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Generation and maintenance of concentration gradients between the mesophyll and bundle sheath in maize leaves

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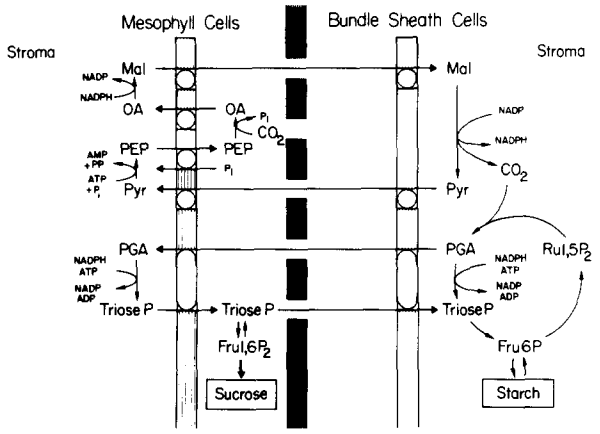
(1) Experiments have been carried out to test the proposal that intercellular transport of carbon occurs by diffusion during photosynthesis in C-4 plants. (2) The intercellular distribution of metabolites has been compared in different conditions. A partial separation of the mesophyll and bundle sheath was obtained by homogenisation in liquid N_2, followed by filtration through nylon nets with differing aperture. (3) Concentration gradients between the bundle sheath and mesophyll were found for 3-phosphoglycerate, triose phosphates, malate and pyruvate during photosynthesis. These gradients are shown to be large enough to allow rapid intercellular transport by diffusion. They disappear when photosynthesis is prevented by removal of light or CO_2. (4) The concentration gradients for triose phosphates and 3-phosphoglycerate are due to the differing capacity of the bundle sheath and mesophyll to reduce 3-phosphoglycerate. (5) The distribution of carbon between the malate/pyruvate and 3-phosphoglycerate/triose phosphate shuttles is flexible, and may be controlled by phospho*enol*pyruvate carboxylase. (6) The maintenance of these large concentration gradients has consequences for the regulation of sucrose synthesis and the Calvin cycle.

Introduction

Two different types of cell cooperate during photosynthesis in C_4 plants. An extreme form of this cooperation is found in NADP-malic enzyme species with agranal chloroplasts in the bundle sheath such as maize. These agranal chloroplasts are deficient in Photosystem II [1,2] and are unable to carry out noncyclic electron transport to produce NADPH, although ATP can be produced by cyclic electron transport. The bundle sheath chloroplasts, however, contain Ru(1,5)P_2 carboxylase and the Calvin cycle. In contrast, the chloroplasts in the mesophyll cells carry out noncyclic as well as cyclic electron transport to produce NADPH and ATP, but do not have the enzymes of the Calvin cycle. Further, while starch accumulation is normally restricted to the bundle sheath chloroplasts [1,2], the synthesis of sucrose is mainly in the cytosol of the mesophyll cells [3]. This intercellular specialization allows a high CO_2 concentration to be maintained in the bundle sheath, while suppressing O_2 evolution in these cells, so that carboxylation of Ru(1,5)P_2 is favoured [1,2], but requires rapid transfer of fixed carbon and energy between the different types of cells.

Abbreviations: C_4, plants carrying out photosynthesis involving incorporation of CO_2 first into organic acids; C_3, plants where photosynthesis involves only the Calvin cycle; DHAP, dihydroxyacetone phosphate; Fru(1,6)P_2, fructose-1,6-diphosphate; Fru(2,6)P_2, fructose-2,6-bisphosphate; P_i, inorganic phosphate; Ru(1,5)P_2, ribulose-1,5-biphosphate; Sedu(1,7)P_2, seduheptulose-1,7-bisphosphate.



Scheme I. Metabolic pathways during photosynthesis in a maize leaf.

Two major intercellular exchanges of metabolites, or shuttle systems, have been proposed [1] (Scheme I). In the first shuttle, an exchange of malate and pyruvate allows NADPH and CO_2 to be supplied to the Calvin cycle in the bundle sheath. Malate moves from the mesophyll into the bundle sheath where it is converted to CO_2, NADPH and pyruvate by NADP-malic enzyme in the chloroplasts. The pyruvate returns to the mesophyll, where it is reconverted to malate in a sequence of reactions involving conversion to phospho*enol*pyruvate by pyruvate phosphate dikinase in the chloroplast, carboxylation by phospho*enol*pyruvate carboxylase in the cytosol, and reduction of oxaloacetate to malate by NADP-malate dehydrogenase in the chloroplast. The CO_2 released in the bundle sheath is refixed by Ru(1,5)P_2 carboxylase, producing two molecules of glycerate-3-*P*, but only one of these can be reduced utilizing the NADPH which has been shuttled into the bundle sheath via the malate/pyruvate exchange. Thus a second shuttle is required, in which the remaining half of the glycerate-3-*P* moves from the bundle sheath to the mesophyll chloroplasts, where it is reduced to triose phosphates. At least two thirds of these triose phosphates have to return to the Calvin cycle in the bundle sheath chloroplast, whereas the remaining third may be removed for synthesis of sucrose in the mesophyll [3].

The bundle sheath is surrounded by a suberized cell wall layer, which provides a barrier to movement by diffusion through the apoplast, but there are numerous plasmodesmatal connections with the mesophyll. For this reason, it has been suggested that intercellular transport occurs by diffusion in the symplasm, and calculations indicated that a concentration gradient of 5–10 mM between the bundle sheath and mesophyll would allow diffusion at the rates required during photosynthesis [1,4]. Although maize leaves contain large amounts of malate, pyruvate, triose phosphates and glycerate-3-*P* [1,5], it was not possible to investigate whether intercellular concentration gradients were present until methods became available to separate the mesophyll and bundle sheath tissues from intact maize leaves. In one recently developed method, leaves were squashed by means of a roller device to yield a sap of almost pure mesophyll which was quenched in perchloric acid within a second [6,7]. In a second approach, maize leaves were rapidly frozen and then partially homogenized under liquid N_2. Particles enriched in bundle sheath or mesophyll were separated by filtration through a series of nylon nets under liquid N_2 [8]. Both these methods showed that most of the glycerate-3-*P* was located in the bundle sheath, while most of the triose phosphates were located in the mesophyll during photosynthesis. On the other hand, no evidence could be found for concentration gradients of pyruvate, and it was suggested that these might be masked by sequestration in non-metabolized pools, or by active transport within a cell. Most of the malate was located in the mesophyll, but this did not provide clear evidence for the presence of a concentration gradient in the symplasm, as much of this malate might be located in the vacuoles of the mesophyll cells [6–8].

