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MITOCHONDRIAL FOLATES AND METHIONYL-tRNA TRANSFORMYLASE ACTIVITY DURING GERMINATION AND EARLY GROWTH OF SEEDS

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Key Word Index—Folate derivatives; methionyl-tRNA transformylase; plant mitochondria; germination.

Abstract—Mitochondria were isolated from the cotyledons of pea (*Pisum sativum* cv Homesteader) and peanut (*Arachis hypogaea* cv Early Spanish) seeds over a 7-day growth period. The rate of mitochondrial oxygen uptake increased 3–4-fold during the first 4 days of growth and parallel changes were observed in the respiratory control and ADP/O ratios. In both species, the total cotyledonary pool of folate derivatives increased 3–4-fold during this period of germination whereas that associated with isolated mitochondria increased 5–10-fold. Until day 3 of growth, the mitochondrial folates were principally polyglutamates of 10-formyltetrahydrofolate but between day 4 and day 7 increasing levels of 5-methyltetrahydrofolate polyglutamates were detected. Pea and peanut mitochondria contained methionyl-tRNA transformylase (EC 2.1.2.9) activity that displayed an absolute requirement for 10-formyltetrahydrofolate. The specific activity of this enzyme rose during germination, reaching maximal levels between days 3 and 4. Isolated pea mitochondria had the ability to incorporate [^{3}H]leucine and [^{35}S]methionine into protein in a reaction that required ADP and malate but was strongly inhibited by chloramphenicol. Organelles isolated after 4 days of germination incorporated leucine at rates *ca* 5-fold greater than shown by mitochondria of 16-hour-old seedlings. The inter-relationships between respiratory activity, mitochondrial formyltetrahydrofolates and methionyl-tRNA transformylase activity suggest a role for organelle protein synthesis during germination of these legume species.

INTRODUCTION

Germination and seedling growth are recognized as periods of dynamic change [1–4]. Thus the transition from dormant to actively growing states is marked by increased metabolic activity as well as by development of characteristic organelle structure [1–4]. This enhanced metabolism includes a rise in respiratory activity as shown by increased oxygen uptake and cytochrome oxidase activity [5–9]. At the ultrastructural level, these changes are paralleled by the appearance of mitochondria which rapidly develop cristae as germination and growth proceed [5, 8, 10, 11].

In legume species, two patterns of mitochondrial development have been proposed [1, 8]. In pea, this development involves repair and activation of pre-existing organelles within the cotyledons [12, 13]. Studies of the Alaska variety of pea [12] suggest that this process may not require protein synthesis, at least during the early stages of germination and growth. However, in the Homesteader variety [13], mitochondrial protein synthesis appears to be important in organelle development, although evidence for increases in the number of mitochondria is lacking. In peanut cotyledons, there is excellent evidence that *de novo* biogenesis of mitochondria occurs during germination [8, 14–16]. This biogenesis involves both mitochondrial and cytoplasmic protein synthesis, a situation in common with other organisms [17, 18].

Formylmethionyl-tRNA^{fmet}, required for the initiation of organelle protein synthesis, is generated by the 10-formyltetrahydrofolate:methionyl-tRNA transformylase reaction [19]. The enzyme has been studied in bacteria [20, 21], fungal mitochondria [22–24], and in the chloroplasts of algae [23–27] and higher plants [28, 29]. The mitochondrial transformylase of higher plants has received little study [30] although, by analogy with other systems, this folate-dependent enzyme must play a key role in organelle protein synthesis.

Earlier studies from this laboratory showed that the mitochondria of pea cotyledons contain a number of metabolically important folate derivatives including 10-HCO-H_4PteGlu [31]. In addition, pea mitochondria have the ability to interconvert formyl-, methylene- and methyltetrahydrofolates [32]. These investigations centred on mitochondria that were isolated after 3–4 days of growth. Earlier stages of seedling development were not analysed. In the present work we have, therefore, examined the mitochondria of pea and peanut cotyledons for changes in folate pools and transformylase activity over a 7-day growth period. Mitochondria of both species developed an ability to generate formylmethionyl-tRNA during germination. In pea, this was accompanied by organelle protein synthesis.

