Subcellular localization of two porphyrin-synthesis enzymes in *Pisum sativum* (pea) and *Arum* (cuckoo-pint) species

Alison G. SMITH

Department of Botany, University of Cambridge, Downing Street, Cambridge CB2 3EA, U.K.

The subcellular location of the two porphyrin-synthesis enzymes 5-aminolaevulinate dehydratase (ALAD) and porphobilinogen deaminase (PBGD) was investigated in *Pisum sativum* (pea) leaves and spadices of *Arum* (cuckoo-pint). Throughout the tissue-fractionation procedures the distribution of the two enzymes paralleled that of the plastid marker enzyme (ADP-glucose pyrophosphorylase), even in *Arum*, a tissue where the synthesis of non-plastid haem is predominant. The distribution of cytosolic marker enzyme (lactate dehydrogenase) was significantly different from that of ALAD and PBGD and, although purified mitochondria from both species had some residual activity, this was always less than contaminating plastid marker enzyme. The results suggest that ALAD and PBGD are exclusively plastid enzymes. The significance of this for the role of plastids in cellular porphyrin synthesis is discussed.

INTRODUCTION

Higher-plant cells contain a number of different porphyrin derivatives located in several compartments. The major one, chlorophyll (Chl), is confined to chloroplasts, but haem is found in mitochondria and peroxisomes, as well as in chloroplasts and other plastids. In addition, although the precise distribution of phytochrome, whose chromophore is a linear tetrapyrrole, is uncertain, immunofluorescent studies have shown it to be evenly distributed throughout the cells of dark-grown tissue (Saunders *et al.*, 1983). It is well established that plastids can make 5-aminolaevulinate (ALA), the precursor of porphyrins, and then make chlorophyll from it (for a review, see Castelfranco & Beale, 1981). Since high levels of ferrochelatase activity have been demonstrated in plastids (Little & Jones, 1976), they can presumably also make haem. However, the question still remains, where is non-plastid porphyrin synthesized?

In greening etiolated barley (*Hordeum*) leaves, both haem and Chl were labelled preferentially by glutamate rather than by glycine (Castelfranco & Jones, 1975). ALA is made from glutamate in chloroplasts, but from glycine in mitochondria (Castelfranco & Beale, 1981). This implies that at least the initial precursor of this haem was made in plastids. However, since the source of the labelled haem was not established, this does not eliminate the possibility that non-plastid haem was made elsewhere, nor indeed that ALA made in the plastids was then exported for the subsequent steps of the pathway.

Some support for the suggestion that other compartments may be involved in porphyrin synthesis comes from the study of cultured peanut (*Arachis hypogaea*) cells, where as much as 2% of the protein they synthesize is the haemoprotein cationic peroxidase. After incubation of the cells in [¹⁴C]ALA for 4 h, most of the label in haem was associated with the mitochondrial rather than the amyloplast fraction (Chibbar & van Huystee, 1986), which led the authors to suggest that the haem for

peroxidase is made in mitochondria rather than in amyloplasts. However, their data cannot rule out the possibility of exchange of mitochondrial haem with that made elsewhere in the cell, and, since no recovery data for the incorporation of [¹⁴C]ALA nor the distribution of marker enzymes were presented, this contention must be viewed with caution.

In addition, although many authors have concluded that porphyrin-synthesis enzymes are found in organelles other than plastids, except for a rigorous study by Little & Jones (1976), which found ferrochelatase activity in both mitochondria and plastids of barley, the assignments of non-plastid locations to ALAD (Nandi & Waygood, 1967), coproporphyrinogen oxidase (Hsu & Miller, 1970) and protoporphyrinogen oxidase (Jacobs & Jacobs, 1987) were not supported by characterization of the subcellular fractions by marker enzymes, nor were recovery data for the enzymes presented.

