Biochem. J. (1972) **128**, 29–40
Printed in Great Britain

Localization and Interconversion of Tetrahydropteroylglutamates in Isolated Pea Mitochondria

By M. T. CLANDININ and E. A. COSSINS
Department of Botany, University of Alberta, Edmonton 7, Alberta, Canada

(*Received* 8 *November* 1971)

1. Mitochondria were extracted from 4-day-old pea cotyledons and purified on a sucrose density gradient. 2. Microbiological assay of the purified mitochondrial fraction with *Lactobacillus casei* (A.T.C.C. 7469), *Streptococcus faecalis* (A.T.C.C. 8043) and *Pediococcus cerevisiae* (A.T.C.C. 8081) revealed a discrete pool of conjugated and unconjugated derivatives of tetrahydropteroylglutamic acid. 3. Solubilization and chromatographic studies of the mitochondrial fraction demonstrated the presence of formylated and methylated derivatives, 10-formyltetrahydropteroylmonoglutamic acid, 5-formyltetrahydropteroylmonoglutamic acid and 5-formyltetrahydropteroyldiglutamic acid being the major derivatives present. 4. The principal mitochondrial pteroylglutamates were labelled when dry seeds were allowed to imbibe [2-^{14}C]pteroylglutamic acid and 5-[*methyl*-^{14}C]-methyltetrahydropteroylmonoglutamic acid. 5. The ability of isolated mitochondria to catalyse oxidation and reduction of tetrahydropteroylglutamic acid derivatives was demonstrated in feeding experiments in which [^{14}C]formaldehyde, [3-^{14}C]serine, sodium [^{14}C]formate, 5-[*methyl*-^{14}C]methyltetrahydropteroylmonoglutamic acid or [2-^{14}C]-glycine served as C_1 donor. In addition, ^{14}C was incorporated into free amino acids related to C_1 metabolism.

In recent years, attention has centred on techniques for isolation and characterization of pteroylglutamate derivatives involved in C_1 metabolism (Bakerman, 1961; Silverman *et al.*, 1961; Sotobayashi *et al.*, 1966; Blakley, 1969; Butterworth *et al.*, 1963; Santini *et al.*, 1964; Iwai & Nakagawa, 1958*a*,*b*; Iwai *et al.*, 1959). Later investigations of higher plants (Roos *et al.*, 1968; Shah & Cossins, 1970; Roos & Cossins, 1971; Cossins & Shah, 1972), involving modifications of these techniques, have revealed that 5-methyl and conjugated derivatives are commonly the principal components of the pteroylglutamate pool. Dodd & Cossins (1969, 1970) have concluded that the former derivatives are important as methyl donors in the new synthesis of methionine during germination.

Okinaka & Iwai (1970) have demonstrated that several key enzymes of pteroylglutamate synthesis are localized in the mitochondria of plants. It has been further suggested that most pteroylglutamate precursors including dihydropteroylglutamic acid are synthesized in the mitochondrion and are transported to the cytoplasm, where further reduction and addition of C_1 units is thought to occur (Okinaka & Iwai, 1970). In contrast with this suggestion, Wang *et al.* (1967) have shown that 10-HCO-H_4PteGlu* is the major constituent of the pteroylglutamate pool of rat liver mitochondria. These authors have suggested, on the basis of enzyme studies, that the new synthesis of methyl groups and interconversion of 10-HCO-H_4PteGlu and 5,10-CH_2-H_4PteGlu do not occur in rat liver mitochondria. Sankar *et al.* (1969) have also reported an association of uncharacterized pteroylglutamates with mitochondria isolated from mouse liver. In preliminary studies with isolated plant mitochondria, derivatives of H_4PteGlu were detected but their synthesis and physiological significance was not examined (Shah *et al.*, 1970).

Work by Tolbert and co-workers (Tolbert, 1963; Tolbert *et al.*, 1968, 1969; Kisaki & Tolbert, 1969; Bruin *et al.*, 1970) has resulted in the proposal of schemes for the utilization of glyoxylate, glycollate and serine, which involve chloroplast, mitochondrial and peroxisomal compartments within the cell. As these reactions are thought to involve at least one reaction of C_1 metabolism, it is clear that pteroylglutamates and enzymes catalysing their interconversion could be associated to some extent with these cellular fractions. Earlier studies from this laboratory (Shah & Cossins, 1969, 1970; Shah *et al.*, 1970; Cossins & Shah, 1972) have substantiated the involvement and localization of pteroylglutamates in the C_1 metabolism of pea chloroplasts.

To assess the possible involvement of mitochondria in the C_1 metabolism of plant tissues, the present work has studied the occurrence and interconversion

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

of pteroylglutamate derivatives in mitochondria isolated from germinating pea cotyledons.

Materials and Methods

Materials

Chemicals. [^{14}C]Formaldehyde, sodium [^{14}C]-formate, [2-^{14}C]PteGlu, 5-[*methyl*-^{14}C]CH_3-H_4PteGlu and L-[3-^{14}C]serine were purchased from Amersham–Searle Corp., Des Plaines, Ill., U.S.A. Other chemicals, of the highest quality commercially available, were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and Sigma Chemical Co., St. Louis, Mo., U.S.A. Tetrahydrofolic acid was purchased exclusively from Sigma.

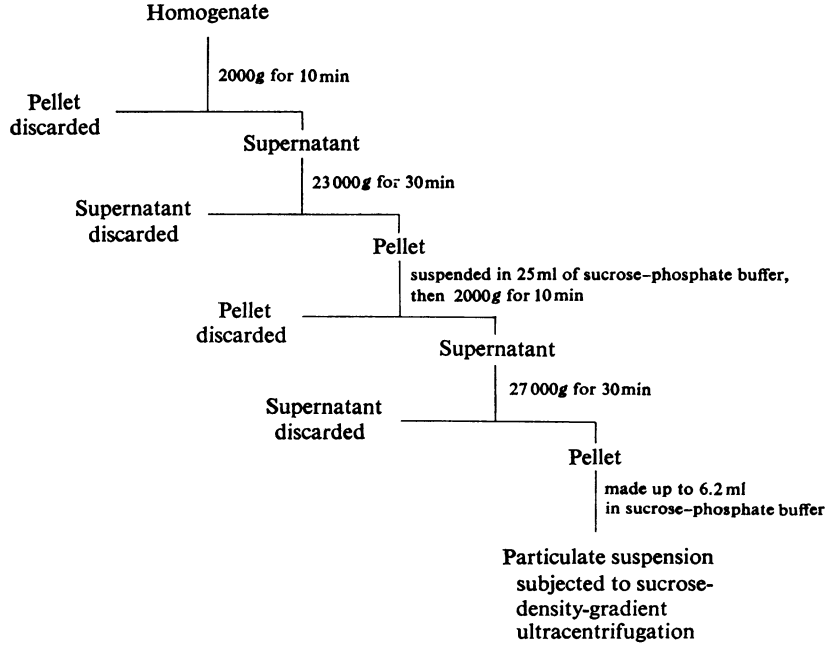
Plant material. Seeds of *Pisum sativum* L. cultivar Homesteader were soaked in deionized water at 25°C. After 30min seeds that had begun to imbibe water were discarded and the remainder were allowed to complete imbibition for a further period of 7h. Seeds that had fully imbibed were then selected and germinated in moist vermiculite in darkness at 25°C for 88h.