RESULTS

Isolation and respiratory activity of mitochondria

Mitochondria were isolated during a 7-day growth period from pea and peanut cotyledons. Purity of the final suspension was assessed by assay of marker enzymes (Table 1). For both mitochondrial sources, contamination

*The abbreviations used for folate derivatives are those suggested by the IUPAC-IUB Commission, e.g. 10-HCO-H_4PteGlu = 10-formyltetrahydropteroylglutamic acid.

Table 1. Particulate enzyme activities before and after purification of mitochondria

Enzyme	Peanut		Pea	
	Homogenate	Gradient-purified mitochondria	Homogenate	Gradient-purified mitochondria
Succinic dehydrogenase				
Sp. act.	0.12	5.6	0.12	1.95
Total units	515	62.5	665	75
% of total units	100	12	100	11.3
Alcohol dehydrogenase				
Sp. act.	2.43	0.83	12.4	6.5
Total units	1.00×10^4	9.33	3.46×10^4	154
% of total units	100	0.09	100	0.44
Catalase				
Sp. act.	699	1.38×10^4	98.3	652
Total units	2.55×10^6	1.21×10^5	2.74×10^5	1.55×10^4
% of total units	100	4.8	100	5.6

Enzyme activities are given as units per mg protein. A unit of activity is defined as μmol substrate utilized per hour. The homogenate is the filtered brei obtained after grinding whole 3-day-old cotyledons.

by other cellular organelles was minimal. For example, cytoplasmic contamination, as judged by alcohol dehydrogenase, was negligible. Similarly, peroxisomes were effectively excluded as 95% of the catalase ativity did not sediment with the mitochondrial fraction. Respiratory activities are shown in Figs 1 and 2. In peanut, mitochondrial ADP/O ratios increased almost 2-fold during the first 3–4 days of germination and respiratory control ratios rose by a slightly greater amount (Fig. 1). The same trends were obvious in pea mitochondria (Fig. 2) although the values were somewhat lower. Since these ratios give a measure of coupling between ATP synthesis and oxygen uptake, it follows that the mitochondria of both species were more efficient in this respect as germination and growth proceeded.

Levels and nature of mitochondrial folates

Earlier studies showed that folate levels in whole pea cotyledons increased during the first 3 days of germination [33]. Changes in mitochondrial folates were not reported, however. In the present study, folates were extracted from mitochondria throughout a 7-day germination period (Figs 3 and 4). In pea (Fig. 3) and peanut (Fig. 4) total folate concentration, as measured by *L. casei*, rose during early germination with the most rapid rise occurring between 0 and 4 days. Parallel assays involving *P. acidilactici* (Figs 3 and 4) provided information on the nature of the mitochondrial folate pool. In this regard, the difference in folate levels detected by the two assay organisms gives a measure of 5-CH_3-H_4PteGlu concentration [33]. Clandinin and Cossins [32] showed that methyl and formylfolates were the principal derivatives of pea mitochondria. Ion exchange chromatography [34] established that this situation also applied to peanut mitochondria (data not shown). It therefore follows that the data for *P. acidilactici* assays (Figs 3 and 4) will be indicative of formylfolate concentrations. Thus in both pea and peanut mitochondria, it was apparent that about 75–80% of the total folate pool was formylated especially after day 2 of growth. These percentages declined in the mitochondria of older tissues. Mitochondrial folates also existed as polyglutamates (Figs 3–6) with peanut having about 65–80% of the total pool highly conjugated. In pea,

