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Distribution of Enzymes in Mesophyll and Parenchyma-Sheath Chloroplasts of Maize Leaves in Relation to the C_4-Dicarboxylic Acid Pathway of Photosynthesis

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1. Mesophyll and parenchyma-sheath chloroplasts of maize leaves were separated by density fractionation in non-aqueous media. 2. An investigation of the distribution of photosynthetic enzymes indicated that the mesophyll chloroplasts probably contain the entire leaf complement of pyruvate,P_i dikinase, NADP-specific malate dehydrogenase, glycerate kinase and nitrite reductase and most of the adenylate kinase and pyrophosphatase. The fractionation pattern of phosphopyruvate carboxylase suggested that this enzyme may be associated with the bounding membrane of mesophyll chloroplasts. 3. Ribulose diphosphate carboxylase, ribose phosphate isomerase, phosphoribulokinase, fructose diphosphate aldolase, alkaline fructose diphosphatase and NADP-specific 'malic' enzyme appear to be wholly localized in the parenchyma-sheath chloroplasts. Phosphoglycerate kinase and NADP-specific glyceraldehyde phosphate dehydrogenase, on the other hand, are distributed approximately equally between the two types of chloroplast. 4. After exposure of illuminated leaves to $^{14}CO_2$ for 25sec., labelled malate, aspartate and 3-phosphoglycerate had similar fractionation patterns, and a large proportion of each was isolated with mesophyll chloroplasts. Labelled fructose phosphates and ribulose phosphates were mainly isolated in fractions containing parenchyma-sheath chloroplasts, and dihydroxyacetone phosphate had a fractionation pattern intermediate between those of C_4 dicarboxylic acids and sugar phosphates. 6. These results indicate that the mesophyll and parenchyma-sheath chloroplasts have a co-operative function in the operation of the C_4-dicarboxylic acid pathway. Possible routes for the transfer of carbon from C_4 dicarboxylic acids to sugars are discussed.

Species in which the C_4-dicarboxylic acid pathway of photosynthesis (Hatch & Slack, 1966) operates possess a characteristic type of leaf anatomy (Johnson & Hatch, 1968; Downton & Tregunna, 1968) and two morphologically distinct types of chloroplast, each restricted to one type of cell (Brown, 1958; Hodge, McLean & Mercer, 1955; Johnson, 1964; Laetsch, 1968). The mesophyll cells of maize and other Panicoid grasses contain chloroplasts that resemble those of plants utilizing the Calvin cycle in size and the presence of grana, but these chloroplasts do not accumulate starch. The chloroplasts of the parenchyma sheath, on the other hand, are devoid of grana, are considerably larger than the mesophyll chloroplasts and contain numerous starch grains. Partial separation of the two types of chloroplasts was achieved by the density fractionation of freeze-dried leaves in non-aqueous media (Slack, 1969), and a study of the distribution of certain chloroplast enzymes suggested that each type of chloroplast may have a different role in photosynthetic carbon dioxide fixation. Ribulose diphosphate carboxylase and phosphoribulokinase appeared to be mainly localized in the parenchyma-sheath chloroplasts, whereas pyruvate,P_i dikinase (Hatch & Slack, 1968, 1969*a*), an enzyme specifically associated with the C_4-dicarboxylic acid pathway, was mainly localized in the mesophyll chloroplasts.

Rhoades & Carvalho (1944) presented evidence

that the starch deposited in the parenchyma-sheath chloroplasts of maize leaves was derived from carbon dioxide fixed by the mesophyll chloroplasts. This observation suggested that the two types of chloroplast may act in a concerted manner in photosynthetic carbon dioxide fixation. The aim of the present studies was to determine the intracellular localization of enzymes and intermediates of the C_4-dicarboxylic acid pathway in an effort to describe the individual functions of the two types of chloroplast.

MATERIALS

Plants of maize (*Zea mays* var. DS606A) were grown in vermiculite culture under natural illumination at 28°. Starch-containing leaves were harvested from 3-week-old plants at 10.30a.m., about 5hr. after sunrise. Destarched leaves were obtained from similar plants that had previously been darkened for 24hr. and illuminated in full sunlight for 30min. immediately before harvest to activate pyruvate,P_i dikinase (Slack, 1968; Hatch & Slack, 1969*a*).

The following enzymes and reagents were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.: aspartate aminotransferase, lactate dehydrogenase, pyruvate kinase, glycerophosphate dehydrogenase–triose phosphate isomerase mixture, NAD-specific glyceraldehyde phosphate dehydrogenase, ribulose 1,5-diphosphate, glyceraldehyde 3-phosphate, oxaloacetic acid, phosphoenolpyruvic acid, $NADP^+$, NADPH, NAD^+, NADH and ATP. Dithiothreitol and *N*-2-hydroxyethylpiperazine-*N'*-ethane-2-sulphonic acid were purchased from Calbiochem, Los Angeles, Calif., U.S.A. Carbon tetrachloride (AnalaR grade), hexane (b.p. 67–70°), sulphanilamide, benzyl viologen and *N*-1-naphthylethylenediamine dihydrochloride were obtained from British Drug Houses Ltd., Poole, Dorset. $NaH^{14}CO_3$ was supplied by The Radiochemical Centre, Amersham, Bucks.

METHODS

Freeze-drying of leaf. Illuminated and destarched leaves were treated in a similar manner. Leaf laminae were frozen either by placing the material into a flask in an ethanol–solid CO_2 bath either directly or after prior freezing of the leaf in liquid N_2. The frozen leaf was broken into small pieces by pounding with a chilled glass rod. Freeze-drying was carried out over P_2O_5 in a freeze-dryer at −25° and 0·05torr. The material was dry after approx. 6hr. and was held over P_2O_5 at −25° until required.