Methods

Homogenization and preparation of particulate fraction. All operations were carried out at 2–4°C. Samples of cotyledons (150g) were homogenized by hand in a meat grinder with 300ml of 0.1M-potassium phosphate buffer (pH7.0 at 5°C) containing 0.5M-sucrose. Radioactive samples were homogenized with a mortar and pestle. The homogenate was passed through six layers of cheesecloth and subjected to differential centrifugation as shown in Scheme 1.

The pellet was suspended in the extraction buffer to a final volume of 6.2ml and portions (1.0ml) were layered on each of six discontinuous sucrose density gradients. Each density gradient consisted of nine layers and was prepared at 4°C by pipetting, in sequence: 1.0ml of 77.2%, 0.5ml of 67.6%, 1.0ml of 64.5%, 3.0ml of 61.5%, 3.0ml of 58.5%, 0.5ml of 52.7%, 0.5ml of 47.0%, 0.5ml of 40.0% and 2.0ml of 26.4% (w/v) sucrose. The gradients, contained in a Spinco SW40 rotor, were centrifuged at 40000rev./min (199000*g* at $r_{av.}$) for 190min in a Beckman Spinco model L2-65B ultracentrifuge and decelerated without braking. Fractions were collected from the bottom of the tube in a cold-room at 2°C, as shown in Table 1. Enzyme assays were performed immediately. Protein was measured by the method of Lowry *et al.* (1951) with crystalline egg albumin as a reference standard.

Enzyme assays. Spectrophotometric assays were done with a Beckman DB recording spectrophotometer. Fumarase (EC 4.2.1.2) was assayed by the



Scheme 1. *Preparation of particulate fractions by differential centrifugation*

Sucrose-phosphate buffer is 0.1M-potassium phosphate (pH7.0 at 5°C) containing 0.5M-sucrose.

Table 1. *Protein content, volume and sucrose concentration of fractions collected from the sucrose density gradient*

The percentage distribution of protein within the gradient is the mean of three separate analyses, run in duplicate. Fraction 5 was found to contain a mean of 253.9 μg of protein/g fresh wt. of cotyledons extracted. Sucrose concentrations were determined by refractometry.

Fraction	Vol. of fraction (ml)	Concn. of sucrose (M)	Distribution of protein (%)
1	1.0	2.26	7.1
2	1.5	1.91	9.3
3	3.0	1.79	13.5
4	3.0	1.71	32.7
5	1.0	1.46	22.8
6	0.5	1.14	2.9
7*	3.0	0.68	11.6

* Uppermost fraction of the gradient.

method of Massey (1952). One unit of activity is defined as the amount of enzyme causing an initial rate of change of E_{300} of 0.01/min at 20°C and pH 7.3.

Succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of Hiatt (1961). One unit of enzyme activity is defined as the amount of enzyme that will cause a decrease of 0.01/min in E_{600} under the conditions specified by Hiatt (1961).

Catalase (EC 1.11.1.6) was determined by following the rate of disappearance of H_2O_2 at 240nm. The reaction was carried out at 25°C in 0.05M-potassium phosphate buffer (pH 7.0). The E_{240} of the substrate solution was initially 0.54. The time required for E_{240} to decrease from 0.45 to 0.40 corresponded to the decomposition of 3.45 μmol of H_2O_2 in 3ml of solution.

Peroxidase (EC 1.11.1.7) activity was assayed by the method of Gregory (1966) and expressed as μmol of ascorbic acid oxidized/min.

Glycollate oxidase (EC 1.1.3.1) was assayed by the method of Zelitch & Ochoa (1953). One enzyme unit is defined as the amount which causes a decrease in E_{620} of 0.01/min.

Serine hydroxymethyltransferase (EC 2.1.2.1) was assayed by the method of Taylor & Weissbach (1965).

5,10-Methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) was assayed by the method of Cossins *et al.* (1970).

10-Formyltetrahydrofolate synthetase (EC 6.3.4.3) was assayed by the method of Hiatt (1965). The extraction buffer included 0.1mM-GSH and 1mM-2-mercaptoethanol as additional constituents. Because the activity of this enzyme was found to vary between extractions, its activity was further checked by a microbiological assay of the N^{10}-formyl derivative formed. In such assays the reaction system of Hiatt (1965) was used with the following modifications. Instead of treating the reaction system with HCl, the pH was raised to 12.0 by addition of 5M-KOH followed by heating for 2min at 60°C to convert 10-HCO-H_4PteGlu into 5-HCO-H_4PteGlu and oxidize H_4PteGlu remaining in the reaction system. The concentrations of 5-HCO-H_4PteGlu were then determined microbiologically by using *Pediococcus cerevisiae* as described below.

Electron microscopy of isolated particles. Fractions from the sucrose density gradient were embedded in agar and fixed at 2°C with 4% (w/v) glutaraldehyde in 0.01M-potassium phosphate buffer (pH 6.8) containing 50% (w/v) sucrose. The agar segments were then post-fixed in unbuffered 1% (w/v) OsO_4 for 30min. The segments were dehydrated in an acetone series and embedded in Epon. During dehydration, the particulate material was stained for 5h in aq. 70% (v/v) acetone containing 1% (w/v) uranyl nitrate. Sections were prepared on a Reichert Om U2 ultra-microtome with a Dupont diamond knife. Light gold sections were mounted on 200-mesh grids and stained with aq. lead citrate for 3min. The grids were then examined with a Phillips EM 200 electron microscope at 60kV (Reynolds, 1963).

Determinations of respiratory control. Determinations of ADP/O ratios were performed with a YSI model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) with succinate and α-oxoglutarate as substrates (Chance & Williams, 1955, 1956).