The present study was carried out in order to determine whether there is more than one location for porphyrin synthesis within the plant cell, or whether it is confined to the plastids. The subcellular distribution of two of the enzymes involved, namely ALAD and porphobilinogen deaminase (PBGD), was investigated in pea and *Arum*. The results indicate that, unlike in animal cells (Elder, 1976), the enzymes are not present in the cytosol, nor are they in mitochondria, but rather they are associated exclusively with the plastids, even in tissues like the spadices of *Arum*, where the synthesis of mitochondrial haem is predominant.

EXPERIMENTAL

Materials

ALA hydrochloride, trypsin and soybean trypsin inhibitor were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Pyruvate, hydroxypyruvate, 3-phosphoglycerate, NAD⁺, NADH, ADP and alkaline

Abbreviations used: ALA, 5-aminolaevulinate; ALAD, 5-aminolaevulinate dehydratase; PBG, porphobilinogen; PBGD, porphobilinogen deaminase; Chl, chlorophyll; NAD-ME, NAD⁺-dependent 'malic' enzyme; ADPG-P, ADP-glucose pyrophosphorylase; PPase, alkaline pyrophosphatase; HPR, hydroxypyruvate reductase; LDH, lactate dehydrogenase.

phosphatase were obtained from Boehringer, Lewes, East Sussex, U.K. [$U^{14}C$]Glucose-1-phosphate and ^{125}I -protein A were from Amersham International, Aylesbury, Bucks., U.K. Porphobilinogen and antibodies to *Euglena* PBGD were gifts from Dr. C. Abell, University Chemical Laboratory, Cambridge, U.K. Antibodies to spinach (*Spinacia oleracea*) ALAD were from Professor H. A. W. Schneider, University of Cologne, Cologne, Federal Republic of Germany.

Plant material

Peas (*Pisum sativum*, var. Feltham First) were sown in compost and grown in complete darkness, or on a 16 h day/8 h night cycle, at 25 °C for 7–8 days. Spadices from *Arum maculatum* L. and *Arum typhonium* L. at the β -stage (ap Rees *et al.*, 1983) were collected from the Botanic Gardens, Cambridge, U.K., and used within 1 h.

Tissue fractionation

Plastid preparation. Green pea leaves were homogenized with a Polytron instrument for 10 s in 0.33 M-sorbitol/5 mM-MgCl₂/50 mM-Tris/HCl (pH 8)/0.1 % bovine serum albumin/1 % 2-mercaptoethanol (GR solution) and then centrifuged at 6000 *g* for 2 min. The crude plastid pellet was resuspended carefully with a small paintbrush in GR solution and, to achieve further purification, layered on to a 20–80 % (v/v) Percoll gradient in GR solution. The gradient was centrifuged at 5000 *g* for 20 min.

Mitochondrial preparation. Etiolated pea leaves or *Arum* spadices were chopped with a razor blade in 0.3 M-sucrose, 25 mM-Hepes/NaOH (pH 7.6)/1 mM-EDTA/0.1 mM-dithiothreitol (SIM solution) for 10–15 min at 4 °C and filtered through eight layers of muslin. After centrifugation at 10000 *g* for 15 min the crude mitochondrial pellet was resuspended carefully in SIM solution with a small paintbrush. For further purification the crude pellet was adjusted to 35 % (w/v) sucrose and then layered on to a 35–55 % (w/v) sucrose gradient over a 65 %-(w/v)-sucrose cushion. The gradients were centrifuged in a Beckman SW40 rotor at 24000 rev./min for 15 min (pea) or 27000 rev./min for 3 h (*Arum*).

Enzyme assays

ALAD activity was measured as the amount of PBG produced after incubation of the extract with 2.5 mM-ALA for 1 h at 37 °C (Schneider, 1970). The incubation was carried out in 100 mM-Hepes (pH 8.2)/12 mM-MgCl₂/5 mM-2-mercaptoethanol/0.02 % Triton X-100. Similarly, PBGD activity was measured as the amount of uroporphyrinogen I produced after incubation of the extract with 0.2 mM-PBG at 37 °C for 1 h (Bogorad, 1962). The incubation mix contained 0.2 M-sodium phosphate (pH 8.0)/0.6 mM-EDTA/0.02 % Triton X-100, except for latency experiments, when the Triton X-100 was added at the end of the incubation.

