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Subcellular Localization of the Pathway of *de Novo* Pyrimidine Nucleotide Biosynthesis in Pea Leaves[1]

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ABSTRACT

The subcellular distribution of the enzymes of *de novo* pyrimidine nucleotide biosynthesis was investigated in pea (*Pisum sativum* L. cv Progress No. 9) leaves. Aspartate carbamoyltransferase, the committed step of the pathway, was found to be strictly confined to the chloroplasts. Dihydro-orotase, orotate phosphoribosyl transferase, and orotidine decarboxylase activities were also found only in the plastids. The remaining enzyme of the pathway, dihydroorotate dehydrogenase, was shown to be mitochondrial.

Pyrimidine nucleotide biosynthesis in all organisms proceeds via the general scheme shown in Figure 1 (7, 12, 18). In bacteria, each of the steps is catalyzed by a separate enzyme; all of these are soluble except DHOdeHase[2], which is membrane-bound (18). In *Neurospora* and yeast, CPSase activity dedicated to pyrimidine biosynthesis resides on the same soluble polypeptide as ACTase activity (7) while the remaining enzymes are separate. DHOdeHase is mitochondrial in these organisms (15); the others are all soluble. Pyrimidine biosynthesis in mammalian cells involves two multifunctional soluble proteins: the CPSase/ACTase/DHOase complex and the OPRTase/ODCase complex. Each consists of just one polypeptide catalyzing multiple reactions (8). The intervening step, dehydrogenation of dihydroorotate, is carried out by an enzyme of the inner mitochondrial membrane (3) which feeds electrons directly to the respiratory chain (5).

Little is known of the organization or subcellular location of this pathway in plants. There is thought to be only one CPSase (19, 20) as in bacteria (18). This activity is found in the plastids of suspension cultured soybean cells and of pea leaves (24, 25). In contrast, the bulk of the OPRTase and ODCase of cultured *Vinca rosea* cells (9) and *Phaseolus mungo* seedlings (1) was found in the high-speed supernatant fraction after differential centrifugation. This was taken as evidence that these enzymes are primarily located in the cytosol. These two activities have recently been shown to reside on a single polypeptide in tomato cells (27). ACTase activity has also been reported to be cytosolic in *Vinca rosea* (9).

Given the lack of information available, we decided to investigate the subcellular location of *de novo* pyrimidine biosynthesis in pea leaves. Using techniques which keep nearly all of the organelles intact, we have been able to demonstrate that four steps of the pathway, catalyzed by ACTase, DHOase, OPRTase, and ODCase, take place in the plastids and the remaining reaction, dehydrogenation of dihydroorotate, occurs in the mitochondria.

MATERIALS AND METHODS

Materials. Percoll and Sephadex were from Pharmacia, Cellulysin and Macerase from Calbiochem, [^{14}C]aspartate and [^{14}C] orotate from Amersham, and Dowex from BioRad. All other biochemicals were from Sigma. Pea (*Pisum sativum* L. cv Progress No. 9) seeds were obtained from Agway Corp. They were germinated and grown in vermiculite for 14 to 20 d on a 12 h light/12 h dark cycle at 21 to 23°C.

Preparation of Chloroplast Extract. Intact chloroplasts were isolated by a modification of the method of Fish and Jagendorf (4). Pea shoots were harvested into a grinding medium which consisted of 330 mM sorbitol, 50 mM Taps-KOH (pH 8.4), 0.05% BSA (w/v), and 5 mM ascorbic acid (added just before use). The shoots were cut into small pieces with scissors and then homogenized in a Polytron homogenizer for about 30 s. The homogenate was filtered through cheesecloth and centrifuged at 1500*g* for 3 min. The pellet was resuspended in a small volume of grinding medium and layered onto a 26-ml linear 25% (v/v) to 92% (v/v) Percoll gradient containing 330 mM sorbitol, 50 mM Taps-KOH (pH 8.4) and gradients of 0.75 to 2.76% (w/v) polyethylene glycol 3350, 0.25 to 0.92% (w/v) Ficoll and 0.12 to 0.46% (w/v) BSA. The gradient was centrifuged at 13,200*g* for 7 min in a swinging bucket rotor. Intact chloroplasts were collected from the lower green band and washed once with grinding medium. The pellet was resuspended in 1 to 2 ml of breaking medium, which consisted of 30 mM Hepes-NaOH (pH 7.2), 25 mM KCl, 5 mM $MgCl_2$. Chl concentration was measured, and the thylakoids were removed by centrifugation in an Eppendorf centrifuge at 12,800*g* for 10 min. The clear supernatant was desalted by centrifuging through a small column of Sephadex G-50 Fine (16) and then used for enzyme assays.