Microbiological assay of pteroylglutamates. Pteroylglutamates were assayed by the 'aseptic plus ascorbate' technique of Bakerman (1961), by using *Lactobacillus casei* (A.T.C.C. 7469), *Streptococcus faecalis* (A.T.C.C. 8043) and *P. cerevisiae* (A.T.C.C. 8081). Standard reference curves were constructed by using PteGlu and 5-HCO-H_4PteGlu. The lactic acid produced after 72h at 37°C was titrated and used as a measure of bacterial growth (Freed, 1966). Correlation of the titration values to standard reference curves, constructed with PteGlu and 5-HCO-H_4PteGlu, allowed determination of the concentration of derivatives.

Chromatography of pteroylglutamate derivatives. Column chromatography of pteroylglutamate derivatives was performed on DEAE-cellulose columns (20cm × 1.8cm) by using a continuous concentration gradient of potassium phosphate buffer (pH6.0) in the presence of ascorbate (Roos *et al.*, 1968; Roos & Cossins, 1971). Pteroylglutamate derivatives were identified by using the basic criteria described earlier (Sengupta & Cossins, 1971; Roos & Cossins, 1971; Cossins & Shah, 1972).

Solubilization of mitochondrial pteroylglutamates. After fractionation of the sucrose density gradient, mitochondrial fractions were immediately subjected to various solubilization treatments. These included: (*a*) sonication at full amplification with a Fisher Ultrasonic Generator model BPO (Blackstone Ultrasonics Inc., Sheffield, Pa., U.S.A.) for 1min at 4°C; (*b*) threefold dilution with 1% (w/v) potassium ascorbate (pH6.0), and incubation with 2% (w/v) sodium deoxycholate for 30min at 4°C; (*c*) threefold dilution with 1% (w/v) potassium ascorbate (pH6.0); (*d*) sonication for 1min followed by treatment with 2% (w/v) deoxycholate for 30min at 4°C; (*e*) treatment with 2% (w/v) deoxycholate without dilution. After such treatments, the fraction was placed over a concentrated sucrose (65%, w/v) layer and centrifuged in a Spinco SW40 rotor at 30000rev./min (119000*g* at $r_{av.}$) for 20min. Pteroylglutamate content of the sedimented and soluble fractions from this step was then assayed microbiologically.

Feeding experiments with ^{14}C. One hundred seeds, selected after the first 30min of imbibition, were allowed to imbibe 20μCi of 5-[*methyl*-^{14}C]CH_3-H_4PteGlu (61μCi/μmol) or 25μCi of [2-^{14}C]PteGlu (55.3μCi/μmol). After uptake of the label, the seeds were allowed to complete imbibition in water as before and germinated in Petri dishes for 88h on moist filter paper in darkness at 25°C.

Radioactive samples were 'counted' in a liquid-scintillation counter (Nuclear–Chicago Corp., Unilux II model). Portions (50–200μl) of the radioactive solutions were 'counted' in 15ml of fluor containing 6.5g of 2,5-diphenyloxazole and 0.65g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/l of dioxan–anisole–dimethoxyethane (6:1:1, by vol.). The counting efficiency was 78% as determined by calibration with a [^{14}C]toluene internal standard. All counts were corrected for background (22c.p.m.) and were regarded as significant only when at least three times this value.

Biosynthesis of pteroylglutamates in vitro. Samples of the mitochondrial fraction (approx. 1.0mg of protein) were incubated at 35°C for 20min with 4nmol of H_4PteGlu under buffered conditions. The specific

Table 2. *Distribution of particulate enzymes within fractions of the sucrose density gradient*

The results are mean values of at least three separate extractions. Enzyme activities are expressed in units/g fresh wt. of cotyledons. n.d., Not detectable.

Enzyme	Fraction no. ... 1	2	3	4	5	6	7
Fumarase							
Activity	0.313	0.95	0.50	1.51	5.28	n.d.	n.d.
Distribution (%)	3.66	11.1	5.88	17.7	61.7	—	—
Succinate dehydrogenase							
Activity	n.d.	n.d.	n.d.	1.16	2.15	n.d.	0.185
Distribution (%)	—	—	—	33.2	61.5	—	5.29
Peroxidase							
Activity	0.052	0.089	0.055	0.188	0.041	0.007	n.d.
Distribution (%)	12.0	20.6	12.7	41.9	9.51	1.56	—
Catalase							
Activity	0.192	0.947	2.92	2.06	2.06	2.18	0.097
Distribution (%)	2.26	11.2	34.4	24.2	24.2	2.57	1.15
Glycollate oxidase							
Activity	0.067	0.151	0.235	4.97	2.04	0.138	1.38
Distribution (%)	<1	1.68	2.62	55.5	22.8	1.81	15.4
Serine hydroxymethyltransferase							
Formaldehyde formed (pmol)	0.339	1.0	0.831	2.07	15.0	2.37	0.349
Distribution (%)	1.40	4.20	3.40	10.4	62.4	9.86	1.50
10-HCO-H_4PteGlu synthetase							
10-HCO-H_4PteGlu formed (nmol)	n.d.	n.d.	n.d.	n.d.	1.81	n.d.	n.d.

Table 3. *Distribution of total pteroylglutamate in fractions of the sucrose density gradient*

Fractions were sonicated and boiled to extract pteroylglutamates. Concentrations are expressed in ng of PteGlu for *L. casei* and ng of 5-HCO-H_4PteGlu for *P. cerevisiae*/g fresh wt. of cotyledons extracted.

	Assay with *L. casei*		Assay with *P. cerevisiae*	
Fraction no.	ng	Distribution (%)	ng	Distribution (%)
1	0.806	2.33	0.493	2.79
2	0.940	2.72	0.806	4.57
3	0.672	1.94	1.08	6.10
4	2.15	6.22	1.75	9.91
5	21.9	63.5	9.43	53.5
6	4.70	13.6	3.13	17.8
7	3.36	9.72	0.940	5.33
Total	34.6	100	17.6	100

quantities of substrates and cofactors added to the reaction systems in the various experiments and their controls are given in the appropriate tables. Reduced FAD was generated *in situ* by incubation of FAD with diaphorase (lipoamide dehydrogenase; Sigma) and NADH for 10min at 35°C before addition of mitochondrial protein. Diaphorase activity was verified by following the oxidation of NADH at 340nm. Decreases in E_{340} were shown to have absolute requirements for diaphorase and FAD.

The biosynthesis of pteroylglutamates was terminated by boiling the reaction system for 2min after addition of 1ml of potassium ascorbate (1.2%, w/v; pH6.0). Samples, containing approx. 1μg of pteroylglutamates, were then chromatographed on DEAE-cellulose. Fractions from the columns were assayed for ^{14}C and the presence of pteroylglutamate derivatives was verified with *L. casei*.