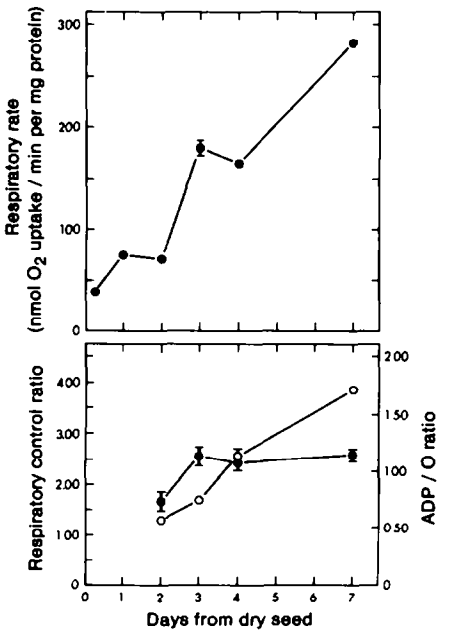

Fig. 1. Changes in the respiratory activity of mitochondria isolated from peanut cotyledons over a 7-day period. Respiratory rate (upper graph) and respiratory control ratios (○——○) and ADP/O ratios (●——●) (lower graph) were determined polarographically using succinate as the substrate. Respiratory rate was measured during state 3 respiration. The values were determined from three replicates per experiment with at least two separate extractions of mitochondria. The points represent mean values ± s.e. Clearly, reproducible, coupled mitochondria were not observed prior to the second day. Data are expressed on a per mg mitochondrial protein basis.

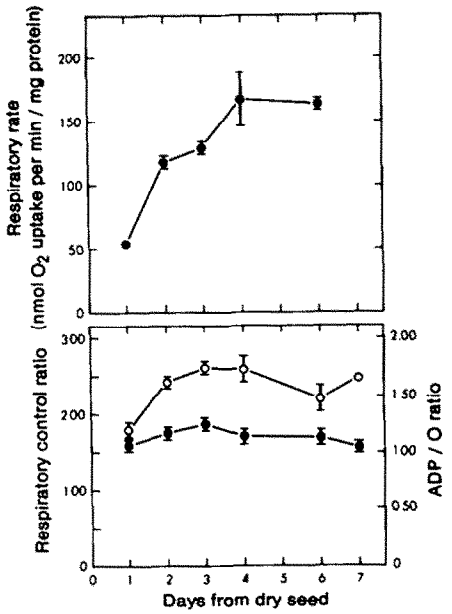

Fig. 2. Changes in the respiratory activity of pea mitochondria over a 7-day period. Values were determined as for peanut mitochondria. Lower graph: respiratory control ratio (○——○), ADP/O ratio (●——●).

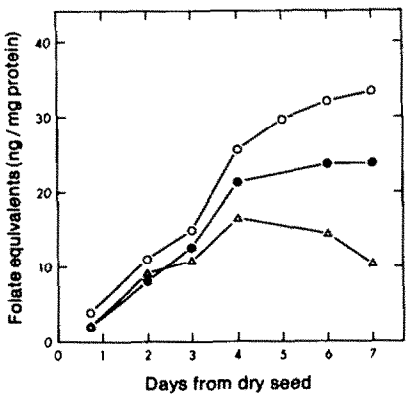

Fig. 3. Changes in pea mitochondrial folate levels. Folates were extracted from isolated mitochondria of various ages. All samples were assayed with *L. casei* before (△——△) and after (○——○) hydrolysis by γ-glutamyl carboxypeptidase. Hydrolysed samples were also assayed with *P. acidilactici* (●——●). Each point represents the mean value of three different aliquots, in duplicate, from one mitochondrial extraction. Separate extractions gave data that differed by less than 10% from the above. Data are expressed as ng folate equivalents per mg of mitochondrial protein.

the corresponding range was 25–35% of total folates. To determine whether these polyglutamate pools may have been partially hydrolysed during extraction, samples of freshly isolated mitochondria were examined for endogenous conjugase activity. The optimal assay conditions described for pea γ-glutamyl carboxypeptidase

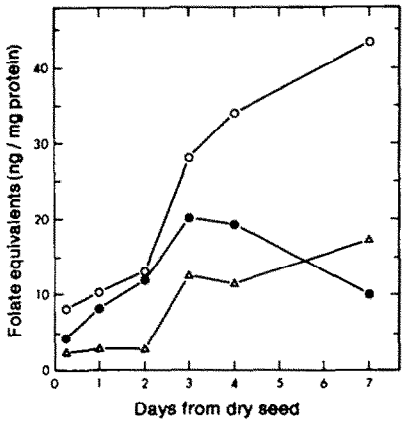

Fig. 4. Changes in peanut mitochondrial folate levels. The levels were determined as for pea mitochondria, with the same number of replicates. Symbols as in Fig. 3.

