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BIOSYNTHESIS OF FOLATE DERIVATIVES *IN VITRO**

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Key Word Index—*Pisum sativum;* Leguminosae; biosynthesis; tetrahydropteroylglutamate; cell-free extracts; methionine.

Abstract—Cell-free extracts of 3-day-old pea cotyledons and root tips have been examined for ability to synthesize derivatives of tetrahydropteroylglutamic acid in reaction systems which included formaldehyde, serine and formate as precursors of one-carbon units. The reaction products were isolated by DEAE-cellulose chromatography and assayed microbiologically using *Lactobacillus casei* and *Pediococcus cerevisiae*. Synthesis of 5-methyltetrahydropteroylglutamic acid was stimulated by addition of NADPH and the presence of FAD in the extraction buffer. 10-Formyl and 5-formyltetrahydropteroylglutamates were formed in all reaction systems but omission of the one-carbon precursor reduced these syntheses. The presence of 5,10-methylenetetrahydropteroylglutamate reductase activity was confirmed by use of ^{14}C-labelled derivatives. The oxidative reaction had a pH optimum of 6·4 and was not inhibited by additions of L-methionine to the reaction system. Cotyledon and root tip extracts also catalyzed the transmethylation of homocysteine utilizing methyl groups from 5-methyltetrahydropteroylmonoglutamate. The reaction was strongly inhibited by L-methionine *in vitro*.

INTRODUCTION

MICROBIOLOGICAL assay of pteroylglutamate derivatives has established that these metabolically important compounds are of widespread occurrence in higher plants.[1–7] In addition, fractionation studies have revealed that mitochondria[8] and chloroplasts[9,10] contain pools of pteroylglutamates which are principally methyl and formyl derivatives of H_4PteGlu.

The central role of pteroylglutamates in the one-carbon metabolism of animal and microbial species is now firmly established.[11] This involvement also applies to plant species

* The abbreviations used are listed in *Biochem. J.* **102,** 15 (1967): e.g. H_4PteGlu = tetrahydropteroylglutamate; 10-HCO-H_4PteGlu = N^{10}-formyltetrahydropteroylmonoglutamate.

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as pteroylglutamates derivatives participate in the biosynthesis of serine[12–14] and methionine[15–17] as well as the decarboxylation of glycine.[18] Furthermore, the effects on plant growth of PteGlu antagonists[19] and inhibitors of pteroylglutamate biosynthesis[20] are consistent with this general conclusion.

Although several key enzymes of one-carbon metabolism are known to occur in plants[19] there have been no reports on the biosynthesis of methylated pteroylglutamates *in vitro* despite the fact that such derivatives are commonly the principal components of the pteroylglutamate pool.[5–7] Studies with micro-organisms and mammalian tissues suggest that 5-CH_3-H_4PteGlu in plants is derived from more oxidized derivatives such as 5,10-CH_2-H_4PteGlu. It is also likely that biogenesis and subsequent transfer of such methyl groups may be regulated by L-methionine.[17,21]

The present paper reports on the ability of pea seedling extracts to synthesize formyl and methyl derivatives of H_4PteGlu and examines the possible regulation of these biosyntheses by L-methionine.

TABLE 1. SYNTHESIS OF PTEROYLGLUTAMATE DERIVATIVES BY COTYLEDON EXTRACTS

		Quantity of pteroylglutamates (μg/reaction system)					
One-carbon source	Omission from complete reaction system	10-HCO-H_4PteGlu	5-HCO-H_4PteGlu	5-CH_3-H_4PteGlu	Unidentified derivative	H_4PteGlu	Total
Formaldehyde	None	9·5	5·9	6·3	13·5	1·4	36·6
	NADPH	1·7	4·4	Absent	2·8	13·9	22·7
	HCHO	0·9	Absent	19·8	Absent	7·9	28·6
Formate	None	3·1	2·4	18·0	Absent	7·3	30·8
	NADPH	2·2	2·0	Absent	Absent	18·1	22·3
	HCOOH	1·0	Absent	19·3	Absent	7·6	27·9
Serine	None	5·6	3·8	13·5	4·3	7·8	35·0
	NADPH	1·4	2·0	Absent	Absent	19·5	22·9
	Serine and pyridoxal-5′-phosphate	0·9	Absent	19·8	Absent	7·9	28·6

Samples (0·3 ml, 20 mg protein) of cotyledon protein (50–80% $(NH_4)_2SO_4$ fraction) were incubated at 32° for 1·5 hr with: 20 μmol potassium phosphate buffer (pH 6·7); 50 μmol 2-mercaptoethanol; 1 μmol FAD; 80 nmol H_4PteGlu, 3·5 μmol NADPH and 12·5 μmol of HCHO, formate or serine as indicated. The formate and serine experiments also included 15 μmol ATP and 0·1 μmol pyridoxal-5′-phosphate respectively. The final volume was 4 ml. Data are in PteGlu equivalents.

