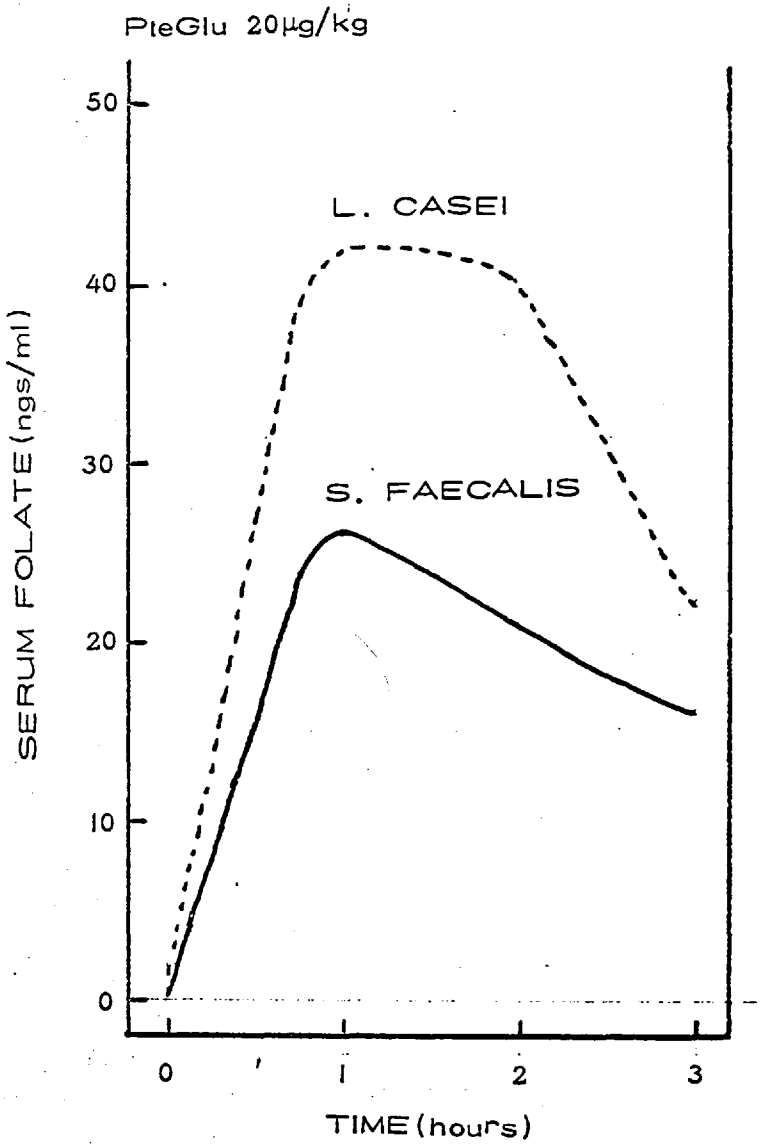

Figure XXV The mean serum folate levels following an oral dose of PteGlu, 20µgs/kg. (13 subjects)

The serum folate zero values have been subtracted from the remaining values.

Table 18.

Serum folate levels after 20 µg/kg PteGlu given orally.*

Subject	Assay organism	Time (after oral dose) 1 hour	2 hours	3 hours
1	L.casei	34	32	25
	S.faecalis	20	30	16
2	L.casei	32	18	13
	S.faecalis	38	20	17
3.	L.casei	47	24	19
	S.faecalis	30	26	18
4	L.casei	47	38	8
	S.faecalis	47	23	5
5	L.casei	35	26	24
	S.faecalis	26	20	20
6	L.casei	30	70	25
	S.faecalis	10	7.5	5
7	L.casei	100	100	55
	S.faecalis	60	62	48
8	L.casei	63	68	35
	S.faecalis	42	44	30
9	L.casei	106	72	34
	S.faecalis	62	19	15
10	L.casei	26	22	14
	S.faecalis	10	7	3
11	L.casei	27	39	31
	S.faecalis	0	6	8
12	L.casei	0	15	26
	S.faecalis	0	13	23
13	L.casei	7	23	25
	S.faecalis	0	10	8
Mean	L.casei	42	41	22
	S.faecalis	26	21	16

* The L.casei serum folate $\overset{zero}{\wedge}$ value has been subtracted from the remaining values.

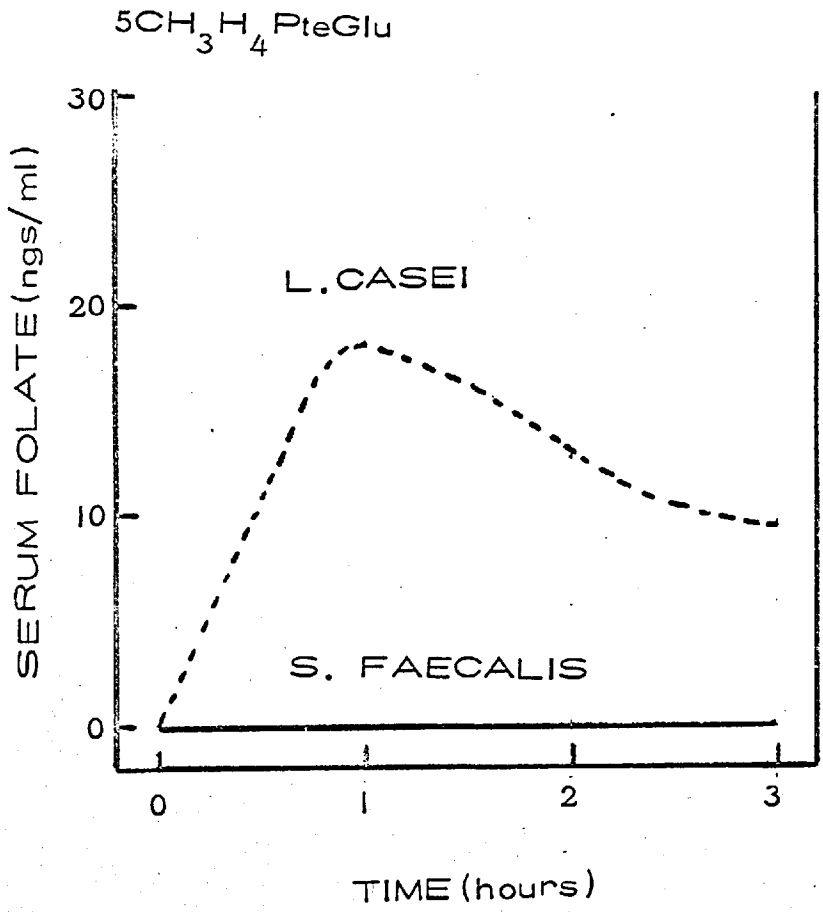

Figure XXVI. The mean serum folate values following an oral dose of 5 CH_3H_4PteGlu 10μg/kg (16 subjects).

The zero serum folate value has been subtracted from the remaining values.

2. Reduced monoglutamates.

A. 5 Methyl tetrahydrofolic acid

16 subjects were tested, the oral dose level being 10 µg/kg as the biologically active L.isomer.

All subjects showed a rise in serum folate levels after $CH_3 H_4$PteGlu when L.casei was used as assay organism although subjects, 3,7,13 and 16 had maximum rises over base line of only 5 ng/ml or less. No S.faecalis activity was detectable (table 19 and fig XXVI)

Table 19.

Serum folate levels after 10 µg/kg $5CH_3\ H_4$PteGlue orally.*

(ng/ml L.casei assay)

	Time after oral dose		
Subject	1st hour	2nd hours	3rd hours
1	7	30	23
2	21	24	22
3	4	1	3
4	13	3	3
5	11	8	5
6	23	15	12
7	5	3	3
8	19	13	7
9	29	15	15
10	31	16	12
11	28	15	12
12	32	15	9
13	3	4	1
14	28	19	9
15	29	17	14
16	3	2	0
Mean	18	12.5	9.4

* The serum folate zero value has been subtracted from the remaining values.

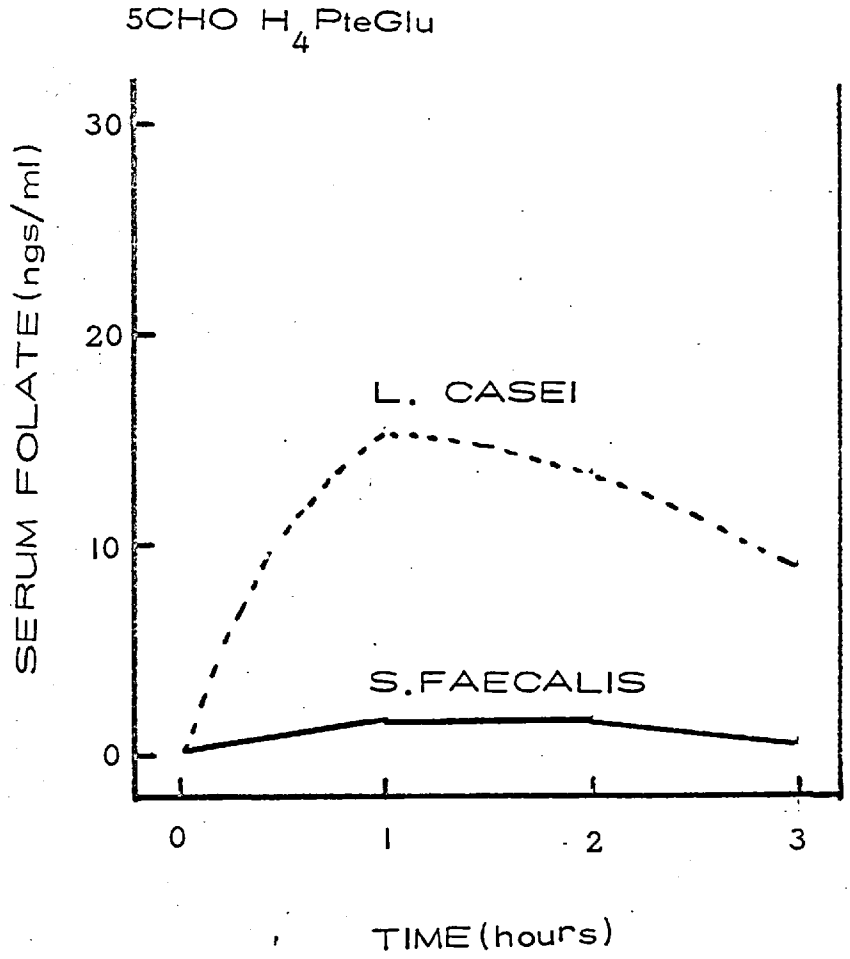

Figure XXVII. The mean serum folate level following an oral dose of 5 CHO H_4PteGlu, 10 µg/kg (13 subjects).

The zero serum folate value has been subtracted from the remaining values.

B. 5 formyl tetrahydrofolic acid.

13 subjects were tested, the oral dose being 10 µg/kg as the active L.isomer. As well as L.casei and S.faecalis, P.cerevisiae, which responds only to non-methylated reduced folates, was used as assay organism.

All thirteen subjects showed a rise in serum folate levels when assayed with L.casei, but only 4 showed any activity when assayed by routine methods using S.faecalis suggesting the presence in the serum of a small amount of PteGlu. There was no growth with P.cerevisiae. (Table 20 and fig XXVII.

C. Dihydrofolic acid.

Absorption studies with this material were carried out on eleven subjects. The dose given was 20 µg/kg, as it was erroneously thought that the chemically prepared material had the 5,6 dihydro-structure, and consequently was a mixture of isomers. In fact, the 7,8 dihydro-structure has beenconfirmed for dihydrofolate (Hillcoat and Blakley, 1964; Pastore, Friedkin and Jardetzky, 1963).

All eleven subjects showed a rise in serum folate levels with L.casei, but there was no activity with S.faecalis using routine and aseptic addition techniques. (Table 21 and fig XXVIII.) Although the compound given orally was fully active with S.faecalis, the assay results showed that the rise in serum folate was due entirely to 5 CH_3 H_4 PteGlu.

Table 20.

Serum folate levels after 10 µg/kg 5 CHO H_4 PteGlu orally.

(ng/ml)*

Subject	Assay Organism	Time after oral dose		
		1 hour	2 hours	3 hours
1	L.casei	10	13	8
	S.faecalis	4	7	0
2	L.casei	16	16	7
	S.faecalis	4	5	4
3	L.casei	12	12	8
	S.faecalis	7	5	5 +
4	L.casei	11	11	11
	S.faecalis	0	0	0
5	L.casei	6	7	11
	S.faecalis	4	4	0
6	L.casei	20	11	8
	S.faecalis	0	0	0
7	L.casei	7	12	5
	S.faecalis	0	0	0
8	L.casei	21	11	9
	S.faecalis	0	0	0
9	L.casei	25	11	15
	S.faecalis	0	0	0
10	L.casei	11	15	12
	S.faecalis	0	0	4
11	L.casei	30	19	15
	S.faecalis	0	0	0
12	L.casei	14	25	10
	S.faecalis	0	0	0
13	L.casei	19	12	19
	S.faecalis	0	0	0
Mean	L.casei	15.5	13.5	9
	S.faecalis	1.5	1.6	0.7

* The L.casei serum folate zero value has been subtracted from the remaining values.

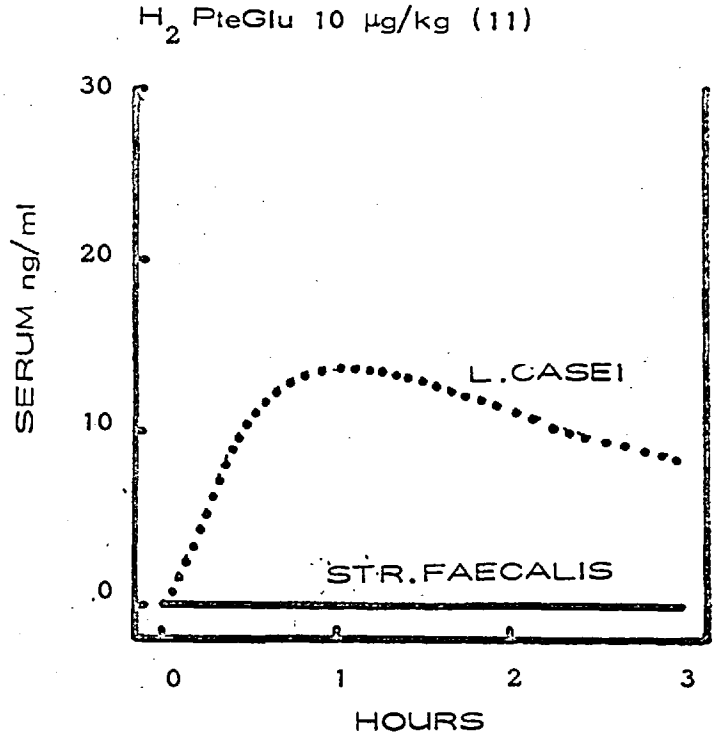

Figure XXVIII - The mean serum folate levels following an oral dose of H_2PteGlu 20µg/kg (11 subjects).
The zero serum folate value has been subtracted from the remaining values.

Table 21.

Serum folate levels after 20 µg/kg H^2PteGlu orally*.

(ng/ml)

		Time after oral dose		
Subject	Assay organism	1 hour	2 hours	3 hours
1	L.casei	9	8	1
2	L.casei	8	4	1
3	L.casei	21	24	21
4	L.casei	30	23	19
5	L.casei	19	14	10
6	L.casei	5	2	1
7	L.casei	6	7	11
8	L.casei	7	14	10
9	L.casei	9	9	9
10	L.casei	27	17	14
11	L.casei	13	8	6
Mean		14	12	9.4

* The serum folate zero value has been subtracted from the remaining values.

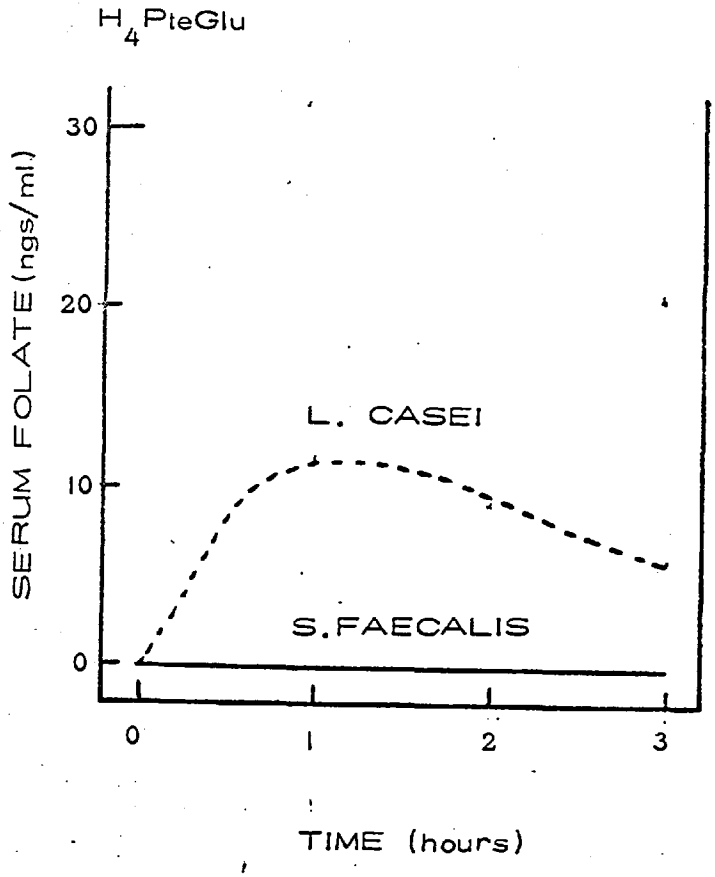

Figure XXIX - The mean serum folate level following an oral dose of H_4PteGlu 10µg/kg (13 subjects).
The zero serum folate value has been subtracted from the remaining values.

D. Tetrahydrofolic acid.

Thirteen subjects were tested during a dose of 10 µg/kg as the active L.isomer.

Again all the subjects showed a rise in serum folate levels on L.casei assay only (Table 22 and fig XXIX.)

Table 22.

Serum folate levels after H_4PteGlu 10µg/kg orally. (L.casei assay) (ng/ml)*

	Time after oral dose		
Subject	1 hour	2 hours	3 hours
1	7	6	4
2	11	7	4
3	8	7	6
4	8	7	2
5	15	8	8
6	9	12	9
7	17	13	8
8	17	14	10
9	2	7	5
10	20	15	9
11	14	8	6
12	11	8	5
13	10	1	0
Mean	11.5	9	6

* The serum folate zero value has been subtracted from the remaining values.

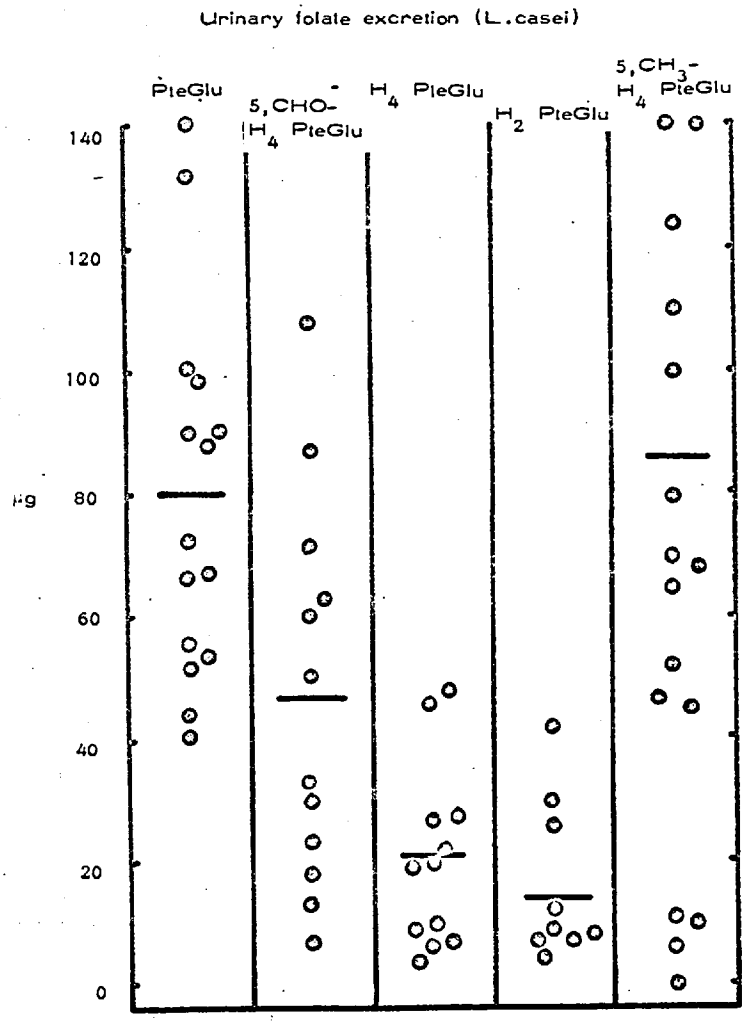

Figure XXX. Urinary folate excretion (L.casei assay) following oral monoglutamyl folates.

The dose level was 10µg/kg with the exception of H_2PteGlu, 20µg/kg.

IV. 3. Urinary excretion of folate after oral monoglutamyl compounds.

Urinary excretion of folate after oral doses of PteGlu and its naturally occuring analogues is shown in table 23 and fig XXX. Levels were highest with L. casei as assay organism, indicating that 5 CH_3 H_4PteGlu was the predominant form excreted irrespective of the oral compound given.

Table 23.

Urinary excretion of folate (μg) after oral monoglutamyl compounds (10 μg/kg) (L.casei assay).