We have extended these studies by comparing the distribution of metabolites in the presence and absence of photosynthetic carbon fluxes. When a metabolite is diffusing from one cell to another, the flux will depend on the size of the concentration gradient. This concentration gradient is built up as a metabolite is rapidly produced in one cell, while being rapidly removed in the other cell. When the photosynthetic reactions which generate the concentration gradient are stopped, the gradient should decrease or disappear. This provides an additional criterion for the existence of diffusion

driven flux, which should allow such fluxes to be identified in spite of the overall measurement being complicated by non-metabolic pools or active transport processes. To inhibit photosynthesis we have used either darkness, or illumination in CO_2 free air. By comparing the distribution in these two conditions it should also be possible to identify any contribution made by light-dependent active transport processes.

Methods

Maize was grown as in Ref. 8. Illumination was provided by two projectors (white light) at 16 cm distance, with a combined ligh intensity of 2500 $mE \cdot m^{-2} \cdot s^{-1}$. CO_2-free air was provided by passage in a closed system through a 20×1 cm column of $Ca(OH)_2$, after bringing the air to 90–100% humidity. Material was quenched in liquid N_2 and the intercellular fraction, extraction, metabolite assays and Fru(2,6)P_2 determination were carried out as in Ref. 8. A 3-fold enrichment between bundle sheath and mesophyll compartments was attained by homogenizing maize leaves under liquid N_2, and separating particles of different size by passing the homogenate in liquid N_2 through nylon nets of varying aperture. The intercellular origin of metabolites was estimated by comparing their distribution with that of marker enzymes in the fractions obtained.

Results and Discussion

Overall metabolite levels

Maize plants were preilluminated for 4–6 h before leaf strips were removed and further incubated for 25 min either in the dark in air, in the light in air, or in the light in CO_2-free air. The total metabolite content after these treatments is shown in Table I. As previously found [6,7] there are considerable changes of glycerate-3-*P* (5-fold), triose phosphates (25-fold) and phospho*enol*-pyruvate (2-fold) between dark and light. These changes in glycerate-3-*P* and triose phosphates are more pronounced than those in leaves or protoplasts from spinach [9,10], pea, wheat or barley [11,12]. In C_3 plants the triose phosphate content is usually below 40 nmol per mg Chl and rarely exceeds 80 nmol per mg Chl. Glycerate-3-*P* varies between 100 and 200 nmol per mg Chl, increasing less than 2-fold on illumination compared to the dark and often remaining unaltered. The contents of glycerate-3-*P* and triose phosphates also decrease when maize leaves are illuminated in CO_2-free air (Table I), but are still high compared with the amounts found in leaves and protoplasts of C_3 plants.

TABLE I

METABOLITE CONTENT OF MAIZE LEAVES

Maize leaves were detached, held in the dark for 2 h and illuminated for 25 min in air or CO_2-free air, or in dark. Water-saturated air was used in all cases. Results are mean ± SE of 3–5 experiments.

	Metabolite content		
	Light $+CO_2$	Light $-CO_2$	Dark
	(nmol per mg Chl)		
Triose phosphates	233 ± 44	136 ± 22	9 ± 3
Glycerate-3-*P*	341 ± 45	65 ± 17	62 ± 28
Pyruvate	211 ± 37	67 ± 27	199 ± 43
Malate	1035 ± 282	594 ± 85	935 ± 23
Phospho*enol*pyruvate	51 ± 19	19 ± 10	22 ± 4
Fru(1,6)P_2	33 ± 7	70 ± 14	4
Glucose-6-phosphate	120 ± 27	107 ± 29	80 ± 35
Fructose-6-phosphate	58 ± 15	38 ± 13	26 ± 11
Ru(1,5)P_2	161 ± 25	276 ± 50	45 ± 8

The content of pyruvate and malate is also substantial and exhibits considerable changes. In our conditions, no large changes were found between light and dark (Table I), but it has previously been shown that pyruvate increases on illumination of maize leaves after a long dark pretreatment [6,7] and also increases transiently after darkening [6,7]. In CO-free air, both pyruvate and malate decrease, suggesting that carbon moves out of the intermediates of the malate/pyruvate shuttle when it is interrupted by restricting phosphoenolpyruvate carboxylase activity (see below for further discussion).

The levels of metabolites associated with the Calvin cycle, such as Ru(1,5)P_2 and Fru(1,6)P_2, are negligible in the dark, but remain high in the absence of CO_2, both the total amounts and the alterations being in this case similar to those found in C_3 plants. The level of hexose phosphates in-

creases only slightly in conditions allowing rapid photosynthesis, as is also the case in C_3 plants [9,12]. The absolute amount of hexose phosphate is also similar to that in C_3 plants. Thus, it appears that maize differs from C_2 plants in having particularly large pools of triose phosphates and glycerate-3-*P* which develop during photosynthesis (see also Refs. 6 and 7), whereas the pools of other inntermediates in the Calvin cycle and sucrose synthesis are not markedly different from those found in C_3 plants.

Intercellular distribution

The intercellular distribution of metabolites was studied in each condition using partial homogenization and filtration under liquid N_2 [8] to provide an estimate of the distribution of a metabolite between the mesophyll and bundle sheath. The distribution of metabolites in the dark, in the light, and in the light in the CO_2-free conditions are summarized in Table II. Triose phosphates, which were mainly in the mesophyll during rapid photosynthesis [6–8], became more equally distributed in CO_2-free air. In the dark the content of triose phosphates was too low to allow accurate measurements of the distribution. The distribution of glycerate-3-P was also equal in CO_2-free conditions and in the dark, but was mainly in the bundle sheath during rapid photosynthesis (see also Refs. 6–8). Pyruvate was equally distributed during rapid photosynthesis (see also Refs. 6–8) and in the dark, was mostly in the mesophyll fraction in the light in CO_2-free conditions. The proportion of malate in the mesophyll decreased in CO_2-free conditions and in the dark, although the changes were not large. However, the total amounts of malate were large, and only a small proportion may be directly involved in photosynthetic metabolism [1].

