PTEROYLGLUTAMATES AND METHIONINE BIOSYNTHESIS IN ISOLATED CHLOROPLASTS

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1. Introduction

L-Methionine has been implicated in plants as a source of methyl groups for synthesis of a variety of compounds including lignin [1], pectin [2], chlorophyll [3] and quinones [4]. While the biosynthesis of L-methionine in plants has been examined in some detail [5, 6] its formation in chloroplasts has not been reported previously despite its established importance in the methylation of chlorophyll [3] and plastoquinone [4].

The present studies have revealed the existence of a chloroplastic pool of several tetrahydropteroylglutamates (H_4PteGlu)*, and the presence, in chloroplasts, of an enzyme system forming methionine from 5-CH_3-H_4PteGlu and homocysteine.

2. Materials and methods

Pea plants (*Pisum sativum* var. Homesteader) were grown for 15 days, and placed in a dark cabinet for 24–36 hr to deplete the starch content. Young developing leaves were deveined and immediately frozen and lyophilized.

Chloroplasts from the freeze-dried leaves were isolated non-aqueously by a modification of a method previously described [7]. The density gradients of CCl_4/*n*-hexane used had specific gravities of 1.34, 1.32 and 1.30. The final chloroplast pellet was dried *in vacuo* at –4°.

H_4PteGlu derivatives were extracted by heating samples of isolated chloroplasts in 0.6% (w/v) potassium ascorbate solution (pH 6.0) at 95° for 10 min [8] followed by ultrasonic treatment [7]. After centrifugation to remove chloroplast debris and precipitated protein, aliquots of the extracts were chromatographed using DEAE-cellulose columns [9]. Fractions (3 ml) of the column effluent were collected and assayed for pteroylglutamate derivatives using *Lactobacillus casei* (A.T.C.C. 7469), *Steptococcus feacalis* (A.T.C.C. 8043) and *Pedicoccus cerevisiae* (A.T.C.C. 8081) [8]. The lactic acid produced after 70 hr incubation at 37° was titrated as a measure of bacterial growth. The amounts of H_4PteGlu derivatives in each fraction were calculated using reference curves [10]. Individual derivatives were identified by co-chromatography with authentic samples, and by using chicken pancreas and hog kidney conjugases [11, 12].

For studies of methionine synthesis, isolated chloroplasts were suspended in 0.15 M potassium phosphate buffer (pH 7.0) and ruptured ultrasonically followed by dialysis at 2° for 16 hr. The dialysed extract was incubated with added cofactors (table 1) at 30° for 1 hr and the reaction terminated by rapid cooling at 0°. An aliquot of the reaction mixture was placed on a column (0.55 cm × 3 cm) of Dowex AG1-X10 resin (Cl^- form). Six fractions, each of 0.2 ml, were then eluted with distilled water. Under the conditions employed, methionine was quantitatively eluted from the column. Each 0.2 ml fraction was then separated by thin-layer chromatography using plates coated with Mn_{300}

* The abbreviations used for pteroylglutamic acid and its derivatives are those suggested by the IUPAC-IUB Commission as listed in the Biochemical Journal, 102 (1967) 15: e.g. 10-HCO-H_4PteGlu = N^{10}-formyltetrahydropteroylmonoglutamate.

Table 1
Requirements for methionine synthesis from 5-CH_3-H_4PteGlu by chloroplast extracts.

Reaction system	^{14}C-Methionine synthesized (dpm/mg protein) X 10^{-4}	
	Crude chloroplast extract	Dialysed chloroplast extract
Complete*	15.15	11.89
Minus enzyme	nil	nil
Boiled enzyme	0.16	0.14
Minus DL-homocysteine	4.2	1.72

* The complete system contained DL-homocysteine, μmoles; 5-[*Me*-^{14}CH_3-H_4PteGlu, 0.016 μmole; potassium phosphate buffer (pH 7.0), 40 μmoles and chloroplast extract (4–5 mg protein).

cellulose, and *n*-butanol-acetic acid-water (60 : 15 : 25 by vol) as the solvent system. Methionine was identified by co-chromatography and by autoradiography. Where ^{14}C-labelled substrate was used, radioactivities were measured by liquid scintillation counting using a fluor containing 7.5 g PPO and 0.65 g dimethyl POPOP dissolved per litre of dioxane: dimethoxyethane:anisole (6 : 1 : 1, by vol). Protein in chloroplast extracts was determined colorimetrically [13] after prior removal of pigments [14].