Chromatography of labelled free amino acids. Amino acids formed from ^{14}C-labelled substrates were separated by using a Beckman model 121 automatic amino acid analyser equipped for stream-division of the column effluent. Before such analysis, a portion of the reaction system was passed through a column (1.2cm×10cm) of Dowex 50W (X8; H^+ form; 100–200 mesh) (Bio-Rad Laboratories, Richmond, Calif., U.S.A.). The column was then washed with deionized water and the amino acids were eluted with 2M-HCl. After removal of the HCl *in vacuo* on a Buchler flash-evaporator, the amino acids were dissolved in 0.067M-sodium citrate buffer (pH2.2) and subjected to chromatography. Acidic and neutral amino acids were eluted from a 54cm bed of Beckman–Spinco P.A. 28 resin by using a sequence of 0.067M-sodium citrate buffer (pH3.15 and 4.22). Basic amino acids were eluted from a 12cm bed of Beckman–Spinco P.A. 35 resin by using 0.127M-sodium citrate buffer (pH5.25). The reagent buffers were adjusted to their respective pH values at 23°C, and elution was carried out at 53°C, at a flow rate of 70ml/h. Radioactive peaks were identified by co-chromatography with internal standards. Radioactive amino acid peaks were further identified by t.l.c. on 20cm×20cm silica gel GF plates (Mandel Scientific Co., Montreal, Que., Canada) with phenol–water (3:1, v/v) and butanol–water (7:3, v/v) as solvent systems.

Results

Fractionation of organelles

After isopycnic non-linear sucrose-density-gradient centrifugation of the particulate fraction, separate bands were detected, which on the basis of their enzyme complements included mitochondria, in fraction 5, and peroxisome-like bodies in fractions 3 and 4 (Table 2). Fraction 5 was characterized as containing mainly intact mitochondria by the presence of comparatively high activities of fumarase and succinate dehydrogenase but low activities of peroxidase and glycollate oxidase. Electron microscopy revealed that this fraction contained intact mitochondria with some contamination by mitochondrial fragments. No micro-bodies could be detected in these sections. When ADP/O ratios were determined with succinate and α-oxoglutarate as substrates, the mitochondrial fraction was found to have respiratory-control values of 2.5 and 4.0 respectively, thus indicating that some degree of integrity existed in the organelles of this fraction.

Localization of pteroylglutamate derivatives and related enzymes

Correlation was found between the distribution of serine hydroxymethyltransferase and those of other enzymes characteristic of mitochondria (Table 2).

Table 4. *Solubilization of pteroylglutamate derivatives from isolated mitochondria*

Treatments were applied to fraction 5 (Table 3). For experimental details see the text.

		Pteroylglutamate solubilized (%)			
		Assay with *L. casei*		Assay with *P. cerevisiae*	
Treatment	Fraction ...	Particulate	Supernatant	Particulate	Supernatant
Osmotic shock		84	16	21	79
Osmotic shock and 2% deoxycholate		<1	99	<1	99
Sonication		15	85	2.6	97.4
Sonication and 2% deoxycholate		<1	99	<1	99
2% Deoxycholate		1	99	3.2	96.8

Although the former enzyme and 10-HCO-H_4PteGlu synthetase were associated with the mitochondrial fraction, 5,10-CH_2-H_4PteGlu dehydrogenase was not detected by the spectrophotometric method. The activity of 10-HCO-H_4PteGlu synthetase, although apparently restricted to the mitochondrial fraction, may be to some extent also present in other particulate fractions, as difficulty was encountered in assay of this enzyme by the spectrophotometric method.

Microbiological assays of total pteroylglutamates indicated that high concentrations occurred in the 27000*g* pellets obtained from 4-day-old cotyledons. Further examination of these by sucrose-density-gradient centrifugation (Table 3) revealed that 63% of the *L. casei* growth response and 54% of that given by *P. cerevisiae* was associated with the mitochondrial fraction. The difference in total values given by these two organisms indicates that methyl and formyl derivatives of H_4PteGlu with possibly different degrees of conjugation were present in this fraction. The presence of pteroylglutamates in the denser fractions of the sucrose gradient suggests that these compounds may also be associated with mitochondrial fragments.

The total pteroylglutamate contents of the mitochondrial fraction as assayed with *L. casei* amounted to approx. 3.5–4% of the total pteroylglutamate content of 4-day-old cotyledons. Repeated washing of the mitochondrial fraction decreased this value by as much as 50%, indicating that these derivatives are readily leached out of this organelle.

Release of pteroylglutamates from mitochondria by solubilizing treatments

Solubilizing treatments, summarized in Table 4, confirmed that the mitochondrial fraction contained a pool of pteroylglutamates that are bound more tightly than can be explained solely by adsorption or diffusion of these compounds during extraction of the mitochondria. Some 85% of the total pteroylglutamates, as measured with *L. casei*, were retained by the mitochondrial debris after osmotic shock, suggesting that the derivatives were in large part membrane-bound. This contention was supported by the observation that, after sonication, 15% of the total pteroylglutamate content was retained by the mitochondrial debris, but this value was decreased after treatment with deoxycholate. With one exception, analogous results were obtained with *P. cerevisiae* after these treatments. In most treatments release of pteroylglutamates to the supernatant was higher when the values were determined with *P. cerevisiae*, indicating that formylated derivatives may be less tightly bound than methylated and/or conjugated derivatives.

Chromatography of mitochondrial pteroylglutamates

Figs. 1(*a*) and 1(*b*) are typical elution patterns of the derivatives present in the mitochondrial fraction. The first major peaks (Fig. 1*a*, peaks a, c and e) possessed growth-promoting properties typical of formyl derivatives and occupied positions in the elution sequence corresponding to authentic 10-HCO-H_4PteGlu, 5-HCO-H_4PteGlu and 5-HCO-H_4PteGlu$_2$ respectively (Roos & Cossins, 1971). The large *L. casei* peak (Fig. 1*b*, peak d) coincided with authentic 5-CH_3-H_4PteGlu. A small shoulder at fractions 76–78 (Fig. 1*b*) may represent H_4PteGlu. Other derivatives present co-chromatographed with the standard derivatives (Roos & Cossins, 1971) and were as follows: peak b, 10-HCO-H_4PteGlu$_2$; peak f, 5-CH_3-H_4PteGlu$_2$. In addition, peaks g–j, identified in earlier work (Roos & Cossins, 1971) as conjugated derivatives, were also present.