Non-aqueous density fractionation. A modification of the stepwise fractionation method described by Smillie (1963) was employed. About 100mg. of freeze-dried leaf was homogenized in 30ml. of a mixture of hexane and carbon tetrachloride (density 1·30) in a glass tissue homogenizer (A. Gallenkamp and Co. Ltd., London E.C.2). The homogenate was filtered through glass wool and the filtrate centrifuged at 12000*g* for 10min. The supernatant contained suspended material of density less than 1·30 and the pellet contained material of density greater than 1·30. This was suspended in 30ml. of a hexane–carbon tetrachloride mixture of density 1·33 and centrifuged as described above to give pelleted material of density greater than 1·33 and a supernatant containing suspended material of density 1·30–1·33. The pellet was further fractionated by using a hexane–carbon tetrachloride mixture of density 1·36 followed by a mixture of density 1·40. An equal volume of hexane was added to the suspensions of different density and the material was pelleted by centrifuging at 5000*g* for 5min. The pellets were suspended in hexane, subsampled, dried *in vacuo* and stored at −15° over silica gel. All other procedures were conducted at 3°.

Enzyme assays. Samples of the fractions were suspended in 100mM-tris–HCl buffer, pH8·3, containing 5mM-$MgCl_2$ and 2·5mM-dithiothreitol. Samples of the suspension were used for the assay of ribulose diphosphate carboxylase (EC 4.1.1.39), phosphopyruvate carboxylase (EC 4.1.1.31) (Slack & Hatch, 1967) and pyruvate,P_i dikinase (Hatch & Slack, 1968). For the assay of other enzymes the supernatant obtained by centrifuging the extracts at 10000*g* for 5min. was used. These enzymes, with the reference to their method of assay, were: phosphoglycerate kinase (EC 2.7.2.3) (Latzko & Gibbs, 1968), fructose diphosphate aldolase (EC 4.1.2.13) (Rutter *et al.* 1966), phosphoribulokinase (EC 2.7.1.19) (Anderson & Fuller, 1967), ribose phosphate isomerase (EC 5.3.1.6) (Axelrod, 1955), NADP-specific 'malic' enzyme (EC 1.1.1.40) in the direction of decarboxylation (Ochoa, 1955), alkaline fructose diphosphatase (EC 3.1.3.11) (Racker & Schroeder, 1958), acid phosphatase (EC 3.1.3.2) (Lowry, 1957), NADP-specific malate dehydrogenase and NAD-specific malate dehydrogenase (EC 1.1.1.37) in the direction of malate formation (Hatch & Slack, 1969*b*), NADP-specific glyceraldehyde phosphate dehydrogenase (EC 1.2.1.9) (Gibbs, 1955), glycerate kinase (EC 2.7.1.31) (radiotracer procedure of Hatch & Slack, 1969*b*), pyrophosphatase (EC 3.6.1.1) and adenylate kinase (EC 2.7.4.3) (Hatch, Slack & Bull, 1969).

For the assay of nitrite reductase (EC 1.6.6.4) fractions were suspended in 40mM-*N*-2-hydroxyethylpiperazine-*N'*-ethane-2-sulphonic acid–NaOH buffer, pH7·5, containing 2·5mM-dithiothreitol and centrifuged at 10000*g* for 5min. Samples of the supernatant were assayed by the procedure of Joy & Hageman (1966) with benzyl viologen and dithionite.

Chlorophyll. The chlorophyll content of resuspended fractions was determined by the procedure of Arnon (1949).

Intracellular localization of photosynthetic intermediates. Plants were illuminated in sunlight for 2hr. before harvest. Lamina segments were removed and illuminated in a photosynthetic chamber (Hatch & Slack, 1966) for 45min. to allow the resumption of a steady rate of photosynthesis. $^{14}CO_2$ was introduced into the chamber and the segments were transferred to liquid N_2 25sec. thereafter. The frozen leaf was freeze-dried and fractionated exactly as described above except that hexane–carbon tetrachloride mixtures of density 1·32, 1·36, 1·40 and 1·43 were used. The fractions were suspended in 0·05M-HCl, stood for 15min. at 0° and neutralized with aq.NH_3. Labelled intermediates were separated and identified by paper chromatography as described previously (Hatch & Slack, 1966; Johnson & Hatch, 1969). The chlorophyll and enzyme contents of the fractions were determined on subsamples suspended in buffer as described above.

Investigations of the composition of the various fractions by light- and electron microscopy. For light-microscope studies fractions were suspended in 0·33M-sorbitol and viewed either under phase contrast, or with direct light after staining with *I*-KI solution. For electron microscopy the freeze-

dried fractions were suspended in 24mM-sodium phosphate buffer, pH7·2, and pelleted in small polythene tubes in a Microfuge (Beckman Instruments) at 4000rev./min. for 2min. The pellets were then fixed in 2% (w/v) osmium tetroxide in 25mM-sodium phosphate buffer, pH7·2, for 1½hr. and washed three times in phosphate buffer before dehydration in ethanol. They were then embedded in an Araldite–Epon mixture (Mollenhauer, 1964) at 85° by vacuum infiltration and allowed to polymerize at 85° for 24hr. Thin sections were cut on a Reichert OUM2 ultramicrotome and stained for 1hr. in 2% (w/v) uranyl acetate in 50% (v/v) ethanol followed by 15min. in lead citrate (Fischke, 1966) before examination in a Philips EM200 electron microscope.