Preparation of Whole Leaf Extract. Pea leaves were homogenized in a medium consisting of 30 mM Hepes-NaOH (pH 7.2), 25 mM KCl, 5 mM $MgCl_2$, and 5 mM ascorbic acid (added just before use). After filtration through cheesecloth, an aliquot was removed for measurement of Chl concentration. The homogenate was then centrifuged 3 times for 10 min each at 12,000*g*, discarding the pellet each time. The final supernatant was desalted in the same manner as the chloroplast extract prior to use in enzyme assays.

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[2] Abbreviations: DHOdeHase, dihydroorotate dehydrogenase (EC 1.3.3.1); ACTase, aspartate carbamoyltransferase (EC 2.1.3.2); CPSase, carbamoyl-phosphate synthetase (EC 6.3.5.5); DHOase, dihydro-orotase (EC 3.5.2.3); OPRTase, orotate phosphoribosyl transferase (EC 2.4.2.10); ODCase, orotidine-5′-phosphate decarboxylase (EC 4.1.1.23); Taps, tris(hydroxymethyl) methylaminopropanesulfonic acid; Mops, 3-(N-morpholine) propanesulfonic acid; Ches, 2-(N-cyclohexylamino) ethanesulfonic acid; DCIP, 2,6-dichlorophenolindophenol; PRPP, 5-phosphoryl-ribose 1-pyrophosphate.

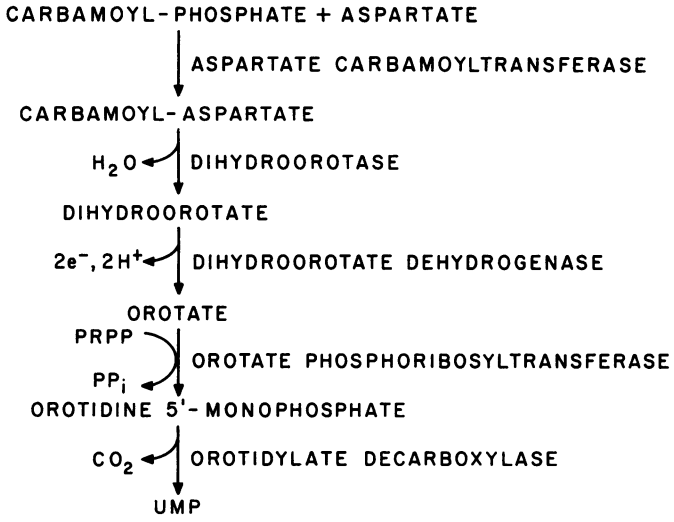

FIG. 1. Pathway of *de novo* pyrimidine nucleotide biosynthesis.

Preparation of Mitochondria. Mitochondria were isolated by a modification of the method of Nishimura *et al.* (17). Pea leaves were ground with a Polytron homogenizer in a medium consisting of 300 mM sorbitol, 10 mM Mops-KOH (pH 7.2), 1 mM EDTA, and 0.1% (w/v) BSA. The homogenate was filtered through cheesecloth and centrifuged at 3,000*g* for 10 min. The pellet was discarded. Thre supernatant was centrifuged for 20 min at 17,300*g*. The pellet from this second spin was resuspended in a small amount of grinding medium and layered on a step gradient of 4 ml 15% (v/v), 4 ml 25% (v/v), and 4 ml 60% (v/v) Percoll, each layer containing 250 mM sucrose, 20 mM Mops-KOH (pH 7.2), and 0.2% (w/v) BSA. This gradient was spun for 30 min at 30,000*g* in a swinging bucket rotor. Mitochondria were collected from the 25%/60% interface.