Roos & Cossins (1971) have suggested that PteGlu may be an intermediate in the synthesis of more

highly reduced compounds such as 5-CH_3-H_4PteGlu in pea cotyledons. To examine this possibility and to determine whether the mitochondrial pool of pteroylglutamates would be derived from such a precursor, [2-^{14}C]PteGlu and 5-[*methyl*-^{14}C]CH_3-H_4PteGlu were supplied during imbibition. Labelled derivatives were detected in the mitochondrial pool after such feeding (Fig. 2). 5-[*methyl*-^{14}C]CH_3-H_4PteGlu gave rise to labelled 10-HCO-H_4PteGlu, 5-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu more readily than in similar experiments involving [2-^{14}C]PteGlu. Labelled PteGlu was incorporated into most of the mitochondrial pteroylglutamates, but the specific radioactivity of these was too low to permit clear resolution of individual derivatives. This finding might be related to the dramatic synthesis of pteroylglutamates that occurs in this tissue (Roos & Cossins, 1971), thus diluting the specific radioactivity of the substrate incorporated.

The presence of pteroylglutamate derivatives and the occurrence of related enzymes in pea mitochondria suggests that these derivatives are interconvertible and may have metabolic significance in the synthesis and metabolism of related amino acids. The presence of serine hydroxymethyltransferase and 10-HCO-H_4PteGlu synthetase suggests that C_1 units can enter the mitochondrial pteroylglutamate pool at the formyl and hydroxymethyl levels of oxidation. A number of experiments were therefore designed to examine ability of isolated pea mitochondria to generate, interconvert and transfer C_1 units via the pteroylglutamate pool.

Biosynthesis of pteroylglutamates in vitro

When the mitochondrial fraction was incubated with [*methylene*-^{14}C]-5,10-CH_2-H_4PteGlu, radioactivity was incorporated into 10-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu (Table 5). In the presence of $NADP^+$ or NAD^+ the labelled substrate was readily oxidized to 10-HCO-H_4PteGlu and smaller amounts of labelled 5-CH_3-H_4PteGlu were formed. Substitution of NADPH for $NADP^+$ increased utilization of the substrate. The presence of $FADH_2$ did not decrease the amount of substrate oxidized to 10-HCO-H_4PteGlu, but there was a marked increase in

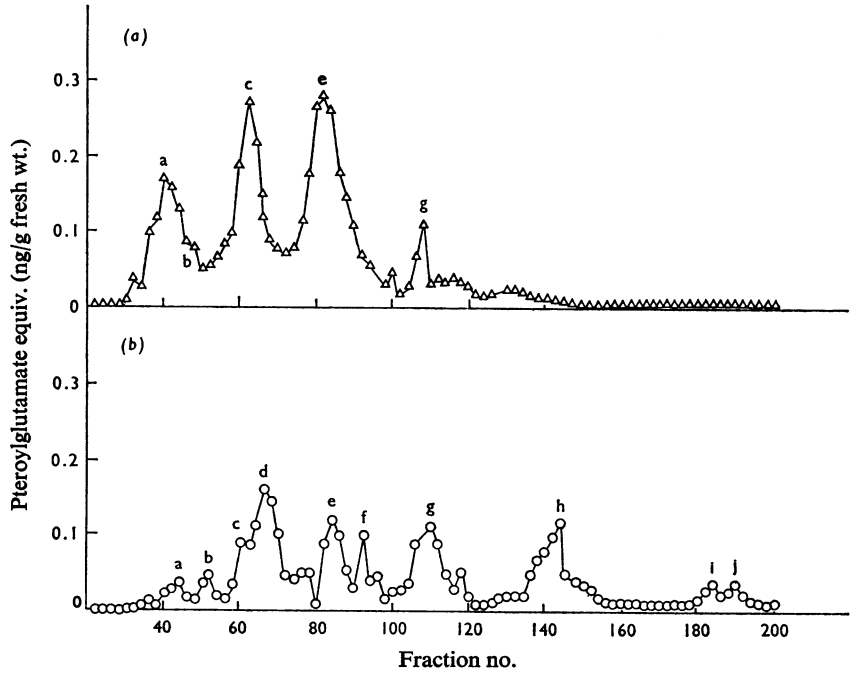


Fig. 1. *Separation of pteroylglutamate derivatives from isolated mitochondria of 4-day-old cotyledons*

After DEAE-cellulose chromatography the fractions (3ml) were assayed for pteroylglutamates by using *P. cerevisiae* (*a*) and *L. casei* (*b*). The derivatives shown are: a, 10-HCO-H_4PteGlu; b, 10-HCO-H_4PteGlu$_2$; c, 5-HCO-H_4PteGlu; d, 5-CH_3-H_4PteGlu; e, 5-HCO-H_4PteGlu$_2$; f, 5-CH_3-H_4PteGlu$_2$; g, h, i and j, unidentified conjugated derivatives.

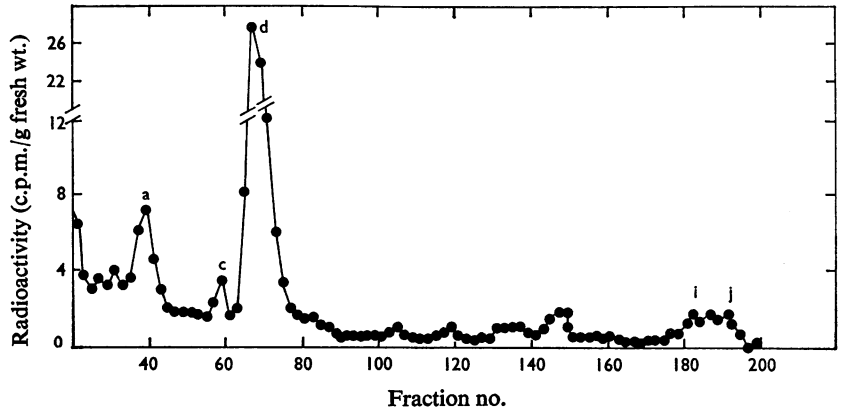


Fig. 2. *Separation of labelled mitochondrial pteroylglutamate derivatives, by column chromatography on DEAE-cellulose, after 5-[methyl-^{14}C]CH_3-H_4PteGlu was supplied to the germinating seeds*

Fractions (3ml) were collected and assayed for ^{14}C. The identified peaks shown are: a, 10-HCO-H_4PteGlu; c, 5-HCO-H_4PteGlu; d, 5-CH_3-H_4PteGlu; i and j, unidentified derivatives.

Table 5. *Utilization of [methylene-^{14}C]-5,10-CH_2-H_4PteGlu by isolated mitochondria*

The complete reaction system contained, in 1.0ml: 40μmol of potassium phosphate buffer (pH7.5), 4.0nmol of H_4PteGlu, 50μCi of [^{14}C]formaldehyde (15μCi/μmol), 1.0μmol of $NADP^+$, 1.0μmol of 2-mercaptoethanol, 0.1μmol of EDTA and mitochondrial fraction. NAD^+, NADH, NADPH and FAD (1.0μmol) were individually substituted for $NADP^+$ as additional controls. When NADH and FAD were combined, 0.30 unit of lipoamide dehydrogenase was added to produce an estimated initial concentration of 1.0mM-$FADH_2$. DL-Homocysteine (1.0μmol) was freshly prepared from the thiolactone. Results are expressed as c.p.m./g fresh wt. of cotyledons.