RESULTS

Composition of fractions isolated from starch-containing leaves. An examination of the fractions by light-microscopy indicated, as previously found (Slack, 1969), that fractions of density less than 1·33 contained chloroplasts free of starch grains, whereas heavier fractions contained starch-containing chloroplasts. As indicated by the distribution of chlorophyll (Table 1) the fractions of density less than 1·30 contained most of the starch-free chloroplasts and the fraction of density 1·36–1·40 most of the starch-containing chloroplasts. Electron micrographs of these two fractions (Plates 1*a* and 1*b*) confirmed the earlier assumption (Slack, 1969) that the chloroplasts of density less than 1·30 were the grana-containing starch-free mesophyll plastids, and the chloroplasts of density greater than 1·36 the starch-containing plastids of the parenchyma sheath that are devoid of grana. The relatively small proportion of the total chlorophyll isolated with the parenchyma-sheath chloroplasts is in agreement with the observations of Rhoades & Carvalho (1944) and Brown (1958) that maize leaves contain fewer parenchyma-sheath chloroplasts than mesophyll chloroplasts and that the former plastids contain less chlorophyll than the latter. Complete separation of the two types of chloroplasts was not achieved, since the fractions of intermediate density contained both types of chloroplast, and fragments of a few grana-containing chloroplasts were observed in the fractions of density 1·36–1·40. A few individual starch grains were present in the fraction of density less than 1·30. The chloroplast lamellae retained their osmophilic properties after non-aqueous isolation, and grana were clearly visible in the mesophyll chloroplasts (Plate 1*b*) although the grana thyalkoids were grossly swollen. The outer membranes of the chloroplasts were absent, but the internal membrane system remained intact so that the structural integrity of many of the chloroplasts was retained although the stroma was absent. However, chloroplast fragments were present in all the preparations, especially in fractions containing the parenchyma-sheath chloroplasts. Numerous free starch grains were present in these fractions, and attached to many were portions of lamellae that had presumably remained associated with the grains when chloroplasts were disrupted. The disruption of the chloroplast envelope and loss of stromal material from chloroplasts prepared in a non-aqueous medium and fixed in polar solvents is in agreement with the findings of Stocking, Shumway, Weier & Greenwood (1968).

Distribution of enzymes in fractions prepared from destarched and starch-containing leaf. It has already been demonstrated that mesophyll and parenchyma-sheath chloroplasts are isolated together when the latter are destarched by holding plants in darkness before harvest (Slack, 1969). With destarched leaves about 90% of the chlorophyll-containing material had a density of less than 1·33 (Table 1). Similarly about 90% of the leaf complement of the enzymes listed, excepting the last three, were present in these fractions. NAD-specific malate dehydrogenase and phosphoglycerate mutase, which are probably present in chloroplasts and other cellular compartments, and acid phosphatase, an extra-chloroplastal enzyme, are included for comparison. Fumarase and invertase, two other enzymes unlikely to be associated with chloroplasts, were distributed in a manner similar to that of NAD-specific malate dehydrogenase and acid phosphatase respectively.

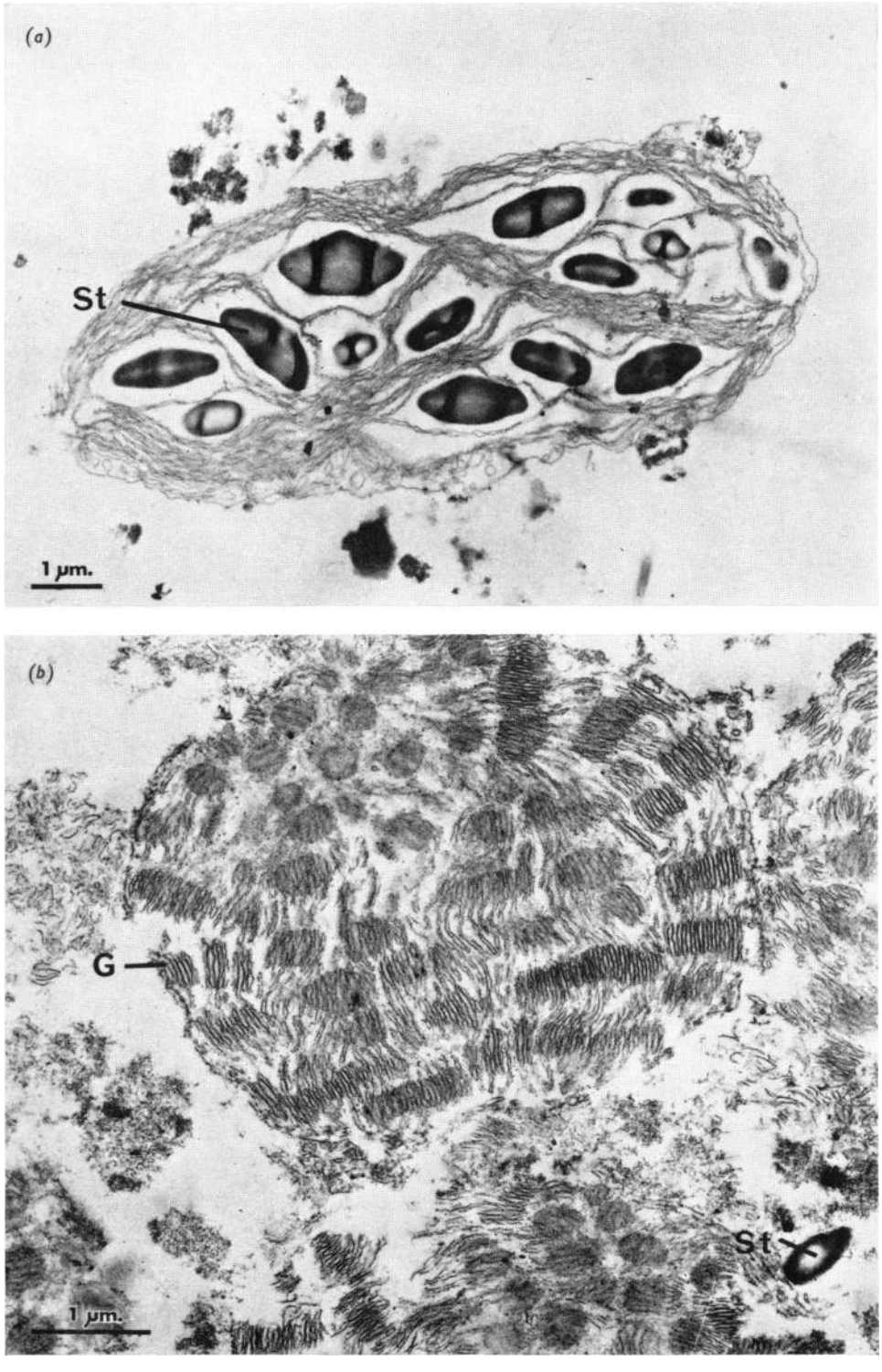
The isolation of the other enzymes in the chlorophyll-containing fractions is consistent with the view that they are associated with chloroplasts. These enzymes have been divided into three groups on the basis of their fractionation patterns obtained with starch-containing leaves. The first group contains the enzymes that were isolated predominantly in fractions containing the mesophyll chloroplasts, i.e. pyruvate,P_i dikinase, NADP-specific malate dehydrogenase and glycerate kinase. The second group includes enzymes present in both types of chloroplasts. However, the distributions of these enzymes between the parenchyma-sheath and mesophyll chloroplasts were obviously different. Adenylate kinase and pyrophosphatase, which are thought to operate in association with pyruvate,P_i dikinase in the conversion of pyruvate into phosphoenolpyruvate (Hatch *et al.* 1969), were mainly associated with the mesophyll chloroplasts, whereas NADP-specific glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase were present in about equal proportions in the two types of chloroplasts. The third group contains ribulose diphosphate carboxylase, phosphoribulokinase, ribose phosphate isomerase, fructose diphosphate aldolase, alkaline fructose diphosphatase and NADP-specific 'malic' enzyme. These enzymes were isolated in the low-density fractions from destarched