Preparation of Protoplasts. Protoplasts were prepared by a modification of the method of Wallsgrove *et al.* (26). Fully expanded pea leaves were harvested and the lower epidermis removed with forceps. The leaves were floated on digestion medium containing 550 mM sorbitol, 1 mM KH_2PO_4 (pH 5.5), and 5 mM $MgCl_2$. After a few min, Cellulysin and Macerase in digestion medium were added to final concentrations of 2% (w/v) and 0.5% (w/v), respectively. The leaves were incubated in the dark at 30°C for 3 h, then the medium was gently swirled and poured through cheesecloth. The leaves were washed once with digestion medium. The filtrate was centrifuged for 2 min at 150*g*. The protoplasts were gently resuspended by swirling the tubes, and layered on a step gradient of 3 ml 0.3 M sucrose, 0.25 M sorbitol, 50 mM Tricine (pH 7.5), and 3 ml 0.7 M sucrose, 50 mM Tricine (pH 7.5). After centrifugation at 150*g* for 4 min, intact protoplasts were collected from the lower interface.

Sucrose Gradient Centrifugation. BSA was added to the protoplasts to a final concentration of approximately 0.1 to 0.2% (w/v). The protoplasts were ruptured by one or two passes through 20 μm or in later experiments 5 μm nylon mesh attached to a syringe. The broken cells were layered on a 14 ml linear gradient of 25% (w/w) to 60% (w/w) sucrose containing 50 mM Tricine-KOH (pH 7.5) in a cellulose nitrate tube. The gradient was centrifuged in a Beckman SW-27 rotor for 4 min at 4,000 rpm and then for 10 min at 10,000 rpm. Gradients were fractionated by upward displacement with 60% (w/w) sucrose.

Enzyme Assays. The reaction mixture for ACTase consisted of 20 mM Ches-NaOH (pH 9.5), 25 mM [^{14}C]aspartate (40 μCi/mmol), 4 mM Li_2-carbamoyl phosphate (freshly dissolved) and extract in a total volume of 120 μl. After incubation at 37°C, the reaction was terminated by addition of 80 μl 5% TCA and the precipitate centrifuged out. A 150 μl aliquot was applied to a 0.3 ml Dowex 50W-X12 (H^+ form) column, and eluted with 0.6 ml water. Under these conditions aspartate remained bound to the column and the product of the reaction, carbamoyl aspartate, was eluted. The eluate was collected in a scintillation vial and counted in a Triton-Toluene based scintillation cocktail.

DHOase was assayed in the reverse direction. The reaction mixture consisted of 50 mM Tris-HCl (pH 8.5), 1 mM dihydroorotate (pH 8.5), and extract in a total volume of 1 ml. After incubation at 37°C, carbamoyl aspartate produced was assayed colorimetrically by the method of Prescott and Jones (22).

DHOdeHase was assayed by Cyt *c* reduction. The reaction mixure contained 100 mM KH_2PO_4 pH 7.0, 10 mM KCN, 0.01% (w/v) Triton X-100, 0.02 mM Cyt *c* and extract in 1 ml. The assay was started by addition of 100 μl of 10 mM dihydroorotate (pH about 7) and the change in *A* at 550 nm recorded. No change in absorbance was seen in the absence of either extract or substrate. DHOdeHase was also assayed by DCIP reduction using a reaction mixture of 100 mM KH_2PO_4 (pH 7.0), 10 mM KCN, 0.1 mM DCIP, 1 mM dihydroorotate (pH about 7), and sample in 1 ml and recording the change in *A* at 600 nm.