[35] were modified to include incubation at pH 7.2. Mitochondria were sonicated and incubated with an excess of yeast folylpolyglutamates for 6 hr. Control systems lacked mitochondrial protein. Subsequent assays of total folates using *L. casei* provided no evidence that either mitochondrial source could catalyse a significant hydrolysis of folylpolyglutamates at pH 7.2.

Extracts of whole cotyledons were also assayed as shown in Figs 5 and 6. In pea, total folates (Fig. 5, open circles) rose 4-fold during 0–3 days of growth and then remained relatively constant. In peanut, total folates rose 4–5-fold during 0–5 days (Fig. 6, open circles) and were about four times the levels found in pea. These trends were also observed when the data were expressed on a per cotyledon basis. In both species, cotyledonary folates were principally methylated and highly conjugated as judged by the differential responses of *L. casei* and *P. acidilactici* and the effects of conjugase treatments respectively.

Methionyl-tRNA transformylase and mitochondrial protein synthesis

Optimal assay conditions were established for the transformylase from both mitochondrial sources. In this regard, the enzyme of both species (Table 2) had absolute requirements for methionyl-tRNA and 10-HCO-H_4PteGlu. Product formation was also dependent on the presence of mitochondrial protein. Omission of Mg^{2+} from the reaction system gave increases in activity of about 35% whereas less product was formed when K^+ was omitted. In other studies (data not shown), activity was found to be maximal at pH 7.6 and to be a linear function of methionyl-tRNA concentration up to 0.5 μM. When the concentration of the folate substrate was varied, activities were maximal at 10 μM but declined markedly above this level. Isolated mitochondria of both species displayed a formylation reaction that proceeded as a linear function of time for up to 30 min at 30°. The optimal concentration of mitochondrial protein in these assays was found to be 50 μg.

The nature of the reaction product was examined by PAGE. In these analyses, migration of the double-labelled product was compared to that of [^{3}H]methionyl-tRNA

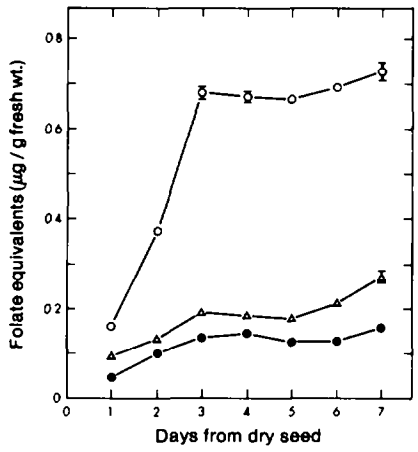

Fig. 5. Changes in pea cotyledon folate levels during the first 7-days of growth. Sample folate levels were determined by microbiological assay using *L. casei* (open symbols) and *P. acidilactici* (closed circles). Samples were assayed using *L. casei* before (△——△) and after (○——○) hydrolysis with pea cotyledon γ-glutamyl carboxypeptidase. Only hydrolysed samples were assayed by *P. acidilactici*. Values are given as equivalents of folic acid for *L. casei*-assayed samples and *l*-5-HCO-H_4PteGlu for *P. acidilactici*-assayed samples. The level of folates in each sample was determined by using three aliquots, in duplicate, from one extraction. A total of three different extractions were made. Each point represents the means ± s.e.