	^{14}C incorporated					
Reaction system	10-HCO-H_4PteGlu	5-CH_3-H_4PteGlu	Serine	Methionine	Histidine	Unknown (1)
Complete	6970	1950	2280	1400	1970	2350
Substituted NAD^+ for $NADP^+$	5050	950	4520	750	2280	2550
Substituted NADH for $NADP^+$	4940	1690	1730	420	2310	1010
Substituted NADPH for $NADP^+$	8090	2310	2620	550	3030	1110
Substituted NADH and $FADH_2$ for $NADP^+$	6200	17570	2620	770	18950	1225
Substituted NADH and homocysteine for $NADP^+$	5170	450	3320	2910	1210	3.5×10^6

the labelling of the methyl derivative, suggesting that 5,10-methylenetetrahydropteroylglutamate reductase occurred in the mitochondrial fraction. When homocysteine was added, substantially less ^{14}C was recovered in 5-CH_3-H_4PteGlu. In addition to these pteroylglutamates serine, methionine, histidine and an unidentified compound were labelled (Table 5). Reducing conditions favoured the production of histidine. When homocysteine was added methionine synthesis was stimulated, whereas the amount of ^{14}C radioactivity incorporated into histidine was significantly decreased.

The ability of the isolated mitochondria to oxidize and reduce C_1 units was further investigated by supplying [3-^{14}C]serine (Table 6). Results similar to the previous experiment were obtained, with two exceptions. First, when serine was the substrate the effects of adding $FADH_2$, and $FADH_2$ plus homocysteine, were different and NADPH was more effective in the synthesis of labelled 5-CH_3-H_4PteGlu.

Table 6. *Incorporation of [3-^{14}C]serine into derivatives of H_4PteGlu and free amino acids by the isolated mitochondria*

The complete reaction system contained, in 1.0ml: 40μmol of potassium phosphate buffer (pH7.5), 4nmol of H_4PteGlu, 0.2μmol of pyridoxal 5'-phosphate, 1.0μmol of $NADP^+$, 4.8μCi of [3-^{14}C]serine (48μCi/μmol), 1.0μmol of 2-mercaptoethanol, 0.1μmol of EDTA and mitochondrial fraction. NADPH was substituted for $NADP^+$ as a control. As an additional supplement to the reaction system, NADPH and FAD were combined with 0.30 unit of lipoamide dehydrogenase in the presence and absence of 1.0μmol of homocysteine. Results are expressed as c.p.m./g fresh wt. of cotyledons. n.d., Not detectable.

	^{14}C incorporated				
Reaction system	10-HCO-H_4PteGlu	5-CH_3-H_4PteGlu	Glycine	Methionine	Unknown (2)
Complete	13300	3000	n.d.	170	n.d.
Minus enzyme	n.d.	n.d.	n.d.	n.d.	n.d.
Substituted NADPH for $NADP^+$	14600	5700	1510	140	n.d.
Substituted NADPH and $FADH_2$ for $NADP^+$	14800	2400	1360	240	630
Substituted NADPH, $FADH_2$ and homocysteine for $NADP^+$	14200	2500	2090	170	n.d.

Table 7. *Incorporation of [^{14}C]formate into derivatives of H_4PteGlu and amino acids*

The complete reaction system contained, in 1.0ml: 22.7μmol of triethanolamine–HCl buffer (pH8.0), 4nmol of H_4PteGlu, 4.38μmol of ATP, 1.0μmol of NADPH, 10μmol of $MgCl_2$, 5.9μCi of [^{14}C]formic acid (0.118μCi/μmol) and mitochondrial fraction. $NADP^+$ (1.0μmol) was substituted for NADPH as an additional supplement to the reaction system. Formic acid was titrated to pH8.0 before addition to the reaction system. Results are expressed as c.p.m./g fresh wt. of cotyledon. n.d., Not detectable.

	^{14}C incorporated				
Reaction system	10-HCO-H_4PteGlu	5-CH_3-H_4PteGlu	Serine	Methionine	Unknown (3)
Complete	n.d.	140	175	525	595
Minus enzyme	n.d.	n.d.	n.d.	n.d.	n.d.
$NADP^+$ substituted for NADPH	4940	n.d.	n.d.	n.d.	n.d.

Secondly, the amounts of ^{14}C in methionine were much lower than in the previous experiment, and this was possibly contingent on the lack of effect of $FADH_2$. An observation arising from this experiment was the synthesis of glycine.

When the incorporation of [^{14}C]formate into H_4PteGlu derivatives and free amino acids was determined (Table 7), it was clear that in the presence of $NADP^+$ all the radioactivity was apparently trapped in 10-HCO-H_4PteGlu. However, when conditions favoured reduction, accumulation of 10-HCO-H_4PteGlu was not observed and some labelling of 5-CH_3-H_4PteGlu, methionine and serine occurred. In similar experiments with 5-[*methyl*-^{14}C]CH_3-H_4PteGlu as substrate, incorporation of radioactivity into serine and methionine was also observed. Mitochondria incubated with [2-^{14}C]glycine (Table 8) incorporated label into 10-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu. In addition, some ability to methylate homocysteine was observed in this experiment.