NAD-ME and HPR activities were measured as described by ap Rees *et al.* (1983), PPase as described by Gross & ap Rees (1986), ADPG-P as described by MacDonald & ap Rees (1983) and LDH as described by Davies & Davies (1972).

Chl determination

Chl was determined by the method of Arnon (1949) after extracting the samples in 80 % (v/v) acetone.

Trypsin-protection experiments

The effect of trypsin on enzyme activity was tested by incubating extracts at room temperature for 45 min with trypsin (5 mg/ml) with and without trypsin inhibitor (7.5 mg/ml) and with and without Triton X-100 (0.05 %). After the incubation, trypsin inhibitor and Triton-X-100 were added to those samples which did not have them, and these treated extracts were assayed for ALAD and PBGD activity as described above. The results were expressed as a percentage of the activity in an untreated sample, that is, one to which trypsin inhibitor, Triton X-100 and trypsin were added immediately before the enzyme assay.

Western blotting

Protein samples were electrophoresed on 15 % (w/v) polyacrylamide gels containing SDS, blotted on to nitrocellulose and then challenged with polyclonal antibodies, essentially as described by Smith & Gray (1984).

RESULTS

Subcellular localization of ALAD and PBGD in green pea leaves

Initial experiments were carried out on 7-day-old green pea leaves, since these contain much higher activities of ALAD and PBGD than do etiolated pea leaves (Smith, 1986). Table 1 shows the distribution of Chl and enzyme activities after the fractionation described in the Experimental section. After the initial 6000 *g* spin the pellet contained 60 % of the Chl, but only 10 % of the soluble plastid marker enzyme ADPG-P (MacDonald & ap Rees, 1983), which suggests that most of the chloroplasts have ruptured during homogenization. Similar results have been observed by others (Ellis, 1977). In addition, 16 % of NAD-ME (mitochondrial marker enzyme; ap Rees *et al.*, 1983) and about 10 % of both ALAD and PBGD activities pelleted. In contrast, virtually all of the cytosolic marker enzyme LDH (ap Rees *et al.*, 1983) remained in the supernatant. Comparison of the distribution of LDH with those of ALAD and PBGD show that they are significantly different (Fisher's *P* value < 0.001). This is highly indicative, although it does not prove definitely, that the two porphyrin-synthesis enzymes are not located in the cytosol.

When intact chloroplasts are purified from the 6000 *g* pellet on Percoll gradients (see the Experimental section), they contain 3.7 % of the total Chl and about 3 % of the total ADPG-P, ALAD and PBGD activities. However, less than 0.5 % of the total mitochondrial marker enzyme is present, despite the fact that it was the major enzyme in the 6000 *g* pellet. This lack of correlation of ALAD and PBGD activities with that of NAD-ME is even more marked if the results are expressed as a percentage of that loaded on the gradients: the chloroplasts contain 20–30 % of the ALAD, PBGD and ADPG-P activities, but only 0.5 % of the NAD-ME.

The conclusion to be drawn from these results is that, in green pea leaves, ALAD and PBGD are confined to

Table 1. Distribution of Chl and marker-enzyme activities after subcellular fractionation of green pea leaves

Leaf homogenate was centrifuged at 6000 *g* for 2 min. The crude chloroplast pellet was further purified on a 20–80 % (v/v) Percoll gradient as described in the Experimental section. The values for the total homogenate are expressed per g fresh weight and the other values are expressed as percentages. The results are means for three experiments $\pm$ S.E.M., except for ADPG-P and Chl, where results are means for two experiments.