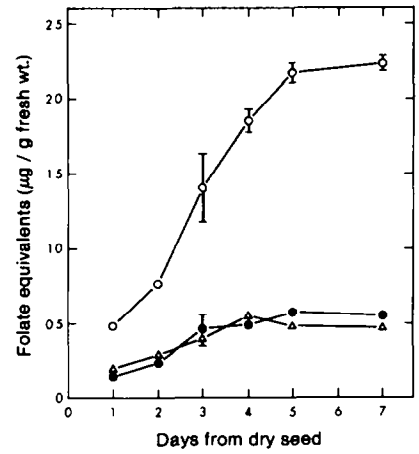

Fig. 6. Changes in peanut cotyledon folate levels during the first 7 days of growth. The levels were determined as for pea cotyledons, with the same number of replicates. Each point represents the mean ± s.e. Symbols: △——△, *L. casei* before and ○——○, after hydrolysis; ●——● *P. acidilactici* after hydrolysis.

and bulk tRNA of *E. coli*. The transformylase product co-migrated with the tRNA standards, exhibiting a distinct tRNA band (data not shown). This band was more heavily labelled when the [^{14}C]folate substrate was included in the transformylase reaction system. Although the formyl group might be bound to tRNA at a location distinct from the methionyl residue, extensive studies of this transformylation reaction in other species [19] suggest that this is unlikely.

Table 2. Requirements for transformylase activity in pea and peanut mitochondria

Reaction system	Enzyme activity* Pea	Peanut
Complete	790	1535
Minus mitochondria	n.d.	n.d.
Heated mitochondria†	n.d.	n.d.
Minus K^+	632	1230
Minus Mg^{2+}	1122	1996
Minus [^{3}H]-met-tRNA	n.d.	n.d.
Minus [^{14}C]-HCO-H_4PteGlu	n.d.	n.d.

*Expressed in pmol fmet-tRNA formed/100 pmol tRNA recovered/mg protein.

†Mitochondrial protein was incubated at 100° prior to assay.

n.d., Not detected.

Transformylase activities were also measured over a 7-day growth period as summarized in Fig. 7. The specific activity in pea mitochondria rose rapidly for the first 4 days but then declined slightly. In peanut, activity rose rapidly after imbibition with maximal specific activities being reached between 3 and 4 days. The subsequent decline in transformylase activity was greater than occurred in pea mitochondria.

The presence of met-tRNA transformylase activity in pea and peanut mitochondria suggests that organelle protein synthesis may occur during germination [18]. This possibility was examined in 4-day-old pea mitochondria as shown in Table 3. The ability to incorporate [^{3}H]leucine and [^{35}S]methionine into TCA precipitable material was assessed in experiments of 30 and 45 min duration respectively. The requirements for incorporation of both amino acids were similar and consistent with a requirement for ATP. Chloramphenicol inhibited the incorporation of both isotopes by *ca* 60%. In contrast, cycloheximide had little effect on the incorporation of methionine. In the complete reaction system, leucine incorporation was found to be a linear function of time for up to 1 hr and of protein concentration up to 2.2 mg per assay. The reaction was also affected by Mg^{2+} concentration with maximal incorporations occurring at 15 mM (data not shown). In related experiments, mitochondria were isolated from pea cotyledons at different stages of germination. These preparations were then assessed for ability to incorporate [^{3}H]leucine using the complete reaction system (see Experimental) and an incubation period of 45 min. Mitochondria from seeds that had been germinated for only 16 hr incorporated an average of 881 dpm leucine/mg protein with a range of 727–1124 dpm/mg protein for two separate mitochondrial isolations. In contrast, mitochondria from 4-day-old cotyledons incorporated an average of 4662 dpm leucine/mg protein with a corresponding range of 3600–5413 dpm. At both ages, the incorporation of leucine was inhibited by chloramphenicol and either drastically reduced or eliminated when ADP was not present in the reaction system.