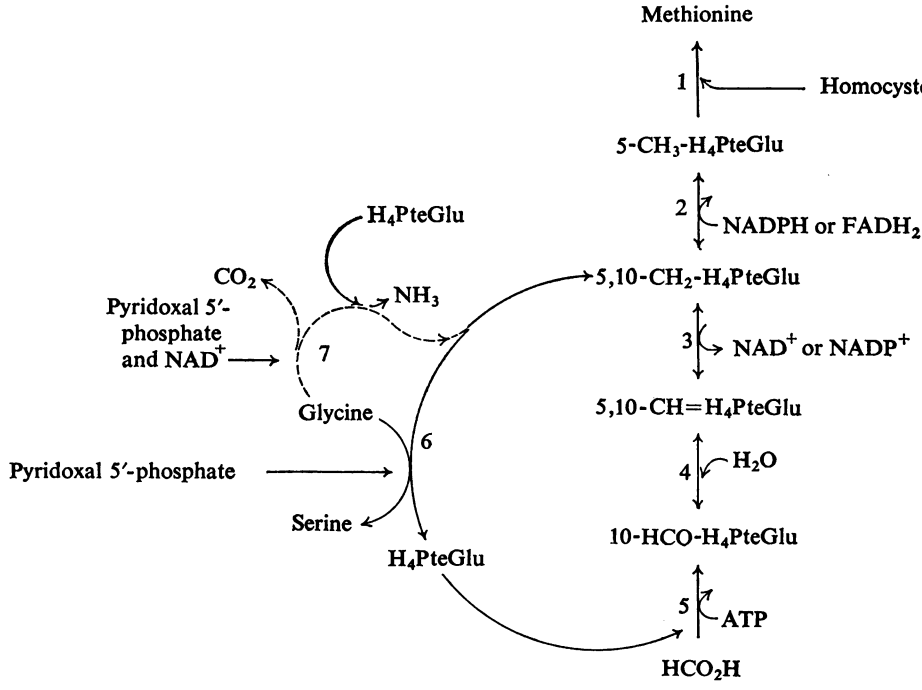
Discussion

On the basis of widely accepted criteria, it is reasonable to conclude that fraction 5 is essentially mitochondrial. The presence of the enzymes serine hydroxymethyltransferase (Scheme 2, reaction 6) and 10-HCO-H_4PteGlu synthetase (Scheme 2, reaction 5) in the isolated mitochondria suggests that at least part of the C_1 metabolism of this tissue is compartmented. This possibility is supported by the presence, in this fraction, of pteroylglutamate derivatives, known to be metabolically important in other tissues. Further support for this contention comes from the

Table 8. *Incorporation of* [2-^{14}C]*glycine into derivatives of* H_4*PteGlu and amino acids*

The complete reaction system contained, in 1.0ml: 40μmol of potassium phosphate buffer (pH7.5), 4nmol of H_4PteGlu, 5.7μCi of [2-^{14}C]glycine (57μCi/μmol), 1.0μmol of 2-mercaptoethanol, 2.0μmol of dithiothreitol, 0.1μmol of pyridoxal 5′-phosphate, 1.0μmol of NAD^+ and mitochondrial fraction. FAD (0.5μmol) combined with 0.30 unit of lipoamide dehydrogenase in the presence and absence of 1.0μmol of homocysteine were used as additional supplements to the reaction system. Results are expressed as c.p.m./g fresh wt. of cotyledons. n.d., Not detectable.

	^{14}C incorporated				
Reaction system	10-HCO-H_4PteGlu	5-CH_3-H_4PteGlu	Serine	Methionine	Unknown (4)
Complete	760	280	101500	n.d.	3600
Minus enzyme	n.d.	n.d.	n.d.	n.d.	n.d.
Plus FAD and diaphorase	n.d.	100	4350	n.d.	360
Plus FAD, diaphorase and homocysteine	n.d.	370	7500	285	n.d.



Scheme 2. *Major* C_1 *transfer reactions leading to serine and methionine synthesis in pea mitochondria*

The broken line indicates that the precise requirements and mechanism of reaction 7 have not been elucidated in plant tissue.

differential binding of the derivatives associated with the mitochondria (Table 4). This binding may result in a spatial organization of the mitochondrial pteroylglutamate pool, which could have physiological significance. Mitochondria from other species have been shown to contain a number of metabolically important pteroylglutamates and in some cases ability to interconvert these has been clearly demonstrated (Noronha & Sreenivasan, 1960; Wang *et al.*, 1967; Sankar *et al.*, 1969). We therefore

conclude that the association and possibly metabolism of certain pteroylglutamates in mitochondria is ubiquitous in higher organisms.

When concentrations of formyl derivatives in mitochondria are expressed on the basis of fresh weight of tissue (approx. 18.0ng/g fresh wt.) and compared with the values determined for whole tissue extracts (105ng/g fresh wt.), it is clear that approx. 17% of the formyl pool is associated with the mitochondria. This value is, however, a minimal one, as formyl derivatives would undoubtedly be lost to the supernatant during isolation of the organelle (Tables 3 and 4). Similar calculations of the distribution of methyl derivatives indicated that they may be largely associated with the soluble components of the cell.

Table 5 shows that isolated pea mitochondria are capable of synthesizing formyl- and methyl-pteroylglutamates from 5,10-CH_2-H_4PteGlu (reactions 3 and 2 respectively). Such syntheses would involve oxidation and reduction of C_1 units. In addition, synthesis of associated amino acids as summarized in Scheme 2 was observed (Tables 5 and 6). When C_1 units were donated to H_4PteGlu at the hydroxymethyl level of oxidation (Tables 5 and 6) subsequent oxidation or reduction was correlated with the presence of nicotinamide nucleotides. The homocysteine-dependent synthesis of methionine, and the production of serine and histidine (Tables 5, 6 and 8), further implicate the mitochondria in the biosynthesis of amino acids related to pteroylglutamate metabolism. Further, the substantial incorporation of ^{14}C into a number of unidentified compounds, particularly under conditions that favour methionine synthesis (Table 5), suggests the operation of other pathways that may also be related to mitochondrial pteroylglutamate metabolism. These other products were not formed in reaction systems containing boiled mitochondrial fraction. However, considering the very high amounts of ^{14}C incorporated in some cases (Table 5) the possibility of non-enzymic reactions cannot be ruled out.

Isolated pea mitochondria also synthesized 5-CH_3-H_4PteGlu from 10-HCO-H_4PteGlu (Table 7), an ability apparently lacking in rat liver mitochondria (Wang *et al.*, 1967). When the reaction conditions favoured oxidation, no synthesis of 5-CH_3-H_4PteGlu occurred, indicating that 5,10-CH_2-H_4PteGlu dehydrogenase and reductase were both instrumental in catalysing this conversion.

The apparent absence of 5,10-CH_2-H_4PteGlu dehydrogenase and reductase in rat liver mitochondria led Wang *et al.* (1967) to conclude that the major significance of mitochondrial serine hydroxymethyltransferase must be related to the reversible interconversion of glycine and serine. It seems likely, however, in pea mitochondria that serine hydroxymethyltransferase, besides functioning in the interconversion of serine and glycine, may act as a source of C_1 units for utilization by the mitochondrial pteroylglutamate pool. Glycine also acted as a source of C_1 units (Table 8) presumably by a reaction involving decarboxylation (Scheme 2, reaction 7).