TABLE II

INTERCELLULAR DISTRIBUTION OF METABOLITES IN MAIZE LEAVES

The distribution was analyzed by partial homogenization and filtration in liquid N_2 of leaf material preincubated as described in Table I. Results are the mean ± SE of 3–5 fractionations of separate plant material. n.d., not determined.

	% of metabolite in mesophyll		
	Light $+CO_2$	Light $-CO_2$	Dark
Triose phosphates	91 ± 8	65 ± 9	n.d.
Glycerate-3-*P*	24 ± 5	59 ± 13	50 ± 13
Malate	67 ± 3	59 ± 10	44 ± 2
Pyruvate	55 ± 20	108 ± 20	53 ± 6
Phospho*enol*pyruvate	73 ± 11	50	40

The overall concentrations present in the bundle sheath and mesophyll in the different conditions are estimated in Table III, assuming that chlorophyll is equally distributed between the mesophyll and bundle sheath (see Refs. 3 and 7) and that the volume of the combined stroma and cytosol resembles that of C_3 plant (for References, see Ref. 12) in being about 40 μl per mg Chl. Our estimates of the concentration gradients between the mesophyll and bundle sheath are summarized in Table IV. These values are approximations, given the limitations of the method, the assumptions made, and the possibility that they may be modified by subcellular compartmentation, but they provide a guide to the concentrations occurring in vivo, and the way in which they change. The question arises to what extent these overall concentration gradients represent a concentration gradient in the symplasm between the two cell types.

Gradients of triose phosphate, glycerate-3-P, malate and pyruvate

There is clear evidence for the presence of symplastic concentration gradients of both glycerate-3-*P* and triose phosphates. Our results (see also Refs. 6–8) reveal that during photosynthesis the concentration gradient of glycerate-3-*P*-from bundle sheath to mesophyll and the reciprocal gradient of triose phosphates are both in the order of 10 mM. These gradients collapse when photosynthesis is prevented in CO_2-free air or in the dark, as expected for symplastic gradients. In conditions where photosynthesis is inhibited, there is no evidence for substantial accumulation of either glycerate-3-*P* or triose phosphates in the cell to which they normally move, suggesting that active transport does not make any substantial contribution to the overall distribution of these two metabolites. Of course, this does not exclude the

TABLE III

ESTIMATED CONCENTRATIONS OF METABOLITES IN MESOPHYLL AND BUNDLESHEATH

The concentrations are calculated from the results of Table I and II, assuming that chlorophyll is equally distributed between the mesophyll and bundlesheath, and that the combined volume of the chloroplast and cytosol is 40 μl per mg Chl. Values are in mM.

	Mesophyll			Bundlesheath		
	Light + CO_2	Light − CO_2	Dark	Light + CO_2	Light − CO_2	Dark
Triose phosphates	11	4	0.3	1	2	0.3
Glycerate-3-*P*	4	2	1.5	13	1.5	1.5
Malate	35	17	20	17	12	25
Pyruvate	6	3	6	5	0.5	5
Phospho*enol*pyruvate	2	0.5	0.5	0.7	0.5	0.7

possibility that, within a cell, the glycerate-3-*P* may be preferentially located in the chloroplast and triose phosphates in the cytosol, as in the case in C_3 species [9,11–13], and any such intracellular heterogeneity would modify the magnitude of the diffusion gradient in the symplasm (see Ref. 8 for fuller discussion). Due to the equilibrium constant of the reaction catalysed by triose phosphate isomerase, the dihydroxyacetone phosphate concentration will be 10–20-fold above that of glyceraldehyde-3-phosphate. Correspondingly, most of the concentration gradient will be due to dihydroxyacetone phosphate.

For malate, an apparent gradient of up to 20 mM is found during photosynthesis, but taken alone this would not provide sound evidence for a symplastic concentration gradient, as it might only reflect the distribution of stored malate in the vacuole or in non-photosynthetic cells. However, this gradient decreases in the dark or in CO_2-free conditions as the amount in the bundle sheath increases and that in the mesophyll decreases. As it is unlikely that stored malate is redistributed between vacuoles of different cells within 20 min, these changes provide evidence for a symplastic concentration gradient with higher concentrations in the mesophyll than in the bundle sheath during photosynthesis.

No overall concentration gradient of this order could be found for pyruvate (see also Refs. 6–8). It is, however, of interest that the remaining pyruvate is restricted to the mesophyll during illumination in the absence of CO_2, while in the dark it remains equally distributed. These results could be explained by an active light-dependent accumulation of pyruvate in the mesophyll cells. In view of the good symplastic connections and rapid diffusion of the other metabolites, it is un-

TABLE IV

DIFFERENCE OF CONCENTRATION BETWEEN THE MESOPHYLL AND BUNDLESHEATH

The values are estimated from Table III (assuming equal distribution between cytosol and chloroplast). The concentration gradients are calculated for the direction mesophyll → bundlesheath (MS → BS) or bundlesheath → mesophyll (BS → MS). A negative sign means a gradient against the direction which the metabolite must move during photosynthesis.

Metabolite	Gradient Direction	Concentration gradient (mM)		
		Light + CO_2	Light − CO_2	Dark
Triose phosphates	MS → BS	10	2	0.0
Glycerate-3-*P*	BS → MS	9	−0.5	0.0
Malate	MS → BS	18	5	−5
Pyruvate	BS → MS	−1	−3	−1

likely that active transport of pyruvate would occur at the site of entry into the mesophyll cells, and a more plausible site would be at uptake into the chloroplasts. Active accumulation of pyruvate in the mesophyll chloroplasts might lower the cytosolic concentration far enough to provide a concentration gradient into the mesophyll cytosol from the bundle sheath that would not be detected by our procedure. Earlier studies have shown uptake of pyruvate into maize mesophyll chloroplast, but did not provide any evidence for a light-dependent accumulation [14]. Recently, however, a light-induced active uptake of pyruvate into maize mesophyll chloroplasts has been demonstrated which has a K_m of under 1 mM for pyruvate [15]. Alternatively, some of the pyruvate pool attributed to the mesophyll cells may actually be located in non-photosynthetic cells. If the pyruvate in the metabolic pools in the photosynthetic cells were reduced to zero in CO_2-free conditions, the residual pyruvate would represent this nonphotosynthetic pool, which would mask any concentration gradient built up in photosynthetic conditions. It might be noted that these two explanations are not mutually exclusive, and that both could provide a concentration gradient of around 5 mM.