Fraction	NAD-ME	ADPG-P	LDH	ALAD	PBGD	Chl
Total homogenate (μ mol/h per g fresh wt. or, for Chl, mg/g fresh wt.)	14.1 $\pm$ 3.3	9.02	1.49 $\pm$ 0.4	2.3 $\pm$ 0.05	0.21 $\pm$ 0.001	0.86
6000 <i>g</i> Supernatant (% of total)	69.4 $\pm$ 9.2	70.1	98.8 $\pm$ 0.7	83.5 $\pm$ 2.3	80.3 $\pm$ 2.9	46.9
6000 <i>g</i> Pellet (% of total)	15.7 $\pm$ 4.0	9.9	0.83 $\pm$ 0.8	9.5 $\pm$ 2.3	8.9 $\pm$ 2.3	59.0
Recovery (% of total)	85.1	80.0	99.6	93.0	89.2	105.9
Purified chloroplasts (% of total)	0.47 $\pm$ 0.17	2.8	–	2.6 $\pm$ 0.3	2.6 $\pm$ 0.3	3.7
(% of that loaded on gradient)	0.52 $\pm$ 0.02	33.8	–	20.9 $\pm$ 1.0	19.4 $\pm$ 1.4	8.2

Table 2. Distribution of enzyme activities after fractionation of crude homogenate of etiolated pea leaves

Leaf homogenate was centrifuged at 10000 *g* for 15 min. The values for 'Total homogenate' are expressed per g fresh weight; other values are expressed as percentages. Results are means $\pm$ S.E.M for several experiments. The number of experiments in which the enzyme activity was measured is given in parentheses.

Fraction	NAD-ME (14)	PPase (7)	LDH (3)	HPR (4)	ALAD (11)	PBGD (13)
Total homogenate (μ mol/h per g fresh wt.)	4.59 $\pm$ 0.5	126.5 $\pm$ 21.2	1.12 $\pm$ 0.3	8.14 $\pm$ 0.7	0.178 $\pm$ 0.03	0.009 $\pm$ 0.001
10000 <i>g</i> Supernatant (% of total)	35.5 $\pm$ 5.0	64.5 $\pm$ 3.6	99.0 $\pm$ 0.1	69.5 $\pm$ 25.9	57.6 $\pm$ 4.5	62.3 $\pm$ 5.9
10000 <i>g</i> Pellet (% of total)	68.5 $\pm$ 5.7	26.6 $\pm$ 3.0	0.0	12.0 $\pm$ 3.6	37.8 $\pm$ 3.52	41.8 $\pm$ 5.4
Recovery (% of total)	104.0	91.1	99.0	81.5	85.4	104.1

the plastids. However, since most porphyrin synthesis in this tissue is likely to be for plastids, the possibility remains that low levels of ALAD and PBGD are present elsewhere in the cell, for instance in the cytoplasm, as in animals (Elder, 1976). Since plastid envelope membranes are relatively fragile, it is almost impossible to prepare plant cytoplasm free from contaminating plastid stroma, but attempts were made to separate mitochondria from chloroplasts by the method of Nash & Wiskich (1983). However, mitochondria could not be prepared free of chloroplasts and peroxisomes from this tissue, possibly because a different variety of pea was being used (Feltham First rather than Massey Gem) and/or younger plants (7 days rather than 10–12 days).

Subcellular localization of ALAD and PBGD in etiolated pea leaves

In order to overcome the contamination problems and also to increase the proportion of synthesis of non-plastid porphyrin to that for plastids, all subsequent experiments were carried out using etiolated or non-green tissue. Since the activity of ADPG-P was low in these tissues, it was difficult to monitor its distribution during the purification procedures. PPase was therefore used as an alternative plastid marker (Gross & ap Rees, 1986), but in a preliminary experiment the distribution