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RESULTS

Synthesis of Formyl and Methyl Pteroylglutamates in vitro

A number of experiments were initially conducted with extracts of 3-day-old pea cotyledons. Three potential one-carbon precursors were examined in reaction systems which would theoretically allow oxidation and reduction of the one-carbon unit (Table 1). Complete systems containing formaldehyde, formate and serine respectively synthesized 5- and 10-HCO-H_4PteGlu in addition to 5-CH_3-H_4PteGlu. An unidentified derivative was also formed in the presence of formaldehyde and serine but was absent in the formate experiments. This derivative was clearly separated from the other products by DEAE-cellulose chromatography and appeared to be an oxidized formyl compound as judged by its ability to support the growth of *Lactobacillus casei* but not that of *Pediococcus cerevisiae*. Control reaction systems containing all components with the exception of cotyledon extract or containing boiled extract were found, by microbiological assay, to contain only H_4PteGlu after the incubation period.

It is clear from Table 1 that synthesis of formyl and methyl pteroylglutamates was decreased by omission of NADPH from the reaction system. Synthesis of the former derivatives was also drastically reduced on omission of the one-carbon source but an appreciable amount of 5-CH_3-H_4PteGlu was still formed. Examination of this latter synthesis in other experiments revealed a dependence on FAD in the extraction buffer. For example, if 3-day-old cotyledons were extracted in buffer not containing FAD and then fractionated with $(NH_4)_2SO_4$ as before, ability to synthesize 5-CH_3-H_4PteGlu was lost even if FAD was added during the incubation at 32°.

The large synthesis of 5-CH_3-H_4PteGlu in the absence of an added one-carbon source (Table 1) may clearly be due to the presence of a suitable precursor in the cotyledon extract. Such a precursor may be liberated from protein during the incubation, since dialysis or Sephadex-G25 treatment of the plant extract did not appreciably reduce the synthesis of 5-CH_3-H_4PteGlu in the absence of formate, formaldehyde or serine. To examine this point, samples of the cotyledon extract were examined for amino acids both before and after incubation with the reaction components at 32° for 1·5 hr. The results of such analyses revealed that 73 nmol of serine were liberated/mg protein after incubation.

The synthesis of formyl pteroylglutamates from formaldehyde and H_4PteGlu was examined in more detail using cotyledon extracts known to be particularly rich in 5,10-CH_2-

TABLE 2. SYNTHESIS OF FORMYL PTEROYLGLUTAMATES BY COTYLEDON EXTRACTS

Derivatives	Quantity (μg reaction system)
10-HCO-H_4PteGlu	125
Unidentified derivative	100
5-HCO-H_4PteGlu	57
H_4PteGlu	43
Total pteroylglutamates recovered	325

The reaction system (3 ml) contained: 16·5 μmol K phosphate buffer (pH 7·5), 12·5 μmol formaldehyde, 0·8 μmol H_4PteGlu, 50 μmol 2-mercaptoethanol and tissue extract (15 mg protein, 60–80% $(NH_4)_2SO_4$ fraction). After addition of 1·8 μmol NADP, the increase in absorbance at 340 nm was followed for 20 min at 32°.

H_4PteGlu dehydrogenase activity.[19] As reported earlier by Cossins *et al.*[19] such extracts readily reduced NADP in a reaction having absolute requirements for H_4PteGlu and formaldehyde. When such systems were examined microbiologically (Table 2) it was clear that 10-HCO-H_4PteGlu was the main product. In addition, significant amounts of 5-HCO-H_4PteGlu and the unidentified derivative were formed. Such extracts, however, failed to synthesize 5-CH_3-H_4PteGlu which may be related to the absence of FAD in the buffer used.

To confirm that formaldehyde, formate and the 3 carbon of serine were incorporated into the one-carbon group of the pteroylglutamates, further experiments were conducted with ^{14}C-labelled precursors. The results of such experiments employing [^{14}C]-formaldehyde are given in Fig. 1. Extracts of the cotyledons and root tips of 3-day-old seedlings clearly had ability to incorporate the label into 10-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu. Smaller levels of radioactivity were also present in 5-HCO-H_4PteGlu (Fig. 1a) but no evidence was obtained for labelling of the unidentified derivative formed in the earlier experiments. In this connection it should be noted however that the concentration of formaldehyde was necessarily very low in this experiment, the main prerequisite being use of substrate with a high specific radioactivity.