3. Results and discussion

3.1. *Pteroylglutamate derivatives in chloroplasts*

Preliminary studies of H_4PteGlu derivatives in isolated chloroplasts demonstrated that the isolation technique was of importance. A comparison of chloroplasts isolated aqueously [15] and non-aqueously showed that the former method resulted in considerable losses of pteroylglutamates. Consequently, all further work was carried out with non-aqueously isolated chloroplasts.

The elution and identification of pteroylglutamates in isolated pea chloroplasts is presented in fig. 1. Several derivatives were present, the major being 10-HCO-H_4PteGlu, 5-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu accounting for 30%, 4%, and 25% respectively of the total pteroylglutamates recovered. In addition, smaller amounts of

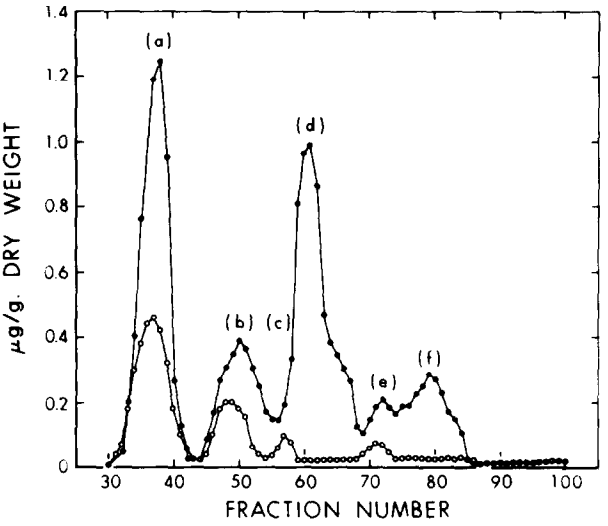


Fig. 1. H_4PteGlu derivatives in chloroplasts.
Assay organism: ●—●, *L. casei*; ○—○, *S. faecalis*.
(a) 10-HCO-H_4PteGlu; (b) 10-HCO-H_4PteGlu$_2$; (c) 5-HCO-H_4PteGlu; (d) 5-CH_3-H_4PteGlu; (e) H_4PteGlu; (f) 5-CH_3-H_4PteGlu$_2$.

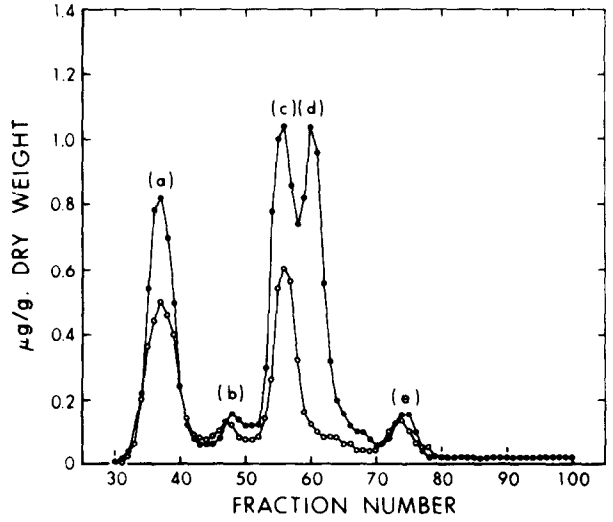


Fig. 2. H_4PteGlu derivatives in chloroplasts after treatment with hog-kidney conjugase. The compounds are as identified in fig. 1.

10-HCO-H_4PteGlu$_2$, 5-CH_3-H_4PteGlu$_2$ and H_4PteGlu were also evident.