Estimation of rates of diffusion of metabolites

Hatch and Osmond [1] have estimated the relation between concentration gradients and the flux rates between the mesophyll and bundle sheath. In Table V, their calculation is used together with the results of Table IV (for pyruvate, see discussion above) to estimate the relative fluxes of several metabolites. In calculating the fluxes, both the magnitude of the concentration gradient as well as the molecular weight has been included (see legend for details). Fluxes of the magnitude of $5 \cdot 10^{-2}$ $\mu mol \cdot cm^{-2} \cdot s^{-1}$ through the plasmodesmata should be adequate to support photosynthesis in maize given that about 3% of the interface between the bundle sheath and the mesophyll is occupied by plasmodesmata [1]. The fluxes we estimate are $2\frac{1}{2}$–6 fold in excess of this value, suggesting that

TABLE V

ESTIMATION OF FLUXES OF METABOLITES ON THE BASIS OF CONCENTRATION GRADIENTS MEASURED IN MAIZE LEAVES

The fluxes are calculated according to the formula

$$J = \frac{aD(C)r_b}{lr_m^2},$$

where a is the fraction of bundlesheath surface occupied by plasmodesmata; D is the diffusion coefficient; C, the concentration gradient between the bundle sheath and mesophyll; l is the length of the bundlesheath cylinder and r_m and r_b are the average radii of the chloroplast layer in mesophyll and bundlesheath, cells, respectively, relative to the central vascular strand (see Ref. 1). A value of $D = 8 \cdot 10^{-6}$ $s^{-1} \cdot cm^{-2}$ is taken for malate [1], and is adjusted for other metabolites on the basis of molecular weight. For CO_2 and HCO_3^-, $D = 10^{-5}$ $s^{-1} \cdot cm^{-2}$ [1]. Values for a, l, r_m and r_b are from Ref. 1. The values for C are taken from Table IV (for pyruvate see text).

Metabolite	C (mM)	Mol. wt.	D (10^{-6} $s^{-1} \cdot cm^{-2}$)	J (10^{-2} $\mu mol \cdot s^{-1} \cdot cm^{-2}$)
Malate	18 [a]	134	8.0	30 [a]
Pyruvate	5	88	12.2	12
Triose phosphates	10 [a]	170	6.3	16 [a]
Glycerate-3-*P*	9	186	5.8	14
Phospho*enol*pyruvate	1.0 [a]	168	6.4	1.6 [a]
Glucose-6-*P*	1.5 [a]	260	4.1	1.3 [a]
Fru2,6P_2	0.005 [a]	344	3.1	0.005 [a]
CO_2 and HCO_3^-	0.7	–	10	0.35

[a] In direction mesophyll → bundlesheath.

diffusion rates would be high enough to cover the rate of photosynthesis even if the assumptions made in our experiments or the calculation are in error by this extent.

The relation between the fluxes estimated in this way and those required during photosynthesis may be affected by a number of other factors. First, since sucrose is synthesized from triose phosphates in the mesophyll the rate of triose phosphate transport may be reduced by up to one-third compared with the other metabolites. Slightly larger fluxes of malate and pyruvate may be needed to compensate for back-diffusion of CO_2, but this is probably minor (see Ref. 1 and below). The movement of pyruvate and glycerate-3-*P* from the bundle sheath to the mesophyll may be aided by a simultaneous movement of water in the transpiration stream; this would equally tend to hinder the movement of malate and triose phosphates back to the bundle sheath. This entrainment effect is likely to be small, however. Water loss in maize can reach 4 $mmol \cdot m^{-2}\ s^{-1}$ on a leaf area basis which, related to the surface of the plasmodesmata in the bundle sheath surface (see legend to Table V), would be a flux of about 7 $\mu mol \cdot cm^{-2} \cdot s^{-1}$, or about 130 $nl \cdot cm^{-2} \cdot s^{-1}$. If a metabolite were present at 10 mM, less than $0.2 \cdot 10^{-2}\ \mu mol \cdot cm^{-2} \cdot s^{-1}$ would be carried, far less than the fluxes driven by the concentration gradients (see Table V).

The fluxes of some other metabolites which are not directly involved in intercellular shuttling are also estimated (Table V). The rate of diffusion of glucose-6-phosphate or phospho*enol*pyruvate would be about 10-fold lower. Fru(2,6)P_2, a signal metabolite present at low concentrations which is involved in control of photosynthetic sucrose synthesis (see Ref. 10), would diffuse very slowly. At such low fluxes, the contribution of the transpiration stream in preventing movement into the bundle sheath could become relatively more important. This may be decisive in accounting for the virtual restriction of Fru(2,6)P_2 to the mesophyll which was reported previously [8]. Finally, for comparison, estimates of the fluxes of CO_2 and bicarbonate back from the bundle sheath to the mesophyll are included from Hatch and Osmond [1]. These are only 1–3% of the fluxes of malate or pyruvate calculated from our measurements of concentration gradient, showing that the back-diffusion of bicarbonate and CO_2 is likely to be minimal in maize leaves.

These results suggest that large concentration gradients of selected metabolites indeed occur between the bundle sheath and mesophyll cells of maize leaves. The remainder of this article will examine how these gradients are generated, and what implications this has for other aspects of photosynthetic metabolism. Several general problems arise. Firstly, generation of a symplastic concentration gradient requires that a metabolite can be rapidly produced despite its being present at a high concentration in one cell type, while being rapidly removed in the other cell type, even though the concentration there is much lower. What specialization of metabolism in the bundle sheath and mesophyll allows this to occur? Secondly, how are the sizes of the metabolite pools and gradients in the two shuttle systems controlled and coordinated? Thirdly, how are the high concentrations of selected metabolites protected, so that they are not dissipated by other reactions? Fourthly, to what extent are other aspects of photosynthetic metabolism modified due to the presence of such high concentrations of selected intermediates?