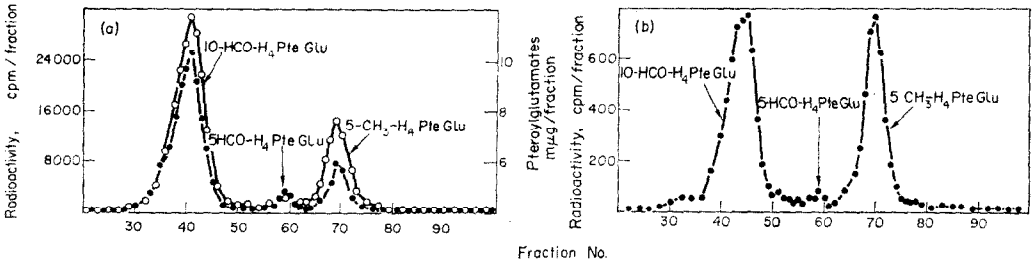

FIG. 1. INCORPORATION OF [^{14}C]-FORMALDEHYDE INTO FORMYL AND METHYL PTEROYLGLUTAMATES. Chromatography of labelled derivatives synthesized by 3-day-old cotyledon extracts (A) and root tip extracts (B). ●—radioactivity; ○—*L. casei* assay.

Studies of 5,10-CH_2-H_4PteGlu *reductase in Pea Seedling Extracts*

The FAD and NADPH requirements for 5-CH_3-H_4PteGlu synthesis suggest that pea cotyledon extracts contain 5,10-CH_2-H_4PteGlu reductase activity. To examine this possibility assays were carried out using (ME-^{14}C)-5-CH_3-H_4PteGlu, FAD and menadione according to the standard procedure of Dickerman and Weissbach.[22] The oxidation product, 5,10-CH_2-H_4PteGlu, readily dissociates into labelled formaldehyde and H_4PteGlu under the condi-

TABLE 3. GENERAL REQUIREMENTS FOR 5,10-CH_2-H_4PteGlu REDUCTASE ACTIVITY

Omission from reaction system	[^{14}C]HCHO formed (cpm/reaction system)
None	4460
FAD	3960
Menadione	2400
HCHO	950
FAD*	420

* FAD was omitted from the extraction buffer and reaction components.

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tions of the assay and may, therefore, be separated from the labelled substrate by ion exchange chromatography. Table 3 summarizes the chief requirements for activity of this enzyme in 3-day-old cotyledon extracts. Similar data were obtained when extracts of 3-day-old root tips were examined. Although omission of FAD from the reaction system caused only a slight reduction in product formation, absence of this dinucleotide from the extraction buffer greatly reduced enzyme activity. Menadione was also required for maximal activity and reaction systems lacking formaldehyde as a trapping agent gave less product than the complete system. Control systems containing only cotyledon extract and substrate failed to produce detectable levels of 5,10-CH_2-H_4PteGlu. Product formation was also found to be linear for the first 5–10 min and activity was reduced to about one-third of the initial rate after 20 min. Product formation was approximately linear with 1·5–3·2 mg protein in the reactions system (3000 and 12 000 cpm respectively in the formaldehyde produced). Activity was maximal at pH 6·4, similar to the optimum for this enzyme in mammalian liver[23] and *E. coli*.[24] Activity was reduced more rapidly at the lower pH values. An examination of the labelled product indicated that it was highly volatile at 45°, was capable of forming an extractable product with dimedon and was recovered in greater amounts when formaldehyde was added during the incubation.

The Effect of L-*Methionine on Pteroylglutamate Enzymes*

It was of interest to determine whether *S*-adenosylmethionine and L-methionine, products of one-carbon metabolism, might affect the activity of the enzymes as has been shown for mammalian[25] and yeast[21,26] systems respectively. Figure 2 summarizes the results of experiments on the possible inhibition by L-methionine of enzymes which catalyze the conversion of 5,10-CH_2-H_4PteGlu to the methyl group of methionine. For comparison, data are also included for the 5,10-CH_2-H_4PteGlu reductase of *Saccharomyces cerevisiae* (ATCC 9763) which is known to be inhibited by L-methionine *in vitro*.[21] It is clear from