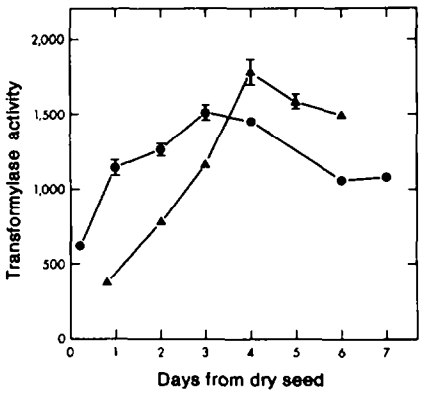

Fig. 7. Changes in the activity of 10-HCO-H_4PteGlu:met-$tRNA^{f\text{-}met}$ transformylase in mitochondria isolated from pea (▲——▲) and peanut (●——●) cotyledons. The activity of the enzyme is given as pmol fmet-$tRNA^{f\text{-}met}$ formed per 100 pmol tRNA recovered/mg mitochondrial protein. Enzyme activity was proportional to protein concentration in all assays, and was determined from at least three protein levels, in duplicate, from one mitochondrial extraction. Each point represents the mean ± s.e. from at least two separate extractions.

Table 3. Requirements for amino acid incorporation by isolated pea mitochondria

	Amino acid incorporation (dpm/mg protein)	
Assay conditions	[^{3}H]Leucine	[^{35}S]Methionine
Complete system	2038	19820
−malate	n.a.	7333
−ADP	n.d.	7135
−ADP, plus ATP	3913	42415
$-Mg^{2+}$	102	n.a.
+Chloramphenicol	734	7730
+Cycloheximide	n.a.	17442

Incorporation of the radioisotope was measured after 30 and 45 minutes in experiments with [^{3}H]leucine and [^{35}S]methionine respectively. ATP was supplied as indicated at 1 mM. The inhibitors were supplied at 100 μg/ml of reaction system. n.a., Not assayed; n.d., not detected.

DISCUSSION

The development of respiratory activity in pea and peanut mitochondria (Figs 1 and 2) was clearly accompanied by changes in the folate pool (Figs 3 and 4) and by increases in the specific activity of methionyl-tRNA transformylase (Fig. 7). In addition, the chloramphenicol-sensitive incorporation of leucine and methionine by pea mitochondria (Table 3) raises the possibility that these changes may be inter-related through a requirement for mitochondrial protein synthesis. Discussion of these possible relationships must logically include a consideration of changes in the mitochondrial pool of 10-HCO-H_4PteGlu as this folate is the established source of C_1 units for the formylation of methionyl-tRNA [19].

The present analyses (Figs 3 and 4) show that HCO-H_4PteGlu derivatives are the major folates of mitochondria isolated from pea and peanut cotyledons during the first 4 days of growth. In both species, this class of mitochondrial folates increased in concentration as growth proceeded to day 4. Furthermore, the increased assay of these folates after conjugase treatments (Figs 3 and 4) implies that they occur within the mitochondria as highly conjugated polyglutamates. In contrast, folate assays of whole cotyledon extracts (Figs 5 and 6) indicated that HCO-H_4PteGlu$_n$ derivatives were a minor part of the total folate pool. In such assays, the large differential response between the two assay bacteria suggests that the cytosolic pool is dominated by 5-CH_3-H_4PteGlu$_n$. Thus, the earlier contention [32] that pea mitochondria contain a discrete pool of largely HCO-H_4PteGlu$_n$ has support from the present study. It is also noteworthy that studies of other eukaryotic systems have provided similar data on mitochondrial folates. Thus in mammalian liver [36, 37], the mitochondrial folates are mainly 10-HCO-H_4PteGlu$_n$, while those of the cytosol are chiefly 5-CH_3-H_4PteGlu$_n$. There is also evidence, from recent studies, that the mitochondrial folate pools of rat liver [38] and *N. crassa*, wild type [39] are 85–95% folylpolyglutamate.

Mitochondrial folates have importance in a number of reactions of C_1 metabolism. In this regard, plant and animal mitochondria contain several folate-dependent enzymes [31]. In yeast, a genetic lesion affecting the expression of a mitochondrial serine hydroxymethyltransferase isoenzyme results in multiple auxotrophies for histidine, methionine, adenine and thymine [40]. These diverse products of C_1 metabolism are therefore normally synthesized by pathways that are dependent on the mitochondrial generation of 5,10-CH_2-H_4PteGlu. In pea mitochondria there is evidence for several folate-dependent reactions including those of glycine–serine interconversion, methionine synthesis and the generation of 10-HCO-H_4PteGlu from the corresponding methylene derivative [31]. On this basis, it is likely that the mitochondria of pea and peanut cotyledons have the ability to generate the folate substrate for methionyl-tRNA transformylase. Conceivably, glycine and serine, arising by proteolysis during germination [1], could provide C_1 units for this reaction.