OPRTase and ODCase were assayed in a coupled system similar to that of Ashihara (1). A half-dram vial was placed within a four-dram vial and 25 μl each of 500 mM Tris-HCl (pH 8.8), 10 mM $MgCl_2$, 6 mM PRPP (freshly dissolved), and 2.5 mM carboxyl[^{14}C]orotate (400 μCi/mmol) were pipetted into the inner vial. A Whatman No. 1 paper wick soaked with 0.1 N NaOH was placed in the outer vial, which was then capped with a serum stopper. The reaction was started by injecting 150 μl extract through the stopper into the inner vial. After incubation at 37°C, the reaction was stopped by injecting 200 μl 5% TCA into the inner well. The vials were left at 37°C for an additional h to allow $^{14}CO_2$ to be trapped. The wicks were then removed and counted in Triton-Toluene cocktail.

Cyt *c* oxidase was assayed as described previously (6) except that 0.1% (w/v) Triton X-100 was substituted for digitonin. Sucrose-phosphate synthase (23), fumarase (14), and catalase (13) were assayed as described previously.

Other Assays. Sucrose concentration was determined using a refractometer. Chl was measured by absorbance in 95% ethanol (28) and protein was estimated using the BioRad protein assay reagent.

RESULTS

Chloroplasts purified on Percoll density gradients showed very little contamination by mitochondria, peroxisomes, or cytosol (Table I). Therefore, if an enzyme activity is found in a chloroplast extract, particularly if a large proportion of the activity is found in this fraction, it is reasonable to conclude that the enzyme is located within the chloroplast *in vivo*.

As shown in Table II, considerable ACTase activity was found

Table I. *Comparison of Marker Enzyme Assays in Whole Leaf and Chloroplast Extracts*

Chloroplasts were prepared as stated in "Materials and Methods." Assays were conducted after osmotic rupture, removal of membranes, and desalting. Whole leaf extract was prepared as stated in "Materials and Methods" and desalted prior to use in assays.

Enzyme	Activity in Whole Leaf Extract	Activity in Chloroplast Extract	% of Total Activity in Chloroplast Extract
Catalase	12.3[a]	1.1[a]	8.9
Fumarase	0.40[a]	0.02[a]	5.0
Sucrose-phosphate synthase	1.29[b]	ND[c]	4

[a] Change in A_{240}/min·mg Chl. [b] μmol/mg Chl·h. [c] ND, not detectable.

Table II. *ACTase Activity in Chloroplasts*

Chloroplasts were prepared as detailed in "Materials and Methods." The complete reaction mixture contained 185 mM glycine-NaOH (pH 10.5), 12.5 mM [^{14}C]aspartate (12 nCi), 2.5 mM Li_2-carbamoyl phosphate and extract in a total volume of 80 μl. Incubation was for 1 h at 37°C in experiment 1 and for 20 min at 37°C in experiment 2.

Treatment	cpm	% of Complete
Experiment 1		
Complete	4921	100
−extract	393	8
−carbamoyl phosphate	400	8
complete + 1.25 mM UMP	1904	39
complete + 1.25 mM CMP	4995	102
Experiment 2		
Complete	2406	100
−extract	345	14
+1 mM UMP	350	14
+1 mM UDP	1013	42
+1 mM UTP	2015	84