Table 1. *Distribution of chlorophyll and enzymes after the non-aqueous density fractionation of destarched and starch-containing leaves*

Leaves were freeze-dried, homogenized and fractionated, and chlorophyll content and enzyme activities were determined, as described in the Methods section. The total activity of each enzyme and the total chlorophyll content represents the sum of the individual fractions. The groups of enzymes are: 1, enzymes associated with mesophyll chloroplasts; 2, enzymes in both types of chloroplasts; 3, enzymes associated with parenchyma-sheath chloroplasts; 4, enzymes largely or completely unassociated with chloroplasts.

	Destarched leaves						Starch-containing leaves					
		Distribution of activity among fractions of different density (%)						Distribution of activity among fractions of different density (%)				
Enzyme	Total activity (μmoles/min.)	<1·30	1·30–1·33	1·33–1·36	1·36–1·40	>1·40	Total activity (μmoles/min.)	<1·30	1·30–1·33	1·33–1·36	1·36–1·40	>1·40
Chlorophyll	82*	62	27	5	3	3	108*	70	6	3	13	8
Group 1												
Pyruvate,P_i dikinase	0·07	56	28	5	5	6	0·20	65	8	5	14	8
Glycerate kinase	0·28	56	29	6	4	5	0·34	70	11	5	7	7
NADP-specific malate dehydrogenase	0·30	61	30	4	3	2	0·32	61	15	8	12	4
Group 2												
Adenylate kinase	6·9	58	26	6	4	6	9·4	54	9	6	15	16
Pyrophosphatase	3·0	47	40	5	8	3	4·8	51	5	4	24	15
NADP-specific glyceraldehyde phosphate dehydrogenase	1·0	51	35	6	5	3	1·5	46	5	5	28	16
Phosphoglycerate kinase	4·1	45	32	9	6	8	6·5	37	9	7	24	23
Group 3												
Ribulose diphosphate carboxylase	0·14	44	41	7	4	4	0·17	8	3	5	51	33
Phosphoribulokinase	2·8	33	35	9	10	12	4·2	8	4	4	44	40
Ribose phosphate isomerase	1·3	43	40	9	5	3	6·1	8	3	5	45	39
Fructose diphosphate aldolase	0·69	40	39	9	6	6	1·0	9	3	5	48	34
Alkaline fructose diphosphatase	0·04	46	47	0	3	3	0·11	6	4	4	62	24
NADP-specific 'malic' enzyme	0·66	43	44	6	3	5	0·60	10	4	5	63	18
Group 4												
NAD-specific malate dehydrogenase	2·3	15	21	13	19	32	3·2	22	11	12	28	28
Acid phosphatase	0·08	4	8	3	8	77	0·09	4	3	3	10	43
Phosphoglycerate mutase	0·26	17	18	11	16	38	0·42	17	8	10	22	80

* Total chlorophyll content (μg.).



EXPLANATION OF PLATE 1

(*a*) Chloroplast from a pellet of fraction, density 1·36–1·40, from starch-containing maize leaves prepared in non-aqueous media. The abundance of starch grains (St) and the absence of grana should be noted. (*b*) Section of a pellet from fraction, density less than 1·30, showing a complete chloroplast with grana (G), fragments of other chloroplasts and a starch grain (St).

leaves, but appeared in fractions containing parenchyma-sheath chloroplasts (density greater than 1·36) with starch-containing leaves.

Qualitatively similar results to those reported in Table 1 were observed in several other experiments; however, some quantitative differences were apparent between individual experiments in the distribution of chlorophyll and enzymes amongst the various fractions. For instance with the starch-containing leaf described in Table 1 ribulose diphosphate carboxylase and other enzymes of the parenchyma-sheath chloroplasts were isolated in fractions of density greater than 1·36 whereas these enzymes were isolated in fractions of density 1·33–1·40 in the experiment described in Table 3. These differences may reflect small differences in the density of the hexane–carbon tetrachloride mixtures employed or be due to variations in the amount of starch in the parenchyma-sheath chloroplasts.

The total activities per unit weight of chlorophyll for most of the enzymes listed in Table 1 from starch-containing and destarched leaves were similar, and also similar to those reported for aqueous extracts of maize leaves (Slack & Hatch, 1967; Hatch & Slack, 1968; Hatch *et al.* 1969). This observation is consistent with the view that the fractionation patterns were not influenced by enzyme inactivation, either during extraction in non-aqueous media or by the dark treatment used to destarch the leaf. However, the activities of pyruvate,P_i dikinase and ribose phosphate isomerase, per unit weight of chlorophyll, were about twofold and fourfold greater respectively in the fractions from starch-containing leaves compared with those from the destarched leaves. The former enzyme is inactivated in darkness and activated by light (Slack, 1968; Hatch & Slack, 1969*a*). Hence the lower activity obtained with destarched leaves probably reflects the incomplete activation of the enzyme by the brief period of illumination before harvest. The reason for the lower activity of ribose phosphate isomerase in destarched leaves is unknown.