PteGlu	5 CHO H_4 PteGlu	H_4 PteGlu	H_2 PteGlu	5 CH_3 H_4 PteGlu
132	60	10	7	110
147	72	110	42	140
66	30	20	30	11
44	13	6	66	100
40	33	22	26	53
56	7	46	6	65
72	18	27	9	10
90	87	27	6	80
98	22.5	7	12	47
101	63	19	81	124
67	57	48	4.5	70
88	108	9		450
24		4		<1
90				45
52				68
				6
Mean 79.8	47	20.5	14.2	86

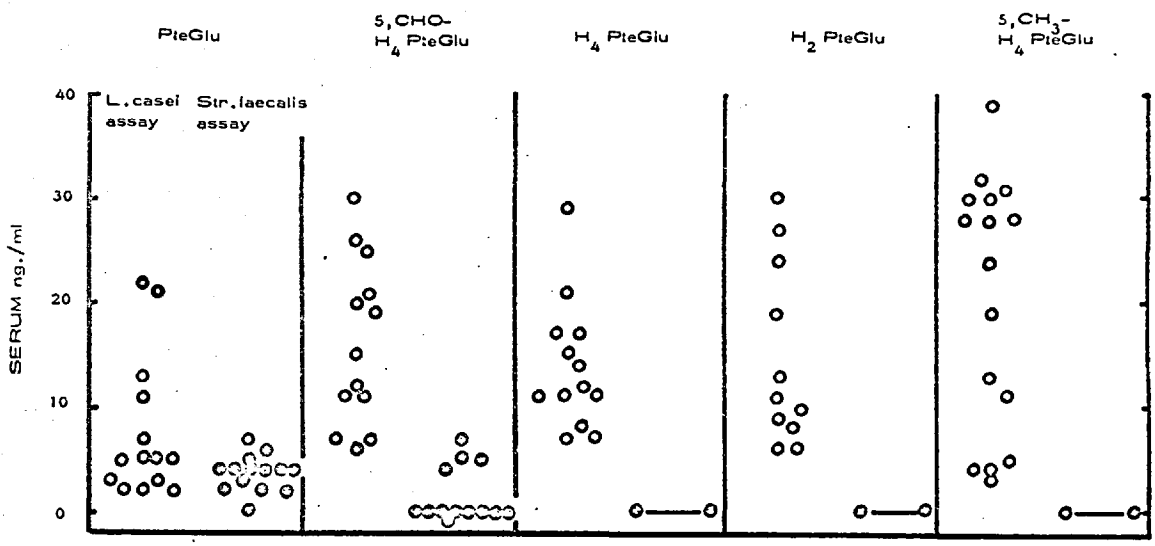

Figure XXXI. The mean serum folate values following oral administration of monoglutamyl folates.

The dose level was 10μg/kg in all cases except H_2PteGlu, 20μg/kg.

IV. 4. Summary of findings:

After oral dosage with 'natural' folates, 5CHO H_4PteGlu 5 CH_3 H_4PteGlu, and H_2 & H_4 PteGlu, the form of folate accumulating in the plasma was the methylated reduced form active for L.casei (fig XXXI). Similarly considerably higher values were obtained with L.casei than with S.faecalis or P.cerevisiae when urinary excretion of folate was assayed.

When PteGlu itself was given by mouth some S.faecalis active material appeared in the plasma, although L.casei activity was markedly higher than that for S.faecalis. Urinary excretion followed the same pattern, that is, the L.casei activity exceeded the S.faecalis activity, although S.faecalis values were greater than those obtained when natural folates were given.

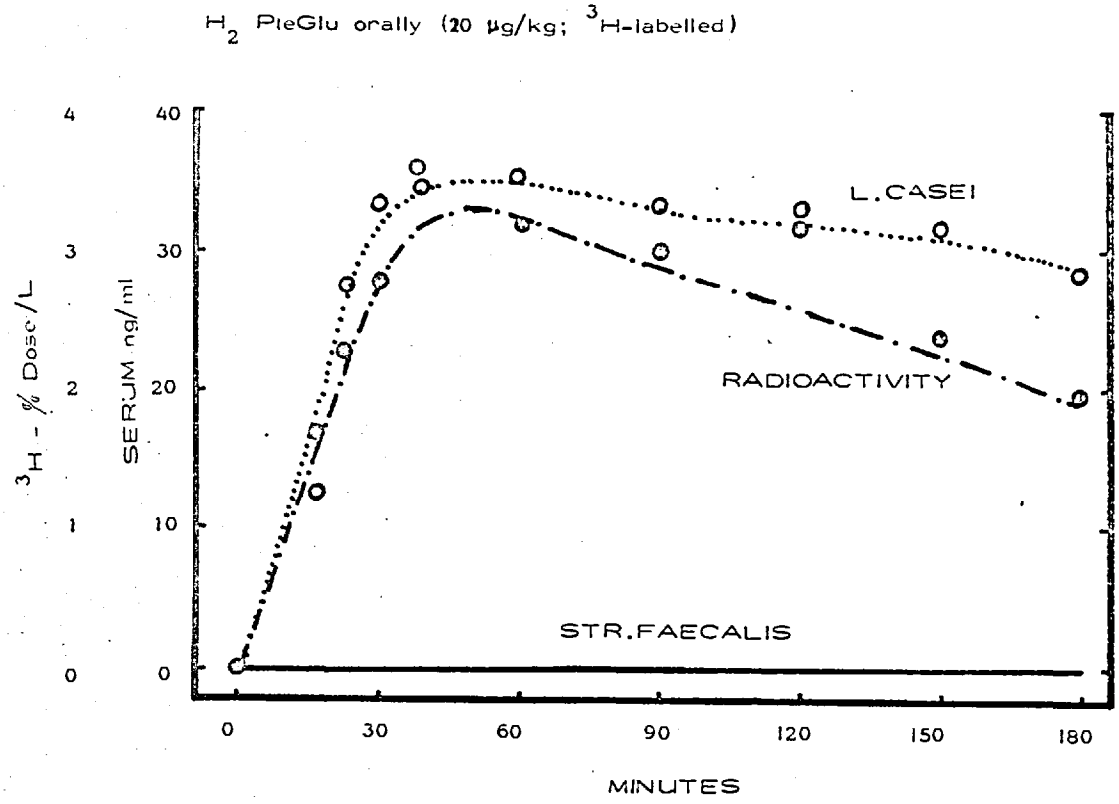

Figure XXXII. The serum folate level after 20µg/kg of tritium-labelled H_2PteGlu given orally.

The L.casei activity of the zero sample has been subtracted from the remaining values.

As both subjects tested showed a similar overall pattern the results from only one subject are presented here.

5. Studies with tritiated reduced folates.

The results reported above indicated that when "natural" folates were given orally the resulting folate compound in plasma was predominantly the reduced methylated form. This could come about by either:

1. Alteration of the oral dose by further reduction and methylation in the cells of the small gut.

2. Absorption of the unaltered compound, followed by its reduction and methylation in tissues such as the liver.

3. Absorption of the unaltered or altered compound which then rapidly exchanges with tissue folate, much of which is 5 CH_3 H_4PteGlu.

To determine which of these possibilities might be correct H_2 and H_4PteGlu were labelled with tritium (section II, 6,B) and its fate following oral and intravenous administration was studied. S.faecalis (for which all 3 compounds are fully active) and L.casei were used as assay organisms, and counting of radioactivity of the plasma samples was carried out as described (section II. 7). L.casei assay results have been derived by subtracting the fasting value from each of the later values, and where applicable subtracting activity due to S.faecalis. The remaining folate activity is that due to 5 CH_3 H_4PteGlu alone.

Table 24.

Absorption of H^3 labelled H_2PteGlu.

		Time after oral dose (minutes)								
		10	20	30	40	69	90	130	150	180
Subject (1)	(L.casei (ng/ml)	3	19	21	30	18	19	14	10	10
	(S.faecalis	0	0	0	0	0	0	0	0	0
	^3H (% dose/litre plasma)	0.9	1.2	2.0	2.8	3.0	2.9	1.8	2.0	2.0
Subject (2)	(L.casei (ng/ml)	13	29	34	37	43	43	34	34	29
	(S.faecalis	0	0	0	0	0	0	0	0	0
	^3H (% dose/litre plasma)	1.7	2.3	2.8	3.6	3.2	3.0	3.2	2.4	2.0

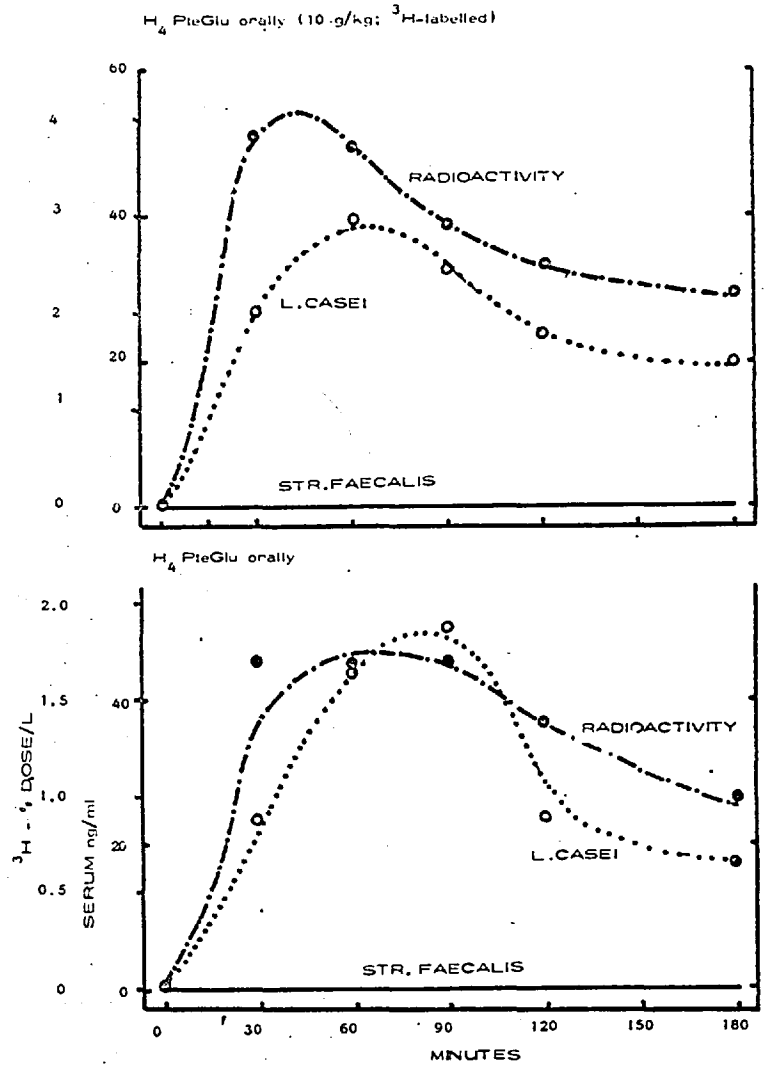

Figure XXXIII. The serum folate level after 10 µg/kg tritium-labelled H_4PteGlu.

The L. casei value of the zero sample has been subtracted.

A. Absorption of tritium labelled H_2 PteGlu.

Two studies were carried out, and the results are shown in table 24 and fig XXXII.
Although on L.casei assay slightly lower peak levels were obtained in one subject than the other, the overall pattern on both microbiological assay and tritium counting was essentially the same. The serum folate activity was measurable with L.casei only, and there was no activity for S.faecalis. Plasma radioactivity corresponded to L.casei activity.

B. Absorption of tritium labelled H_4 PteGlu.

The results of the two studies carried out are set out in table 25 and fig XXXIII.

The pattern of results was essentially similar to those found with H_2PteGlu the only plasma folate activity detected being that due to an L.casei active factor, the appearance of which was paralleled by the tritium count rate.

Conclusions.

After oral dihydrofolate the rise in plasma folate levels was measurable by L.casei only there being no increased S.faecalis activity. Thus the compound in plasma was not only a methylated form, but also had been further reduced from a dihydro- to a tetrahydrofolate compound, as 5 CH_3 H_2PteGlu

Table 25.

Absorption of H^3 - labelled H_4PteGlu.

	30	60	90	120	150
Subject (1) (L.casei	24	44	50	24	17
(S.faecalis	0	0	0	0	0
^3H (% dose/litre plasma	1.7	1.7	1.6	1.4	1.0
Subject (2) (L.casei	27	39	33	24	20
(S.faecalis	0	0	0	0	0
^3H (% dose/litre plasma	3.8	3.7	2.5	2.5	2.2

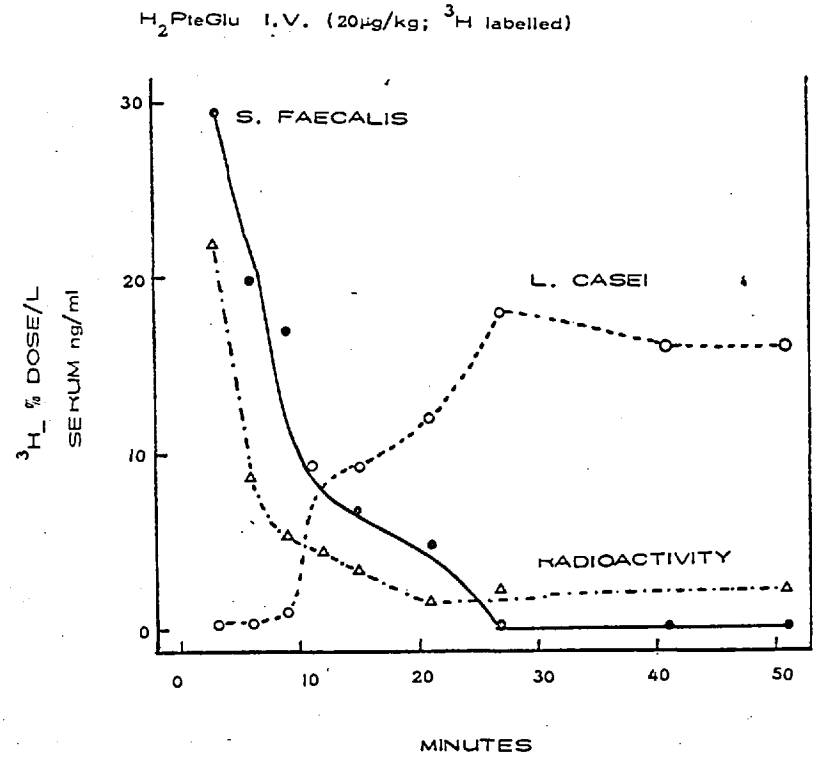

Figure XXXIV - The intravenous clearance of tritium-labelled H_2PteGlu 20µg/kg.
L.casei activity has been derived by subtracting the 0 minute L. casei value from the remaining values, and by subtracting activity due to S. faecalis. The remaining folate activity is that due to 5 CH_3 H_4Pte Glu alone.

is not available to L.casei (Blakley, 1969). With tetrahydrofolate administration the results were essentially the same, an L.casei active material only appearing in the plasma.

That this methylated reduced folate did not arise by exchange with tissue folate was shown by the change in plasma radioactivity, which corresponded to L.casei activity. If the mechanism of increased plasma methyltetrahydrofolate levels had been one of simple exchange plasma radioactivity would not have followed the rise in L.casei activity as the L.casei values would be due to unlabelled tissue folate.

C. Intravenous clearance of tritium labelled reduced folate.

The absorption studies presented above, which showed that exchange with tissue folate was not the mechanism by which 5 CH_3 H_4PteGlu appeared in plasma, irrespective of the oral compound given, left two possible explanations for the pathway of absorption of natural folates, viz. alteration of the dose in the gut wall, or its absorption unchanged followed by reduction and methylation in body tissue (1 and 2 page 160). This was investigated by measuring the clearance of intravenously administered tritiated H_2 and H_4PteGlu, that is, bypassing any action of the gut wall.

The clearance of both compounds showed a similar overall pattern (tables 26 and 27). The L.casei serum values have been derived by subtracting growth activity for

Table 26.

I.V. Clearance of ^{3}H - H_2PteGlu (20µg/kg body weight)

Time after IV. dose (minutes)	assay organisms (1) L. casei* (total activity)	(2) S. faecalis	(1)-(2) = $5CH_3H_4$-PteGlu	% Radioactivity-litre plasma.
3	26	29	3	22
6	20	20	0	8.9
9	18	17	1	5.7
12	17	7.5	9.5	4.7
15	17	7.5	9.5	3.5
21	17	5	12	1.8
27	18	0	18	2.7
41	16	0	16	4.1
51	16	0	16	2.3

* The value of the fasting sample has been subtracted. Values in ng/ml.

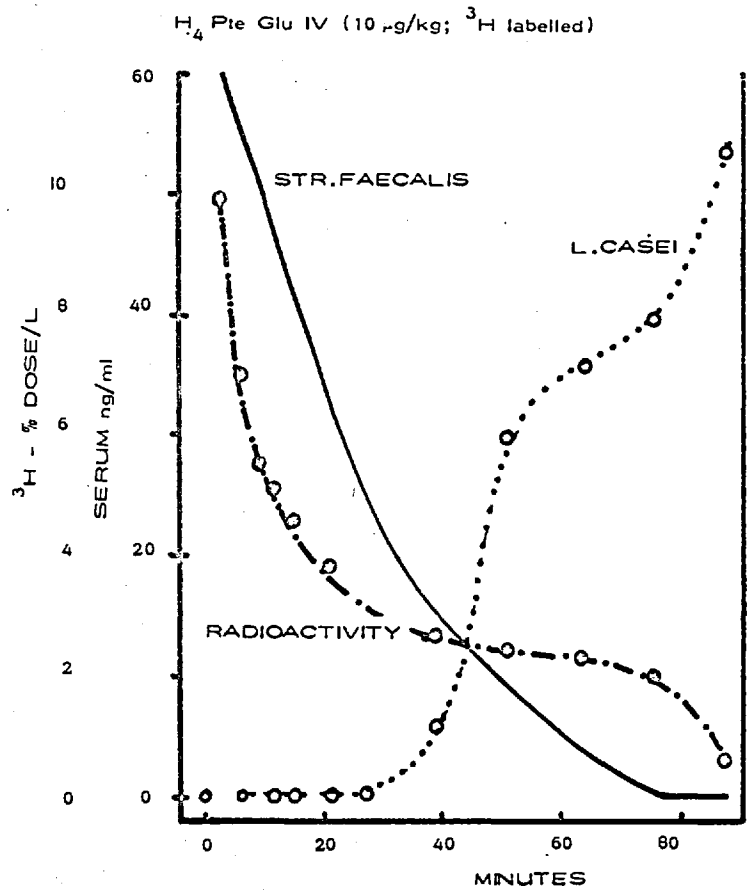

Figure XXXV - The intravenous clearance of tritium-labelled H_4PteGlu, 10µg/kg.
L.casei activity has been derived by subtracting the o minute L.casei value from the remaining values and by subtracting activity due to S.faecalis. The remaining folate activity is that due to 5 CH_3 H_4PteGlu alone.

S.faecalis (H_2 and H_4PteGlu) from the total L.casei activity (H_2 or H_4PteGlu + 5 CH_3 H_4PteGlu), in order to arrive at a value for 5 CH_3 H_4PteGlu alone.

The labelled injected doses of tritiated folates, both of which are active for S.faecalis, disappeared rapidly from plasma and were replaced by non-radioactive, L.casei active 5 CH_3 H_4PteGlu (figures XXXIV and XXXV).

This indicated a rapid and complete exchange between parenteral and tissue folate, and showed that the reduction and methylation of similar doses, given orally, must have therefore taken place in the gut wall.

Table 27.

I.V. Clearance of ^{3}H - H_4PteGlu (10 µg/kg body weight).

Time (minutes)	Assay organisms (1) L.casei	(2) S.faecalis	(1)-(2) = 5 CH_3H_4PteGlu	% Radio-activity per litre plasma
3	58	60	0	9.5
6	54	52	0	7
9	50	52	0	5.5
12	47	48	0	5.1
15	45	46	0	4.6
21	29	33	0	3.8
27	21	22	0	3.0
39	21	15	6	2.7
51	40	10	30	2.4
64	40	4	36	2.3
76	40	0	40	2.0
88	54	0	54	0.6

Discussion:

The intestinal absorption of PteGlu has been well documented (Denko, 1951, Spray & Witts, 1952, Clark 1953; Girdwood 1953; Chanarin et al 1958; Anderson et al 1960; Chanarin & Bennett 1962; Klipstein 1963; Baker, Frank, Feingold, Ziffer, Gellene, Leevy & Sobotka, 1965; Hepner, Booth, Cowan, Hoffbrand & Mollin, 1968). These studies are however of little relevance to the physiological absorption of dietary folate, firstly because the bulk of food folate is in the form of reduced methyl and formyl compounds, and secondly because the oral dose given by these workers varied from 500-5000 µg PteGlu, an amount considerably in excess of that found in the normal diet. It is also debatable whether PteGlu can be considered a "natural" folate, or arises by the oxidative degradation of reduced folates, as it constituted only approximately 10 per cent of total folate in the diet analysed in section III, a value within the experimental error of assay techniques.

From the evidence presented here it is apparent that, although some pteroylglutamic acid is absorbed unchanged, some is also reduced and methylated in the gut wall during normal absorption. These results differ from those reported by Whitehead and Cooper (1967) who noted the absorption of unaltered folic acid, since folic acid was present in samples of portal blood after oral dosage. Butterworth, Baugh and Krumdieck (1969), giving doses of 4 mgms. PteGlu, also reported absorption of unaltered folic acid, but noted that one

patient had a higher urinary L.casei than S.faecalis activity "suggesting metabolic conversion of labelled material to a form such as N^5-methyltetrahydrofolate before excretion". Baker et al (1965) concluded that after a 5 mgm dose of PteGlu, conversion to a metabollically active folate took place, but these workers did not provide any evidence that the 5 CH_3 H_4PteGlu found in plasma arose by conversion of the test dose rather than by exchange with tissue methylfolate.

Oral administration of "natural" folate resulted in absorption, as determined by the increase in plasma folate levels, equivalent to that obtained with PteGlu, (fig XXXI) but there are significant differences in the manner in which PteGlu and the natural folates are absorbed. This is probably related to the substrate specificity of the enzyme dihydrofolate reductase, which rapidly converts H_2 to H_4PteGlu, but reduces PteGlu to H_2 PteGlu at only about one tenth of the rate of the former reaction, particularly at physiological pH (Zakrzewski & Nicholl, 1960). Some forms of dihydrofolate reductase may even fail to react with PteGlu (Nath & Greenberg 1961).

Thus PteGlu was only partly reduced, and appeared in the blood in approximately equal quantitites of unchanged PteGlu (S.faecalis active) and 5 CH_3 H_4PteGlu (L.casei active). On the other hand H_2PteGlu was completely reduced to H_4PteGlu, and all the orally administered forms passed to the blood as 5 CH_3 H_4PteGlu.