Kisaki *et al.* (1971) have demonstrated decarboxylation of glycine in plant mitochondria. In conjunction with the glycollate pathway, the occurrence of such 'glycine decarboxylase' activity could result in the generation of C_1 units at the oxidation level of 5,10-CH_2-H_4PteGlu. This type of reaction has been fully characterized by Klein & Sagers (1966*a*,*b*, 1967*a*,*b*) in *Peptococcus glycinophilus*. A similar reaction has been demonstrated by Kikuchi and coworkers in rat liver mitochondria (Motokawa & Kikuchi, 1969*a*,*b*; Sato *et al.*, 1969*a*,*b*; Motokawa *et al.*, 1970; Yoshida & Kikuchi, 1970). The possibility that an analogous system for decarboxylation of glycine may occur in isolated pea mitochondria was suggested by the synthesis of [^{14}C]glycine from [3-^{14}C]serine (Table 6) and by the ability of the mitochondria to synthesize formyl- and methyl-pteroylglutamates from [2-^{14}C]glycine (Table 8). The possible role of this reaction in the biosynthesis of glycine, however, remains to be elucidated.

This work was supported by grants-in-aid of research awarded to E. A. C. by the National Research Council of Canada. M. T. C. was the recipient of a National Research Council of Canada Post-Graduate Bursary and a Gulf Oil Canada Limited Post-Graduate Fellowship during these investigations.

References

Bakerman, H. A. (1961) *Anal. Biochem.* **2**, 558
Blakley, R. L. (1969) *Biochem. Folic Acid Relat. Pteridines* 8
Bruin, W. J., Nelson, E. B. & Tolbert, N. E. (1970) *Plant Physiol.* **46**, 386
Butterworth, C. E., Santini, R., Jr. & Frommeyer, W. B. (1963) *J. Clin. Invest.* **42**, 1929
Chance, B. & Williams, G. R. (1955) *J. Biol. Chem.* **217**, 383
Chance, B. & Williams, G. R. (1956) *Advan. Enzymol. Relat. Subj. Biochem.* **17**, 65
Cossins, E. A. & Shah, S. P. J. (1972) *Phytochemistry* **11**, 587
Cossins, E. A., Wong, K. F. & Roos, A. J. (1970) *Phytochemistry* **9**, 1463
Dodd, W. A. & Cossins, E. A. (1969) *Arch. Biochem. Biophys.* **133**, 216
Dodd, W. A. & Cossins, E. A. (1970) *Biochim. Biophys. Acta* **201**, 461
Freed, M. (1966) in *Methods of Vitamin Assay* (Freed, M., ed.), chapter 2, p. 223, Interscience, New York
Gregory, R. P. F. (1966) *Biochem. J.* **101**, 582
Hiatt, A. J. (1961) *Plant Physiol.* **36**, 552
Hiatt, A. J. (1965) *Plant Physiol.* **40**, 184

Iwai, K. & Nakagawa, S. (1958*a*) *Mem. Res. Inst. Food Sci. Kyoto Univ.* **15**, 40
Iwai, K. & Nakagawa, S. (1958*b*) *Mem. Res. Inst. Food Sci. Kyoto Univ.* **15**, 49
Iwai, K., Nakagawa, S. & Okinaka, O. (1959) *Mem. Res. Inst. Food Sci. Kyoto Univ.* **19**, 17
Kisaki, T. & Tolbert, N. E. (1969) *Plant Physiol.* **44**, 242
Kisaki, T., Imai, A. & Tolbert, N. E. (1971) *Plant Cell Physiol.* **12**, 267
Klein, S. M. & Sagers, R. D. (1966*a*) *J. Biol. Chem.* **241**, 197
Klein, S. M. & Sagers, R. D. (1966*b*) *J. Biol. Chem.* **241**, 206
Klein, S. M. & Sagers, R. D. (1967*a*) *J. Biol. Chem.* **242**, 297
Klein, S. M. & Sagers, R. D. (1967*b*) *J. Biol. Chem.* **242**, 301
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265
Massey, V. (1952) *Methods Enzymol.* **1**, 729
Motokawa, Y. & Kikuchi, G. (1969*a*) *J. Biochem.* (*Tokyo*) **65**, 71
Motokawa, Y. & Kikuchi, G. (1969*b*) *Arch. Biochem. Biophys.* **135**, 402
Motokawa, Y., Hiraga, K., Kochi, H. & Kikuchi, G. (1970) *Biochem. Biophys. Res. Commun.* **38**, 771
Noronha, J. M. & Sreenivasan, A. (1960) *Biochim. Biophys. Acta* **44**, 64
Okinaka, O. & Iwai, K. (1970) *J. Vitaminol.* (*Kyoto*) **16**, 196
Reynolds, E. S. (1963) *J. Cell Biol.* **17**, 208
Roos, A. J., Spronk, A. M. & Cossins, E. A. (1968) *Can. J. Biochem.* **46**, 1533
Roos, A. J. & Cossins, E. A. (1971) *Biochem. J.* **125**, 17
Sankar, D. V., Geisler, A. & Rosza, P. W. (1969) *Experientia* **25**, 691
Santini, R., Jr., Brewster, C. & Butterworth, C. E., Jr. (1964) *Amer. J. Clin. Nutr.* **14**, 205
Sato, T., Kochi, H., Motokawa, Y., Kawasaki, H. & Kikuchi, G. (1969*a*) *J. Biochem.* (*Tokyo*) **65**, 63
Sato, T., Kochi, H., Sato, N. & Kikuchi, G. (1969*b*) *J. Biochem.* (*Tokyo*) **65**, 77
Sengupta, U. K. & Cossins, E. A. (1971) *Phytochemistry* **10**, 1723
Shah, S. P. J. & Cossins, E. A. (1969) *Phytochemistry* **9**, 1545
Shah, S. P. J. & Cossins, E. A. (1970) *FEBS Lett.* **7**, 267
Shah, S. P. J., Roos, A. J. & Cossins, E. A. (1970) *Chem. Biol. Pteridines, Proc. Int. Symp. 4th. 1969*, 305
Silverman, M., Law, L. W. & Kaufman, G. (1961) *J. Biol. Chem.* **236**, 2530
Sotobayashi, H., Rosen, F. & Nichol, C. A. (1966) *Biochemistry* **5**, 3878
Taylor, R. T. & Weissbach, H. (1965) *Anal. Biochem.* **13**, 80
Tolbert, N. E. (1963) *Nat. Acad. Sci. Nat. Res. Counc. Publ.* no. 1145
Tolbert, N. E., Oeser, A., Kisaki, T., Hageman, R. H. & Yamazaki, R. K. (1968) *J. Biol. Chem.* **243**, 5179
Tolbert, N. E., Oeser, A., Yamazaki, R. K., Hageman, R. H. & Kisaki, T. (1969) *Plant Physiol.* **44**, 135
Wang, F. K., Koch, J. & Stokstad, E. L. R. (1967) *Biochem. Z.* **346**, 458
Yoshida, T. & Kikuchi, G. (1970) *Arch. Biochem. Biophys.* **139**, 380
Zelitch, I. & Ochoa, S. (1953) *J. Biol. Chem.* **201**, 707