Localization of phosphopyruvate carboxylase. Phosphopyruvate carboxylase has been implicated in the C_4-dicarboxylic acid pathway (Slack & Hatch, 1967; Johnson & Hatch, 1968), but, unlike other photosynthetic enzymes, the manner in which the enzyme was distributed between non-aqueously isolated fractions was variable. In certain experiments the enzyme was isolated with chloroplasts, but in others most of the activity appeared in fractions of higher density (Table 2). The patterns of distribution of pyruvate,P_i dikinase are included for comparison. It was thought initially that the rate at which the leaf was frozen may have had some influence on the fractionation pattern of phosphopyruvate carboxylase.

In one experiment with destarched leaves, in which the leaves were frozen in liquid nitrogen, the fractionation patterns of phosphopyruvate carboxylase and pyruvate,P_i dikinase were similar (Expt. 2), whereas the fractionation patterns of the two enzymes were completely different with leaves subjected to a slower freezing procedure (Expt. 1). There was, however, little effect of freezing rate on the manner in which phosphopyruvate carboxylase was fractionated from starch-containing leaves (Expts. 3 and 4). In separate fractionations of samples from a single batch of freeze-dried leaves variable fractionation patterns were obtained for phosphopyruvate carboxylase, although the

Table 2. *Comparison of the distribution of phosphopyruvate carboxylase and pyruvate,P_i dikinase after the non-aqueous fractionation of destarched and starch-containing leaves*

Leaves used in Expts. 1 and 2 were destarched and those used in Expts. 3, 4 and 5 contained starch. In Expts. 1 and 3 the leaves were frozen in flasks immersed in an ethanol– solid CO_2 mixture and in Expts. 2, 4 and 5 by immersion in liquid N_2. The procedures for freeze-drying, density fractionation and enzyme assays were similar for all experiments, and as described in the Methods section, except that mixtures with a higher range of density were used in Expt. 5. (*a*) Phosphopyruvate carboxylase; (*b*) pyruvate,P_i dikinase.

	Distribution of activity in fractions (%)									Distribution of activity in fractions (%)	
	Expt. 1		Expt. 2		Expt. 3		Expt. 4			Expt. 5	
Density	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	Density	(*a*)	(*b*)
<1·30	11	56	46	56	2	65	2	45	<1·32	52	69
1·30–1·33	23	28	31	30	1	8	5	16	1·32–1·36	25	19
1·33–1·36	6	5	10	6	3	5	13	10	1·36–1·40	19	11
1·36–1·40	17	5	10	6	22	14	47	22	1·40–1·43	3	2
>1·40	42	6	2	2	72	8	32	6	>1·43	1	1

patterns for other enzymes were constant. This suggests that the manner in which phosphopyruvate carboxylase is fractionated may be influenced by an as yet unidentified factor of either homogenization or fractionation. In Expt. 5 a range of hexane–carbon tetrachloride mixtures of slightly greater density was employed and in this experiment the distribution pattern of phosphopyruvate carboxylase was similar to that of pyruvate,P_i dikinase, with a large proportion of the enzyme in fractions containing mesophyll chloroplasts.

Intracellular localization of nitrite reductase. Nitrite reductase has been shown to be associated with the chloroplasts of destarched maize leaves isolated in non-aqueous media (Ritenour, Joy, Bunning & Hageman, 1967). Ferredoxin is believed to be the natural electron donor for the enzyme (Losada, Ramirez, Paneque & Del Campo, 1965; Joy & Hageman, 1966). The fractionation pattern of nitrite reductase obtained with starch-containing leaves was compared with that of chlorophyll, pyruvate,P_i dikinase, ribulose diphosphate carboxylase and NADP-specific glyceraldehyde phosphate dehydrogenase (Table 3). The two lightest fractions contained about 80% of the activity of both pyruvate,P_i dikinase and nitrite reductase, and similar proportions of these enzymes were isolated in the fractions containing predominantly parenchyma-sheath chloroplasts. These results suggest that the mesophyll chloroplasts contain a large part of the leaf complement of nitrite reductase.

Distribution of photosynthetic intermediates in fractions isolated in non-aqueous media. Non-aqueous chloroplast isolation has been used extensively to investigate the movement of photosynthetic intermediates from the chloroplasts to the cytoplasm during Calvin-cycle photosynthesis (Urbach, Hudson, Ullrich, Santarius & Heber, 1965; Heber, Santarius, Hudson & Hallier, 1967). We have attempted to use this technique to investigate the distribution of photosynthetic intermediates between the mesophyll and parenchyma-sheath chloroplasts of maize leaves. Initial experiments indicated that a relatively large but constant proportion of all the intermediates examined was lost from the chloroplasts when leaves were frozen in light petroleum at −80°. However, it was found that the leakage of intermediates from chloroplasts could be decreased by freezing leaves in liquid nitrogen and by using the density range 1·32–1·43 to fractionate the leaf tissue. The fractionation patterns for a number of photosynthetic intermediates obtained with maize leaves that had been exposed to $^{14}CO_2$-labelled air for 25 sec. are shown in Fig. 1. Chlorophyll, pyruvate,P_i dikinase (an enzyme present in mesophyll chloroplasts) and fructose diphosphate aldolase (an enzyme present in parenchyma-sheath chloroplasts) are included for comparison. The fractionation patterns of these enzymes suggests that the low-density fractions contained more parenchyma-sheath chloroplasts than in the experiments described above. Labelled malate, aspartate and 3-phosphoglycerate had almost identical fractionation patterns with a preponderance of these labelled intermediates in the low-density fractions containing mesophyll chloroplasts. On the other hand, labelled hexose phosphates and ribulose phosphates were fractionated in a similar manner to fructose diphosphate aldolase, the fractions of density 1·36–1·40 containing the largest proportion of these labelled intermediates. The fractionation pattern of dihydroxyacetone phosphate was intermediate between that of the dicarboxylic acids and the sugar phosphates. Qualitatively similar results were obtained in other

Table 3. *Distribution of chlorophyll, nitrite reductase, pyruvate,P_i dikinase, ribulose diphosphate carboxylase and NADP-specific glyceraldehyde phosphate dehydrogenase after the non-aqueous fractionation of starch-containing leaves*

Leaves were frozen in a flask cooled with an ethanol–solid CO_2 mixture, then freeze-dried, homogenized and fractionated, and chlorophyll content and enzyme activity were determined as described in the Methods section. The total activity of each enzyme and the total chlorophyll content represents the sum of the individual fractions.