was identical with that of ADPG-P (results not shown). Table 2 shows the enzyme activities and their distribution after centrifugation at 10000 *g* of a homogenate of etiolated pea leaves. Again the distribution of ALAD and PBGD is significantly different from that of the cytosolic marker, LDH (Fisher's *P* value < 0.001). The 10000 *g* pellet can be regarded as a crude mitochondrial preparation, since 68 % of the mitochondrial marker (NAD-ME), but only 12 and 27 % of the peroxisome and plastid markers [HPR (ap Rees *et al.*, 1983) and PPase (Gross & ap Rees, 1986) respectively] were recovered in this fraction, and there was no detectable cytoplasmic contamination. Both ALAD and PBGD activities are much lower than in green leaves, but a higher proportion pelleted. A trypsin-protection experiment was carried out to determine whether the ALAD and PBGD activities in this pellet were protected by a membrane (Table 3). Both enzymes were more sensitive to trypsin treatment of the pellet in the presence of Triton than in its absence, suggesting that about 40 % of each enzyme is in a membrane-bound organelle. However, all the NAD-ME activity in this pellet exhibited latency (as defined by ap Rees *et al.*, 1983), that is, there was no measurable activity in the absence of Triton X-100. In addition, an equivalent amount of plastid marker was present in the 10000 *g* pellet, so it is possible that the ALAD and

Table 3. Effect of trypsin on ALAD and PBGD activities in etiolated pea leaves

The 10000 g pellet from centrifugation of etiolated pea leaves was treated with trypsin as described in the Experimental section.

Treatment	Activity (% of that in untreated sample)	
	ALAD	PBGD
Trypsin	51	61
Trypsin + trypsin inhibitor	81	95
Trypsin + Triton X-100	11	24
Trypsin + trypsin inhibitor + Triton X-100	73	80

Fig. 1. (a) Distribution of NAD-ME (◆), HPR (●) and PPase (■) activities in a sucrose gradient of pea 10000 g pellet (see the Experimental section), and (b) distribution of ALAD (◇) and PBGD (△) activities in the same gradient

The gradient was divided into 12 1 ml fractions, starting from the top, and the gradient pellet (fraction 13).

PBGD are protected by the plastid, rather than the mitochondrial, membrane.

To try to resolve this question, a method was developed to separate mitochondria from etioplasts. The 10000 g pellet was layered on to a sucrose gradient as described in the Experimental section. It was found to be important to centrifuge the gradients for the minimum time possible

in order to resolve the two organelles. After 15 min centrifugation there was a fluffy white band in the middle of the gradient and a yellow band on the 65% cushion. When the gradient was fractionated from the top into 1 ml aliquots, which were then tested for enzyme activity (Fig. 1a), the peak of NAD-ME and, to a lesser extent, most of the peroxisome marker (HPR) corresponded to the white band (fractions 7–9), and the yellow band (fraction 11) contained a peak of PPase activity. The gradient pellet (fraction 13) comprised starch and other debris, including material that was not fully resuspended in the crude 10000 g pellet.

The distributions of ALAD and PBGD parallel that of the plastid marker (Fig. 1b). Although there was residual activity in pooled fractions 7 and 8 (mitochondria with some peroxisomes), this was less than the contamination by the plastid marker (Table 4). In addition, although all the NAD-ME in this fraction showed latency, none of the PBGD was latent. Since ALA crosses membranes easily, it was not possible to conduct latency experiments with ALAD. Finally, when proteins from this purified mitochondrial fraction were subjected to Western-blot analysis using antibodies to spinach ALAD or *Euglena* PBGD, there was no detectable antigen present, although the 10000 g supernatant and, to a lesser extent, the 10000 g pellet contained both ALAD and PBGD protein (results not shown). Although it is possible that a putative mitochondrial enzyme would not cross-react with these antibodies, the latter cross-reacts with the enzyme purified from a number of different sources including the bacterium *Rhodospseudomonas spheroides*, algae and ferns (Schneider & Liedgens, 1981). From these results it can be concluded that there is no ALAD or PBGD in pea mitochondria. Although the lack of correlation of the distribution of these enzymes with that of the cytosolic marker enzyme does not prove that they are absent from the cytosol, this result, taken together with the fact that their distribution parallels that of the plastids, provides substantial support for the idea that they are indeed confined to these latter organelles.