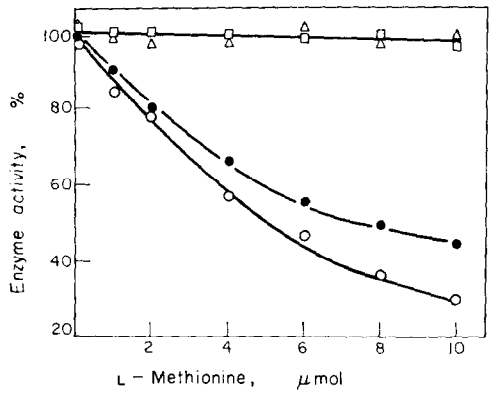

FIG. 2. THE EFFECT OF L-METHIONINE ON 5,10-CH_2-H_4PteGlu REDUCTASE AND 5-CH_3-H_4PteGlu: HOMOCYSTEINE TRANSMETHYLASE ACTIVITIES.
Reductase activities of cotyledon (□) and root tip (△) extracts; ●—reductase activity of *Saccharomyces cerevisiae* extracts; ○— transmethylase activity of root tip extracts.

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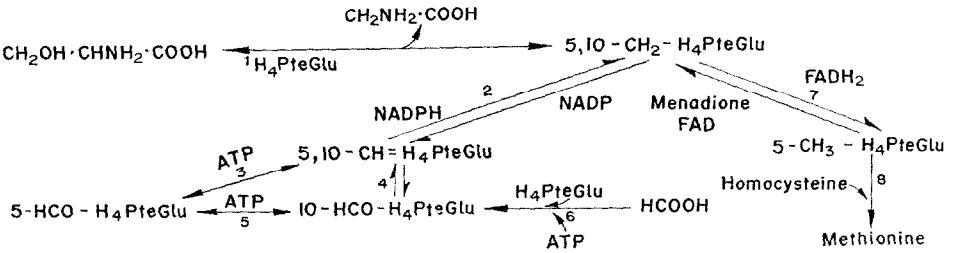
Fig. 2 that the corresponding reductase of pea cotyledons and root tips is not affected by relatively high concentrations of L-methionine. The possibility that the reductase was 'desensitized' during isolation and $(NH_4)_2SO_4$ fractionation appears unlikely as no inhibition was observed when cell-free extracts were examined before $(NH_4)_2SO_4$ fractionation. In further assays *S*-adenosylmethionine (10–200 nmol) was added to the reaction system; in no case was inhibition observed. In agreement with an earlier report[17] regarding pea cotyledon 5-CH_3-H_4PteGlu:homocysteine transmethylase, this enzyme from pea root tips was inhibited by L-methionine. In contrast, the transmethylase from *S. cerevisiae* is not inhibited by this amino acid *in vitro* but is repressed *in vivo*.[27]

The possible effect of L-methionine on methylene and formyl pteroylglutamate biosynthesis was examined in two ways. Firstly, the effect of methionine on production of one-carbon units from serine was investigated by measurement of serine hydroxymethyltransferase activity in crude and partially purified cell-free extracts. No inhibition of this enzyme was observed when L-methionine was added to the reaction systems to a final concentration of 1–10 μmol/ml. Secondly, the synthesis of 5- and 10-HCO-H_4PteGlu from [^{14}C]-formaldehyde was examined in the presence of 1–5 μmol/ml of L-methionine using complete reaction systems containing the components given in Table 1. Addition of L-methionine did not significantly alter the incorporation of label into these products. Similar results were obtained for reaction systems which included unfractionated cell-free extracts.

DISCUSSION

The general requirements for synthesis of formyl and methyl pteroylglutamates from formaldehyde, formate and serine (Table 1) are consistent with operation of the reactions outlined in Scheme 1. It is clear that 5,10-CH_2-H_4PteGlu arising from serine or generated by the non-enzymic condensation of formaldehyde and H_4PteGlu was oxidized and reduced to 10-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu respectively. These syntheses imply that the extracts contain 5,10-CH=H_4PteGlu cyclohydrase (E.C. 3.5.4.9), (Scheme 1,



SCHEME 1. MAJOR PATHWAYS OF PTEROYLGLUTAMATE METABOLISM IN PEA COTYLEDONS.

reaction 4) and 5,10-CH_2-H_4PteGlu reductase (E.C. 1.1.1.68) (Scheme 1, reaction 7), enzymes which have not previously been reported for plants. 5-HCO-H_4PteGlu formed in reaction systems containing formate, formaldehyde and serine respectively (Tables 1 and 2) may have arisen enzymically by operation of 5-HCO-H_4PteGlu cyclodehydrase (Scheme 1, reaction 3) and 5-HCO:10-HCO-H_4PteGlu mutase (Scheme 1, reaction 5), or alternatively may have been formed non-enzymically from 10-HCO-H_4PteGlu during boiling of the reaction system.[28] As the enzymic formation of 5-HCO-H_4PteGlu requires ATP, the second alternative appears more likely.