Enzyme	Total activity (μmoles/min.)	Distribution of activity among fractions of different density (%)				
		<1·30	1·30–1·33	1·33–1·36	1·36–1·40	>1·40
Chlorophyll	206 *	73	8	10	8	1
Nitrite reductase	0·04	64	16	11	7	2
Pyruvate,P_i dikinase	0·48	74	7	8	9	2
Ribulose diphosphate carboxylase	0·27	9	2	37	46	6
NADP-specific glyceraldehyde phosphate dehydrogenase	2·9	44	5	20	23	8

* Chlorophyll content (μg.).

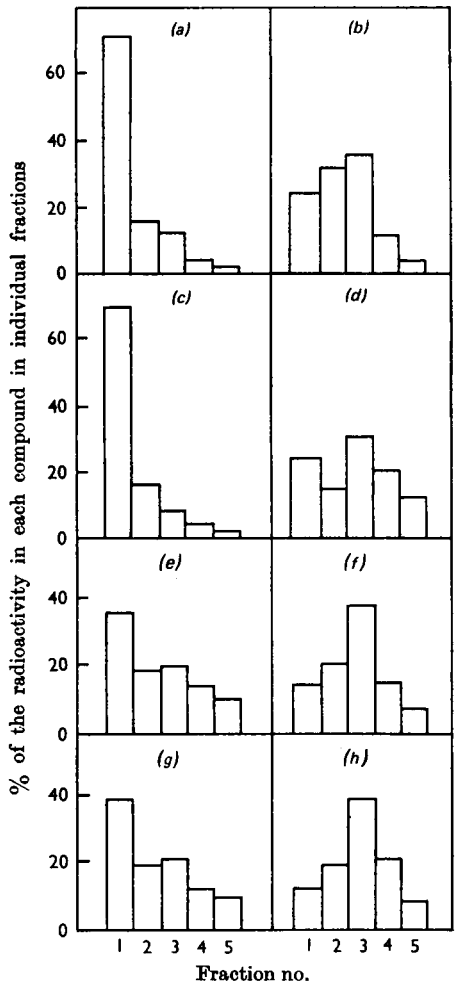


Fig. 1. Distribution of photosynthetic intermediates, chlorophyll and two photosynthetic enzymes obtained by density fractionation in non-aqueous media. Plants were illuminated for 2hr. before harvest, and detached leaves subsequently illuminated at 8000ft.-candles for 45min. before the introduction of $^{14}CO_2$. Experimental details and procedures for the isolation and identification of compounds are described in the Methods section. Fraction numbers 1–5 represent densities of $<1{\cdot}32$, 1·32–1·36, 1·36–1·40, 1·40–1·43 and $>1{\cdot}43$ respectively. (*a*) Chlorophyll; (*b*) fructose diphosphate aldolase; (*c*) pyruvate,P_i dikinase; (*d*) dihydroxyacetone phosphate; (*e*) malate + aspartate; (*f*) hexose phosphates; (*g*) 3-phosphoglycerate; (*h*) ribulose phosphates.

experiments, but the portion of each radioactive intermediate in fractions of density greater than 1·40 was generally larger than shown in Fig. 1. We suggest that the photosynthetic intermediates isolated in these heavy fractions represent the leakage of low-molecular-weight compounds from the chloroplasts during either freeze-drying or homogenization of the leaf.

DISCUSSION

The complete separation of the mesophyll and parenchyma-sheath chloroplasts by non-aqueous density fractionation is rendered difficult by several practical problems. The loss of starch grains from parenchyma-sheath chloroplasts during homogenization would lower the density of these chloroplasts to that of the mesophyll chloroplasts. In addition, the adsorption of cytoplasmic or other cellular material on the mesophyll chloroplasts can be expected to increase the density of these plastids. The duration of the illumination period before freeze-drying is also critical, since only poor separation was obtained when the illumination period was too short to allow the accumulation of adequate amounts of starch in the parenchyma-sheath chloroplasts. Prolonged illumination, which might have been expected to enhance the difference between the density of the two types of plastid, was found to increase the density of the mesophyll chloroplasts.

Electron-microscope studies of the fractions described in Table 1 showed that the mesophyll chloroplasts were contaminated by a few parenchyma-sheath chloroplasts and vice versa. Hence it is probable that the small amounts of pyruvate,P_i dikinase, NADP-specific malate dehydrogenase and glycerate kinase present in fractions containing parenchyma-sheath chloroplasts reflect the contamination of these fractions by mesophyll chloroplasts. Similarly it is probable that fructose diphosphate aldolase, alkaline fructose diphosphatase, ribulose diphosphate carboxylase, phosphoribulokinase, ribose phosphate isomerase and NADP-specific 'malic' enzyme are localized entirely in the parenchyma-sheath chloroplasts. On the other hand, adenylate kinase, pyrophosphatase, phosphoglycerate kinase and NADP-specific glyceraldehyde phosphate dehydrogenase were associated with both mesophyll and parenchyma-sheath chloroplasts. The first two enzymes appear to be predominantly located in the mesophyll chloroplasts. Slack (1969) suggested that NADP-specific glyceraldehyde phosphate dehydrogenase was mainly located in the mesophyll chloroplasts. However, the present studies, in which a more complete separation of the two types of chloroplast was achieved, indicate that this enzyme and phosphoglycerate kinase are distributed approximately equally between the mesophyll and parenchyma-sheath chloroplasts. It is noteworthy that the activities of adenylate kinase and pyrophosphatase in leaves of species