Subcellular localization of ALAD and PBGD in *Arum* spadices

Despite the results obtained above, the fact remains that in etiolated leaves the potential for the synthesis of plastid porphyrin is much greater than that for non-plastid porphyrin. It is conceivable, therefore, that an alternative pathway exists, but is not detectable in these tissues. To test this hypothesis, an alternative tissue was studied. The spadices of *Arum*, just before thermogenesis, show a massive increase in the activity of tricarboxylic-acid-cycle enzymes (ap Rees *et al.*, 1983). It is likely, then, that in this tissue the biogenesis of mitochondria will predominate over that of plastids, such that synthesis

Table 4. Proportion of loaded activity recovered in fractions from a sucrose gradient (see Fig. 1) of etiolated pea leaves

Fraction	Loaded activity recovered (%)				
	NAD-ME	PPase	HPR	ALAD	PBGD
7+8 (mitochondria)	38.7	4.0	10.0	0.6	2.9
11 (plastids)	3.5	10.4	4.5	8.0	21.0
13 (gradient pellet)	2.3	25.9	5.3	25.0	27.0

Table 5. Distribution of enzyme activities after subcellular fractionation of *Arum* spadices

The homogenate of *Arum* spadices was centrifuged at 10000 *g*. The crude mitochondrial pellet was purified further on 35–55%-(w/v)-sucrose gradients as described in the Experimental section. The results for the total homogenate are expressed per g fresh weight; the other values are expressed as percentages. Values are means $\pm$ S.E.M. for six experiments, except where indicated (see footnotes).

Fraction	NAD-ME	PPase	LDH*	ALAD	PBGD†
Total homogenate (μ mol/h per g fresh wt.)	64.6 $\pm$ 10.6	1026.0 $\pm$ 440.0	4.12 $\pm$ 0.9	0.507 $\pm$ 0.17	0.024 $\pm$ 0.005
10000 <i>g</i> Supernatant (% of total)	15.1 $\pm$ 5.1	68.7 $\pm$ 5.8	99.1 $\pm$ 0.7	78.3 $\pm$ 3.6	73.6 $\pm$ 8.6
10000 <i>g</i> pellet (% of total)	90.6 $\pm$ 5.2	27.2 $\pm$ 3.8	0.8 $\pm$ 0.5	21.7 $\pm$ 4.8	32.3 $\pm$ 5.9
Recovery (% of total)	105.7	95.9	99.9	100.0	106.8
Purified mitochondria (% of total)‡	35.0	3.8	—	0.42	1.6
(% of that loaded on gradient)‡	55.0	9.1	—	2.1	4.5

* Mean for three experiments.

† Mean for four experiments.

‡ Mean for two experiments.

of mitochondrial haem will be greater than that for plastids. Interestingly, the activities per g fresh weight of both ALAD and PBGD in this tissue (Table 5) were twice those found in etiolated pea leaves (Table 2), although about 5–10-fold less than in green pea leaves (Table 1).

The crude mitochondrial fraction (10000 *g* pellet), containing 90 % of the mitochondrial marker, routinely had one-third of the plastids and similar amounts of ALAD and PBGD activities (22 and 33 % respectively). Table 6 shows the results of a trypsin-protection experiment on this pellet. The apparent stimulation of PBGD activity by the treatments makes it difficult to assess the effect of trypsin on this enzyme; the reason for this stimulation is unknown, but was observed each time the experiment was performed. Nevertheless, it is clear that about 40 % of the ALAD activity in this pellet was protected by a membrane from trypsin-attack. Similarly, in a latency experiment, less than 50 % of the PBGD activity was assayable in the absence of Triton-X-100, which suggests that both enzymes are in a similar location in this preparation. However, as for etiolated

peas, this is unlikely to be the mitochondria, since more than 90 % of the NAD-ME was latent, and the recoverable activities of the two porphyrin-synthesis enzymes are comparable with the extent of contamination by the plastid marker enzyme.