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The lack of 5-CH_3-H_4PteGlu synthesis when extracts were prepared in the absence of FAD (Table 2) suggests that plant 5,10-CH_2-H_4PteGlu reductase, like the enzyme from *Escherichia coli*,[24] is partially inactivated when isolated under these conditions. It is likely that this enzyme contains protein-bound FAD which is readily lost during fractionation of the extracts with ammonium sulphate. In preliminary experiments designed to purify this reductase from pea root tips, (Sengupta and Cossins, unpublished data) stimulation of activity by FAD has been observed following ammonium sulphate fractionation and dialysis. Considering the general properties of other 5-CH_3-H_4PteGlu reductases, it is likely that FAD, after accepting hydrogen from NADPH, acts as an electron donor during synthesis of 5-CH_3-H_4PteGlu. This suggestion is supported by the observation (Table 3) that the reverse reaction proceeded without addition of NADP but was stimulated by addition of menadione. The latter electron acceptor presumably favoured oxidation of the substrate by reoxidizing $FADH_2$.

The lack of inhibition of 5-CH_3-H_4PteGlu reductase activity by methionine (Fig. 2) and 5-adenosylmethionine is in contrast to earlier investigations of this reductase from *Saccharomyces*[21] and rat liver[25] respectively. In bacteria, synthesis of this enzyme is controlled by methionine.[29] If the serine hydroxymethyltransferase reaction is the major point of entry for one-carbon units into the pteroylglutamate pool,[11] it is clear that cellular mechanisms must exist whereby the resulting 5,10-CH_2-H_4PteGlu can be efficiently utilized for purine, thymidylate and methionine biosynthesis. In this connection, product inhibition of 5-CH_3-H_4PteGlu:homocysteine transmethylase (Fig. 2) may have some physiological significance in regulating flow of methyl groups from the pteroylglutamate pool. In addition, reversibility of 5,10-CH_2-H_4PteGlu reductase would be expected to limit this flow as 5-CH_3-H_4PteGlu accumulates. Methionine might also regulate the biosynthesis of homocysteine as is well documented for *Saccharomyces*.[30,31]

Finally, it is possible that methionine regulates the one-carbon metabolism of plants in a selective manner. The lack of effect of L-methionine on serine hydroxymethyltransferase and on synthesis of formyl pteroylglutamates suggests that other pathways for utilization of 5,10-CH_2-H_4PteGlu such as purine and thymidylate biosynthesis are regulated by mechanisms distinct form those controlling methyl group biogenesis.

EXPERIMENTAL

Chemicals. Tetrahydropteroylglutamic acid was obtained from Sigma Chemical Company, St. Louis, U.S.A. One-carbon derivatives of H_4PteGlu were synthesized chemically.[7] [Methyl-^{14}C]-5-CH_3-H_4PteGlu [3-^{14}C]-serine and [^{14}C]-formaldehyde were obtained from the Radiochemical Centre, Amersham, England.

Preparation of cell-free extracts. Surface-sterilized seeds of *Pisum sativum* L. cv. Homesteader were germinated in vermiculite at 25° for 3 days.[5] Samples (10 g) of the cotyledons were ground at 2° in a mortar with 10 ml of 1 mM K phosphate (pH 6·7) containing 1 mM EDTA, 10 mM 2-mercaptoethanol and 10 mg/l. FAD. Samples (200) of 3-day-old pea root tips (5 mm apical segments) were ground at 2° in 10 ml of 50 mM K phosphate (pH 7·5) containing 5 mM 2-mercaptoethanol. In both cases the homogenates were centrifuged at 18×10^3 *g* for 20 min. In the majority of experiments the supernatants were fractionated by addition of saturated $(NH_4)_2SO_4$ solution (see Tables), dissolved in the extraction buffer and dialyzed against this buffer for 24 hr at 2°. Extracts rich in 5,10-CH_2-H_4PteGlu dehydrogenase activity were prepared as described by Cossins *et al.*[19] followed by fractionation with saturated $(NH_4)_2SO_4$ solution. Protein precipitated between 60 and 80% of saturation was redissolved in the extraction buffer. *Saccharomyces cerevisiae* (ATCC 9763) was cultured[21] and cell-free extracts were prepared after lyophilization.[21] Protein was determined by the method of Lowry *et al.*[32]