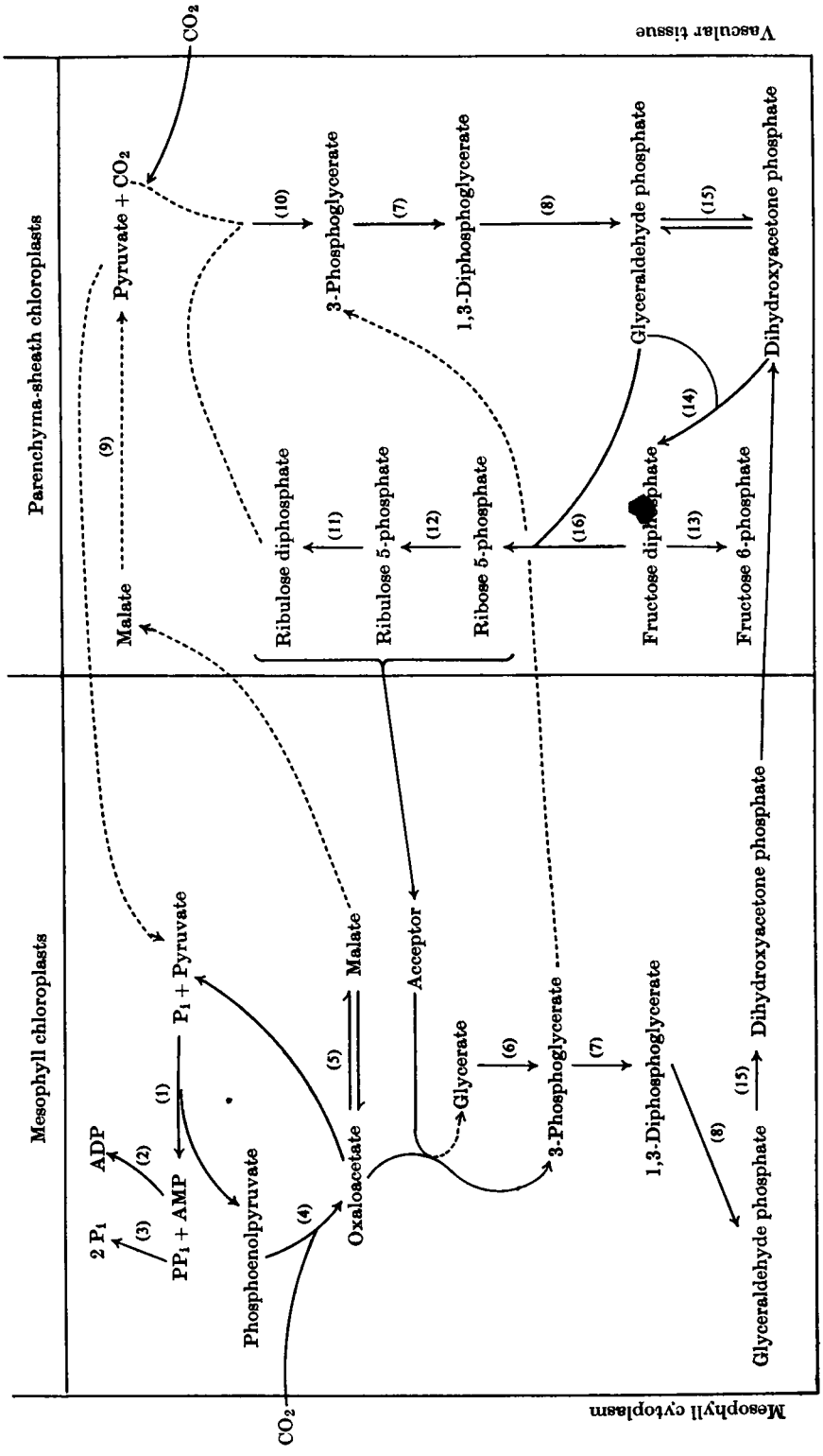
that photosynthesize via the C_4-dicarboxylic acid pathway are about 60-fold and tenfold greater respectively than their activities in leaves of species that utilize the Calvin cycle (Hatch *et al.* 1969).

We have previously reported that phosphopyruvate carboxylase fractionated from destarched maize leaves in a manner characteristic of a chloroplast enzyme when the density range 1·35–1·40 was employed (Slack & Hatch, 1967). In the present studies, however, the fractionation pattern obtained for this enzyme was variable. In one experiment with destarched leaves phosphopyruvate carboxylase was isolated in the chloroplast-containing fractions, and in other experiments with starch-containing leaves the enzyme appeared to be mainly associated with mesophyll chloroplasts. In several other experiments variable proportions of the enzyme were isolated with cytoplasmic enzymes. These observations suggest that phosphopyruvate carboxylase is mainly associated with the mesophyll chloroplasts and that the enzyme is localized at some site from which it is readily released during non-aqueous isolation. The electron-microscope studies carried out on fractions described in Table 1 indicated that the chloroplast membranes were removed during isolation, and in this particular experiment the enzyme was isolated together with the 'cytoplasmic' enzymes. An attractive explanation for the variable results obtained for phosphopyruvate carboxylase is that it is located in, or on, the double outer membranes of mesophyll chloroplasts, and that the degree of retention of this membrane varied from one experiment to another.