The same pattern is seen when mitochondria are purified further on sucrose gradients (Table 5). Whether the activities are expressed as a percentage of the total or of that loaded on the gradient, any ALAD and PBGD activity can be accounted for by the presence of contaminating plastids. In addition, as before, the distribution of the cytosolic marker enzyme LDH was significantly different from that for the two porphyrin-synthesizing enzymes (Fisher's *P* value < 0.001).

Thus, even in a tissue such as *Arum* spadix, where the synthesis of plastid porphyrin is likely to be less important than for other organelles, there is no appreciable ALAD or PBGD activity in the mitochondria or the cytosol; rather these activities seem to be confined to the plastids.

DISCUSSION

The results presented here show that, throughout the fractionation of cells from green and etiolated pea leaves and spadices of *Arum*, the distribution of the activities of the two porphyrin-synthesis enzymes, ALAD and PBGD, are significantly different from that of the cytosolic marker enzyme and, although there is residual activity in purified mitochondrial preparations, this is always less than the degree of contamination by plastids. Conversely, there is a strong correlation between the distribution of these enzymes and the plastid marker. The conclusion to be drawn, therefore, is that most, if not all, of the ALAD and PBGD is confined to the plastids, presumably in the stroma. This is in contrast with the claim that ALAD is in the mitochondria of wheat (*Triticum*) leaves (Nandi & Waygood, 1967), but the mitochondrial fraction in this case was 'remaining particles' after preparation of intact chloroplasts. The authors themselves noted that this fraction contained broken chloroplasts as well as mitochondria, so any

Table 6. Effect of trypsin on ALAD and PBGD activities in *Arum* spadices

The 10000 *g* pellet from the centrifugation of *Arum* spadix homogenate were treated with trypsin as described in the Experimental section.

Treatment	Activity (% of that in untreated sample)	
	ALAD	PBGD
Trypsin	51	164
Trypsin + trypsin inhibitor	76	270
Trypsin + Triton X-100	12	29
Trypsin + trypsin inhibitor + Triton X-100	81	264

ALAD activity may be attributable to this contamination.

However, it is difficult to exclude categorically the presence of ALAD and PBGD elsewhere in the cell, even though their distribution during cell fractionation parallels that of the plastids and is significantly different from that of the cytosolic marker enzyme. Nevertheless, this would represent a very small proportion of that in the plastids, even in tissues where plastid porphyrin synthesis is relatively unimportant, such as *Arum* spadices. This suggests that plastids play a role in the synthesis of porphyrin for the rest of the cell and that, despite the fact that ALA can readily cross the plastid envelope, it is unlikely to be the substance exported, since the next two enzymes involved in its metabolism are confined to the plastid. This means that a tetrapyrrole of some sort must be the candidate for export. This is not an impossible task, since in animal cells the last stages, at least, of haem synthesis are mitochondrial and so haem must cross the mitochondrial membrane to form cytochrome *P*-450 in the endoplasmic reticulum, catalase and peroxidase in the peroxisomes, and so on. In order to resolve further the question of the site of non-plastid porphyrin synthesis, information regarding other enzymes in the pathway is necessary. There is good evidence for ferrochelatase in barley mitochondria (Little & Jones, 1976), but although protoporphyrinogen oxidase has been purified from barley mitochondria, its properties were identical with the enzyme from barley chloroplasts (Jacobs & Jacobs, 1987). Since no estimations of the total activity within the cell were made, nor was the extent of plastid contamination of the mitochondrial fraction determined, it is not possible to be certain that the enzyme really is present in mitochondria. Clearly it is essential to establish definitely the location of the other porphyrin-synthesis enzymes in order to obtain an overall view of porphyrin synthesis within the plant cell.