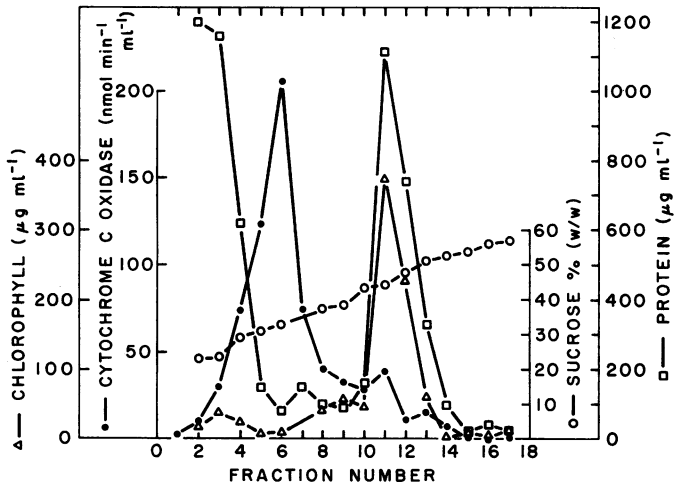

FIG. 2. Distribution of Chl, protein, and Cyt-oxidase activity following sucrose density gradient centrifugation of ruptured pea leaf protoplasts. Fraction 1 represents the top of the gradient.

in pea chloroplast extracts. Activity was dependent upon the presence of both substrate (carbamoyl phosphate) and extract. Low levels of uridine nucleotides were inhibitory, but cytidine nucleotides had no effect, as previously reported for ACTase from other plant sources (21, 29). The order of inhibition by uridine nucleotides was UMP > UDP > UTP as found for the enzyme from mung beans (21). The pH optimum was at 9.5 with a secondary maximum at about 8.0 (data not shown); this finding is consistent with what is known of the wheat germ enzyme (29). Based on comparison of activity in chloroplast extracts with that in crude leaf homogenates, most of the ACTase activity appears to be chloroplastic (data not shown).

To investigate the possibility that ACTase might be strictly plastidic, an extract in which most of the organelles were intact was required. This was obtained by preparing protoplasts and breaking them gently by passage through 20 or 5 μm nylon mesh. These broken protoplasts were then fractionated on sucrose density gradients. Figure 2 shows a typical gradient.

Cytosolic proteins remain in the volume loaded onto the gradient and, in agreement with the results of Wallsgrove *et al.* (26), the various organelles were well separated; mitochondria were found to band at about 33% sucrose, peroxisomes (by catalase activity, not shown in Figure) at about 38% sucrose and intact chloroplasts at about 45% sucrose. Broken chloroplasts band just above the intact ones; in general, however, 90% or more of the Chl on the gradient is found in the intact plastic band.

It is conceivable that enzymes might be inactivated at the top of the gradient where cytosolic and vacuolar contents are mixed. While we cannot eliminate the possibility of some extremely sensitive activity, there was no general inactivation in these fractions; on those gradients which had a larger than usual proportion of broken chloroplsts we had no difficulty detecting enzyme activities in the fractions at the top of the gradient. When ACTase was assayed on fractions from a sucrose density gradient, only one peak of activity was found, coinciding with the Chl peak (Fig. 3). This indicates that all the ACTase activity in these pea leaf cells is in the chloroplasts.

The second enzyme of the pathway, DHOase, was also found in chloroplast extracts (Table III). Again, the reaction was de-

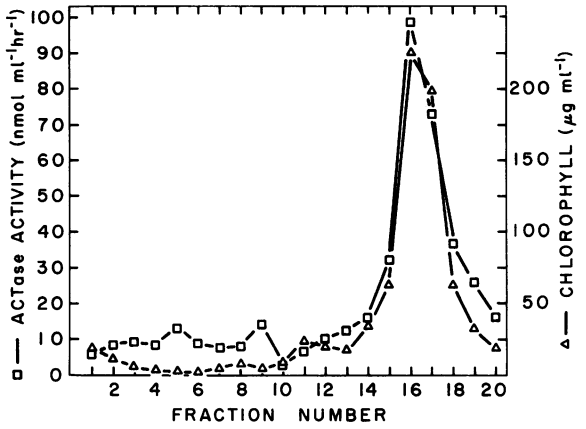

FIG. 3. Distribution of aspartate carbamoyltransferase activity on a sucrose density gradient of ruptured pea leaf protoplasts.

Table III. *DHOase Activity in Chloroplasts*

The complete reaction mixture contained 50 mM Tris-HCl (pH 8.5), 1 mM dihydroorotate (pH 8.5) and extract in a total volume of 1 ml. Incubation was at 37°C for 30 min.