The present studies indicate that the operation of the C_4-dicarboxylic acid pathway requires the concerted function of the mesophyll and parenchyma-sheath chloroplasts. Enzymes that catalyse the formation of the carbon dioxide acceptor, phosphoenolpyruvate, and the incorporation of carbon dioxide into C_4 dicarboxylic acids are present in the mesophyll chloroplasts, whereas the parenchyma-sheath chloroplasts contain enzymes operative in the conversion of triose phosphates into fructose 6-phosphate. The present studies suggest two potential routes for the transfer of carbon from the C_4 dicarboxylic acids to 3-phosphoglycerate that are consistent with earlier labelling studies (Hatch & Slack, 1966; Hatch, Slack & Johnson, 1967; Johnson & Hatch, 1969). These are outlined in Scheme 1. The distribution of photosynthetic intermediates was consistent with the view that 3-phosphoglycerate is formed in the mesophyll chloroplasts. These plastids, however, do not contain ribulose diphosphate carboxylase, and we therefore suggest that 3-phosphoglycerate is formed by a transcarboxylation reaction of the type proposed earlier (Hatch & Slack, 1966). The localization of glycerate kinase in the mesophyll chloroplasts gives tentative support to the view that glycerate is a product of this reaction. Both the mesophyll and parenchyma-sheath chloroplasts contain enzymes that catalyse the conversion of 3-phosphoglycerate into triose phosphates; therefore there are two possibilities with regard to the identity of the C_3 compound transferred to the parenchyma-sheath chloroplasts. The fractionation pattern of dihydroxyacetone phosphate, which was intermediate between that of 3-phosphoglycerate and hexose phosphates, suggests that at least part of the 3-phosphoglycerate is reduced in the mesophyll chloroplasts and that triose phosphates are subsequently moved to the parenchyma-sheath chloroplasts. It is envisaged that the carboxyl acceptor for the transcarboxylation reaction is formed in the parenchyma-sheath chloroplasts and transferred to the mesophyll chloroplasts to maintain a carbon balance between the two types of chloroplasts.

An alternative route for the transfer of carbon from C_4 dicarboxylic acids to 3-phosphoglycerate was suggested by the localization of NADP-specific malate dehydrogenase in the mesophyll chloroplasts, and of ribulose diphosphate carboxylase and NADP-specific 'malic' enzyme in the parenchyma-sheath chloroplasts. The activity of the latter enzyme in leaves of maize and other Panicoid grasses is considerably greater than in leaves of species that utilize the Calvin cycle (Slack & Hatch, 1967). We tentatively suggest (Scheme 1) that oxaloacetate might be reduced in the mesophyll chloroplasts by NADP-specific malate dehydrogenase, and the malate formed subsequently transferred to the parenchyma-sheath chloroplasts. NADP-specific 'malic' enzyme could then catalyse the decarboxylation of malate, and ribulose diphosphate carboxylase bring about the fixation of the released carbon dioxide into 3-phosphoglycerate. To maintain a carbon balance during the operation of such a pathway we envisage that pyruvate would be returned to mesophyll chloroplasts. It is significant that the transfer of malate to the parenchyma-sheath chloroplasts would serve as a transport mechanism for both carbon dioxide and reducing power. However, the fractionation patterns of the photosynthetic intermediates, discussed above, were not consistent with operation of this pathway under the conditions employed. It is probable that the pathway constitutes only a minor route for the fixation of carbon dioxide into 3-phosphoglycerate, since the quantity of ribulose diphosphate carboxylase in leaves of plants utilizing the C_4-dicarboxylic acid pathway is small and quite inadequate to account for the observed rates of photosynthesis (Slack & Hatch, 1967).

It is noteworthy that the parenchyma-sheath chloroplasts contain all the enzymes specifically associated with the Calvin cycle, and therefore are



Scheme 1. Possible interrelationships of mesophyll and parenchyma-sheath chloroplasts in the operation of the C_4-dicarboxylic acid pathway. The scheme shows only the sequence of intermediates, not complete reactions. Numbers refer to the enzyme that catalyses each particular reaction: (1) pyruvate,P_i dikinase; (2) adenylate kinase; (3) pyrophosphatase; (4) phosphopyruvate carboxylase; (5) NADP-specific malate dehydrogenase; (6) glycerate kinase; (7) phosphoglycerate kinase; (8) NADP-specific glyceraldehyde phosphate dehydrogenase; (9) NADP-specific 'malic' enzyme; (10) ribulose diphosphate carboxylase; (11) phosphoribulokinase; (12) ribose phosphate isomerase; (13) alkaline fructose diphosphatase; (14) fructose diphosphate aldolase; (15) triose phosphate isomerase; (16) reactions similar to those operative in the Calvin cycle. Unidentified reactions are not numbered. Possible alternative routes of carbon flow are indicated with broken lines.

probably capable of photosynthesizing autonomously via this cycle. However, $^{14}CO_2$-labelling studies have indicated that the carbon that enters 3-phosphoglycerate is derived from C-4 of the C_4 dicarboxylic acids (Hatch & Slack, 1966; Hatch *et al.* 1967; Johnson & Hatch, 1969). A possible explanation for this anomaly is that the parenchyma-sheath chloroplasts may be relatively inaccessible to carbon dioxide entering the leaf since the parenchyma sheath is surrounded by a layer of tightly packed chloroplast-containing mesophyll cells (Brown, 1958). The parenchyma-sheath chloroplasts, however, may function independently of the mesophyll chloroplasts to fix any respiratory carbon dioxide that diffuses from the vascular tissue.

The operation of the C_4-dicarboxylic acid pathway as depicted in Scheme 1 implies the rapid movement of photosynthetic intermediates between chloroplasts situated in adjacent cells, and hence the possibility of physical connexions between the chloroplasts. It is noteworthy that numerous plasmodesmata have been observed in the cell walls separating the mesophyll and parenchyma-sheath cells of maize (Johnson, 1964) and sugar cane (Laetsch & Price, 1969). Rosado-Alberio, Weier & Stocking (1968) have described a well-developed peripheral reticulum in the mesophyll chloroplasts of maize that is connected directly with the grana by fret channels. They suggest that such a structure could facilitate the movement of photosynthetic intermediates within the chloroplasts. Similar peripheral reticula have been observed in both the mesophyll and parenchyma-sheath chloroplasts of *Atriplex lentiformis* and *Amaranthus edulis* (Laetsch, 1968), in which the C_4-dicarboxylic acid pathway operates. However, no peripheral reticulum was observed in chloroplasts of *Atriplex lastata*, in which the Calvin cycle is operative (C. B. Osmond, J. H. Troughton & D. J. Goodchild, unpublished work). It is possible that this peripheral reticulum, which is apparently restricted to chloroplasts of plants utilizing the C_4-dicarboxylic acid pathway, may represent the terminals of tubules connecting the two types of chloroplast.

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