In common with the methionyl-tRNA transformylases of other species [19], those of pea and peanut mitochondria utilized a monoglutamate folate substrate (Table 2). To date, long chain polyglutamates have not been examined as substrates of this enzyme. However, the bacterial transformylase [21] has equal affinity for 10-HCO-H_4PteGlu and the corresponding triglutamate. Studies of other folate-dependent enzymes [41] suggest that the transformylase may display greater affinities for this substrate when the glutamyl chain length exceeds three. In the light of the occurrence of highly conjugated folates in plant (Figs 3 and 4) and animal [38] mitochondria, the specificity of this enzyme warrants further study. Also of interest would be the assay of this plant enzyme in the presence of homologous methionyl-tRNA rather than the *E. coli* substrate used in the present work. It should be noted that the enzyme of *Euglena* chloroplasts [25] has an equal affinity for *E. coli* methionyl-tRNA or that isolated from *Euglena* cells. On the other hand, the yeast enzyme displays a preference for the native substrate [19].

It seems likely that the methionyl-tRNA transformylases of pea and peanut (Fig. 7) have a direct role in the initiation of organelle protein synthesis [19]. In pea, the incorporation of amino acids by isolated mitochondria (Table 3) had requirements that are indicative of peptide synthesis. It is therefore reasonable to conclude that the cotyledonary mitochondria of both species have the ability to form proteins during germination. As noted in the Introduction, this conclusion has support in the literature.

Although the nature of such mitochondrial proteins has not been fully elucidated, it is clear that the characteristic increase in respiratory activity of germinating seeds can be accompanied by a *de novo* synthesis of cytochrome oxidase [42]. However, in the Alaska variety of pea, this enzyme is initially generated from pre-existing subunits that can be detected in the dry seed [43]. As growth proceeds, in the present Homesteader variety, it is conceivable that this and other mitochondrial proteins may be formed by a *de novo* synthesis in which formyl-methionyl-tRNA^{fmet} acts in peptide chain initiation.

EXPERIMENTAL

Chemicals. Na [^{14}C]formate, L-[^{3}H-Me]methionine, L-[^{35}S]methionine and L-[4, 5-^{3}H]leucine were purchased from Amersham Corp. Bulk tRNA from *Escherichia coli* MRE 600 was supplied by Boehringer Mannheim. All other analytical grade chemicals were supplied by either Sigma or by Fisher Scientific.

Growth of plant material. Seeds of pea (*Pisum sativum* L. cv Homesteader) were surface sterilized by soaking for 5 min in 1% sodium hypochlorite containing Tween 80. After rinsing in distilled water, the seeds were soaked in sterile distilled water for 18 hr and then sown in moist, sterile vermiculite. Seeds of peanut (*Arachis hypogaea* L. cv Early Spanish), treated with a fungicide by the supplier (Stokes Seeds, Ontario), were soaked in sterile distilled water for 6 hr, rinsed and sown in moist, sterile vermiculite. Both species were grown at 25° in darkness. In all experiments, zero time was taken as the start of the soaking period.

Isolation of mitochondria and assessment of respiratory activity. Pea mitochondria were isolated and purified according to Johnson-Flanagan and Spencer [44]. Peanut mitochondria were isolated [8] and purified by discontinuous sucrose density gradient centrifugation [44]. The biochemical purity of these preparations was assessed by assay of marker enzymes including catalase [32], alcohol dehydrogenase [45] and succinic dehydrogenase [46]. Respiratory rates, ADP/O ratios and respiratory control ratios were determined polarographically using succinate as substrate [5]. Respiratory control and ADP/O ratios were calculated according to Chance and Williams [47].