Treatment	A_{466}	Apparent Carbamyl Aspartate Formed	% of Complete
		nmol	
Complete	0.730	86	100
−extract	0.055	13	15
−dihydroorotate	0.021	5	6

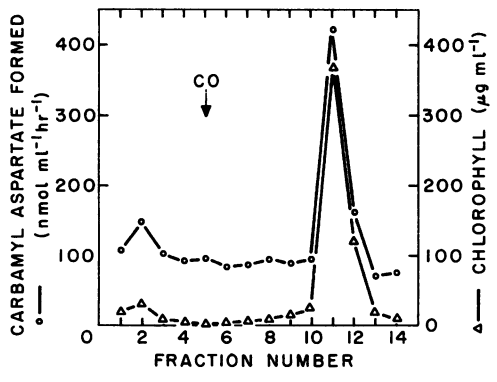

FIG. 4. Distribution of dihydroorotase activity on a sucrose density gradient of ruptured pea leaf protoplasts. The arrow indicates the location of the peak of Cyt oxidase activity.

pendent on addition of extract and subtrate. The relatively high absorbance in the absence of extract was apparently due to the presence of a chromogenic substance, probably carbamoyl-aspartate, in the dihydroorotate used. The assay was linear with amount of extract and with time to 30 min (data not shown). Assay of sucrose gradient fractions indicated that this enzyme also is strictly chloroplastic (Fig. 4).

DHOdeHase was assayed in two ways: by reduction of DCIP or of Cyt *c*. Using the DCIP reduction assay of Karibian (11), activity was found to be associated with Percoll-purified mitochondria, but only a trace of activity was associated with chloroplasts (data not shown). No activity was found if NAD or NADP were used as electron acceptors. Because of a high and variable dihydroorotate-independent DCIP-reducing activity, this assay could not be used on sucrose gradient fractions. To demonstrate the mitochondrial nature of this enzyme positively, therefore, we developed an assay based on reduction of Cyt *c* via the respiratory electron transport chain in the presence of sufficient KCN to inhibit Cyt *c* oxidase. Cyt *c* reduction in this assay was completely dependent on extract and dihydroorotate and was completely inhibited by 5 μg/ml of Antimycin A, which blocks electron transport between Cyt *b* and *c* (Table IV).

These data provide convincing evidence that there is DHOdeHase activity in the mitochondria. Although we cannot rule out the possibility that this activity is also present in some other compartment, the fact that no activity is found using NAD or NADP as electron acceptors, and that without KCN no DCIP reduction could be observed, makes this unlikely.

The final two activities of the pathway, OPRTase and ODCase, were assayed together in a coupled system. Percoll-purified chloroplasts exhibited OPRTase/ODCase activity which was dependent on the presence of extract and PRPP (Table V) and was linear with time for at least 60 min. The pH optimum was broad and centered at 8.5 (data not shown), consistent with data for tomato (27) and black gram (1).

When OPRTase/ODCase was assayed on sucrose gradient fractions of green pea leaf cells, two peaks of activity were observed (Fig. 5). This could mean that this activity is found in both the cytosol and chloroplasts. However, it is also possible that the activity at the top of the gradient originates from the small proportion of chloroplasts which were broken during preparation. Since the assay is based on the release of very small amounts of $^{14}CO_2$ from carboxyl-labeled orotate, any refixation of $^{14}CO_2$ by ribulosebisphosphate carboxylase would cause enzymic activity to be underestimated. Both the carboxylase and the substrate, ribulose bisphosphate, are localized in relatively high concentrations in the stroma of intact chloroplasts, but are considerably diluted in the cytosol fraction at the top of the gradient, which contains only a small amount of stroma from the new broken chloroplasts. This selective refixation of $^{14}CO_2$ would diminish apparent activity most in the chloroplasts, and thus would make the peak of activity at the top of the gradient appear to represent a disproportionately large fraction of the total. To test this possibility, we made protoplasts from etiolated pea leaves, which should contain little carboxylase activity. These cells were broken and fractionated on a sucrose gradient; the OPRTase ODCase activity from this gradient is shown in Figure 6. One large peak of activity was seen in the plastid density region, and a much smaller peak occurred near the top of the gradient; this suggests that these activities are truly plastidic. Although we cannot exclude the possibility that one of the enzymes my also be found in some other compartment, the recent finding that the two activities reside on a single polypeptide in tomato cells (27) makes this extremely unikely.