It would appear therefore that in the course of normal absorption dietary monoglutamyl folates pass through the biochemical pathways described here, namely reduction and methylation in the gut wall, and are passed on to the blood as CH_3H_4PteGlu.

Section V.

The intestinal absorption of polyglutamyl folates, and tests for conjugase inhibitors.

5.1. Introduction.

Some three quarters of dietary folates are polyglutamyl compounds, the pteroic acid moiety being linked to a variable number of glutamic acid residues. The number of residues exceeds three, since triglutamates support the growth of L.casei which polyglutamyl compounds do not, and a compound having seven glutamic acid residues has been described (Pfiffner, Calkins, Bloom & O'Dell, 1946).

In this section studies on the absorption of polyglutamyl forms of folate are described. Yeast concentrate (section II, 6) served as the source of polyglutamate.

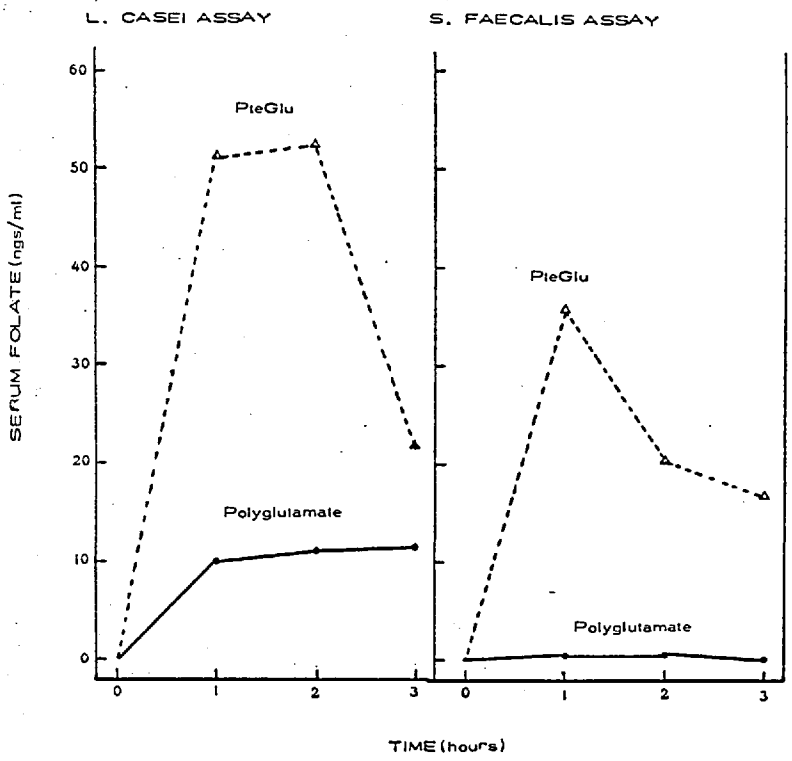

Figure XXXVI - The mean serum folate levels following oral PteGlu or polyglutamyl folate, 20µg/kg. The L. casei serum folate zero value has been subtracted from the remaining values.

2. Absorption studies with yeast concentrates.

A. Normal subjects (8)

Each subject was given 15 mgm PteGlu orally 36 hours before reach test. An oral dose of 20 µg/kg. of PteGlu and subsequently an equimolar dose of yeast polyglutamate, assuming that the compound was largely heptaglutamate, was given and serum folate levels and urinary folate excretion were measured after each compound. Substantially higher serum folate levels (tables 28 and 29 and figure XXXVI were obtained after monoglutamate than after the polyglutamyl compounds, although in subject 8 absorption of both compounds was almost identical. The mean maximum increase over the baseline folate level was 58.7 with PteGlu, and 14.5 ng with polyglutamate (L.casei assay).

Table 28.

Serum and Urinary folate levels after 20 μg/kg orally of PteGlu or polyglutamyl folate (L.casei assay)*.

Subject	Oral dose	Serum folate (ng/ml) 1h	2h	3h	4h	Urine (μg)
1	PteGlu	30	80	5	-	176
	polyglutamate	6	6	5	3	8.6
2	PteGlu	100	100	30	-	-
	polyglutamate	8	8	5	-	4.5
3	PteGlu	63	68	45	-	375
	polyglutamate	8	8	0	0	18
4	PteGlu	106	72	34	-	575
	polyglutamate	17	9	11	0	9
5	PteGlu	26	22	14	-	440
	polyglutamate	0	14	14	9	29
6	PteGlu	27	39	31	-	166
	polyglutamate	18	18	6	0	20
7	PteGlu	0	15	26	-	180
	polyglutamate	5	8	13	10	8
8	PteGlu	7	23	25	-	203
	polyglutamate	8	32	24	24	96
mean	PteGlu	51.3	52.4	26.2	-	302
	polyglutamate	10	13	11.1	11.5	24.3

* The base line serum folate values have been subtracted from the remaining values.

Table 29.

Serum and Urinary folate levels after 20 µg/kg orally of PteGlu or polyglutamyl folate (S.faecalis assay)*.

Subject	Oral dose	Serum folate (ng/ml) 1½	2½	3½	4½	Urine (µg)
1	PteGlu	0.5	7.5	5	-	52
	polyglutamate	0	0	0	0	0.8
2	PteGlu	60	62	48	-	-
	polyglutamate	0	0	0	-	2
3	PteGlu	37	39	25	-	162
	polyglutamate	0	0	0	0	3.4
4	PteGlu	62	19	15	-	575
	polyglutamate	0	0	0	0	7.2
5	PteGlu	10	7	3	-	160
	polyglutamate	0	0	0	0	2
6	PteGlu	0	6	8	-	-
	polyglutamate	2.5	0	0	0	5
7	PteGlu	0	13	23	-	112
	polyglutamate	0	0	0	0	1.0
8	PteGlu	0	10	8	-	139
	polyglutamate	0	3	0	0	14
Mean	PteGlu	35.9	20.4	17	-	200
	polyglutamate	0.3	0.4	0	0	4.4

* The base line serum folate values have been subtracted from the remaining values. It was zero in all but 2 tests.

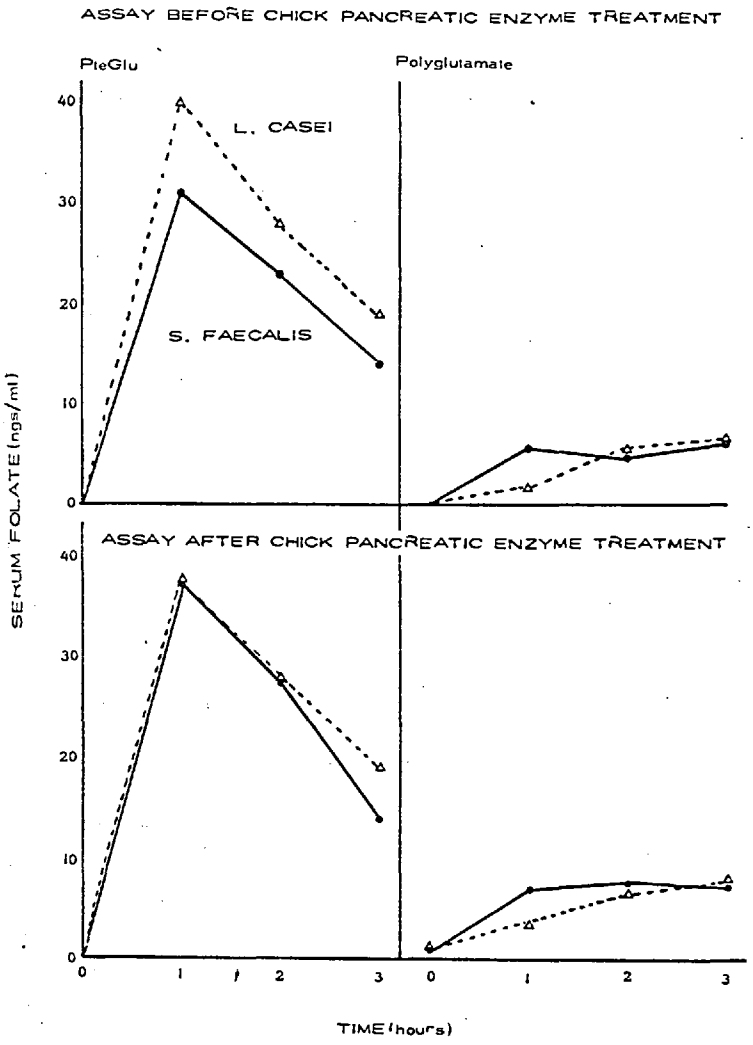

Figure XXXVII. The mean serum folate levels following oral PteGlu or polyglutamyl folate (20μg/kg) before and after treatment of the serum with chick pancreatic enzyme.

S.faecalis assays on the sera showed the same pattern as that reported in Section (IV). After PteGlu, increased S. faecalis levels were obtained, although these were lower than when using L.casei, but after yeast polyglutamyl folate (predominantly methyl and formyl conjugates) no S.faecalis activity was detected. The rise in serum folate when L. casei only was used as test organism suggests the presence in plasma of methyl folate only.

Mean urinary folate excretion after PteGlu was 302 µg (L.casei) and 200 µg (S.faecalis). After polyglutamyl folate the mean values were 24 µg with L.casei and 4.4 µg with S.faecalis. These levels confirm the relatively poor absorption of polyglutamate compared with monoglutamate. Subject 8 however excreted 96 µg after yeast dosage (L.casei)

The rise in "Free" folate activity in serum after oral polyglutamate indicated that the material had been deconjugated during absorption, as the compound itself is unavailable to the assay organisms without prior enzyme treatment. These increased levels were unlikely to have been due to the small amount of free folate in the yeast preparations.

B. <u>The effect of exogenous conjugase on sera and urines.</u>

To determine whether any polyglutamate had been absorbed unchanged, sera and urine from 5 subjects were incubated with chick pancreatic enzyme and re-assayed.

Table 30.

Serum and urinary folate levels following oral heptaglutamate and incubation of serum and urine specimens with chick pancreatic conjugase (L.casei assay).

Subject	Conjugase	Serum folate (ng/ml) 0'	1'	2'	3'	Urine (µg)
9	-	3	6	17	21	2.6
	+	5	10	16	20	2.9
10	-	5	10	18	14	5.5
	+	8	13	16	12	6.5
11	-	3	4	4	6	3.9
	+	4	7	7	7	3.9
12	-	11	11	10	11	6.8
	+	11	9	16	9	9.5
13	-	5	5	5	7	2.2
	+	2	4	6	9	3.7

The difference in assay value before and after conjugase treatment (table 30 and fig XXXVII) was not significant ($P = 0.55$) and it was concluded that there was no evidence for absorption of unchanged polyglutamate.

Table 31.

Serum and urinary folate levels after PteGlu, polyglutamyl folate ($PteGlu_x$) and deconjugated polyglutamyl folate ($PteGlu_x$+) 20 µg/kg - (L.casei assay)*.

Subject	Oral dose	Serum folate 1h	2h	3h	Urine (µg)
1	PteGlu	30	80	5	176
	$PteGlu_x$	6	6	5	8.6
	$PteGlu_x$+	16	7	8	164
2	PteGlu	100	100	30	-
	$PteGlu_x$	8	8	5	4.5
	$PteGlu_x$+	16	20	15	7.3
3	PteGlu	63	68	45	375
	$PteGlu_x$	8	8	0	18
	$PteGlu_x$+	16	9	10	59
4	PteGlu	106	72	34	575
	$PteGlu_x$	17	9	11	29
	$PteGlu_x$+	53	68	38	137
5	PteGlu	26	22	14	440
	$PteGlu_x$	0	14	14	29
	$PteGlu_x$+	45	25	27	208
6	PteGlu	27	39	31	166
	$PteGlu_x$	18	18	6	20
	$PteGlu_x$+	16	7	-	-
7	PteGlu	0	15	26	180
	$PteGlu_x$	5	8	13	8
	$PteGlu_x$+	9	18	11	112
8	PteGlu	7	23	25	203
	$PteGlu_x$	8	32	24	96
	$PteGlu_x$+	23	40	15	83
Mean	PteGlu	51.3	52.4	26.2	302
	$PteGlu_x$	10	13	11.1	24.3
	$PteGlu_x$+	23.7	24.2	17.7	110

*The base line serum folate values have been subtracted from the remaining values.

C. Polyglutamyl folate absorption after preliminary treatment of the oral dose with conjugase enzyme.

Yeast concentrate preparations were treated with chick pancreatic conjugase prior to oral dosage. Where possible the treated yeast preparation used for each subject came from the same batch as the untreated yeast previously given.

Deconjugation of the yeast folates from poly-to mono- or di-glutamyl compounds resulted in improved absorption (table 31) although the serum folate levels did not reach those attained with PteGlu. The mean serum values were twice that encountered after feeding polyglutamate but only reached 50% of the level obtained after oral PteGlu. In subjects 5 and 8 however the serum levels obtained were almost equivalent after deconjugated yeast and PteGlu.

Using S.faecalis as assay organism little increase in serum folate was detected, subjects 1,5,6 & 8 attaining minimal rises only.

Urinary excretion of folate compounds also improved after deconjugated polyglutamates, the mean values being 111 µg (L.casei) and 12.6 µg (S.faecalis). These figures represented a 3-4 fold increase over those following conjugated folate.

D. Summary of findings.

Using L.casei as assay organism serum folate levels

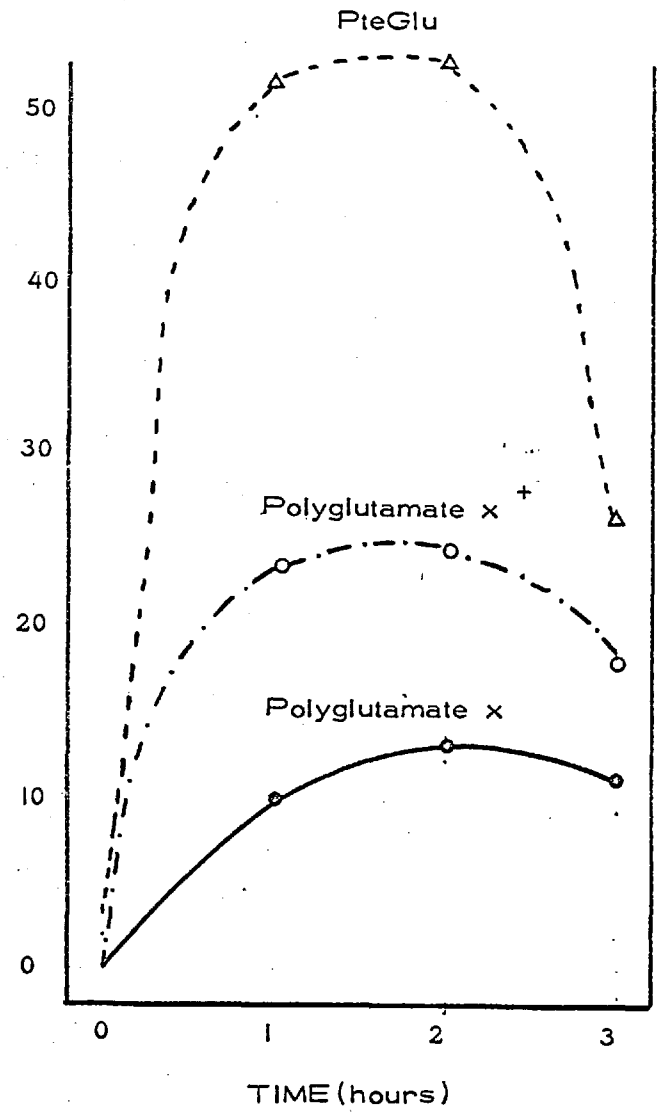

Figure XXXVIII - The mean serum folate levels (L.casei assay) following oral PteGlu, yeast polyglutamyl folate (x) and yeast polyglutamyl folate previously treated with chick pancreatic conjugase (x^+).
The serum folate zero values have been subtracted from the remaining values.

after oral polyglutamyl folates were markedly lower than those attained after monoglutamyl dosage. (figure XXXVIII).

Urinary excretion of folate was lower after polyglutamate than after oral PteGlu. (table 32). These observations suggest poorer absorption of the polyglutamates.

Treatment with chick pancreatic conjugase of the serum and urine samples obtained after oral yeast did not result in statistically significant increases in folate values. Thus polyglutamate appears to be absorbed only after removal of the glutamic acid chain, and long chain polyglutamates do not reach the blood from the gut.

There was little or no rise in serum folate on S.faecalis assay following oral polyglutamate, suggesting that the folate residues released after conjugase treatment were dihydro- or tetrahydro- forms, and hence methylated during absorption (section IV).

Table 32.

Mean maximal folate levels following mono-or polyglutamyl compounds.

Compound	L.casei		S.faecalis	
	Serum	Urine(μg)	Serum	Urine (μg)
PteGlu	58.7	302	29	200
yeast polyglutamate	14.5	24.3	0	4.4
Deconjugated poly-glutamate	29.9	110	1.8	12.6

Table 33.

Serum and urinary folate levels after oral PteGlu and polyglutamyl folate (20µg/kg) in patients with pernicious anaemia (L.casei assay)*.

Subject	Oral dose	Serum folate (ng/ml) 1t	2t	3t	Urine (µg)
Du	PteGlu	38	26	18	480
	polygluta-mate	2	6	-	17
Tho	PteGlu	36	24	20	270
	polygluta-mate	30	21	-	28
Wi	PteGlu	40	44	20	260
	Polygluta-mate	0	10	0	4
Wa	PteGlu	10	8	5	100
	Polygluta-mate	7	4	7	11
Br	PteGlu	17	7	0	
	Polygluta-mate	15	18	22	12
Mean	PteGlu	28.2	20.2	16	280
	Polygluta-mate	13.5	11.8	9.6	14.4

*The base line serum folate values have been subtracted from the remaining values.

3. Polyglutamyl folate absorption in pernicious anaemia.

Human intestinal conjugase has an optimum pH of 4.5 (Hoffbrand and Necheles, 1968; Rosenberg, Godwin, Streiff and Castle, 1968, Rosenberg, Streiff, Godwin and Castle 1969). It has been suggested that when food mixed with gastric acid secretion passes out of the pylorus down the duodenum and jejunum the pH may fall transiently as low as pH 5.0 before returning to relative neutrality (Rhodes and Prestwich, 1966). If this were so some splitting of polyglutamyl folates could take place in the intestinal lumen.

In pernicious anaemia the pH will remain at neutrality and hence polyglutamyl folate absorptions were carried out in patients with pernicious anaemia to test this hypothesis.

Five patients, all of whom had proven pernicious anaemia, were studied.

PteGlu, absorption appeared lower than in normal subjects but the difference was not significant ($P. < 0.1 > 0.05$) The mean maximal serum folate increase was 30 ng, as compared with 59 ng in controls. After polyglutamyl absorption the mean maximal serum folate level was 15 ng, being essentially the same as the level of 14 ng found in normal subjects (table 33).

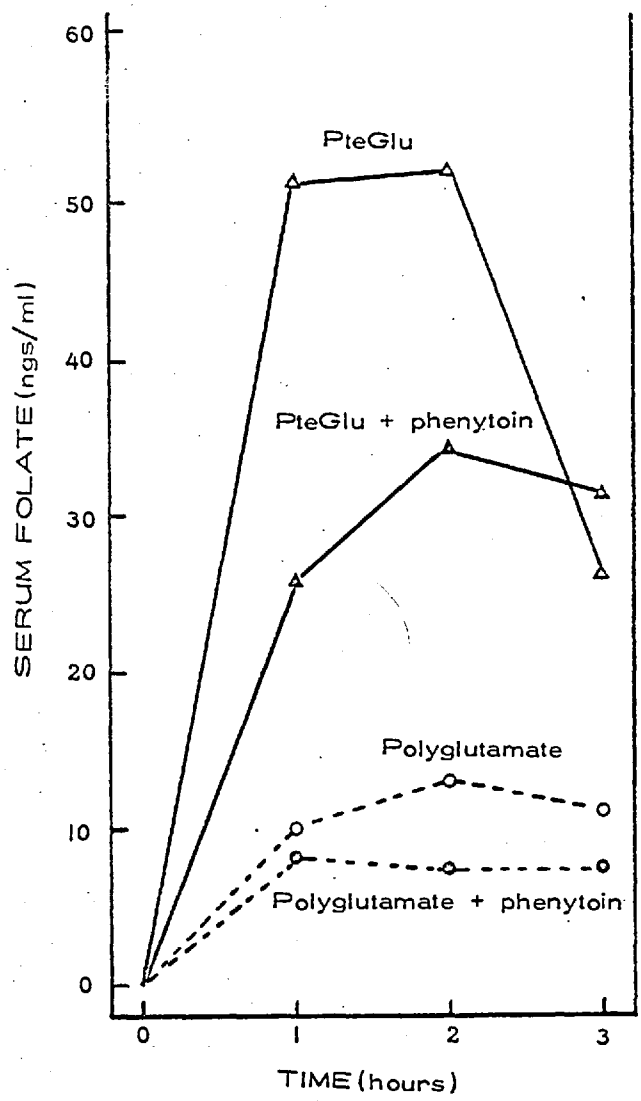

Figure XXXIX. The mean serum folate levels following oral doses of PteGlu and polyglutamyl folate given both with and without oral phenytoin (L. casei assay)

The serum folate zero values have been subtracted from the remaining values.

4. The effect of phenytoin on folate absorption.

The anticonvulsant drug phenytoin (diphenylhydantoin, Dilantin, Epanutin) has been reported to act as an inhibitor of intestinal conjugase activity (Hoffbrand and Necheles, 1968; Rosenberg, Godwin, Streiff & Castle, 1968), resulting in impaired absorption of polyglutamyl folates. This hypothesis was tested in in vivo absorption studies and in an in vitro system (page 195).

The effect of a single 100 mgm dose of phenytoin, given orally at the same time as both PteGlu and yeast concentrate, was studied in the same 8 subjects as previously.

The results are shown in table 34.

A. PteGlu.

The mean maximal rise in serum folate was 59 ng after PteGlu alone, and 41 ng after PteGlu with phenytoin (L.casei assay). Using S.faecalis the values were 28 ng and 22 ng. respectively.