Folate extraction and assay. Cotyledon folates were extracted in ascorbate [33] and stored at −25° until assayed. Mitochondrial folates were extracted by sonication, adjusted to 10% ascorbate (pH 6.0) and incubated at 100° for 10 min. Denatured protein was removed by centrifugation. Aliquots of these folate extracts were treated with plant γ-glutamyl carboxypeptidase [33] to hydrolyse native folylpolyglutamates. Folate contents were determined microbiologically [48, 49] using *Lactobacillus casei* (ATCC 7469) and *Pediococcus acidilactici* (ATCC 8081).

Preparation of labelled substrates. 10-[^{14}C]-HCO-H_4PteGlu was synthesized enzymically [50] with formyltetrahydrofolate synthetase isolated from *Clostridium cylindrosporum* (ATCC 7905) after growth in defined medium [51]. Methionyl-tRNA synthetase was isolated from *E. coli* (ATCC 10798) as previously described [52]. The reaction system for synthesis of [^{3}H]-met-tRNA was modified from ref. [52] and contained: 100 mM HEPES (pH 7.6), 10 mM $MgCl_2$, 10 mM KCl, 2 mM mercaptoethanol, 2 mM ATP, 60 A_{260} units/ml *E. coli* bulk tRNA, 22 μM L-[^{3}H-methyl]-methionine (510 μCi/μmol) and *ca* 240 μg/ml enzyme. The labelled product was separated by three phenol extractions and an overnight EtOH precipitation at −25°. Radiochemical purity of [^{3}H]methionine was periodically checked by automated amino acid analysis and [^{3}H]methionyl-tRNA was prepared 1 day prior to use in transformylase assays.

Assay of 10-HCO-H_4*PteGlu:methionyl-tRNA*^{fmet} *transformylase.* The assay was based on that of Crosti *et al.* [25]. The reaction mixture contained 150 mM HEPES (pH 7.6), 20 mM KCl, 20 mM mercaptoethanol, 0.1 mM EDTA, 0.55 μM [^{3}H]met-tRNA [dissolved in 60 mM HEPES (pH 7.6), 15 mM KCl, 15 mM $MgCl_2$], 10 μM 10-[^{14}C]-HCO-H_4PteGlu and 0.2–1 mg/ml protein. The reaction was terminated after 30 min at 30° by addition of H_2O satd PhOH. NaOAc (pH 4.8) was then added to a final concentration of 0.3 M. The formylated product was recovered by three PhOH extractions and two EtOH precipitations; the latter in a solid CO_2–EtOH bath. After dissolving in H_2O, radioactivity of the product was assessed by liquid scintillation counting. The formylated product was also subjected to PAGE. The 1 mm, 12% gels were prepared in 50 mM NaOAc (pH 4) and run at 11 V/cm until the dye front was *ca* 1 cm from the bottom. tRNA was visualized by staining with ethidium bromide. Labelled tRNAs were detected by fluorography as described by Laskey [53].

Mitochondrial protein synthesis. Incorporation of L-[^{3}H]leucine and [^{35}S]methionine into mitochondrial protein [54] was examined at 25°. The reaction mixture (0.5 ml) contained 0.25 M mannitol, 12.5 mM HEPES, 5 mM KH_2PO_4, 15 mM $MgCl_2$, 12.5 mM KCl, 20 mM malate, 2 mM ADP, 25 μM amino acid mixture (minus leucine or methionine) and 1 μCi [^{3}H]leucine (136 Ci/mMol) or [^{35}S]methionine. The final pH was 7.2 and mitochondria (*ca* 750 μg protein) were added to initiate the reaction. After incubation for periods up to 1 hr, the samples were assessed for labelled protein [55]. Bacterial contamination was monitored by plating samples of the complete reaction mixture after incubation, on Plate Count Agar (Difco). Colonies were counted after 48 hr at 37° and were routinely found to be less than 2000 per assay, a level previously shown to have no significant affect on the observed rates of amino acid incorporation [54].

Protein determination. The method of Lowry *et al.* [56] was employed with bovine serum albumin (Fraction V, Calbiochem) as standard.

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