Table IV. *DHOdeHase Activity in Mitochondria*

Mitochondria were prepared as stated in "Materials and Methods." The complete reaction mixture contained 100 mM KPO_4 (pH 7.0), 1 mM dihydroorotate, 10 mM KCN, 0.02 mM Cyt *c*, 0.01% (w/v) Triton X-100, and extract in a total volume of 1 ml.

Treatment	A_{550}/min
Complete	0.051
−dihydroorotate	−0.003
−extract	0.000
+5 μg antimycin a	−0.004

Table V. *OPRT/ODCase Activity in Chloroplasts*

The complete reaction mixture contained 50 mM Tris-HCl (pH 8.8), 1 mM $MgCl_2$, 0.6 mM PRPP, and 0.25 mM carboxyl-^{14}C-orotate. Incubation was at 37°C for 30 min.

Treatment	cpm	% of Complete
Complete	2386	100
−extract	115	5
−PRPP	196	8

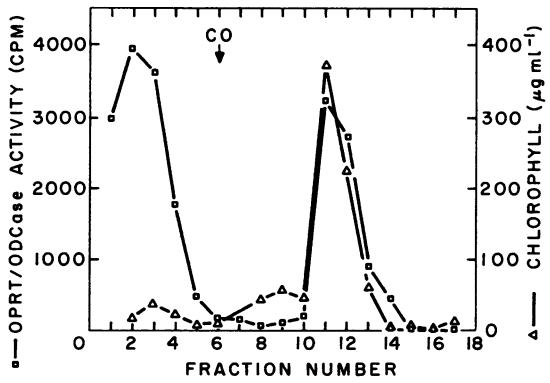

FIG. 5. Distribution of OPRTase/ODCase activity on a sucrose density gradient of ruptured pea leaf protoplasts. The peak of Cyt oxidase activity was in the fraction indicated by the arrow. The assay for OPRTase/ODCase activity was incubated for 2 h.

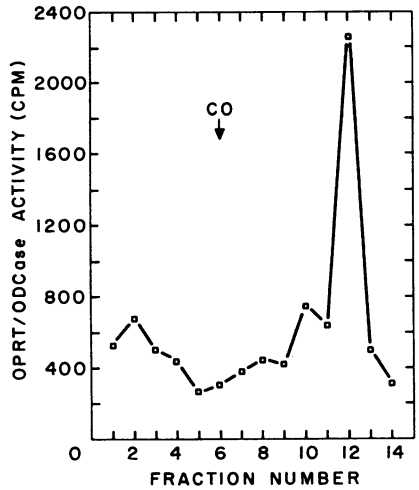

FIG. 6. Distribution of OPRTase/ODCase activity on a sucrose density gradient of rutured protoplasts from etiolated pea leaves. Plants were grown jn the dark for 11 d before harvesting. Protoplasts were prepared and run on a sucrose gradient in the same manner as those from green leaves. The OPRTase/ODCase assay was run for 2 h. The arrow indicates the fraction which showed the peak of Cyt oxidase activity.

DISCUSSION

To date, characterization of the pyrimidine nucleotide biosynthetic pathway in plants has been limited. Athough the pathway

as found in mammals and bacteria has also been shown to occur in higher plants (12), there has been little work on the subcellular localization of this pathway. The results presented here demonstrate that ACTase, DHOase, OPRTase, and ODCase activities are confined to the plastids of pea leaves, while DHOdeHase is mitochondrial. These results conflict with those of Ashihara and colleagues, who reported that ACTase, OPRTase and ODCase in *Vinca rosea* (9), and OPRTase and ODCase in *Phaseolus mungo* (1) were predominantly cytosolic. In their studies, homogenates were fractionated by differential centrifugation. Since they did find a small amount of each activity in the low speed pellet, and since no marker enzyme distributions were reported, the discrepancy between our results and theirs may be explained by breakage of a large proportion of the organelles in their homogenization procedure.