Driving forces for the malate-pyruvate shuttle

The mesophyll cells possess an efficient system for removing pyruvate and generating malate. The light-dependent active uptake of pyruvate in the chloroplasts (see above) is followed by a light-activated [16,17] conversion to phosphoenolpyruvate in an irreversible reaction catalysed by pyruvate phosphate dikinase using ATP produced by photophosphorylation. Phosphoenolpyruvate is carboxylated in the cytosol, again by an irreversible reaction, producing oxalacetate which is taken up into the chloroplast by a carrier system with a high V_{max} and low K_m [18], and reduced to malate using NADPH from the photochemical reactions. The uptake of oxalacetate, the production of NADPH by electron transport, and the equilibrium position of the malate dehydrogenase reactions will all favour generation of a high concentration of malate. It is not known whether the export of malate from the chloroplasts to the cytosol is energised.

Malate is converted to pyruvate in the bundle

sheath in a reversible reaction catalysed by NADP malic enzyme in which NADPH and CO_2 are produced. This reaction will only occur if the products can be removed. The CO_2 is removed by Ru(1,5)P_2 carboxylase, but it is thought that a substantial concentration of CO_2 is nevertheless present in the bundle sheath [1,2]. Therefore, maintaining a low NADPH/NADP ratio may be important if rapid decarboxylation is to occur. NADP-malic C_4 plants typically show a reduction, if not loss, of grana in their bundle sheath [2]. Suppression of NADPH formation by linear electron transport may be unavoidable, if the reaction catalysed by NADP-malic enzyme is to proceed rapidly in the decarboxylation direction. Removal of NADPH would also favour decarboxylation. In isolated bundle sheath preparations, glycerate-3-*P* stimulates conversion of malate to pyruvate [20,21], presumably by providing a strong sink for NADPH. Similarly, the high glycerate-3-*P* found in the bundle sheath of leaves (Table III) should favour decarboxylation. Our results suggest that the bundle sheath may contain more malate than pyruvate (Table III), which would also favour decarboxylation. However, more studies are needed to clarify how decarboxylation is controlled. For example, aspartate stimulates malate decarboxylation by bundle sheath strands [22] and chloroplasts (Jenkins, C. and Boag, S., unpublished data). Although the low amount and equal distribution of aspartate (see Ref. 8) precludes direct participation in malate-pyruvate shuttle, aspartate may still exert a regulatory role. It is also unknown whether the movement of pyruvate or malate across the bundle sheath chloroplast envelope membrane occurs via active transport.

Glycerate-3-P reduction drives diffusion of glycerate-3-P and triose phosphates

For glycerate-3-*P* and triose phosphate, concentration gradients are generated by the differing capacity of the mesophyll and bundle sheath to reduce glycerate-3-*P*. The glycerate-3-*P*/triose phosphate quotients in the bundle sheath and mesophyll are summarized in Table VI. The quotient is far higher in the bundle sheath, suggesting a restriction on glycerate-3-*P* reduction in these cells. As both the bundle sheath and mesophyll contain ample activity of the enzymes to catalyze glycerate-3-*P* reduction [1], their differing capacity for glycerate-3-*P* reduction probably reflects their ability to generate ATP and NADPH. Glycerate-3-*P* reduction is a reversible reaction which can be driven by raised ATP/ADP or NADPH/NADP quotients, or by accumulation of glycerate-3-*P* to high levels [21]. Thus, the glycerate-3-*P*/triose phosphate quotient may be taken as an indicator of the extent to which the generation of ATP and NADPH, or the provision of glycerate-3-*P*, is limiting this reaction. During rapid photosynthesis in leaves of C_3 species the overall glycerate-3-*P*/triose phosphate ratio varies between 4 and 5 [9,11], in contrast to a value of 13 in the maize bundle sheath, and 0.5 in the maize mesophyll (Table VI). This provides evidence that the ATP/ADP and NADPH/NADP ratios are lower in the bundle sheath and higher in the mesophyll during photosynthesis than in a leaf from a C_3 plant.

In view of the specialization in the maize leaf, where the bundle sheath is deficient in electron transport, while the mesophyll is photochemically competent, such differences could be expected. NADPH must be imported into the bundle sheath,

TABLE VI

QUOTIENTS OF METABOLITES IN THE MESOPHYLL AND BUNDLESHEATH CELLS

The results are calculated from the results of Tables I and II.

Quotient	Cell Type	Light $+CO_2$	Light $-CO_2$	Dark
Glycerate-3-*P*/triose phosphates	meosphyll	0.4	0.5	5
	bundlesheath	13	0.6	5
Glycerate-3-*P*/phospho*enol*pyruvate	mesophyll	2	4	3
	bundlesheath	19	3	2

TABLE VII

ADENINE NUCLEOTIDES IN BUNDLE SHEATH AND MESOPHYLL OF MAIZE LEAVES DURING PHOTOSYNTHESIS

Maize leaves were quenched in liquid N_2 after 15 min illumination, and were fractionated as in Methods. Results are mean ± SE of three experiments. Values are in nmol per mg Chl.

	% of total		Amount		ATP/ADP ratio
	ATP	ADP	ATP	ADP	
Whole leaf	≡100	≡100	54 ± 1.1	10 ± 1.7	5.4
Bundlesheath	33 ± 2	70 ± 7	20 ± 1.1	7 ± 0.4	2.9
Mesophyll	63 ± 2	30 ± 7	34 ± 0.3	3 ± 1.1	10.3

and ATP can only be generated by cyclic electron transport [1,2]. In Table VII the intercellular distribution of ATP and ADP is shown for maize leaves during rapid photosynthesis. The ATP/ADP quotient in the mesophyll is considerably higher than that in the bundle sheath. Comparing these values with those for leaves [9,23,24] or protoplasts [25,26] from C_3 plants reveals that the maize mesophyll cells possess a very high ATP/ADP quotient compared with that during C_3 photosynthesis, while the values in the bundle sheath are comparatively low. These measurements of the ATP/ADP and glycerate-3-*P*/triose phosphate quotients apply to the whole cell and do not provide any direct value for the conditions in the stroma. Nevertheless, any marked change in the ATP/ADP ratio is likely to reflect an alteration in the stroma as, at least in C_3 plants [25,26], the ADP in the cytosol is very low and does not alter greatly.