Urinary excretion of folates was 302 µg following PteGlu, as compared with 465 µg following PteGlu and phenytoin (L.casei assay), and 200 µg and 243 µg respectively on S.faecalis assay.

B. Polyglutamyl folate.

Phenytoin given with polyglutamyl folate produced an almost identical mean maximal serum rise as that reached with polyglutamyl folate alone (Lcasei assay). Serum S.faecalis activity was undetectable in both groups.

Table 34.

Serum and urinary folate levels afte PteGlu and polyglutamyl folate (20μg/kg) with and without phenytoin, 100 mgms, given orally (L.casei assay)*.

Subject	Oral dose	Phenytoin	Serum folate (ng/ml) 1½	2½	3½	Urine (μg)
1	PteGlu	-	30	80	5	176
		+	25	30	17	-
	Polygluta-mate	-	6	6	5	8.6
		+	28	0	0	6
2	PteGlu	-	100	100	30	-
		+	18	58	45	84
	Polygluta-mate	-	8	8	5	4.5
		+	11	0	0	3.3
3	PteGlu	-	63	68	45	375
		+	28	23	23	1030
	Polygluta-mate	-	8	8	0	18
		+	3	1	0	11
4	PteGlu	-	106	72	34	575
		+	48	34	30	316
	Polygluta-mate	-	17	9	11	9
		+	5	5	2	29
5	PteGlu	-	26	22	14	440
		+	24	27	12	385
	Polygluta-mate	-	0	14	14	29
		+	8	12	30	44
6	PteGlu	-	27	39	31	166
		+	50	-	30	-
	Polygluta-mate	-	18	18	6	20
		+	8	5	5	8
7	PteGlu	-	0	15	26	180
		+	7	6	20	224
	Polygluta-mate.	-	5	8	13	8
		+	0	4	7	6
8	PteGlu	-	7	23	25	203
		+	2	64	75	750
	Polygluta-mate	-	8	32	24	96
		+	3	31	16	35
Mean	PteGlu	-	51.3	52.4	26.2	302
		+	26	34.6	31.5	465
	Polygluta-mate	-	10	13	11.1	24.3
		+	8.2	7.2	7.5	17.7

* The base line serum folate values have been subtracted from the remaining values.

After oral polyglutamyl folate alone urinary folate excretion was 24.3 µg, and 17.7 µg following polyglutamyl folate and phenytoin, (L.casei) and 4.4 and 4.5 µg with and without the drug using S.faecalis.

C. Summary of findings.

The results summarised in table 35 and figure XXXIX do not support the hypothesis that phenytoin had any effect on conjugase enzymes in vivo. Oral administration of phenytoin had no statistically significant effect on absorption of PteGlu ($P = < 0.2 > 0.1$) or polyglutamyl folate as determined by serum folate rises. There was also no statistically significant difference in urinary folate excretion following phenytoin compared with values obtained without the drug ($P = < 0.3 > 0.2$).

Table 35.

Mean maximal serum and urinary folate following PteGlu and polyglutamyl folate (20 µg/kg) with and without phenytoin (L.casei assay).

Compound	Phenytoin	Serum folate	Urinary folate (µg)
PteGlu	-	58.7	302
	+	41	465
Polyglutamyl folate	-	14.5	24.3
	+	15.6	17.7

5. In vitro studies on the effect of phenytoin on conjugase enzyme, and studies on possible conjugase inhibitors in yeast preparation.

Hoffbrand & Necheles (1968) and Rosenberg, Godwin Streiff and Castle (1968) have suggested that phenytoin acts as an inhibitor of conjugase activity. The presence of conjugase inhibitors was postulated by Bethell, Myers, Andrews, Swendseid, Bird & Brown (1947) to explain some anomalous results they obtained in absorption tests using yeast. Hoffbrand (1969) reported that he was able to separate conjugase inhibitory fractions from polyglutamyl folate in yeast by column chromatography, and that such inhibitors would account for the poorer absorption with polyglutamates of the kind reported in this study.

To test this hypothesis the effect of these "inhibitors" was determined. The folate in human red blood cells is a polyglutamate, and this is not available for microbiological assay with L.casei until it is first exposed to the action of a conjugase enzyme at an acid pH. Such an enzyme is present in all human plasmas. Like intestinal conjugase its pH optimum is 4.5. When whole blood is lysed at this pH the conjugase rapidly releases monoglutamate from polyglutamate, which is then available for assay, with L.casei. The results of a typical experiment are shown in table 36. wherein a haemolysate prepared from carfully washed red cells contained only 30 ng activity for L.casei. After the same haemolysate had been exposed to its own plasma, at pH 4.5, the folate activity increased to 400 ng/ml.

Table 36.

The release of assayable folate from red blood cells by. plasma conjugase.

	Red cell folate (ng/ml)
1) Washed red cell haemolysate	30
2) Same haemolysate after addition of its own plasma (pH 4.5)	400
3) plasma alone	4

In this study phenytoin, and fractions obtained from the column chromatography of yeast extracts, were added to the plasma - red cell haemolysates to determine whether they could interfere with the action of the plasma conjugase in releasing monoglutamyl folate. The tests were done in duplicate and the assays of monoglutamyl folate released were done in triplicate.

A. Test system. Washed normal human red cells were lysed in distilled water, centrifuged and the supernatant diluted in 1% ascorbate, pH 4.5, such that the final concentration was approximately 1:50 and 0.1 mls of this was used as the substrate.

The test system adopted is shown in table 37, the order of addition of the materials being:

1. plasma
2. phenytoin, yeast fraction or water
3. red cell haemolysate.

Components 1 & 2 were mixed and allowed to stand for

Table 37.

Volume	A	B	C
0.5 mls	test material	test material	dist. H_2O
0.1 "	red cells	dist. H_2O	red cells
0.5 "	plasma	plasma	plasma

Table 38.

System	Fraction number 1	3	5	16	17	18
A. Plasma + Yeast + red cells	57	94	140	80	38	86
B. Plasma + Yeast	20	23	80	29	10	40
C. Plasma + red cells	30	75	68	43	29	56
D. C - (A - B)	-7	+4	+8	-8	+1	+10

20 minutes at room temperature. Component 3 (red cell polyglutamate) was added and the whole diluted and assayed as for routine red cell folate measurement.

If the phenytoin or yeast fraction exhibited no inhibitory action on plasma conjugase then the value for A (red cells, phenytoin or yeast, and plasma) minus the value for B (yeast on phenytoin and plasma), would equal C (red cells and plasma)

B. Tests for conjugase inhibition by yeast fractions.

Six yeast fractions were tested, three from an early stage of column elution comprising mainly monoglutamyl folate, and three which were polyglutamyl folates. The fractions were diluted if necessary to enable their folate content to fall within the L.casei assay range, and various dilutions were tested for inhibitory activity.

Results None of the yeast eluates lessened to any significant degree the amount of assayable folate released by the action of plasma conjugase on the red cell substrate (table 38). The differences in the figures obtained were all within those imposed by the limitations of microbiological assay.

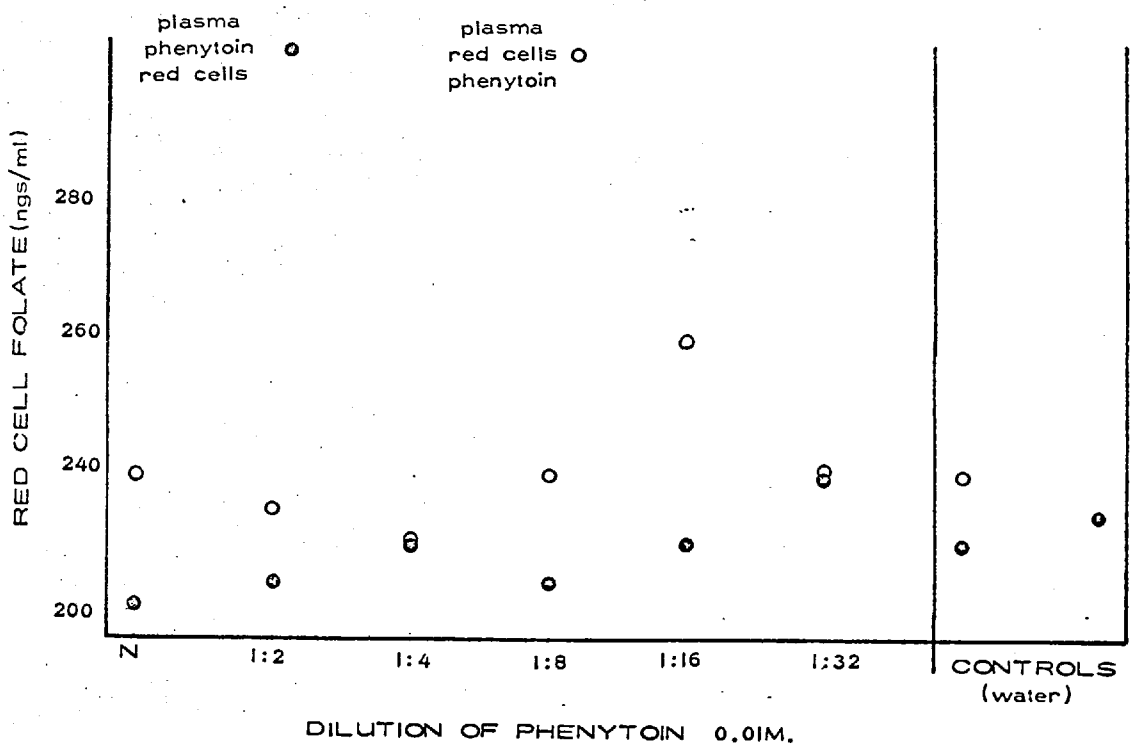

Figure XL. Red cell folate values following tests for inhibition of plasma conjugase activity by phenytoin.

Each value shown is the mean of three tests.

● test system

○ control system

C. Test for conjugase inhibition by phenytoin.

An 0.01 M solution of phenytoin (diphenylhydantoin sodium, MW 274.25) was used in these studies.

The test system emplyed was that described above, and the effect of varying the concentration of phenytoin, and also of plasma conjugase, was determined.

i. Variation of phenytoin concentration. The 0.01 M solution was used as such, and also in doubling dilutions to a concentration of 1/32. A control system was employed for each series of tests consisting of plasma + red cells with distilled water added instead of phenytoin solution. In addition any effect phenytoin itself may have had on the assay organism was determined by including a further batch of controls, one for each concentration of phenytoin used. These consisted of plasma + red cells, with the appropriate dilution of phenytoin added subsequently.

Results. These are shown in table 39 and figure XL. No inhibitory effect of phenytoin on plasma conjugase could be detected at any of the dilutions employed, the differences in assay values all being within the experimental error of the assay.

ii. Variation of plasma conjugase concentration: The effect of varying the concentration of plasma conjugase, while keeping the concentration of phenytoin constant (0.01 M) was also

Table 39.

The effect of varying dilutions of phenytoin on the release of red cell folate by plasma conjugase.

Concentration of phenytoin	Red cell folate (ng/ml)	
	Test	Control
0.01 M	200	230
1/2	210	210
1/4	220	240
1/8	210	240
1/16	220	260
1/32	240	240
nil	220	230

Table 40.

The effect of phenytoin on the release of red cell folate by diluted plasma conjugase.

Plasma concentration	Red cell folate (ng/ml)	
	Test	Control
undiluted	260	255
1:5	240	240
1:10	215	210
1:25	175	175
1:50	165	170
1:100	135	155

determined. Plasma was used concentrated, and also diluted in 1% ascorbate, pH 4.5 at concentrations of 1:5, 1:10, 1:25, 1:50 and 1:100. Control tubes containing plasma, red cell and distilled water were included in the test system for each dilution of plasma used.

Results.

Although the release of monoglutamyl folate from the red cell polyglutamyl folate was, as might be expected, lower with the diluted plasma, again no inhibitory effect of phenytoin on plasma conjugase could be detected. (table 40).

D. Summary of findings.

No inhibitory effect on the ability of plasma conjugase to release assayable folate from its conjugate in red blood cells could be detected with either yeast column eluates or a solution of phenytoin using the *in vitro* test system described here. Thus it would appear that the lower serum folate values obtained after oral administration of yeast polyglutamyl folates as compared with those obtained after oral monoglutamyl folate (PteGlu) were unlikely to be due to the presence of an inhibitor of intestinal conjugase activity in the yeast preparations.

The results obtained when phenytoin was tested *in vitro* confirmed those found by *in vivo* studies, no inhibition of conjugase activity being detectable by either method.

Discussion.

Polyglutamyl compounds were among the first folate derivatives to be described (Stokstad, 1943, Binkley et al 1944) and have been shown to comprise some three quarters of man's daily dietary folate intake. However the absorption of folate polyglutamates has not been extensively studied in man, due primarily to the difficulties involved in obtaining a suitable test material of adequate folate activity. The yeast concentrates used in this study contained a reasonable approximation of the folates found in dietary material, mainly methyl and formyl forms and their conjugates, with the difference that the yeast material consisted of almost pure (97%) polyglutamyl folates. The increased serum folate levels after oral dosage with the compound would have been unlikely to be due to the small amount of monoglutamyl folate present.

The observations reported here have indicated relatively poor absorption in man of dietary polyglutamyl folate as compared with the monoglutamyl forms, although in the chick and rat polyglutamates appear to be fully utilized (Jukes, 1955, Cropper and Scott 1966; Kodicek and Carpenter (1948).

Initial studies with yeast polyglutamyl folates were concerned primarily with haematological responses to the material in deficiency states such as megaloblastic anaemia of pregnancy (Wills, 1931) and pernicious anaemia (Welch, Heinle, Nelson and Nelson, 1946; Bethell, Meyers, Andrews, Swendseid, Bird and Brown, 1947).. The responses noted by Wills, who used an autolyzed yeast preparation, may well have been due to the monoglutamyl folate in the material, since the free folate content is greater in autolysed yeast (Chanarin 1969).

Welch et al, and Bethell et al, employing crystalline pteroylheptaglutamic acid obtained from yeast, reported cases who failed to respond to the conjugate and then responded to an equivalent amount of PteGlu. In normal subjects however, they stated that urinary excretion of folates after yeast pteroylheptaglutamic acid was dependent on the prescence or abscence of a conjugase inhibitor in the oral material. In the absence of an inhibitor urinary folate excretion was the same with both the free vitamin and polyglutamate implying equivalent absorption of both (Swendseid, Bird, Brown and Bethell, 1947). Spray, and Spray & Witts (1952) compared the absorption of PteGlu and yeast polyglutamate (1 mgm) and noted poor absorption of the yeast folate, together with low urinary folate excretion.

In none of these studies mentioned was the <u>in vitro</u> effect of conjugase on the serum or urine samples determined. Cooperman and Luhby (1965) reported one case, given yeast polyglutamate equivalent to 2.46 mgms of folic acid, in whom moderate serum folate rises were obtained on routine L.casei assay, these values increasing strikingly after treatment of the serum with chick pancreatic conjugase. The same pattern was obtained on urine assay. They postulated therefore the absorption and excretion of unaltered polyglutamate from the gastrointestinal tract. Banerjee and Chatterjea (1966 and 1968) have also reported the presence in normal human serum of polyglutamyl folates, detectable only after enzymic digestion. These studies conflict with the result of Herbert, Larrabee and Buchanan (1962) Rosenberg, Streiff,

Godwin and Castle (1969) and those reported here where conjugase treatment of serum samples failed to produce any significant change in serum folate levels.

Jandl and Lear (1956), studying the uptake of yeast folate, found that some 25% of folate in untreated yeast was absorbed, and this was increased to 60% when the yeast was pretreated with conjugase enzyme, but that even this was not as well absorbed as an equivalent dose of PteGlu. The data obtained here on the absorption of polyglutamate first treated with enzyme confirm this finding.

More recent studies have utilized polyglutamyl folate obtained after DEAE chromatographic fractionation of yeast. Streiff and Rosenberg (1967) reported the effect of 10 µg orally of such a preparation, claiming serum folate increases of 70% of those obtained with PteGlu. This dose would appear however to be too small to have an effect on plasma folate levels. Hoffbrand and Necheles (1968), Rosenberg, Godwin, Streiff and Castle (1968) Rosenberg, Streiff, Godwin and Castle (1969) and Hoffbrand Necheles, Maldonada, Horta, and Santini (1969) all employed 200 µg of polyglutamate orally, and all reported increased serum folate levels. Where a comparison was made with PteGlu absorption the polyglutamyl induced folate increases almost parallel the PteGlu rises. In these studies approximately 1000 µg polyglutamate was used and at this dose level the polyglutamate was less well absorbed.

The only study reported utilizing a pure synthetic polyglutamyl folate is that of Butterworth, Baugh and Krumdieck (1969). Giving oral doses of 4.41 mgms of PteGlu heptaglutamate, variously labelled with ^{14}C, they showed that deconjugation of the material took place during the digestive process, and concluded that "50-70%

of ingested heptaglutamate is available to man inasmuch as it was not lost in the faeces". These were in the nature of preliminary observations and data on the recovery of ^{14}C polyglutamate added to faeces was not given.

That increased serum folate after oral polyglutamate is due to the presence of directly assayable mono (or tri)-glutamyl folate would confirm the view of Baker, Frank and Sobotka (1964) that natural polyglutamyl folates must be deconjugated for absorption to occur. Santini, Berger, Berdasco, Sheehy, Aviles and Davila (1962) and Klipstein (1967) noted the presence of conjugase in human duodenal aspirates, while Streiff and Rosenberg (1967) and Rosenberg, Streiff, Godwin and Castle (1969) proposed, on evidence obtained from the deconjugating activity of rat intestinal mucosa, that hydrolysis of polyglutamates to "free" folates occured within the intestinal cells. In guinea pigs Hoffbrand and Peters (1969) localized conjugase activity to the subcellular mucosal fractions. These reports vary on the pH optimum of the conjugase enzyme. Although neither Santini et al or Klipstein specifically mention this point, their assays were performed at pH 6-7. The human conjugase described by Rosenberg et al (1968), Hoffbrand and Necheles, (1968) and Hoffbrand et al (1969) however was stated to have a pH optimum of 4.6

The absorption studies reported here do not throw much light on the site of intestinal deconjugating activity. However the fact that absorption of polyglutamate was identical in both normal and pernicious anaemia subjects would imply localization of conjugase activity in the

gut wall rather than the lumen. The achlorhydric pernicious anaemia subjects would be expected tohave near enutral intestinal juice, at which conjugase would be largely inactive, yet the absorption of polyglutamate by this group paralleled exactly that found in normal subjects.

The folate deficient anaemia found in some subjects receiving anticonvulsant therapy was stated by Hoffbrand and Necheles (1968) and Rosenberg, Godwin, Streiff and Castle (1968) to be due to an inhibitor of intestinal conjugase activity, thus resulting in impaired absorption of folate polyglutamates. The results obtained in the present absorption studies on 8 subjects did not confirm this finding, there being no statistically significant difference in the absorpt ion of either mono- or polyglutamyl folate given with phenytoin compared with dosage without the drug. Baugh and Krumdieck (1969) could not detect phenytoin inhibition of intestinal conjugase by <u>in vitro</u> experiments.

The lowered polyglutamate absorption, inrelation to that of monoglutamate, found in this study is unlikely to be due to the presence of an inhibitor in the yeast concentrate used. Although the presence of such an inhibitor in yeast, characterized as a polypeptide of p-aminobenzoic acid by Sims and Totter (1947), has been reported by Bird, Robbins, Vandenbelt, and Piffner (1946) Swendseid, Bird, Brown and Bethell (1947) and Hoffbrand (1969), the test system described here was unable to detect any decline in the activity of plasma conjugase in the presence of added yeast.

Section VI.

The utilization of polyglutamyl folate.

1. Introduction

In Section V it was shown that the rise in serum folate following polyglutamyl folates is approximately 25% of the rise found with the monoglutamyl form.

As there is close correlation between dietary folate intake and red cell folate levels (Chanarin, Rothman Perry and Stratfull, 1968) the extent of utilization of polyglutamyl folates was studied in seventeen subjects by comparing red cell folate levels attained after daily oral supplementation with either PteGlu, or yeast polyglutamyl folate (yeast tablets B.P.). The dose level was 100µg as the monoglutamate or its equivalent as polyglutamate (5gms of yeast daily supplying 102µg of polyglutamate).

The red cell folates, taken monthly in those subjects receiving PteGlu and more frequently in those receiving polyglutamyl folate, were assayed in a single assay at the end of the trial.

Table 41.

Red cell folate values following a daily oral supplement of PteGlu - 100 µg

Subject	Red cell folate (ng/ml)				
	zero	1 month	2 months	3 months	4 months
1	273	315	365	326	292
2	203	218	305	321	333
3	310	318	422	470	442
4	367	318	485	412	400
5	188	152	254	247	250
6	128	136	207	183	194
7	118	150	213	192	201
8	212	272	395	310	338
9	156	190	230	219	250
10	105	120	157	175	195
Mean	206	218	302	285	290

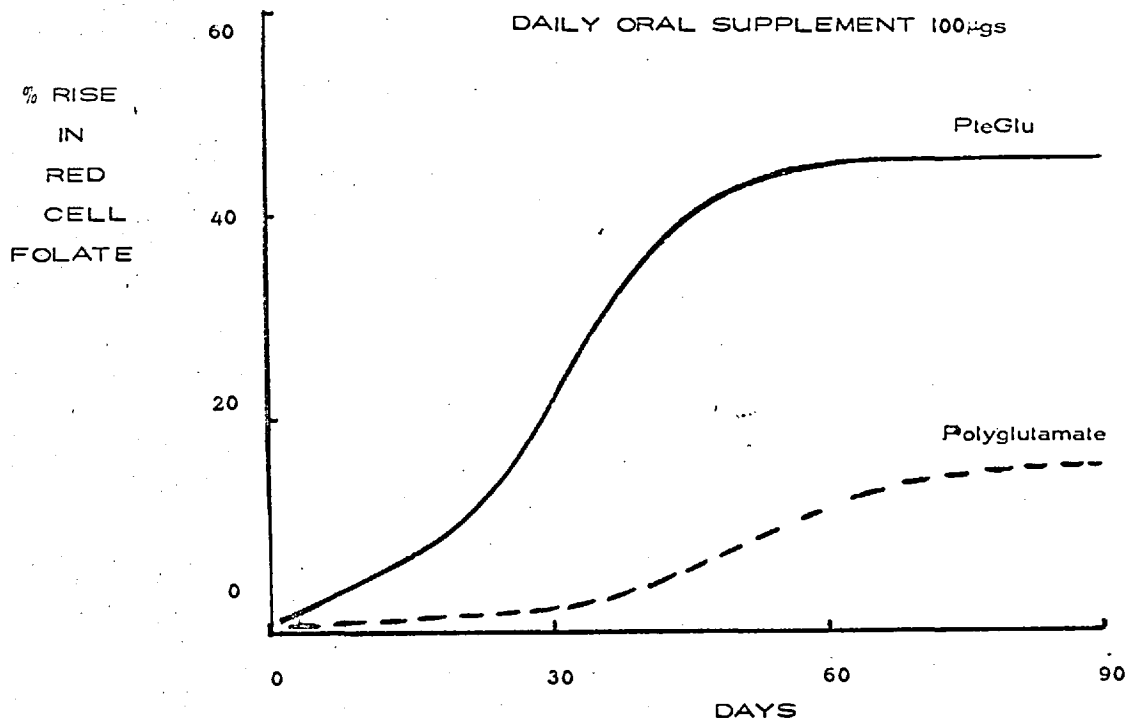

Figure XLI - The relative change in red cell folate levels in 12 normal subjects given 100 µg PteGlu daily, and in 6 normal subjects given 100 µg yeast polyglutamyl folate.