Treatment of the chloroplast extract with hog-kidney conjugase resulted in a 21% increase in total pteroylglutamates when assayed with *L. casei*. Separation of the derivatives on DEAE-cellulose (fig. 2) showed that the levels of 5-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu were considerably increased, suggesting that these compounds are, to some degree, conjugated in chloroplasts. No evidence was obtained for the presence of 5-CH_3-H_4PteGlu$_3$. PteGlu and H_2PteGlu were not detected in chloroplasts either before or after conjugase treatment.

3.2. *Biosynthesis of methionine by isolated chloroplasts*

As chloroplasts contain a pool of 5-CH_3-H_4PteGlu, the possibility exists that this compound may be implicated in the biosynthesis of methionine as is known for other organisms [16, 17]. This possibility was examined by assaying chloroplast extracts for ability to catalyze the transmethylation of homocysteine according to the following equation:

5-CH_3-H_4PteGlu + homocysteine

→ Methionine + H_4PteGlu. (1)

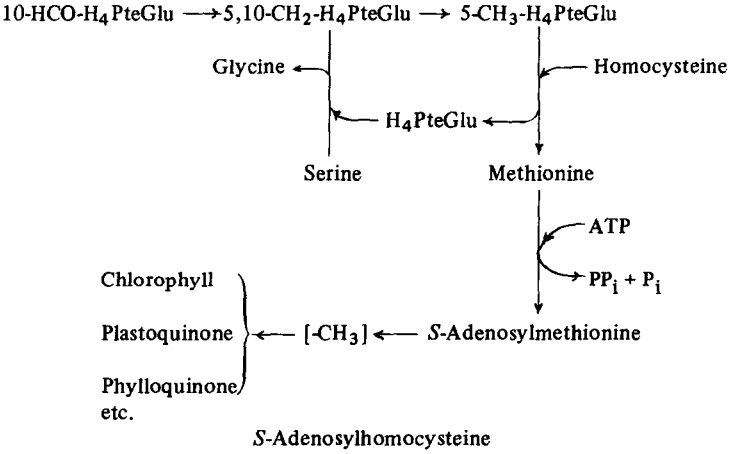
The results of such assays are summarized in table 1. It is clear that the crude extract of isolated chloroplasts was capable of synthesizing methionine in the absence of added homocysteine; this ability was, however, considerably reduced when the chloroplast extract was dialysed prior to incubation. A remarkably greater incorporation of 5-[*Me*-^{14}C] CH_3-H_4PteGlu into methionine was observed when the reaction system, either with crude or dialysed extract, contained added homocysteine. The reaction was essentially enzymic as very little radioactivity appeared in methionine in presence of a boiled enzyme preparation.

On the basis of the present work it appears that pea chloroplasts have ability to synthesize methionine using 5-CH_3-H_4PteGlu as a source of methyl groups. The results are, therefore, different from an earlier report [16] claiming that 5-CH_3-H_4PteGlu$_3$ but not 5-CH_3-H_4PteGlu was the donor of methyl groups for methionine synthesis in whole pea leaf extracts. Although later reports have clearly shown that 5-CH_3-H_4PteGlu$_3$ is a more efficient methyl donor than 5-CH_3-H_4PteGlu [18], the absence of the former compound in pea chloroplasts and the large pool of the latter (fig. 1) suggest that the reaction summarized by equation (1) is of physiological significance in pea chloroplasts.

Exogenously supplied methionine has been shown to be an efficient precursor of the methyl groups of both phylloquinone and plastoquinone in young greening leaves [19]. The reactions, which appeared to occur within the chloroplast, would presumably

Scheme 1

Possible interrelationships between pteroylglutamates, methionine and *S*-adenosylmethionine in chloroplasts.



involve the intermediary formation of *S*-adenosylmethionine (SAM). Direct evidence for the involvement of SAM in the methylation of chlorophyll precursors is documented [3, 20], but the production of this methyl donor by chloroplasts has not been reported. Preliminary studies in our laboratory have shown that SAM is synthesized by chloroplasts from methionine by a reaction dependent on ATP. It appears likely therefore, that a *de novo* synthesis of methyl groups occurs within the chloroplast by the pathway shown in Scheme 1. Present studies are aimed at elucidating the partial reactions of this sequence.

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