Thus, the high glycerate-3-*P*/triose phosphate quotient in the bundle sheath reflects an inbalance between rapid production of glycerate-3-*P* by carboxylation of Ru(1,5)P_2, and the availability of ATP and NADPH. In agreement, when production of glycerate-3-*P* in the bundle sheath is restricted in CO_2-free conditions, the glycerate-3-*P*/triose phosphate ratio in the bundle sheath decreases (Table VI) to values comparable with those in the mesophyll, or those found for C_3 plants in limiting CO_2 [10,12]. In contrast, the glycerate-3-*P*/triose phosphate quotient in the mesophyll remains unaltered, as expected if the rate of glycerate-3-*P* reduction in the mesophyll is limited by the rate at which glycerate-3-*P* diffuses into the mesophyll from the bundle sheath, rather than by the availability of photochemically derived ATP or NADPH. It is likely that diffusion is also limiting for the movement of triose phosphates back to the bundle sheath. The level of triose phosphates in the bundle sheath may rise in CO_2-free air (Table III, see also below) suggesting that the availability of triose phosphates could even limit the activity of the stromal fructosebisphosphatase in the bundle sheath during rapid photosynthesis. This highlights the need for an effective control over other reactions which utilize these metabolites and, potentially, could inhibit photosynthesis by depleting these pools and reducing the rate of diffusion.

Maintenance of high triose phosphate and glycerate-3-P concentrations

The major enzymes which would remove triose phosphates are the stromal and cytosolic fructosebisphosphatases. The compartmentation and properties of the fructosebisphosphatases in maize leaves are discussed in more detail elsewhere [3,8]. The stromal fructosebisphosphatase is restricted to bundle sheath chloroplasts [1,3], where it provides a sink for triose phosphates and contributes to maintaining the low triose phosphate concentrations in the bundle sheath (Table I). In contrast, the cytosolic fructosebisphosphatase is located in the mesophyll [3], but has a 5–15-fold lower affinity for Fru(1,6)P_2 than the enzyme from C_3 plants [8]. A regulatory metabolite called Fru(2,6)P_2 has been shown to play a central role in regulation of the cytosolic fructosebisphosphatase in spinach. When photosynthesis is inhibited the Fru(2,6)P_2 content rises and inhibits the cytosolic fructosebisphosphatase [10]. A similar situation is found in maize. In a typical experiment, 73 ± 10 pmol

Fru(2,6)P_2 · mg Chl was found during rapid photosynthesis. This increased to 205 ± 10 pmol · mg Chl when CO_2 was removed or to 189 ± 14 pmol · mg Chl in the dark. This inhibition by Fru(2,6)P_2, in combination with the low substrate affinity of the cytosolic fructosebisphosphatase from maize [8], accounts for the high level of triose phosphates found in the mesophyll of maize leaves. The effectiveness of the control of the cytosolic fructosebisphosphatase is revealed under CO_2-free conditions, when the triose phosphate concentration in the mesophyll cell remains as high as 4 mM (Table III) although photosynthesis and sucrose synthesis are inhibited. This is still 2–3-fold above the concentration present in a C_3 leaf during rapid photosynthesis [9,10].

As discussed elsewhere [8,27], the cytosolic fructosebisphosphatase may be viewed as a valve, which sets a threshold level at which carbon starts to be withdrawn from the intermediate pools of photosynthesis for production of sucrose. This allows the withdrawal of triose phosphates for production of sucrose to be reconciled with the maintenance of the pools of triose phosphates and of intermediates linked via equilibrium reactions (e.g., Fru(1,6)P_2, Sedu(1,7)P_2, pentose phosphates) at concentrations which will allow rapid turnover of the Calvin cycle. In maize, the situation is more complex than in C_3 plants, as the intercellular concentration gradients must be maintained, as well as the concentrations of the Calvin cycle intermediates. Correspondingly, in maize the threshold level at which the cytosolic Fru(1,6*P*)ase 'valve' starts to remove triose phosphates for synthesis of sucrose is significantly higher than that in C_3 plants such as spinach and wheat. This protects the high concentration of triose phosphates needed in the mesophyll for intercellular diffusion.

Reactions which could deplete glycerate-3-*P* also appear to be subject to control. When the pools of glycerate-3-*P* and phospho*enol*pyruvate are in equilibrium, a glycerate-3-*P*/phospho*enol*pyruvate quotient of 2–4 is expected, as is found in all conditions in both mesophyll and the bundle sheath with the exception of the bundle sheath during rapid photosynthesis (Table V). In this case, the quotient of about 20 provides evidence that the interconversion is restricted. The reason for this is unclear as the bundle sheath contains significant activities of phosphoglycerate mutase and enolase [28,29]. Since phospho*enol*pyruvate and glycerate-3-*P* are readily interconvertible in the mesophyll, the activity of phospho*enol*pyruvate carboxylase must be regulated. Due to the very high activity of phospho*enol*pyruvate carboxylase in the mesophyll of C_4 plants, a small inbalance between the rate at which phospho*enol*pyruvate is utilised by phospho*enol*pyruvate carboxylase and the rate at which pyruvate moves to the mesophyll would result in rapid shifting of carbon between the malate/pyruvate shuttle and the glycerate-3-*P*/triose phosphate shuttle.

Control of the balance between the malate/pyruvate and glycerate-3-P/triose phosphate shuttles

Maximal rates of photosynthesis in maize will require the relative pool sizes of the malate/pyruvate and the glycerate-3-*P*/triose phosphate shuttles to be balanced. If the content of malate or pyruvate is too low, the size of the concentration gradient between the bundle sheath and mesophyll will be restricted, and the transport of CO_2 or redox equivalents into the bundle sheath may then limit photosynthesis. In such conditions, the pools of malate and pyruvate could be built up by withdrawal of carbon from the Calvin cycle if phosphoenolpyruvate carboxylase activity temporarily exceeded the rate at which pyruvate was returning to the mesophyll. Some of the glycerate-3-*P* entering the mesophyll would then be used to build up the pools of the malate/pyruvate shuttle, rather than being reduced to triose phosphates in the chloroplast. On the other hand, if the concentration of phosphorylated intermediates were low, this might restrict the turnover of the Calvin cycle or the intercellular diffusion of glycerate-3-*P* and triose phosphates. In these conditions, carbon could be transferred from malate or pyruvate to the pools of phosphorylated intermediates if the activity of phospho*enol*pyruvate carboxylase was lower than the rate at which pyruvate was returning to the mesophyll. The pools of phosphorylated intermediates could also be built up by autocatalysis or by degradation of storage carbohydrates, but this would still require phospho*enol*pyruvate carboxylase to be controlled, so that all the