2. The effect of long term oral folate supplementation on red cell folate levels.

In the twelve subjects given oral monoglutamyl folate (PteGlu), there was a slow rise in red cell folate values over two months to reach a plateau some 45% above baseline levels. Following withdrawal of the 100 µg daily folate supplement red cell folates showed a slow decline starting after three months, suggesting reutilization of the augmented body stores (table 41, figure XLI).

In the six subjects given oral yeast polyglutamate the increase was only 16% at the end of the test period, although there was an unexplained rise in red cell folate values two weeks after beginning the study (table 42, figure XLI).

Thus polyglutamyl folate given orally was incorporated into red cells at one third of the level of $PteGlu_3$ as this is likely to be due to the quantities of each of these substances absorbed from the gut.

Table 42.

Red cell folate values following a daily oral supplement of polyglutamyl folate (100μg)

Subject	Red cell folate (ng/ml) zero	2 weeks	4 weeks	8 weeks	12 weeks
1	264	265	-	198	235
2	220	341	238	236	275
3	242	280	315	264	-
4	217	200	235	241	256
5	174	176	173	180	197
6	190	430	-	412	382
Mean	234	282	240	255	269

3. Discussion

The data presented here on incorporation of folate into red cells suggested that polyglutamate is utilized to about one third of the extent of monoglutamate. On this basis, if absorption is one third of the 80% absorption reported for PteGlu (Anderson, Belcher, Chanarin and Mollin, 1960) some 27% of polyglutamate is taken up. From the values reported in Section III for daily dietary folate intake (160µg "free" and 576µg "total") this would suggest a folate utilization of 128µg as mono-, di- or triglutamyl folate (80% of 160µg), together with a further 140µg as polyglutamate (27% of 576µg), giving a total average folate intake in man of 270µg daily.

There are several reports in the literature concerning the utilization of natural folates in folate deficient conditions. Suarez, Welch, Heinle, Saurez and Nelson (1946) described the effectiveness of yeast polyglutamates in tropical sprue, while Baumslag and Metz (1964) treated megaloblastic anaemia of pregnancy with large quantities of lettuce, containing both formyl folate and conjugated folates. Similar observations were made by Butterworth, Brewster, Perez-Santiago and Santini (quoted by Butterworth, 1968) using soups prepared from lettuce, asparagus and spinach. In all cases it appeared that the amount of polyglutamate required to produce a response was greater than that required when monoglutamyl folate was used. However it would appear

that, contrary to Herbert's (1963) claim that microbiological assay of dietary material by L.casei without enzymatic digestion is equivalent to human requirements, at least half the folate absorbed is obtained from the polyglutamyl folate portion of the diet.

Section VII.

Summary of findings, and conclusions.

The data reported in this study have been discussed in the context of the relevant literature at the end of each section (Section III, page 131; Section IV, page 171; Section V, page 203 ; Section VI, page 214). The following is a summary of the findings obtained, and the conclusions drawn from these findings are presented.

Man's average daily dietary folate intake was shown to consist of 160μg of mono-, di-, and triglutamyl folate, and 516μg of polyglutamyl folate. Chromatographic fractionation of a representative meal showed the presence of formyl folates, largely 5CHO H_4 PteGlu, and a small amount of PteGlu and $PteGlu_3$. These latter compounds were probably derived from oxidative degradation of other folate derivatives. The main component was reduced methylated folate, which constituted approximately 65% of the folate in the diet.

Absorption studies with naturally occurring monoglutamyl folates resulted in the appearance in the blood of increased levels of $5CH_3$ H_4PteGlu irrespective of the nature of the orally administered derivative. The use of tritium-labelled reduced folates, given both orally and intravenously, showed that reduction and methylation of the oral folate dose took place in the gut wall, and not in tissues such as the liver, or by an exchange mechanism with tissue folate stores. It was concluded that, in the course of normal absorption, all dietary folate was reduced and methylated in the gut wall, and passed on to the blood as 5 CH_3 H_4PteGlu.

Polyglutamyl folate was found not to be as well absorbed as the monoglutamyl form. The form of folate in plasma following oral polyglutamate was monoglutamyl 5 CH_3 H_4PteGlu, suggesting that the material was absorbed following removal of the peptide chain, presumably by the action of an intestinal conjugase. The pH optimum of human conjugase is 4.5. Absorption studies on patients with pernicious anaemia, whose intestinal juice might be expected to be closer to a neutral pH (at which conjugase would be inactive) was essentially the same as that found in normal subjects, the conclusion being that intestinal conjugase activity was located in the gut wall rather than the gut lumen. Thus the poorer absorption of polyglutamyl folate was felt to be due to several factors, acting either singly or in combination.

Firstly, there is possibly a limitation of transfer of the large polyglutamyl folate molecule (M.W. approx. 1200) into the cells lining the villi of the gut, where conjugase activity is localised.

Secondly, the degree to which human intestinal conjugase is effective in removing long chain glutamyl residues from the complex polyglutamyl folates found in the natural materials is uncertain. Ineffective deconjugation of a proportion of ingested folate could account for the lower monoglutamyl folate blood levels found after oral polyglutamate as compared with monoglutamate.

Thirdly, the relative efficiency of the mechanism of reduction and methylation during absorption of different folate

compounds, described in Section IV, is also unknown. It appears from the data presented in Section IV that equivalent doses of methyl and formyl compounds resulted in higher blood folate levels than those reached following unsubstituted PteGlu. In addition. when H_2PteGlu was given at the same dose level as polyglutamyl folate (20µg/kg) the blood levels reached in both cases were similar (mean maximal level following polyglutamate 14.5ng; H_2PteGlu 16.4ng). It could be concluded therefore that some naturally occurring folate compounds are more efficiently reduced and methylated than others, which may explain why even deconjugation of polyglutamyl folates prior to oral dosage failed to produce blood folate rises equivalent to those following PteGlu. This latter finding could however also be due in part to incomplete deconjugation of the polyglutamyl folate dose by chick pancreatic enzyme (Section II, page 98).

In vitro and in vivo tests using the anticonvulsant drug phenytoin showed no inhibition of human conjugase activity. The yeast polyglutamyl folate used in absorption studies was also tested by an in vitro method for conjugase inhibition but no inhibitory effect could be detected. It is unlikely therefore that the poorer absorption of polyglutamyl folate reported here was due to inhibition of conjugase activity by substances in the oral dose given.

When a daily oral supplement of 100µg of mono- or polyglutamyl folate was given over a three month period to each of two groups of normal subjects the red cell folate levels rose in both cases. The rise attained with PteGlu was some 45% above base line, and with polyglutamate, 16%. These data suggest that polyglutamyl folate is utilised to approximately one-third of the

monoglutamyl form. A figure of 80% absorption of PteGlu has been reported for normal subjects, and on this basis it may be concluded that, from the values of 160μg "free" folate and 516μg polyglutamyl folate found for human dietary intake, approximately 270μg of folate daily is utilized by man.

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Normal Dietary Folate, Iron, and Protein Intake, with Particular Reference to Pregnancy

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Estimates for the folate content of a normal daily diet range from 53 μg. (Read *et al.*, 1965), 52 to 97 μg. (Pace *et al.*, 1960), 62 μg. (Denko *et al.*, 1946), 47 μg. for a poor diet, 157 μg. for a low-cost diet, and 193 μg. for a high-cost diet (Mangay Chung *et al.*, 1961), 101 μg. for elderly patients in hospitals, 145 μg. for the elderly at home, and 223 μg. for young controls (Hurdle, 1967), 157 μg. as "free" folate and 689 μg. as total folate (Butterworth *et al.*, 1963), 400 μg. (Jukes, 1961), 380 μg. for a rural and 650 μg. for an urban diet (Santini *et al.*, 1962) to 1,000 to 1,500 μg. (Jandl and Lear, 1956). Some of these data have been compiled from food tables (generally unreliable as regards folate values); others by direct microbiological assay of food extracts with either *Streptococcus faecalis* or *Lactobacillus casei*; and, finally, some data have been derived by microbiological assay after treatment of the food with enzymes that release complex forms of folate not directly available for assay. Further, food folate is labile, being lost on storage, and over 90% of the folate may be lost during cooking. Failure to use appropriate preservatives in preparing food samples may also have led to marked loss of folate before assay. The marked variation in the published data for food folate reflects these uncertainties in technique.

As part of a study on the folate requirements in pregnancy the dietary folate intake was determined by assay of food as cooked and consumed by women attending the antenatal clinic at St. Mary's Hospital. Iron and protein content were also

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measured. Assay of vitamin B_{12} could not be done, as the ascorbate preservative destroyed some half to two-thirds of added vitamin B_{12}.

There is a greater consensus about the dietary content of iron and protein than of folate. The National Food Survey Committee (1966) found a daily iron intake of 14.1 mg. in 1964, and a recent survey by Davis *et al.* (1967) also noted a mean iron intake of 14.1 mg. each day by women.

Methods

Collection of food.—Food for assay was collected in a weighed 5-litre polyethylene container with 500 ml. of 0.1 M phosphate buffer and 3% ascorbate at a final *p*H of 7. At the end of each 24-hour food collection the container was returned to the laboratory, weighed, its contents homogenized, and 100-ml. aliquots stored at −20° C.

Treatment of Food Homogenate before Folate Assay.—Triplicate quantities of food were processed. Four grammes of the food homogenate were made to 50 ml. in 0.1 M phosphate buffer containing 0.1% ascorbate at *p*H 7, and heated at 121° C. for 15 minutes. Half (25 ml.) was removed for assay of "free" folate. This was made up to 200 ml. with distilled water, filtered, and the clear filtrate retained for assay. The second portion (2 g. in 25 ml.) was mixed with 25 mg. of powdered chick pancreas (Difco), suspended in 2 ml. of phosphate-ascorbate buffer, and 1 ml. of freshly prepared 2.5% ascorbic acid added. After incubation for 18 hours at 37° C. a further 1 ml. of 2.5% ascorbate was added and the mixture autoclaved at 121° C. for five minutes. The mixture was finally made up to 200 ml. with water, filtered, and the clear filtrate retained for assay. The *p*H and amounts of ascorbate used had been found to give the highest yield of folate and satisfactory recovery of added folate both in the "free" and in the polyglutamate form.

Controls for Folate Assay.—Folate content of chick pancreas was subtracted by carrying out the determination with pancreatic extract in equivalent amounts of buffer instead of food homogenate, the preparation being treated in the same way as the food homogenate. Food homogenate (4 g.) was also assayed after the addition of 0.5 g. of standard yeast preparation known to contain 20 μg. of the polyglutamate form of folate and 0.3 μg. of "free" folate. The mean recovery of polyglutamate was 104% of the amount added and the recovery of free folate was 113% (Fig. 1).

Microbiological Assay.—All samples after preparation were assayed with *Lactobacillus casei* by the methods described by Toepfer *et al.* (1951) with 1 g. of ascorbate added to every

500 ml. of medium. Many were also assayed with *Str. faecalis* as the test organism.

Iron Estimation.—Six millilitres of food homogenate was transferred to a silica crucible previously cleaned with dilute hydrochloric acid and ashed in a muffle furnace. After cooling, 2 ml. of concentrated hydrochloric acid was added and the crucible was covered with a watch-glass and heated gently for 30 minutes ; it was then cooled and the contents were filtered into a graduated flask and diluted to 100 ml. with distilled

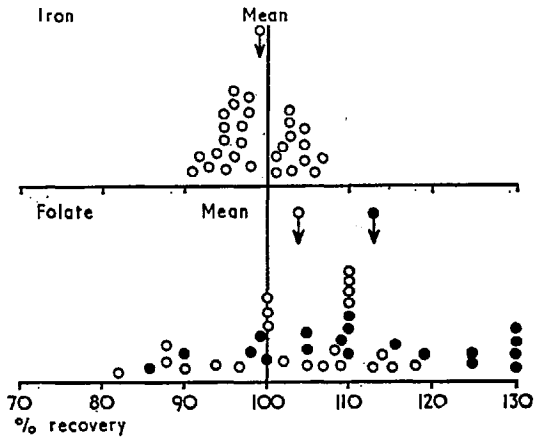

FIG. 1.—Percentage recovery of folate in the form of yeast and ferrous sulphate added to a homogenate of a 24-hour food collection (● "free" folate ; O total folate).

water. Ten millilitres of this solution was taken and to this 3 ml. of acetate buffer, 2 ml. of hydroquinone solution, and 2 ml. of αa'-dipyrolyl solution were added and mixed. The optical density was measured at 520 mμ. A blank determination in reagents alone was used in the reference cell, and the amount of iron present was read from a standard graph. In each experiment two standard solutions were measured as controls, and duplicate samples were taken for analysis. The iron content was also assayed after the addition of 1 ml. of a solution of 50 μg. of $FeSO_4$/100 ml. to the homogenate, and the recovery ranged from 91 to 107%, with a mean of 99% (Fig. 1).

Protein Estimation.—A microKjeldahl method was employed. Duplicate samples of 0.2 ml. of food homogenate were transferred to digestion tubes, 2 ml. of digestion mixture was added, and the tubes were placed on a heating rack. The mixture was allowed to digest until the contents of the tube were colourless. This took one to three hours. Two blank tubes

containing 2 ml. of digestion mixture only were used as controls. The contents were cooled and transferred to the Kjeldahl apparatus with 10 ml. of 40% NaOH. The ammonia produced was collected into a flask containing 5 ml. of boric acid and indicator solution and then titrated against N/70 H_2SO_4.

Patients Co-operating in Study

Pregnant women attending the antenatal clinic at St. Mary's Hospital were asked to prepare all their food in duplicate for a period of seven days. Each was given £5 to cover additional expenditure in that period. The equivalent of any uneaten food was removed from the duplicate plate before the contents were placed in the container with ascorbate-buffer. All foods, fruit, sweets, alcoholic and other drinks, etc., consumed were also added to the container, which was stored in the cold or in a cool place. Each patient wrote down the items of diet she had consumed each day. In all, 16 women carried out daily food collections.

In addition red cell and serum folate estimations were carried out at about the 15th, 30th, and 37th week of pregnancy and a marrow aspiration was performed at the end of pregnancy to determine the character of haemopoiesis and the state of iron stores. These were all healthy women who were gaining weight at the normal rate throughout pregnancy and had a normal-sized foetus at term.

As these women were taking part in a trial designed to determine folate requirements in pregnancy some were having tablets containing 260 mg. of ferrous fumarate and 100 μg. of folate once daily and the rest were having tablets containing only iron. These tablets were not added to the food collection.

Ten 24-hour hospital diets were also assayed. Each diet was collected from one of the wards as it was being issued for consumption to patients. All these studies were carried out between February and September 1967.

Results

Subjects Studied.—Race, age, parity, and husband's occupation are shown in Table I. All the patients maintained adequate haemoglobin levels throughout pregnancy. All had received 1 g. of intravenous iron before the 20th week of pregnancy, all had been given oral iron throughout pregnancy, all had normal serum iron levels throughout pregnancy, and all had demonstrable iron in the marrow at the 38th week. Seven out of the 16 patients had received 100 μg. of folate daily as an oral supplement throughout pregnancy. These women had higher red cell folate levels than the other ten women who had only

TABLE I.—*Subjects Studied*

Subject	Race	Age	Parity	Husband's Occupation	Hb at 38 w. (g./100 ml.)	Red Cell Folate (mμg./ml.)		
						± 15 w.	30 w.	38 w.
1*	Irish m. to Indian	24	0+1	Industrial designer	14·3	174	157	209
2*	English	23	0	Motor mechanic	12·2	120	132	180
3	English	23	0	Motor mechanic	12·0	202	62	51
4	Scottish	22	0	Engineer	12·2	168	97	96
5	English	37	1	Brewer	11·8	137	84	133
6*	English	29	0	Architect	12·4	282	328	250
7	English	29	0+1	Salesman	12·2	150	80	77
8*	English	26	0	Draughtsman	12·8	144	105	144
9*	English	20	0	Shipbroker	14·6	121	112	139
10	South American m. to Welshman	28	0	Land developer	12·2	106	226	220
11	English	21	0	Accountant	14·2	140	65	136
12	Irish m. to Pakistani	34	0	Photographer	13·6	123	174	50
13	East African	22	0+2	Research chemist	13·4	234	189	129
14	Burmese m. to English	25	1+1	Sales manager	11·2	146	178	96
15	English	22	1	Lorry driver	11·8	252	150	126
16*	English	27	0	Press photographer	11·1	220	410	375

* Received 100-μg. supplemental folate throughout pregnancy.

TABLE II.—*Daily Dietary Iron (mg.) and Protein (g.) Intake in Pregnancy*

Subject	Mean		Wednesday		Thursday		Friday		Saturday		Sunday		Monday		Tuesday	
	Fe	Pr	Fe	Pr	Fe	Pr	Fe	Pr	Fe	Pr	Fe	Pr	Fe	Pr	Fe	Pr
1	11·5	81·6	13·6	74·2	7·0	98·6	6·5	91·8	24·3	109·3	4·8	49·5	13·2	84·8	10·7	63·4
2	13·7	69·8	—	—	14·0	72·4	16·1	90·0	6·4	76·7	8·3	55·5	17·2	82·3	10·1	42·3
3	17·8	69·5	14·3	59·9	15·8	65·6	15·6	73·3	24·2	73·5	9·6	49·8	22·5	87·4	23·0	77·2
4	10·9	71·6	15·2	66·0	9·1	68·1	9·7	73·7	8·8	82·2	9·8	78·9	8·9	70·1	14·9	62·2
5																
6	13·7	64·1	16·3	69·0	5·1	56·4	20·2	52·8	19·5	51·5	14·1	78·7	7·3	65·3	13·3	74·8
7	8·9	91·2	9·4	89·5	9·3	111·3	7·3	77·4	8·9	99·4	10·0	82·0	12·0	96·8	5·7	87·4
8	13·4	65·5	13·3	64·2	8·3	63·5	7·6	84·7	21·5	47·9	10·5	78·3	11·1	67·5	21·6	52·6
9																
10	9·9	55·1	14·8	73·5	11·0	65·7	6·4	36·1	14·0	68·0	7·7	42·6	7·7	63·8	7·7	35·8
11	31·4	67·8	32·6	62·5	12·8	69·8	31·5	83·8	38·1	89·0	37·4	81·6	26·2	88·0	41·3	66·7
12	17·7	67·5	21·9	71·1	4·0	66·8	3·7	63·6	49·6	57·3	6·9	62·4	25·6	82·5	12·4	68·8
13	12·6	58·7	13·6	57·2	12·3	68·3	14·5	58·2	14·5	63·1	10·7	50·6	12·0	49·1	10·7	64·6
14	9·0	60·5	5·9	37·6	8·1	59·7	6·9	51·2	8·1	39·7	13·9	92·9	9·8	68·1	10·3	74·2
15	18·5	67·0	24·0	55·5	25·8	90·4	17·2	70·0	34·1	106	14·9	44·1	7·3	45·0	6·4	58·0
16	11·0	70·4	8·0	50·8	7·5	66·4	10·1	61·9	17·9	90·1	12·2	73·0	11·4	73·2	9·7	77·4
Mean	14·2	69·1														

an iron supplement (Table I). All had normoblastic haemopoiesis at the end of pregnancy.

Iron Intake.—The iron content of 97 separate 24-hour food collections was measured (Table II). The mean iron content was 14.2 mg. The mean intake per patient (column 2, Table II) varied from 8.9 to 31.4 mg./day and the daily variation was even greater—from 3.7 to 49.6 mg.

TABLE III.—*Dietary Folate Content Assayed with L. casei (μg/24 Hours)*

Subject	"Free"*		"Total"*	
	Mean	Range	Mean	Range
1	202	115–290	830	499–1,650
2†	101	24–270	945	570–1,430
3	133	70–180	357	210–430
4	141	52–197	607	310–1,150
5	53	38–78	198	109–282
6	271	60–350	1,051	650–2,070
7	162	114–226	361	260–455
8	172	44–430	265	180–465
9	96	27–422	387	167–1,000
10	199	150–245	820	520–1,150
11	161	21–400	849	240–1,950
12	115	58–285	355	240–625
13	265	105–340	1,041	315–1,420
14	97	47–132	529	90–1,237
15	98	27–202	616	290–1,140
16	296	190–400	1,615	1,090–2,300
Mean	160		676	

* "Free" and "Total" equal folate assay before and after incubation with chick pancreatic extract.
† 6 days' collection; all others 7 days' collection.

Protein Intake.—The mean protein intake by a Kjeldahl method on 97 24-hour food collections (Table II) was 69 g., with a range from 55 to 91 g. Daily variations were wider, from 36 to 111 g.

Folate Intake.—The mean folate content of 111 24-hour diets in the absence of pancreatic enzyme was 160 μg. when *L. casei* was used as the assay organism (Table III) and 676 μg. after the use of enzyme. The mean "free" folate intake in individual patients ranged from 53 to 296 μg. daily and day-to-day variations ranged from 21 to 430 μg. as free folate and from 90 to 2,300 μg. as total folate. Much lower values were obtained when *Str. faecalis* was used as the test organism (Table IV), with a mean of 61 μg. as free folate and a mean of 245 μg. as total folate.