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Synthesis of pteroylglutamates in vitro. Reaction systems containing H_4PteGlu, a potential one-carbon donor and coenzymes were incubated with the extracts in the presence of 2-mercaptoethanol to prevent oxidative degradation of tetrahydro derivatives. The reactions were terminated by heating to 100° for 10 min. After removal of precipitated protein the reaction mixtures were diluted by addition of ice-cold 0·5% K-ascorbate (pH 6·0) to give approximately 120 ng pteroylglutamates per ml, and stored at −20°.

Chromatography and assay of pteroylglutamates. Derivatives synthesized by cell-free extracts were isolated by DEAE-cellulose column chromatography.[33] The concentration of derivatives in fractions collected from the columns was determined microbiologically[34] using *Lactobacillus casei* (ATCC 7469) and *Pediococcus cerevisiae* (ATCC 8081). Bacterial growth was measured by titration of the lactic acid after 72 hr incubation at 37° using authentic PteGlu and 5-HCO-H_4PteGlu for calibration.[7] The basic criteria used by Sengupta and Cossins[35] were applied in the identification of individual pteroylglutamates.

Assay of pteroylglutamate enzymes. (a) 5,10-CH_2-H_4PteGlu *dehydrogenase* (E.C. 1.5.1.5) activity was assayed by following the production of NADPH at 340 nm.[19] Pteroylglutamate products were examined after heating the reaction mixtures at 100° for 10 min, dilution with 0·5% K-ascorbate (pH 6·0) and chromatography as described above. (b) 5,10-CH_2-H_4PteGlu *reductase* (E.C. 1.1.1.68) activity was measured.[22] The reaction system, contained in a total volume of 0·32 ml, 10 μmol K phosphate buffer (pH 7·4), 5 nmol FAD, 5 nmol menadione, 5 μmol HCHO, 0·1 μCi [methyl-^{14}C]-5-CH_3-H_4PteGlu (1 μCi/0·016 μmol) and plant extract containing approximately 0·45 mg protein (50–80% $(NH_4)_2SO_4$ fraction). Control systems contained all components with the exception of the cell-free extract. The reaction was carried out at 30° for 30 min and terminated by cooling to 0°. The cooled reaction mixture was immediately placed on a column (0·5 × 2·5 cm) of Dowex AG IX 10 resin (Cl^- form) and labelled HCHO was recovered by washing the column with three aliquots of 0·5 ml H_2O. The effluent was collected in a scintillation vial. The labelled substrate was retained by the column. (c) *Serine hydroxymethyltransferase* (E.C. 2.1.2.1) activity was assayed[36] using dialyzed cell-free extracts. The reaction system, in a total of 1 ml, contained 1 μmol pyridoxal-5′-phosphate, 1 μmol H_4PteGlu, 0·5 μCi [3-^{14}C]-serine (8·5 μCi/μmol), 25 μmol K phosphate buffer (pH 7·4) and cell-free extract (1·5 mg protein). The reaction was initiated by addition of serine, and after incubation at 30° for 30 min was terminated by addition of 0·3 ml of 100 mM Na acetate (pH 4·5), 0·2 ml 100 mM HCHO and 0·3 ml 400 mM dimedone (in 50% EtOH). After heating at 100° for 5 min followed by cooling the dimedon adduct was extracted with toluene and assayed for ^{14}C. Serine hydroxymethyltransferase was also assayed by measuring glycine formation in reaction systems containing $(NH_4)_2SO_4$ fractionated (20–60% fraction) cell-free extract. The reaction components, 5 μmol L-serine, 0·2 μmol H_4PteGlu, 2 μmol pyridoxal-5′-phosphate, 50 μmol K phosphate buffer (pH 7·5) containing 5 mM 2-mercaptoethanol and cell-free extract (0·65 mg protein), total volume 2 ml, were incubated at 30° for 30 min. Glycine formed in the reaction was measured in an amino acid analyzer. (d) 5-CH_3-H_4PteGlu-*homocysteine transmethylase* activity was assayed[17] using $(NH_4)_2SO_4$ fractionated (20–80% fraction) cell-free extracts.

Assay of ^{14}C. Radioactivity was measured by liquid scintillation counting.[16] All counts were corrected for background.

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