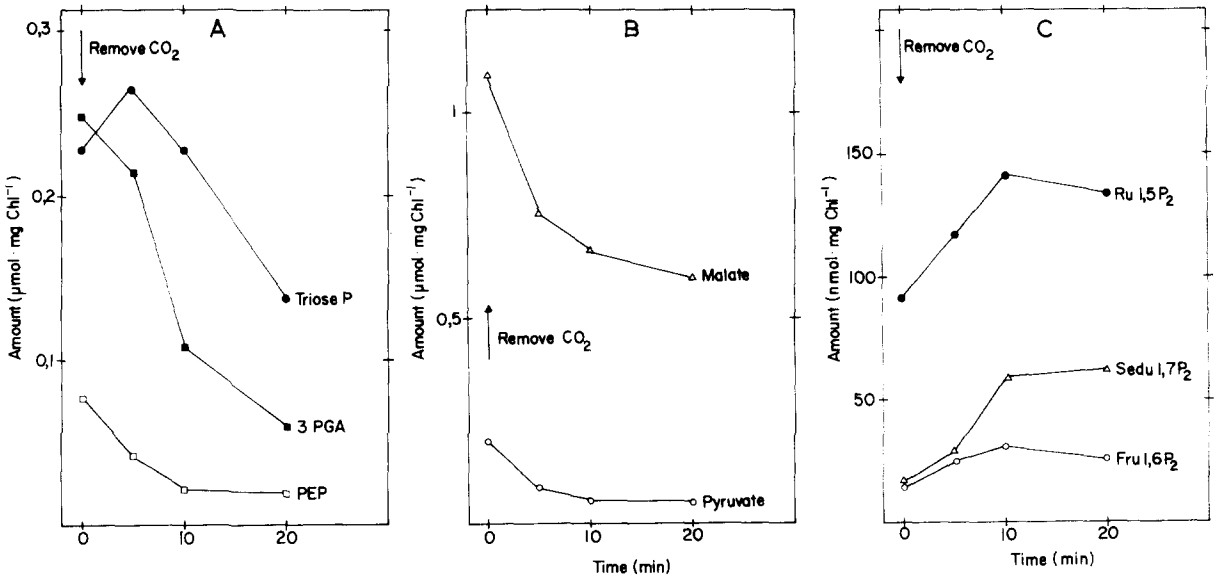


Fig. 1. Alterations of metabolites in maize leaves during a transition in the light from air to CO_2-free conditions. Leaves were preilluminated for 3 h before starting the experiment. (A) Malate and pyruvate; (B) Triose phosphates (DHAP), glycerate-3-*P* (PGA) and phosphoenolpyruvate (PEP); (C) Fru(1,6)P_2, Sedu(1,6)P_2 and Ru(1,5)P_2.

glycerate-3-*P* entering the mesophyll cells was reduced and returned to the bundle sheath.

In earlier studies, darkening maize leaves led to a rapid increase of pyruvate and decrease of the Calvin cycle pools [5]. In contrast, during the induction of photosynthesis carbon moves from malate and pyruvate into the pools of phosphorylated intermediates [29]. This ability to move

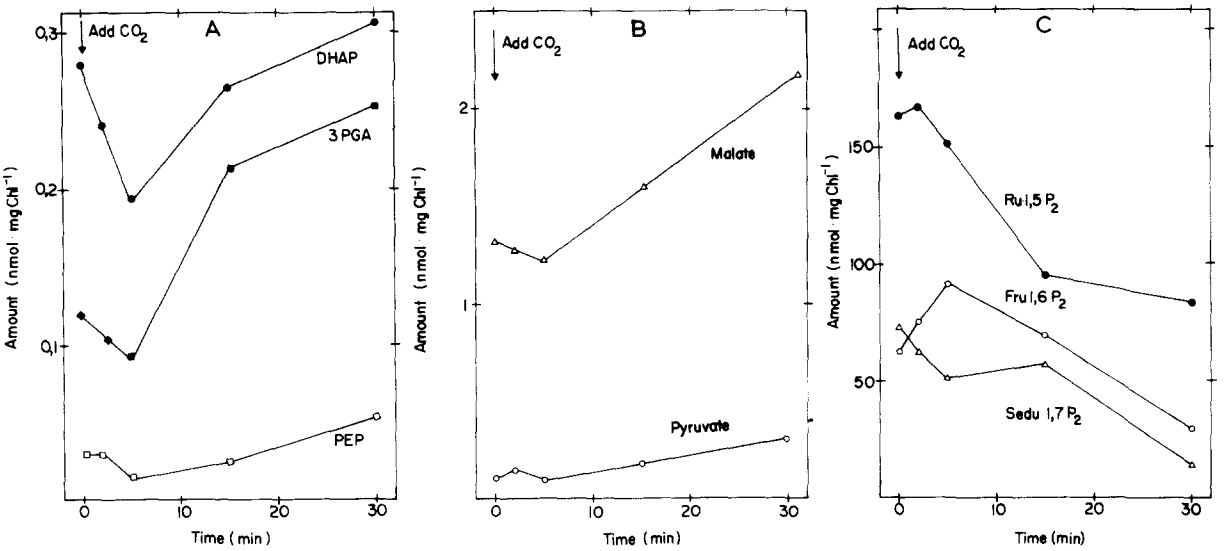


Fig. 2. Alterations of metabolites in maize leaves during a transition from CO_2-free condition to air in the light. (A) Malate and pyruvate; (B) Triose phosphates (DHAP), glycerate-3-*P* (PGA) and phosphoenolpyruvate (PEP); (C) Fru(1,6)P_2, Sedu(1,7)P_2 and Ru(1,5)P_2.

carbon between the pools involved in the different shuttles is further illustrated in Figs. 1 and 2 (see also Table I). Illuminated leaves were transferred from air to CO_2-free air (Fig. 1) or were transferred from CO_2-free conditions back to air again (Fig. 2). After transfer to CO_2-free air the levels of malate and pyruvate decrease for 5–10 min and then stabilize (Fig. 1A). The remaining carbon may partly be in non-photosynthetic pools. On the other hand, the levels of most of the metabolites in the Calvin cycle rise (Fig. 1B and C) although glycerate-3-*P* and, later, triose phosphates, are depleted (Fig. 1B). On returning to air, the pools of malate and pyruvate are built up again (Fig. 2A). The pools of Calvin cycle intermediates, triose phosphates and glycerate-3-*P* even drop initially (Fig. 2B and C), before rising as the onset of photosynthesis allows them to be built up. These alterations in organic acids and phosphorylated intermediates will not be solely due to fluxes catalysed by phospho*enol*pyruvate carboxylase. For example, photorespiration (see also Ref. 12) may be responsible for the gradual depletion of phosphorylated intermediates in CO_2-free conditions, and carbon transfer will also occur between storage carbohydrates and the pools of phosphorylated intermediates. Nevertheless, our results, together with those in light-dark transition [5,29], do show how the pool sizes involved in the shuttles can vary.