Hospital Diet.—The mean folate, iron, and protein content of 10 separate 24-hour hospital diets (Table V) was 117 and 487 μg. for free and total folate as assayed with *L. casei*, 11.3 mg. for iron content, and 70.4 g. for protein content.

Correlation of Dietary Folate Intake and Red Cell Folate Levels.—There was a good correlaton between the red cell folate level on the one hand and both the free and total folate content

TABLE IV.—*Dietary Folate as Assayed with Str. faecalis* (μg./24 Hours)*

Subject	"Free"		"Total"	
	Mean	Range	Mean	Range
1	57	22–91	104	52–178
2	52	19–80	492	130–800
4	70	40–92	216	154–316
9	34	11–77	184	122–246
12	61	31–96	106	72–132
13	75	25–123	322	84–446
Mean	61		245	

* 35 food collections were assayed.

TABLE V.—*Folate, Iron, and Protein Content of a 24-hour Hospital Diet*

Day	Folate (μg.)		Iron (mg.)	Protein (g.)
	Free	Total		
1	120	427	20·2	78·2
2	33	280	9·7	78·7
3	165	294	9·6	62·3
4	102	174	13·2	49·4
5	112	298	6·5	52·7
6	68	465	12·7	83·2
7	157	690	6·8	72·7
8	152	465	5·6	59·8
9	100	1,000	11·4	89·9
10	156	780	16·9	77·4
Mean	117	487	11·3	70·4

FIG. 2.—Comparison of folate intake and red cell folate level at 37th to 38th week in 16 healthy pregnant women.

of the diet (Fig. 2). For free folate $r=0.82$ and $P=0.001$ and for total folate $r=0.86$ and $P=0.001$.

Discussion

The mean iron intake in our group of women (14.2 mg.) is the same as that found in previous studies (Davis *et al.*, 1967). The protein intake (69 g.) is less than that recommended in pregnancy and is far less than the recommendation of 110 g.

in the latter half of pregnancy (B.M.A. 1950). However, the women in this study underwent a normal pregnancy and gave birth to average-sized infants.

The folate content found is in agreement with that determined in American diets by Butterworth *et al.* (1963) using *L. casei* (Table VI). Further, there is close agreement with the American workers when *Str. faecalis* is used as the test organism. In the present study mean values of 61 and 245 μg. for free and total folate were found in 111 diets, and Butterworth *et al.* (1963) recorded values of 52 and 184 μg. for 17 American diets. Butterworth *et al.* (1963), like ourselves, assayed aliquots of complete 24-hour food collections. Hurdle (1967) assayed the free folate content of a large variety of cooked foods with *L. casei* and therefrom calculated the free folate content of diets. The free folate intake of eight healthy young controls was 223 μg. (range 161 to 297) and of 16 elderly people at home 145 μg. (range 95 to 251). These values too are essentially in agreement with the values found by ourselves and by Butterworth's group.

Higher values are obtained with *L. casei* than with *Str. faecalis* because the former is able to utilize both methylfolates and triglutamates and there is a general consensus that values obtained with *L. casei* represent a more realistic estimate of folate content. Though substances such as thymidine may replace the folate requirement for *L. casei* this is not an important factor in the assay of biological material and foodstuffs. Thus a folate-free diet results in almost complete disappearance of growth-promoting material from rat liver for *L. casei*, indicating that other substances are not present in such material in sufficient amount to overcome the need for folate (Bennett *et al.*, 1964).

The availability to man of the two forms of folate termed "free" and "polyglutamate" is not known. Free folate as assayed with *L. casei* comprises monoglutamate and triglutamate forms as the formyl and methyl derivatives as well as some free pteroylglutamic acid. The other forms have seven (heptaglutamate) or more glutamic acid residues. These aspects are reviewed by Butterworth (1968). On the average some 85% of a small dose of tritium-labelled pteroylglutamic acid is absorbed (Anderson *et al.*, 1960). There is no similar information available about other forms of folate. Evidence from haematological responses suggest a less adequate effect with

TABLE VI.—*Folate Content of 24-hour Diet Assayed with L. casei*

	Free Folate	Total Folate
Present study	160 μg.	676 μg.
Butterworth *et al.* (1963)	157 μg.	689 μg.

a heptaglutamate as in yeast given orally when compared with an equimolar amount of pteroylglutamic acid (unpublished observations).

The red cell folate level is a valuable estimate of the folate status of an individual. Fig. 2 shows that there is a significant correlation between this level and the folate intake.

Hospital diets supplied substantially less folate than home diets, and, surprisingly, less iron but similar amounts of protein.

Summary

Assay of 111 24-hour food collections prepared for home consumption showed a mean folate content of 160 μg. as free folate and 676 μg. as total folate (*Lactobacillus casei* assay), a mean iron content of 14.2 mg. a day, and a mean protein content of 69 g. There was a highly significant correlation between the folate intake and the red cell folate level.

Corresponding values for 10 daily collections of a hospital diet were 117 μg. of free and 487 μg. of total folate, 11.3 mg. of iron, and 70.4 g. of protein intake a day.

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Absorption and Utilization of Polyglutamyl Forms of Folate in Man

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Summary: Some three-quarters of folate compounds in a normal mixed diet have a chain of seven glutamic acid residues (polyglutamates). The extent to which these forms of folate are absorbed and utilized by man is of considerable nutritional importance. These studies indicate that the polyglutamate forms were absorbed and utilized to about one-third of the extent of simpler (monoglutamate) forms, as judged by the serum folate levels after oral equimolar doses, by the amount incorporated into red cells in long-term studies, by the amount excreted into the urine, and by their capacity to initiate haematological responses in patients with folate-deficient megaloblastic anaemias.

Introduction

Folate compounds may be divided into two groups, the one having a single glutamic acid residue such as pteroylglutamic acid (PteGlu) and the other a chain of seven glutamic acid residues—that is, pteroylheptaglutamate ($PteGlu_7$) (Binkley *et al.*, 1944 ; Pfiffner, Calkins, Bloom, and O'Dell, 1946). The simpler forms, with the addition of a form having three glutamic acid residues (pteroyltriglutamate), are able to support the growth of microbiological assay organisms such as *Lactobacillus casei*, and these forms have been collectively termed "free" folates. These forms are readily absorbed from the gut (Anderson, Belcher, Chanarin, and Mollin, 1960), and they constitute some 25% of the folates in a normal cooked diet (Butterworth, Santini, and Frommeyer, 1963 ; Chanarin, Rothman, Perry, and Stratfull, 1968). The remaining 75% of dietary folate is not detected by microbiological assay until the glutamic acid chain has been removed by the action of a

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"conjugase" enzyme most conveniently obtained from chick pancreas. The extent to which this form of folate ($PteGlu_7$) is absorbed and the manner in which it is absorbed and utilized by man are not known at the present time. The purpose of this study was to obtain information on these aspects of folate metabolism.

Materials and Methods

Preparation of Heptaglutamate.—Yeast is a rich source of folate containing almost all the folate in the heptaglutamate forms. Brewer's yeast was kindly donated by the Guinness brewery, and 500 g. was made up to 2 litres in 1% aqueous ascorbate. This was heated for five minutes at 15 lb. pressure and the *p*H adjusted to 4 with glacial acetic acid. The folate was absorbed on to 250 g. of charcoal partially deactivated by the addition of stearic acid (Asatoor and Dalgliesh, 1956) and allowed to stand, with occasional stirring, for one hour. The charcoal was filtered through a Buchner funnel, washed with a litre of distilled water, and folate was eluted by washing with 2 litres of ammoniacal ethanol. The eluate was evaporated to dryness in a rotary evaporator and the dried residue stored in the dark. This procedure resulted in recovery of 50 to 60% and fifteenfold concentration of folate. An average of 97% was in the form of $PteGlu_7$ and some 3% as free folate.

Preparation of Chick Pancreatic Extract.—Commercially available dried chick pancreas proved unsatisfactory. Fresh chick pancreas from 10 animals was obtained and transported to the laboratory in the frozen state. It was cut into small pieces and ground in a mortar in the presence of 5 volumes of acetone. This was repeated with a further aliquot of acetone, the pancreatic extract being dried at room temperature and stored at 4° C. Further removal of folates and other interfering substances was carried out as described by Iwai, Luttner, and Toennies (1964) by absorption with Amberlite IR 400 for one hour.

Preparation of Samples for Assay.—Samples of heptaglutamate in aqueous suspension (0.1 ml.) were incubated with 0.1 ml. of conjugase suspension in 10 ml. of 0.1 M phosphate buffer *p*H 7.2 containing 600 mg. of ascorbate/100 ml. for five hours at 37° C. Controls of the specimen without conjugase and of conjugase alone were treated in the same way. After incubation the samples were brought to *p*H 5.7 with HCl, heated for 10 minutes at 10 lb. pressure, filtered, and the clear filtrate retained for assay. The folate value of the specimen containing chick pancreatic extract alone was subtracted from the value of specimen of heptaglutamate plus enzyme. The mean recovery of yeast heptaglutamate added to serum and

assayed in this manner was 99% of the expected value, with a range from 78 to 121% in 18 assays.

Preparations of Folate.—Solutions of folate for absorption tests were prepared from dried synthetic pteroylglutamic acid dissolved in 0.2 N NaOH at a concentration of 2 mg./ml. Folate tablets containing 100 μg. of pteroylglutamic acid were prepared by the hospital pharmacy. Yeast tablets containing 300 mg. of dried yeast, so that 17 tablets supplied the equivalent of 100 μg. of PteGlu as heptaglutamate and 3.2 μg. of PteGlu, were used as a source of heptaglutamate in long-term studies and in haematological response studies.

Microbiological Assay Methods.—Assays of serum, red cell folate, and the various folate preparations were carried out by the methods described by the U.S. Association of Official Agricultural Chemists, dehydrated media being used. One gramme of ascorbate was added to every 500 ml. of medium used in the *L. casei* assay. Red cells were prepared for assay by the method of Hoffbrand, Newcombe, and Mollin (1966). Sera for assay were deproteinized in 0.1 M phosphate ascorbate buffer at *p*H 5.7 with a 1 in 50 dilution of serum. Organisms used were *L. casei* and *Streptococcus faecalis*.

Plan of Study

The purpose of the study was to compare in man the manner in which equimolar amounts of PteGlu and PteGlu$_7$ (1) were absorbed, measuring serum folate levels after 0.045 μM per kg. oral doses of each equivalent to 20 μg. of PteGlu per kg.; (2) were excreted into the urine after these same doses; (3) were utilized, by noting the effects on red cell folate levels in long-term studies; and (4) induced haematological responses when given sequentially to folate-deficient patients with megaloblastic anaemia.

Intestinal Absorption of PteGlu and PteGlu$_7$

Absorption studies with both compounds were carried out on five healthy members of the laboratory staff. The subjects were not saturated with folate before the study and a few days elapsed between each test. Following collection of a fasting blood sample an oral dose of 0.045 μM of folate per kg. was given and blood samples were collected at hourly intervals. The sera were assayed with *Str. faecalis* and *L. casei* both before and after enzyme treatment. The results (see Tables I and II and Fig. 1) show that much higher serum folate values were obtained with PteGlu than with PteGlu$_7$. The peak level in all subjects with PteGlu was evident in the first hourly specimen, whereas it was reached only in the third hour with PteGlu$_7$.

TABLE I.—*Serum Folate Levels After 0.045 μM Folate per kg. Orally Either as Pteroylglutamic Acid or Pteroylheptaglutamic Acid*

Subject	Oral Dose (μg.) PteGlu	Oral Dose (μg.) $PteGlu_7$	Assay	'Conjugase' Enzyme	Serum Folate (mμg./ml.) 0	1 hr	2 hr	3 hr	Urine (μg.)
1	1,160	0	*Str. faec.*	No	0	20	30	16	40
				Yes	0	30	30	18	65
			L. casei	No	4	38	36	29	157
				Yes	6	36	33	35	—
	32	1,140	*Str. faec.*	No	0	4	8	16	0·49
				Yes	0	7	14	14	0·77
			L. casei	No	3	6	17	21	2·6
				Yes	5	10	16	25	2·9
2	1,450	0	*Str. faec.*	No	0	32	18	13	46
				Yes	0	32	18	4	46
			L. casei	No	3	41	23	20	75
				Yes	9	33	24	15	75
	59	1,400	*Str. faec.*	No	0	13	8	8	0·4
				Yes	0	12	8	3	0·45
			L. casei	No	5	10	18	14	5·5
				Yes	8	13	16	12	6·5
3	1,260	0	*Str. faec.*	No	0	30	26	18	127
				Yes	0	48	26	30	150
			L. casei	No	3	50	27	22	180
				Yes	4	48	38	16	240
	38	1,220	*Str. faec.*	No	0	0	0	0	1·3
				Yes	0	2	0	0	1·3
			L. casei	No	3	4	4	6	3·9
				Yes	4	7	7	7	3·9
4	1,550	0	*Str. faec.*	No	0	47	23	5	350
				Yes	0	43	32	10	350
			L. casei	No	8	53	46	16	420
				Yes	16	61	41	19	448
	31	1,520	*Str. faec.*	No	0	10	6	10	1·5
				Yes	0	12	7	10	1·5
			L. casei	No	11	11	10	11	6·8
				Yes	11	9	16	9	9·5
5	1,480	0	*Str. faec.*	No	0	26	20	20	230
				Yes	0	36	26	30	230
			L. casei	No	3	38	29	27	232
				Yes	17	33	24	22	300
	44	1,440	*Str. faec.*	No	0	0	0	0	1·1
				Yes	0	3	10	8	2·4
			L. casei	No	5	5	5	7	2·2
				Yes	2		6	9	3·7

A substantial amount of folate in plasma following oral heptaglutamate appeared directly available to the assay organisms, and hence was probably absorbed after deconjugation. Some was probably absorbed as PteGlu, since there were slightly higher levels throughout after enzyme treatment of the serum samples, though these differences were not statistically significant.

At one hour after PteGlu, higher values were obtained with *Str. faecalis* than with *L. casei*, suggesting the possibility that some pteroic acid (Pte) was present in this sample, though again the differences did not reach statistical significance.

With PteGlu, higher values were obtained with *L. casei* than with *Str. faecalis* before enzyme treatment, suggesting that *L. casei* was also responding to displaced tissue folates not

available to *Str. faecalis* (Chanarin and McLean, 1967). These differences disappeared after enzyme treatment.

TABLE II.—*Mean Folate Absorption After Equimolar Oral Doses of Monoglutamate and Heptaglutamate Forms of Folate* (0.045 μM/kg.)

	Organism	Conjugase	0	1 hr	2 hr	3 hr	Urine (μg.)
			(mμg./ml.)				
PteGlu	Str. faec.	No	0	31	23	14	159
		Yes	0	38	26	19	168
	*L. casei	No	0	40	28	19	213
		Yes	6	38	28	14	266
$PteGlu_7$	*Str. faec.*	No	0	5·4	4·4	6·8	0·97
		Yes	0	7·4	7·8	7·0	1·28
	*L. casei	No	0	1·8	5·6	6·4	4·2
		Yes	1·2	3·8	6·8	8·0	5·3

* The *L. casei* serum folate value in the fasting specimen has been subtracted from all the values.

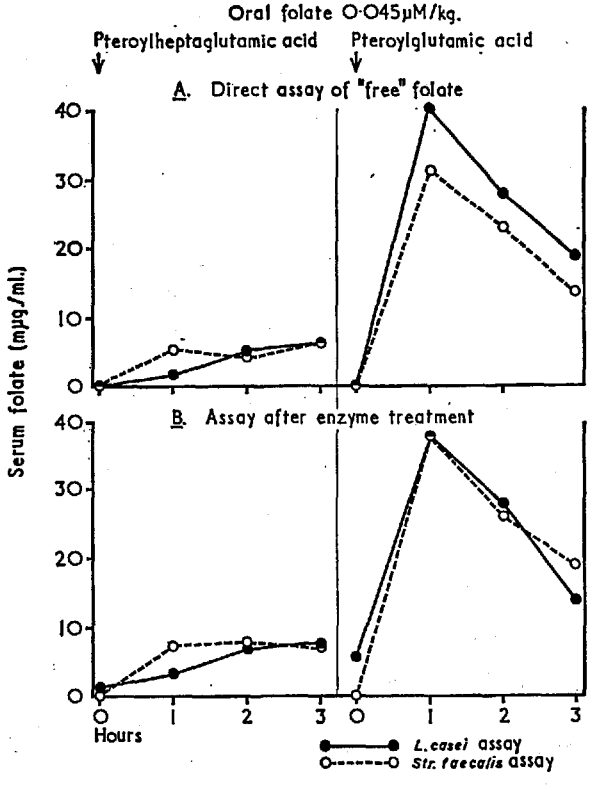

FIG. 1.—Serum folate levels after 0.045 μM folate per kg. as monoglutamate and heptaglutamate.

The conclusion was that $PteGlu_7$ is poorly absorbed by man as compared with PteGlu, that a large proportion is absorbed after removal of the peptide chain, but that some may be absorbed unchanged, particularly in certain subjects (Subject 5). Absorption studies in two patients with idiopathic steatorrhoea appeared to show a more severe impairment of absorption with PteGlu than with $PteGlu_7$.

The results obtained are not likely to have been influenced by the small amount of free folate in the $PteGlu_7$ preparations, since doses of this magnitude (20 to 60 μg.) do not produce measurable changes in serum folate levels given orally or even intravenously.

Urinary Excretion of Folate after Oral Doses

Urine was collected for six hours after oral folate into a bottle containing about 0.5 g. of dry ascorbate. Following oral doses of 0.045 μM/kg. body weight a mean of 159 to 266 μg. appeared in the urine after PteGlu (Tables I and II), and with $PteGlu_7$ this reached only 5 μg. after enzyme treatment. This confirms the poor intestinal absorption of heptaglutamate and good absorption of monoglutamate, of which some 20% of the oral dose was excreted in the urine.

Effect of Daily Supplement on Red Cell Folate Levels

The folate content of red cells is a good indication of the folate status of the individual and is closely correlated with the dietary intake of folate (Chanarin *et al.*, 1968). Eighteen healthy members of the hospital and laboratory staff were asked to take a daily folate supplement of 0.227 μM. Twelve had one tablet containing 100 μg. (0.227 μM) PteGlu daily for five months and six had 17 tablets of yeast (5.1 g.) supplying the equivalent amount of $PteGlu_7$ daily for three months. Blood for red cell folate estimation was taken before the start of the study and at monthly intervals for eight months in those receiving PteGlu and more frequently in those receiving $PteGlu_7$. All the specimens were stored at $-20°$ C. and assayed simultaneously. The results (Fig. 2) show that with PteGlu there was a slow rise in red cell folate over two months to reach a plateau some 45% above baseline levels, but with $PteGlu_7$ the increase was only 16%. On this basis oral heptaglutamate was utilized to one-third of the extent of monoglutamate. In retrospect we would have liked to have more than one initial baseline red cell folate level in the heptaglutamate study, since there were unexplained transient rises in red cell folate in the two weeks after the start of the study.

Following withdrawal of the 100-μg. folate supplement red cell folate showed a slow decline starting after three months, suggesting reutilization of the augmented folate stores.

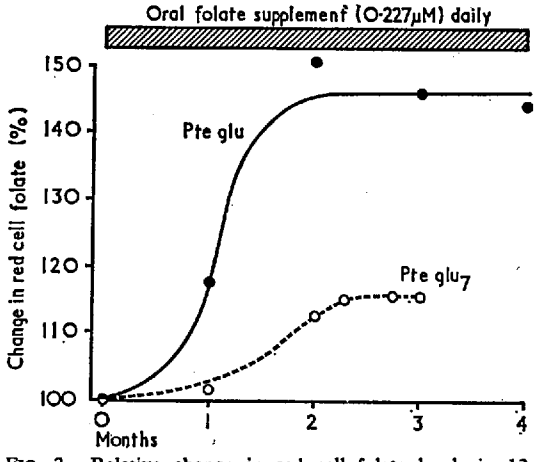

FIG. 2.—Relative change in red cell folate levels in 12 healthy subjects receiving 0.227 μM (100 μg.) pteroylglutamic acid daily and in six healthy subjects receiving 0.227 μM pteroylheptaglutamic acid daily.

Comparison of Haematological Response

Five patients with megaloblastic anaemia due to folate deficiency were treated with yeast tablets supplying the "free" folate equivalent of 50 to 200 μg. of folate daily. Two of these patients had idiopathic steatorrhoea, and neither showed any response to 36 yeast tablets (supplying the equivalent of 200 μg. as heptaglutamate daily). Two other patients were old women with nutritional folate deficiency, and the third a post-gastrectomy patient whose megaloblastic anaemia was due to folate deficiency (? nutritional). One failed to respond to 12 yeast tablets daily, but two others had reticulocyte responses to $PteGlu_7$. In these two the marrow remained megaloblastic and was followed by a second reticulocyte response when an equimolar (or smaller) amount of PteGlu was substituted. The second response was associated with a fall in the serum iron and return of a normoblastic marrow. The response in the 76-year-old postgastrectomy patient is shown in Fig. 3.

These responses confirm relatively poor utilization by mar of $PteGlu_7$ and better utilization of simpler forms of folate.