From the results presented here and from the discussion of the results of other authors, it is apparent that biochemical techniques are not sufficiently rigorous to ascertain unambiguously the presence of an enzyme in a second subcellular compartment, particularly if this represents only a small proportion of the total activity with the cell. One possible alternative approach is that of molecular biology. As more information becomes available on the sequences of proteins in different subcellular compartments, it is clear that, whereas cytoplasmic enzymes are synthesized essentially as the mature protein, those destined for other locations within the cell are synthesized as precursors with *N*-terminal extensions (Chua & Schmidt, 1979; von Heijne, 1984). In addition,

the leader or signal sequences are characteristic for the organelle for which the proteins are destined. Isolation of cDNA clones for porphyrin-synthesis enzymes should allow determination of which leader sequences, if any, are present, and whether there are any differences in the 3' untranslated regions. Should any differences be found, then one can conclude (i) that multiple forms of these enzymes are present and possibly also (ii) the subcellular compartments in which they are localized.

I am grateful to Mr. Simon Wombwell and Mrs. Kathy Hardy for excellent technical assistance, to Dr. Tom ap Rees for generous help and advice, and to Mrs. Yolande Smith for typing the manuscript.

REFERENCES

- ap Rees, T., Bryce, J. H., Wilson, P. M., Green, J. H. (1983) *Arch. Biochem. Biophys.* **227**, 511–521
- Arnon, D. I. (1949) *Plant Physiol.* **24**, 1–15
- Bogorad, L. (1962) *Methods Enzymol.* **5**, 885–891
- Castelfranco, P. A. & Beale, S. I. (1981) in *The Biochemistry of Plants* (Hatch, M. D. & Boardman, N. K., eds.), vol. 8, pp. 376–421, Academic Press, New York
- Castelfranco, P. A. & Jones, O. T. G. (1975) *Plant Physiol.* **55**, 485–490
- Chibbar, R. N. & van Huystee, R. B. (1986) *Phytochemistry* **25**, 585–587
- Chua, N.-H. & Schmidt, G. W. (1979) *J. Cell Biol.* **81**, 461–483
- Davies, D. D. & Davies, S. (1972) *Biochem. J.* **129**, 831–839
- Elder, G. H. (1976) *Essays Med. Biochem.* **2**, 75–114
- Ellis, R. J. (1977) *Biochim. Biophys. Acta* **463**, 185–215
- Gross, P. & ap Rees, T. (1986) *Planta* **167**, 140–145
- Hsu, W. P. & Miller, G. W. (1970) *Biochem. J.* **117**, 215–220
- Jacobs, J. M. & Jacobs, N. J. (1987) *Biochem. J.* **244**, 219–224
- Little, H. N. & Jones, O. T. G. (1976) *Biochem. J.* **156**, 309–314
- MacDonald, F. & ap Rees, T. (1983) *Biochem. Biophys. Acta* **755**, 81–89
- Nandi, D. L. & Waygood, E. R. (1967) *Can. J. Biochem.* **45**, 327–336
- Nash, D. & Wiskich, J. T. (1983) *Plant Physiol.* **71**, 627–634
- Saunders, M. J., Cordonnier, M. M., Palevitz, B. A. & Pratt, L. H. (1983) *Planta* **159**, 543–553
- Schneider, H. A. W. (1970) *Z. Pflanzenphysiol.* **62**, 328–345
- Schneider, H. A. W. & Liedgens, W. (1981) *Z. Naturforsch.* **36c**, 40–50
- Smith, A. G. (1986) in *Regulation of Chloroplast Differentiation* (Akoyonoglou, G. & Senger, H., eds.), pp. 49–54, Alan R. Liss, New York
- Smith, A. G. & Gray, J. C. (1984) *Mol. Gen. Genet.* **194**, 471–476
- von Heijne, G. (1984) *EMBO J.* **3**, 2315–2318