Kapoor and Waygood (10) reported a soluble orotate-dependent NADH-oxidizing activity in wheat embryos. They believed that this activity was the DHOdeHase of this tissue operating in the reverse direction. We did not find any DHOdeHase activity when NAD or NADP was used as electron acceptor, although we did not try to run the reaction in the reverse direction. Activity was detected in a mitochondria-enriched fraction using dihydroorotate dependent DCIP reduction as the assay, but only in the presence of KCN, indicating that electrons were preferentially transferred to the respiratory chain. Demonstration of a dihydroorotate-dependent Cyt *c* reductase activity, inhibited by antimycin A, provided further evidence that pea leaf DHOdeHase is closely linked to the mitochondrial electron transport chain. DHOdeHase of fungi (15) and mammals (3) is known to be a mitochondrial enzyme, feeding electrons into the respiratory chain. Even in bacteria, the biosynthetic DHOdeHase is membrane-bound and uses O_2 or ferricyanide as the final electron acceptor (18). The only well-documented exception to this pattern is an inducible, degradative DHOdeHase in bacteria such as *Zymobacterium oroticum* which appears only when the organism is grown on orotate as the sole carbon source. This enzyme is soluble and interacts with NADH. However, even in these organisms, the biosynthetic enzyme is membrane-bound and linked to O_2 (18).

The subcellular organization of the pyrimidine biosynthetic pathway in pea leaves demonstrated here demands that several intermediates and products be in rapid equilibrium across the chloroplast envelope. In order for the pathway to operate efficiently, dihydroorotate must leave the chloroplast and orotate re-enter. Access to the DHOdeHase does not introduce another barrier because the enzyme is located on the outer surface of the inner mitochondrial membrane, where it is freely accessible to small molecules (3). The pools of UMP outside and inside the chloroplast must be in equilibrium for feedback regulation of ACTase to work effectively, and the products of the pathway must be made available to other compartments of the cell. A carrier capable of concentrating pyrimidine bases or nucleosides from the medium into isolated chloroplasts has been reported (2). These authors did not investigate transport of pyrimidine nucleotides.

The subcellular organization of pyrimidine biosynthesis is different in bacteria, fungi, and animals; plants seem to have yet another organizational scheme. In prokaryotes, each of the activities is on a separate polypeptide; none of the genes is closely linked (18). In eukaryotes, though, some of these genes have fused to form multifunctional polypeptides. Fungi and animals each have two carbamoyl phosphate synthetases; one dedicated to pyrimidine biosynthesis, the other to arginine synthesis or the urea cycle. The pyrimidine pathway enzyme is fused with ACTase in fungi (7) and with ACTase and DHOase in animals (8). Animals also have a second multifunctional polypeptide, containing OPRTase and ODCase activities. Only one CPSase has been detected in plants, providing carbamoyl phosphate for both the pyrimidine and arginine pathways. In agreement with the observations noted previously (24, 25) we have detected this enzyme and the committed reaction of arginine biosynthesis, ornithine carbamoyltransferase, in pea chloroplasts (H. D. Doremus, A. T. Jagendorf, unpublished data). The fact that one CPSase feeds both carbamoyltransferases makes it unlikely that it is fused to the ACTase. There is also no conclusive evidence as to whether ACTase and DHOase activities from higher plants are closely linked. On the other hand, OPRTase and ODCase in tomato cells are known to reside on a single polypeptide (27).

Pyrimidine biosynthesis is largely cytosolic in animal and fungal cells, but as we have shown, these reactions occur only in the chloroplasts of pea leaves. The evolutionary origin and functional significance of this difference in subcellular organization are questions of continuing interest.

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