Discussion

The observations in this study indicate poor absorption and utilization of dietary heptaglutamate as compared with mono-

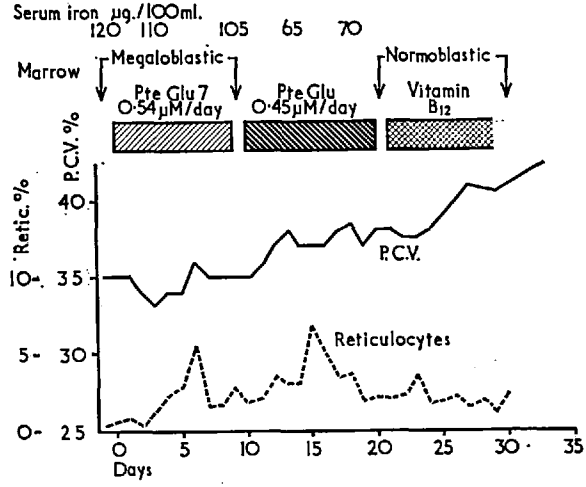

FIG. 3.—Haematological response in patient with megaloblastic anaemia due to folate deficiency first to heptaglutamate then to a slightly smaller dose of monoglutamate, and finally to vitamin B_{12}.

glutamate. The free folate content of a large number of daily diets was 160 µg., and on the basis of 80% absorption shown with tritium-labelled folate (though in fasting subjects) a maximum absorption of some 128 µg. can be anticipated. The data on incorporation of folate into red cells suggest that $PteGlu_7$ is used to about one-third of the extent of PteGlu. On this basis, if absorption is one-third of the 80% absorbed with PteGlu, some 27% of $PteGlu_7$ is taken up. This would suggest a folate utilization of 128 µg. as free folate and a further 140 µg. (27% of 516 µg. of $PteGlu_7$ in an average diet) as $PteGlu_7$, giving a total average folate intake in man of 270 µg. daily expressed in terms of PteGlu.

Man differs from some other species in his relatively poor utilization of heptaglutamate (M.W. 1216) as compared with monoglutamate (M.W. 441). In the chick heptaglutamate appears to be fully utilized (Jukes, 1955).

Jandl and Lear (1956), studying the uptake of yeast folate, found, as we did, that 25% of folate in unaltered yeast was absorbed, and this was increased to 60% when the yeast was pretreated with conjugase enzyme, but that even this was not as well absorbed as an equivalent of pteroylglutamic acid.

Spray (1952) compared the absorption of pteroylglutamic acid (1 mg.) and yeast folate in equivalent amount and noted poor absorption of the yeast folate. Though he did not explore the effect of enzyme on the serum folate level our observations show that this would not have changed the overall pattern

significantly. Spray (1952) and Spray and Witts (1952) also showed that with oral yeast only some 2 μg. of folate appeared in the urine as compared with some 69 μg. after pteroylglutamic acid. More recent studies have been those of Cooperman and Luhby (1965), who in a single case claimed high plasma levels of unaltered heptaglutamate after oral dosage, and Streiff and Rosenberg (1967), who gave only 10 μg. of Pte Glu_7 orally—too small a dose to have an effect on plasma levels.

Haematological responses to oral yeast therapy have been well known since the observation of Wills (1931) on the treatment of megaloblastic anaemia in pregnancy in Bombay. She, and most workers, used Marmite, an autolysed yeast which has a relatively higher proportion of free folate. Wintrobe (1939), in a review, concluded that one-third of patients showed some response to oral yeast, given in doses of often up to 45 g. of Marmite daily, supplying about 12 μg. of folate per g., of which 2 μg. might be free folate. Many of the responses could be largely attributed to the free folate content of the autolysed yeast preparation.

Both Welch, Heinle, Nelson, and Nelson (1946) and Bethell, Meyers, Andrews, Swendseid, Bird, and Brown (1947) reported examples of pernicious anaemia patients who failed to respond to polyglutamyl forms of folate and then responded to the equivalent amount of pteroylglutamic acid.

The absorption of some 270 μg. daily from a normal diet does not represent a vast excess of intake over requirement. This quantity is inadequate to meet the requirements of normal pregnancy—for example, where some additional 100 μg. of folate daily has to be supplied in order to prevent a progressive decline in red cell folate levels (Hansen and Rybo, 1967; Chanarin *et al.*, 1968).

The suggestion that 50 μg. of folate represents the normal requirement was put forward by Herbert (1962). We feel that the data in that paper have been misinterpreted. All three subjects in the study who received from 25 to 100 μg. of folate daily showed a declining serum folate value—that is, all had a lower level at the end of the study than at the beginning—indicating that up to 100 μg. of folate daily was not enough to maintain serum folate levels. That the serum folate level in the only subject on 25 μg. of folate daily fell to below 5 mμg./ml. is hardly relevant, as the levels were falling in all the three subjects, and had the study been continued no doubt the levels would have declined to 5 mμg. or any other level chosen as meaningful. It seems more important that in that widely misquoted study insufficient folate was taken by all three volunteers to sustain their own normal folate values, and the only conclusion that can be drawn is that the requirement was greater than 100 μg. daily.

Other studies have been concerned wth the absorption of large doses of synthetic diglutamate and triglutamate forms of folate, but these do not throw light on utilization of food folate (Baker *et al.*, 1965).

Attention has also been focused on the amount of folate required to produce an adequate haematological response in folate-deficient megaloblastic anaemia. This amount has varied from 25 to 800 μg. daily, depending on whether there is any increased folate requirement or not, and most patients respond to 200 μg. There is, however, no evidence which indicates that the dose producing a haematological response is necessarily the same as the amount of folate required each day to maintain the normal physiological status.

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EVIDENCE FOR REDUCTION AND METHYLATION OF FOLATE IN THE INTESTINE DURING NORMAL ABSORPTION

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EVIDENCE FOR REDUCTION AND METHYLATION OF FOLATE IN THE INTESTINE DURING NORMAL ABSORPTION

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Summary Dihydrofolate (H_2PteGlu) or tetrahydrofolate (H_4PteGlu), given orally, appears in the blood as a *Lactobacillus casei*-active form of folate—namely, methyltetrahydrofolate (CH_3H_4PteGlu). The same dose of folate given intravenously rapidly exchanges with tissue methylfolate. The results indicate that folate is reduced and methylated in the gut wall during its physiological absorption.

DURING studies on the absorption of folate analogues in man we have found that the form of folate appearing in the blood is often different from that given by mouth. Thus when dihydrofolate or tetrahydrofolate, both of which fully support the growth of the assay organism *Streptococcus fæcalis*, is given orally, the form of folate appearing in the blood is active in supporting the growth of *Lactobacillus casei* only, and not *Str. fæcalis*. Eleven medical students were given 10 μg. per kg. of dihydropteroylglutamic acid orally. The serum-folate level, assayed with *L. casei*, rose; but the capacity of the serum to support the growth of *Str. fæcalis* was unchanged (fig. 1). This suggests that the form of folate in the blood is 5-methyltetrahydrofolate, which is the monoglutamate form active only with *L. casei*.

The increase in the methylfolate levels in plasma could arise by an exchange between absorbed folate

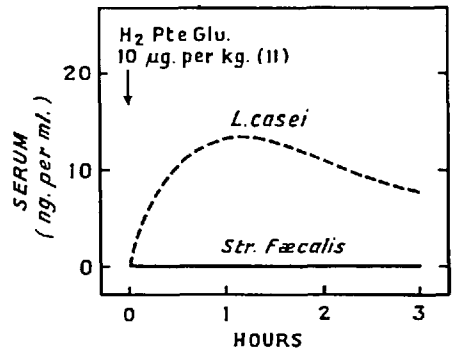

Fig. 1—Change in serum-folate level following an oral dose of 10 µg. per kg. of dihydropteroylglutamic acid in eleven medical students.

Each subject had received 20 mg. pteroylglutamic by mouth 64 hours before the absorption test to " saturate " the tissues and standardise the rate of plasma clearance of absorbed folate. An aseptic addition method was used for *Str. fæcalis* assay since reduced folate derivatives are labile to heat.

and tissue-folate which is largely in the methylform, or by alteration of the oral folate in the gut wall or tissues. An exchange between tissue methyltetrahydrofolate and injected pteroylglutamic acid was demonstrated by McLean and Chanarin.[1] To explore this further we have compared the fate of reduced folate analogues—dihydropteroylglutamic acid (H_2PteGlu) and tetrahydropteroylglutamic acid (H_4PteGlu)—labelled with tritium in the benzene-ring portion of the *p*-aminobenzoic-acid moiety, given either intravenously or orally.

2–3 µg. of tritium-labelled pteroylglutamic acid (60–80 µCi) was added to each individual dose of pteroylglutamic acid in solution (10 µg. per kg.) and reduced to either dihydropteroylglutamic acid by the method of Blakley [2] or to tetrahydropteroylglutamic acid by the method of Silverman and Noronha [3] immediately before use. Tritium in plasma was counted by adding 1·0 ml. of the plasma sample to 1·0 ml. ' Hyamine '; the mixture was incubated at 37°C overnight and added to 15 ml. of scintillator ('PPO' and 'POPOP' in ethanol and toluene). This was counted in a Packard tricarb automatic scintillation spectrometer. Microbiological assay was performed using dehydrated (Difco) media and *L. casei* and *Str. fæcalis* as test organisms.

When tritium-labelled tetrahydrofolic acid was given intravenously to a healthy subject it rapidly disappeared

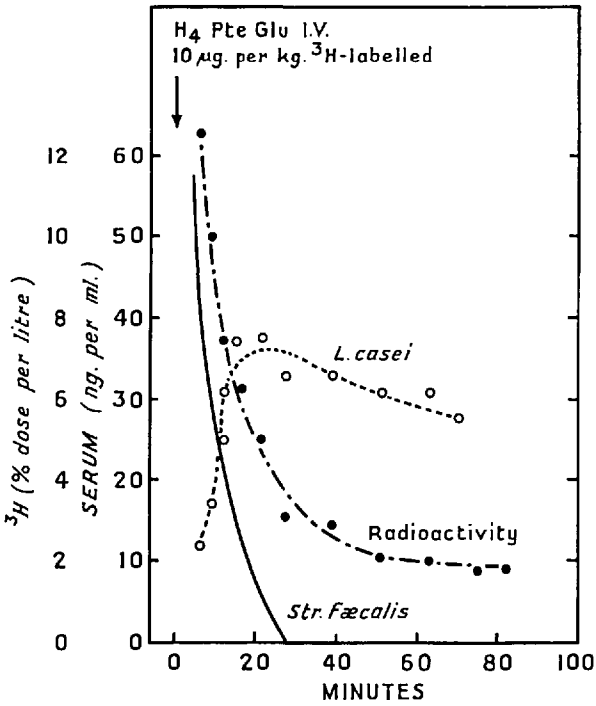

Fig. 2—Changes in serum-folate level and in plasma radioactivity following an intravenous dose of tetrahydropteroylglutamic acid (10 µg. per kg.) labelled with tritium in a healthy subject.

Rapid disappearance of activity for *Str. fæcalis* (assayed by an aseptic addition technique) and of radioactivity is accompanied by a rise in plasma activity for *L. casei*. This indicates that there is an exchange between the labelled injected folate and unlabelled tissue-methyltetrahydrofolate.

from the plasma (fig. 2) and was replaced by an unlabelled *L. casei*-active form of folate which was almost certainly 5-methyltetrahydropteroylglutamic acid. In fig. 2 the *L. casei* folate levels have been derived by first subtracting the preinjection fasting serum-folate level from all samples and thereafter subtracting the results obtained with the *Str. fæcalis* assay from the higher assay result obtained with *L. casei*. This was necessary only in earlier samples since there was no *Str. fæcalis* activity with the later specimens.

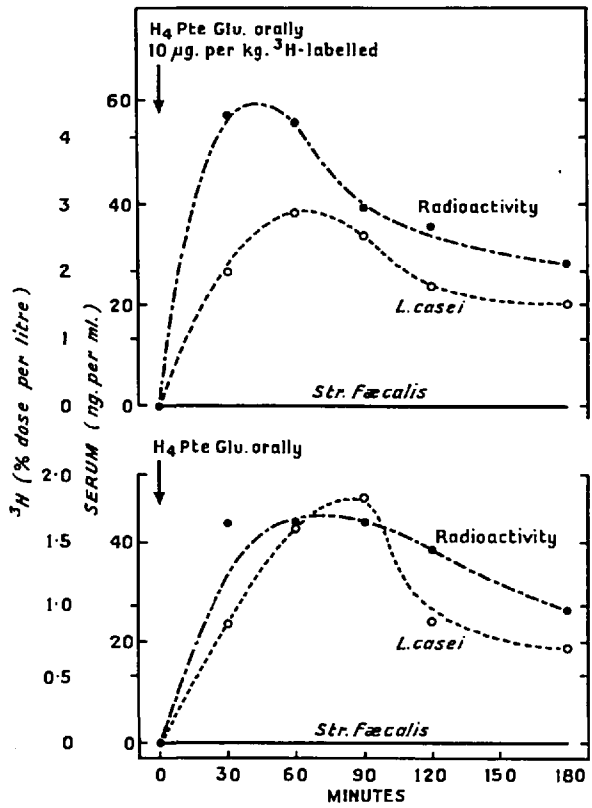

Fig. 3—Changes in serum-folate level and in plasma radioactivity following an oral dose of 10 µg. per kg. of tetrahydropteroylglutamic acid labelled with tritium in 2 healthy subjects.

The rise in plasma radioactivity corresponds to the rise in *L. casei* activity. Although the oral dose is fully active for *Str. fæcalis* there is no *Str. fæcalis* activity in plasma, suggesting that the H_4PteGlu has been methylated during absorption.

By contrast when reduced folate was given by mouth, radioactivity in plasma corresponded to the *L. casei* activity—that is, the orally administered dose had been altered to an *L. casei* active form (fig. 3). Since this does not happen when the same dose is given intravenously, methylation of folate must have taken place in the gut wall.

The same result was obtained when dihydrofolate

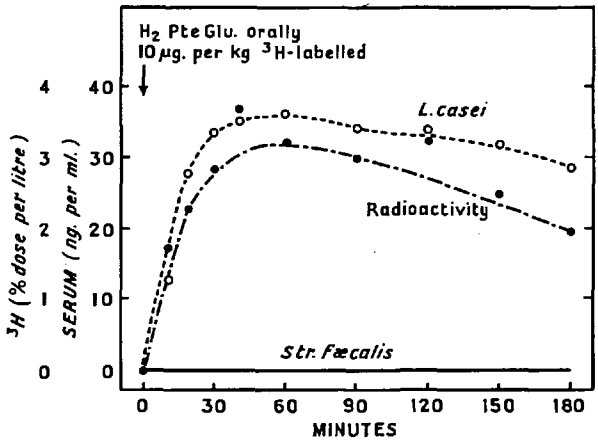

Fig. 4—Changes in the serum-folate level and plasma radioactivity following an oral dose of dihydropteroylglutamic acid labelled with tritium in a healthy subject.

The rise in plasma radioactivity corresponds to the rise in *L. casei* activity only, and suggests further reduction and methylation of the oral dose during absorption.

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was given orally, indicating that in this case folate was both further reduced and methylated before being passed to the blood (figs. 1 and 4).

Our results are supported by the observation in rats that methotrexate, which prevents reduction of folate, inhibits folate absorption.[4,5] They may also explain the observation that calf jejunal extract improved folate absorption in intestinal malabsorption syndrome.[6] We have observed that reduced forms of folate are well absorbed by patients with intestinal malabsorption syndrome. Should the calf jejunal extract contain folate reductase, it may well reduce a significant proportion of the oral dose and so facilitate its absorption.

Our data, however, are in partial disagreement with those of Whitehead and Cooper [7] who reported that in man pteroylglutamic acid was transported unchanged from the small gut to the liver.

Pteroylglutamic acid itself is only partially reduced and methylated during its absorption, because the enzyme dihydrofolate reductase, which efficiently converts dihydrofolate to tetrahydrofolate, reduces folate itself to dihydrofolate at a much slower rate.

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Intestinal Absorption of Reduced Folate Compounds in Man

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SUMMARY. Oral administration of folate compounds was followed by a rise in *L. casei* active factors in blood. This was due to reduction and methylation of the folate compounds in the small gut and this was demonstrated by the use of dihydropteroylglutamic acid labelled with tritium.

Parenteral administration of tritium-labelled folate analogues was followed by a rapid exchange with unlabelled *L. casei* active folate from tissues.

Almost all studies on the intestinal absorption of folate have been concerned with absorption of pteroylglutamic acid (Girdwood, 1953; Chanarin *et al*, 1958; Anderson *et al*, 1960; Johns *et al*, 1961; Chanarin & Bennett, 1962; Klipstein, 1963; Hepner *et al*, 1968). These studies have provided satisfactory clinical tests for folate absorption in patients with intestinal malabsorption syndrome; they have possibly thrown some light on the site of folate absorption but have contributed little to our understanding of folate absorption under more physiological circumstances. The reason for this is that pteroylglutamic acid is a minor component of dietary folate. It is one of the folate analogues directly available on *Streptococcus faecalis* assay and all these analogues together constitute only 9% of dietary folate (Chanarin *et al*, 1968). Secondly, in carrying out folate absorption tests it is necessary to give quantities of the order of 2000 μg of pteroylglutamic acid or more which is about 100-fold the amount of pteroylglutamic acid that might be present in a single meal. Even this small amount of pteroylglutamic acid in the diet has probably been derived by oxidative degradation of reduced folate analogues which alone take part in coenzyme activity. Thus the observations of Whitehead & Cooper (1967) and Butterworth *et al* (1969) that a large amount of orally administered pteroylglutamic acid or its polyglutamate form was largely absorbed unchanged as pteroylglutamic acid, while correct under the conditions of these experiments, do not throw light on handling of the reduced formyl and methyl folate analogues which constitute the bulk of dietary folate.

We have studied the absorption of reduced folate analogues, that is, dihydropteroylglutamic acid and tetrahydropteroylglutamic acid as such, as well as the formyl and methyl derivatives of the latter compound. In some cases tritium-labelled folate was used. Some of our results have been published as a preliminary communication (Chanarin & Perry, 1969) and a full account is presented in this paper.

MATERIALS AND METHODS

Subjects Studied

Observations were made on 69 medical students. Each was given 20 mg pteroylglutamic acid orally on Friday afternoon to achieve some degree of 'saturation' of tissues for folate

compounds and an absorption test was carried out on Monday morning some 68 hr later. The dose of folate was 10 μg/kg body weight as the L-isomer in the case of the reduced folate derivates with the exception of dihydrofolate which was given as 20 μg/kg as we erroneously thought that it was diastereoisomer. The subjects were asked to have a light breakfast on the morning of the test. A blood sample was collected before the oral dose was given and at hourly intervals for 3 hr thereafter. The urine was collected for 6 hr after the oral dose into a container to which had been added approximately 1 g of dry ascorbic acid.

A small number of studies were carried out on subjects who had undergone minor surgery. The procedure was explained in some detail to the patients who agreed to co-operate in these studies. Absorption tests were also carried out on three patients with intestinal malabsorption syndrome of the type associated with a sensitivity to wheat gluten.

Preparation of Folate Analogues

Tritium-labelled pteroylglutamic acid (^{3}*H-PteGlu*). Folic acid labelled in the 3 and 5 positions of the phenyl group was purchased from the Radiochemical Centre, Amersham. Its specific activity was approximately 60 μCi/μg. About 80 μCi was used in each test, the dose being prepared by adding between 1 and 2 μg of labelled folate to non-radioactive pteroylglutamic acid (10 or 20 μg/kg) and then further reduction carried out as required.

Dihydropteroylglutamic acid (H_2*PteGlu*) was prepared by reduction of pteroylglutamic acid at pH 6 with sodium dithionite in the presence of 10% ascorbate and H_2PteGlu precipitated at pH 2.8 in the cold (Blakeley, 1960). The absorption spectrum of the product at pH 13.0 was that of H_2PteGlu with a maximum at 284 nm and disappearance of the peak at 365 nm found with PteGlu.

Tetrahydropteroylglutamic acid (H_4*PteGlu*) was prepared by reduction of pteroylglutamic acid with sodium dithionite in the presence of 10% ascorbate at 75°C and pH 6.0 (Silverman & Noronha, 1961). In one study involving absorption tests in 15 subjects H_4PteGlu purchased from Sigma Chemical Co. was used.

5-Formyltetrahydropteroylglutamic acid (5,*CHO-H*$_4$*PteGlu*) was purchased from Cyanamid Co.

5-Methyltetrahydropteroylglutamic acid (5,CH_3-H_4*PteGlu*) was prepared from the 5-formyl derivative by conversion to 5,10-methenyltetrahydrofolic acid and further reduction of the methenyl group to methyl was carried out with borohydride (Chanarin & Perry, 1967).

Microbiological Assay Methods

All specimens were assayed with *Lactobacillus casei* and *Streptococcus faecalis* and in some cases with *Pediococcus cerevisiae*. Dehydrated culture media (Difco) were used. An aseptic addition technique (Bakerman, 1961) was used for the assay of labile reduced folate compounds. Diluted plasma with 1.0 ml 10% ascorbate at pH 6.0 was added to sterile assay medium to make a final volume of 10 ml.

Plasma samples for *Str. faecalis* assay were also diluted in distilled water without ascorbate, double strength medium added and the samples heated at 116°C for 10 min. Allowance for the turbidity of the sample was made by including an uninoculated tube for each plasma sample in the assay. The turbidity in this tube was subtracted from the mean turbidity of three inoculated tubes for the same plasma sample. This procedure was used to assay pteroyl-

glutamic acid content of samples, labile reduced compounds being lost during autoclaving.

Samples for *L. casei* assay were heated at 116°C for 10 min after 1 in 50 dilution in 0.1 M phosphate buffer pH 5.7 with 0.15 g ascorbate per 100 ml. The clear filtrate was assayed. One gram ascorbate was added to each 500 ml of assay medium for *L. casei* assay. This procedure detected all folate derivatives including methylfolate. The results with this method were similar to those obtained by an aseptic addition technique.

Measurement of Tritium-Activity

One ml plasma was mixed with 1.0 ml Hyamine (*p*-(diisobutylcresoxyethoxyethyl) dimethyl-benzylammonium hydroxide) and left at 37°C for 18 hr. This was added to 15 ml of scintillator made up of toluene 47.9 ml, ethanol 50 ml and PPO and POPOP solution 2.1 ml (PPO: 2,5-diphenyloxazole 50 g; POPOP: 1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene 0.625 g, toluene 500 ml). Each sample was counted in a Packard Tricarb automatic scintillation spectrometer at − 5°C for 50 min.

The tritium activity of plasma samples were expressed as a percentage of the oral or parenteral dose per litre plasma. A portion of the dose in each test was retained, diluted 1 in 50 in water and 0.1 ml added to 1.0 ml of the patients plasma collected before the start of the test. This was then treated with Hyamine, counted as described, and plasma counts per litre expressed as a percentage of the counts obtained with this standard.

Experimental Studies

Absorption of folate analogues. The absorption of PteGlu, H_2PteGlu, H_4PteGlu, 5,CHO-H_4PteGlu and 5,CH_3-H_4PteGlu was studied in five groups of medical students, there being 11–16 students in each group. Plasma levels of folate and urinary excretion of folate were measured.

Absorption of tritium-labelled folate analogues. Observations were made in four patients, two being given ^{3}H-H_2PteGlu and two ^{3}H-H_4PteGlu. The oral dose was 20 μg and 10 μg/kg respectively. An indwelling heparinized needle and two-way tap was left *in situ* and following the oral dose samples were taken at 10 min intervals for 40 min, another at 60 min and thereafter at 30 min intervals up to 3 hr.

Plasma clearance of tritium-labelled folate analogues. In order to determine whether changes to the folate molecule during absorption occurred in the gut or tissues other than the gut, or was exchanged with tissue folate, the same dose of ^{3}H-H_4PteGlu (10 μg/kg) was given intravenously and heparinized blood samples were collected from an indwelling needle and two-way tap in the other arm. Samples were collected at 3 min intervals for 15 min, 6 min intervals to 27 min and 12 min intervals to 1½ hr. These tests were carried out on three patients convalescing from minor surgery.

RESULTS

Absorption of PteGlu

All 15 subjects showed a rise in serum folate levels above base line when assayed with both *L. casei* and *Str. faecalis* (Table I). These results are also shown in Figs 1 and 2 and in these the serum *L. casei* level in the zero blood sample has been subtracted from the folate level in the other samples. The levels with *L. casei* are consistently higher than those obtained

TABLE I. The absorption of folate analogues

Analogue and assay organism	*No. of subjects*	*Mean serum folate (ng/ml)*				*Mean urinary excretion (μg)*
		0 hr	*1 hr*	*2 hr*	*3 hr*	
PteGlu						
L. casei	15	13.0	21.7	20.8	20.0	79.8
Str. faecalis	15	0	4.8	1.1	0.3	18.0
H_2PteGlu						
L. casei	11	9.2	23.1	20.5	18.6	14.2
Str. faecalis	11	0	0	0	0	4.6
H_4PteGlu						
L. casei	13	10.5	20.7	20.9	16.2	20.5
Str. faecalis	13	0	0	0	0	3.4
5,CHO-H_4PteGlu						
L. casei	13	12.0	27.6	25.5	21.5	47.1
Str. faecalis	13	0	1.5	1.6	1.0	6.5
P. cerevisiae	13	0	0	0	0	10.9
5,CH_3-H_4PteGlu						
L. casei	16	11.9	30.9	24.4	21.2	62.0
Str. faecalis	16	0	0	0	0	9.7

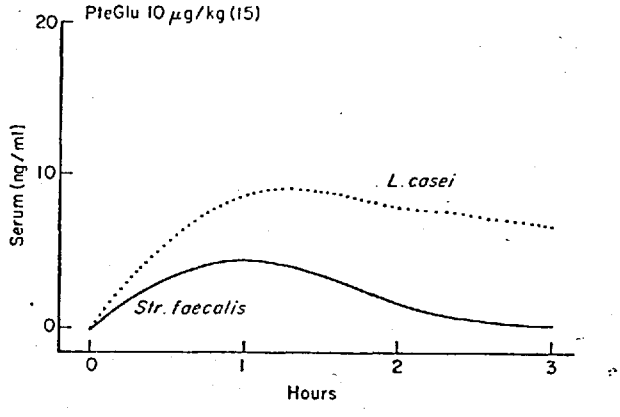

FIG 1. The mean serum folate levels in 15 medical students, following oral doses of 10 μg/kg body weight of pteroylglutamic acid.

with *Str. faecalis* suggesting that a substantial proportion of folate in blood is in the form of 5,CH_3-H_4PteGlu, but that a significant amount of PteGlu is absorbed unchanged.

Absorption of 5,CHO-H_4PteGlu

All 13 subjects showed a rise in serum folate levels when assayed with *L. casei* but only four showed a rise on *Str. faecalis* assay (Table I and Fig 2). The *Str. faecalis* activity was present after heating in the absence of ascorbate suggesting that it was PteGlu. There was no growth with *P. cerevisiae*.

Absorption of H_2PteGlu

All 11 subjects showed a rise in serum folate levels with *L. casei* assay but there was no change in serum folate levels with *Str. faecalis* using both aseptic addition and other techniques. Thus the rise in serum folate is due entirely to an increased level of 5,CH_3-H_4PteGlu

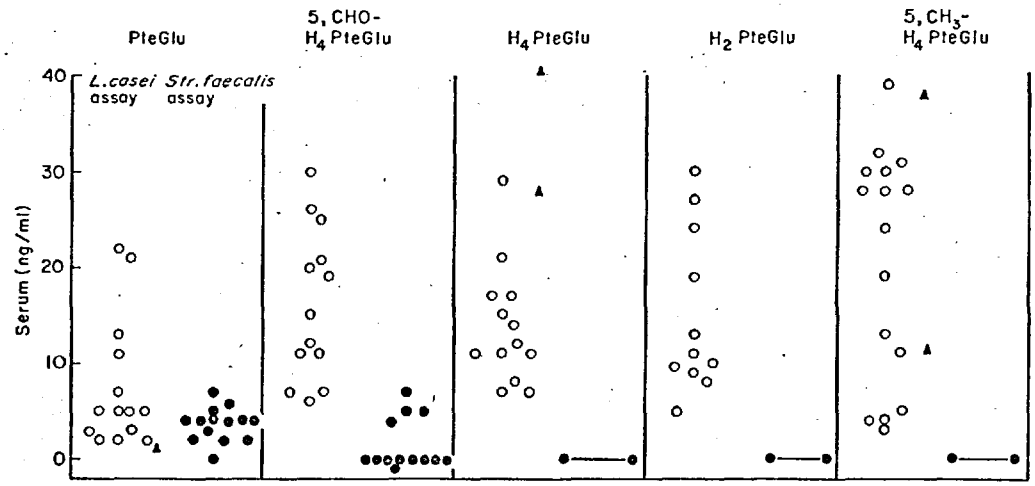

FIG 2. The peak serum folate level following an oral dose of a folate analogue in healthy medical students. The open circle (○) is the serum level assayed with *L. casei* after subtracting the serum folate level in the zero sample. The closed circle (●) is the serum folate level assayed with *Str. faecalis*. The closed triangle (▲) is the peak serum folate level in patients with untreated adult coeliac disease. The oral dose of H_2PteGlu was 20 μg/kg body weight. The other compounds were given as 10 μg/kg or as 10 μg/kg of L-isomer.

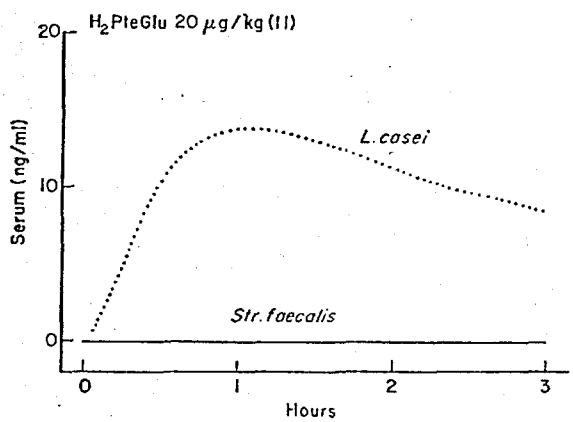

FIG 3. The mean serum folate levels in 11 medical students following oral doses of 20 μg/kg body weight of dihydropteroylglutamic acid.

although the compound given orally, H_2PteGlu, is fully active in supporting the growth of *Str. faecalis* and was shown to be so when added to plasma *in vitro*. The results are set out in Table I and Figs 2 and 3.

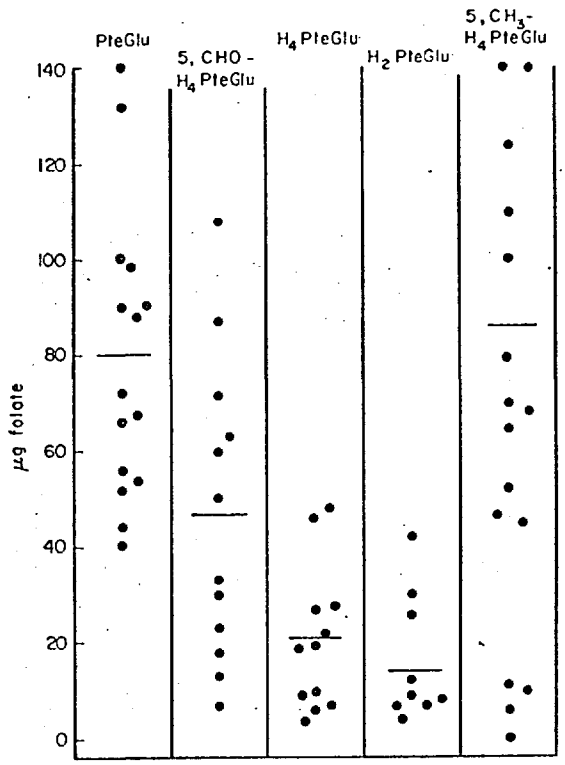

FIG 4. The total urinary excretion of folate (μg) following oral administration assayed with *L. casei*.

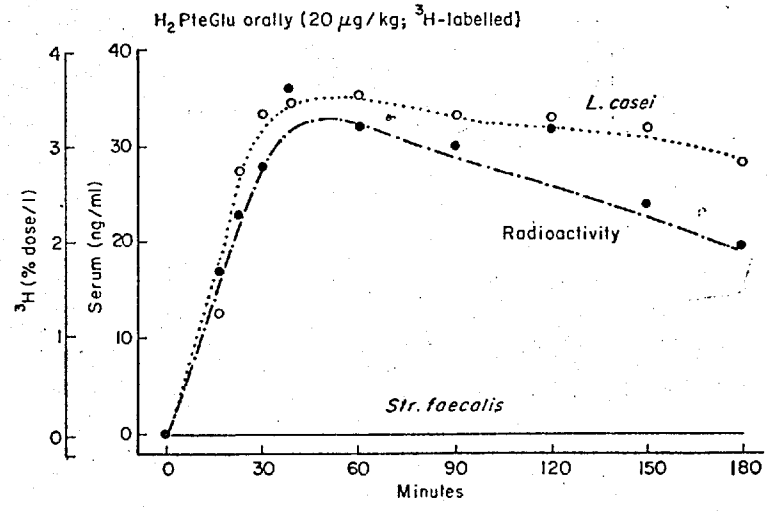

FIG 5. The serum folate level after 20 μg/kg dihydropteroylglutamic acid labelled with tritium. The *L. casei* activity of the zero sample has been subtracted. Although the oral dose is fully active with *Str. faecalis*, the folate appearing in plasma is active only with *L. casei*, indicating that it has been further reduced and methylated.

Absorption of H_4PteGlu

Thirteen subjects were tested and all showed a rise in serum folate activity only on *L. casei* assay (Table I and Fig 2).

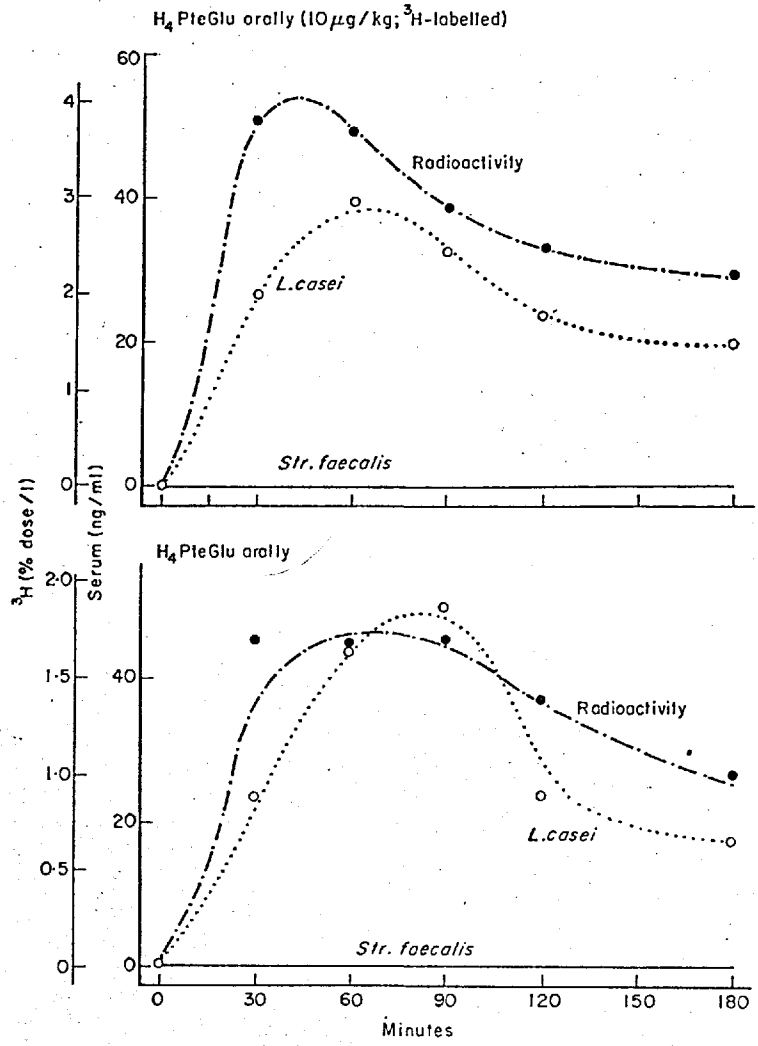

FIG 6. The serum folate levels in two subjects after oral doses of 10 µg/kg tetrahydropteroylglutamic acid labelled with tritium. The *L. casei* activity of the zero sample has been subtracted. Although the oral dose is fully active with *Str. faecalis* the folate appearing in plasma is active only with *L. casei* indicating that it has been methylated.

Urinary Excretion of Folate

The urinary excretion of folate after oral doses of the various analogues is shown in Table I and Fig 4. With all the analogues a considerably higher excretion was obtained by *L. casei* assay indicating that 5,CH_3-H_4PteGlu was the predominant component.

Studies with Tritium-Labelled Folate Compounds

When 'natural' folate analogues are given by mouth the form of folate accumulating in plasma is the reduced 5,methyl derivative active for *L. casei*. This could come about by either:

(a) Alteration of the oral dose in the cells of the small gut by further reduction and methylation.

(b) Absorption of an unaltered folate compound followed by its rapid reduction and methylation in tissues such as the liver.

(c) Absorption of an unaltered or altered folate compound which then rapidly exchanges with tissue folate, much of which is $5,CH_3\text{-}H_4PteGlu$.

To determine which of these possibilities was correct $H_2PteGlu$ and $H_4PteGlu$ labelled with tritium was prepared and its fate following oral and intravenous administration was noted.

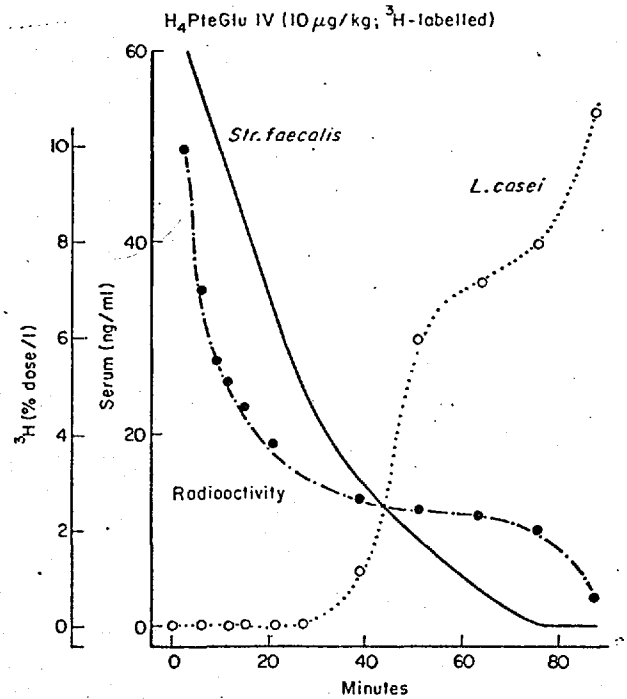

FIG 7. Tetrahydropteroylglutamic acid (10 µg/kg) labelled with tritium has been given intravenously at 0 min. Its rapid disappearance from plasma is measured by decline in radioactivity of plasma and decline of *Str. faecalis* activity. *L. casei* activity has been derived by subtracting the 0 min *L. casei* activity from all specimens and by subtracting activity due to *Str. faecalis*. The remaining folate activity is that supporting the growth of *L. casei* alone (5,methyltetrahydropteroylglutamic acid). The *L. casei* folate activity rises in plasma as the parenteral dose disappears indicating an exchange between parenteral and tissue folate.

Absorption of Tritium-Labelled $H_2PteGlu$

Two studies were carried out. The results in these were almost identical and only one has been set out in detail in Fig 5. As noted in Fig 3, the rise in serum folate activity (the activity

in the zero sample has been subtracted) was detected only with *L. casei* and there was no change in activity for *Str. faecalis*. The change in plasma radioactivity corresponded to *L. casei* activity (Fig 5) indicating that the $5,CH_3$-H_4PteGlu in the plasma was the result of reduction and methylation of the oral dose (either a or b above) and had not arisen by an exchange with tissue methylfolate.

Absorption of Tritium-Labelled H_4PteGlu

The two studies performed are shown in Fig 6 and the patterns are essentially similar in all respects to that found with dihydrofolate. They indicate that the orally administered compound appeared in the blood as the methyl form which was active only with *L. casei*.

Intravenous Clearance of Tritium-Labelled H_4PteGlu

All the studies using intravenous tritium-labelled tetrahydrofolate yielded very similar results and one example is set out in detail in Fig 7. This 80-year-old lady had a serum vitamin B_{12} level of 230 pg/ml, a serum folate of 10 ng/ml and a red cell folate level of 425 ng/ml.

The *L. casei* serum values have been derived by subtracting growth activity for *Str. faecalis* (H_4PteGlu) from total *L. casei* activity (H_4PteGlu + $5,CH_3$-H_4PteGlu) in order to arrive at a value for $5,CH_3$-H_4PteGlu alone. It is this value which is shown in Fig 7. The labelled injected dose of H_4PteGlu (active for *Str. faecalis*) disappears rapidly from plasma and is replaced by non-radioactive methylfolate (active for *L. casei*). This indicates a rapid and complete exchange between parenteral and tissue folate and indicates that reduction and methylation of the orally administered dose must take place in the gut wall.

Observations in Adult Coeliac Disease

We have only had a limited opportunity to study absorption of reduced folate analogues in this disorder. Almost all these patients show impaired absorption of PteGlu even when the dose is reduced to 10 μg/kg. Observations in two patients (Fig 2) show that there was no impairment of absorption of H_4PteGlu and $5,CH_3$-H_4PteGlu as compared to healthy subjects.

DISCUSSION

The evidence that has been presented in this paper indicates that dihydrofolate is reduced to the tetrahydro-form and then methylated in the small gut during its intestinal absorption. Tetrahydrofolate, which is already reduced, is methylated. The metabolism of reduced forms of folate varies with the route of administration. Reduction and methylation take place when these compounds are given by mouth. When given parenterally, however, these compounds exchange with folate in body tissue, that is, when tritium-labelled dihydrofolate or tetrahydrofolate is given orally the labelled folate persists in plasma; when given by injection the labelled folate disappears from plasma and is replaced by an unlabelled form. It is probable that the enzyme, dihydrofolate reductase, is concerned with reduction of folate in the small gut since it has been shown, at least in rats, that folate absorption is inhibited by methotrexate, which binds to dihydrofolate reductase irreversably (Burgen & Goldberg, 1962; Hepner, 1969).

Our limited observations in patients with intestinal malabsorption syndrome indicate

that the reduced forms of folate when given at the 10 μg/kg dose level appear to be as well absorbed as in healthy subjects whereas the absorption of pteroylglutamic acid remains impaired. If so, it would appear that loss of villous surface is accompanied by loss of the capacity to reduce folate.

The exchange between parenteral folate and tissue folate is of great interest, but since absorbed folate is normally in the reduced and methylated form, it would not appear to play a significant role other than perhaps to ensure that any non-methylated folates are rapidly abstracted from the blood.

There are significant differences in the manner in which unnatural forms of folate such as pteroylglutamic acid and natural forms such as the reduced folate analogues are absorbed. This is probably related to the substrate specificity of the enzyme, dihydrofolate reductase. This enzyme rapidly converts H_2PteGlu to H_4PteGlu but reduces folate to dihydrofolate (PteGlu→H_2PteGlu) at only about one-tenth of the rate of the former reaction (Zakrewski & Nicol, 1960). Some forms of dihydrofolate reductase may even fail to react with pteroylglutamic acid (Nath & Greenberg, 1961). Thus reduced forms of folate given orally in our studies were completely reduced to H_4PteGlu and passed to the blood as 5,CH_3-H_4PteGlu. Pteroylglutamic acid, by contrast, was only partly reduced and appeared in the blood in approximately equal amounts of unchanged PteGlu active for *Str. faecalis* and material active for *L. casei* as well, that is, both unchanged PteGlu and some that has been reduced and methylated (Fig 1). Both Whitehead & Cooper (1967) and Butterworth *et al* (1969) gave from 1 to 4.4 mg pteroylglutamic acid by mouth and both pointed out that this compound largely entered the blood unchanged, although Butterworth *et al* (1969) suggested that reduction and methylation of a portion of this oral dose did occur.

A very small proportion of dietary folate is in the form of pteroylglutamic acid (perhaps 5%) and even this is probably the result of oxidation of reduced forms. All folate is split to monoglutamate forms during absorption (Streiff & Rosenberg, 1967; Perry & Chanarin, 1968; Butterworth *et al*, 1969; Rosenberg *et al*, 1969; Hoffbrand *et al*, 1969) and at least in yeast these are largely formyl and methyl derivatives of dihydro and tetrahydrofolates (Schertel *et al*, 1965). In the study reported by Butterworth *et al* (1963) unfortunately ascorbate was omitted during manipulation of the diet and hence it was not possible to determine the proportion of reduced folate derivates. Thus the bulk of ingested folate normally passes through the biochemical pathways we have described, namely, reduction and methylation, and under physiological conditions only 5,methyltetrahydrofolate reaches the blood.

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