The results of Tables I–IV point to two ways in which the distribution of carbon may be balanced between the two shuttle systems. First, the consumption of ATP and NADPH during the conversion of pyruvate to malate must be coordinated with the requirements of glycerate-3-*P* reduction. If glycerate-3-*P* reduction were restricted, then accumulation of this compound and phospho*enol*pyruvate would favour diversion of carbon towards the pools of organic acids. This may occur in limiting light or after darkening, and also if the pools of phosphorylated intermediates are very high so that high triose phosphates or low P_i restrict the reduction of glycerate-3-*P*. However, mesophyll cells seem capable of maintaining very high quotients of ATP/ADP and NADPH/NADP during rapid photosynthesis, so that glycerate-3-*P* reduction occurs rapidly in the presence of a very low glycerate-3-*P*/triose phosphate quotient. These high ATP/ADP and NADPH/NADP quotients may be partly due to the absence of the Calvin cycle in the mesophyll, but they also imply that other enzymes which consume ATP (Pyruvate phophate dikinase) and NADPH (NADP malate dehydrogenase) are controlled to ensure that they do not lower ATP or NADPH to a point where glycerate-3-*P* starts to accumulate. This may be why pyruvate phosphate dikinase is regulated by phosphorylation in response to the adenylate status [16,17], rather than by thioredoxin. Similarly, regulation of NADP malate dehydrogenase by an interaction of NADPH and thioredoxin [16,30] may ensure that ample NADPH remains available for glycerate-3-*P* reduction.

Secondly, control of phosphoenolpyruvate carboxylase may play a crucial role in controlling the distribution of carbon between the two shuttle systems. C_4 plants contain a unique high K_m form of phospho*enol*pyruvate carboxylase which is activated by glucose-6-phosphate and triose phosphate (specifically, dihydroxyacetone phosphate), and inhibited by malate, all being effective in 1–5 mM concentrations [2,13,32]. Our results show that the mesophyll contains 2–3 mM glucose-6-phosphate (see Ref. 8), up to 10 mM triose phosphate and 10–20 mM malate (Table IV), and that these metabolite concentrations vary during photosynthesis. It is tempting to suggest that accumulation of triose phosphates and glucose-6-phosphate in the mesophyll acts as a signal that the Calvin cycle pools are full, so that more of the glycerate-3-*P* is diverted towards pyruvate and malate by increased phospho*enol*pyruvate carboxylase activity. Conversely, accumulation of malate would tend to inhibit phospho*enol*pyruvate carboxylase, so that more carbon is recycled to produce Ru(1,5)P_2 to act as acceptor for CO_2, or is used to produce sucrose. Further studies of phospho*enol*pyruvate carboxylase should reveal how far the alteration of metabolites observed in vivo would regulate the activity of phospho*enol*-pyruvate carboxylase and control the distribution of carbon between the PGA/triose phosphate shuttle and the malate/pyruvate shuttle.

Implications of the shuttles for control of chloroplast metabolism

The regulation of the cytosolic fructose-bisphosphatase and phospho*enol*pyruvate carboxylase provide two examples of how the generation of the high concentrations of metabolites needed for intercellular transport requires modification of metabolism in the cytosol. The maintenance of high glycerate-3-*P*/triose phosphate quotients in the bundle sheath also has implications for the control of the Calvin cycle and starch metabolism in the chloroplasts.

In the C_3 chloroplast, glycerate-3-*P* reduction is restricted as the supply of ATP decrease, due to the mass action effect [33] and because the K_m for ATP of phosphoglycerate kinase is relatively high [34]. The other major sink for ATP in the stroma in phosphoribulokinase, which has high activity and a low K_m for ATP [34,35]. Rapid photosynthesis would require the rates of these reactions to be balanced so that the ATP/ADP ratio remains high enough to allow rapid reduction of glycerate-3-*P*. In spinach, phosphoribulokinase is inhibited by ADP and glycerate-3-*P* [35], decreasing its activity in conditions when glycerate-3-*P* reduction is restricted by shortage of ATP, and allowing the activity of these ATP-consuming reactions to be balanced. In the maize bundle sheath far higher glycerate-3-*P* concentrations and lower ATP/ADP quotients are present during photosynthesis and the properties of phosphoribulokinase are presumably altered to allow it to operate in these conditions.

Starch synthesis is stimulated by increasing glycerate-3-*P*/P_i quotients, which activate ADP-glucose pyrophosphorylase [36]. Our results would predict that the sensitivity of enzymes from maize leaves to this quotient is different from that in C_3 plants. Unless starch synthesis is to occur rapidly in all conditions in maize, the bundle sheath ADP-glucose pyrophosphorylase should require higher glycerate-3-*P*/P_i ratios before it becomes active, conversely increased sensitivity would be needed in the mesophyll. Such alterations in the properties of the isolated ADP-glucose pyrophosphorylase have recently been found (Preiss, J. personal communication). It is also possible to understand why the bundle sheath chloroplasts have been selected as the major site for starch accumulation in maize leaves; this is favoured by the high glycerate-3-*P*, as well as by the low activity or absence of the stromal fructosebisphosphatase in the mesophyll.

The levels of sugar phosphates in maize leaves also provide evidence for in situ regulatory mechanisms in the Calvin cycle which have not previously been recognised in C_3 plants. In CO_2-free conditions the levels of Fru(1,6)P_2 and Sedu(1,7)P_2 rise 2–3 fold (Fig. 1C), which is more than the increase of Ru(1,5)P_2. This provides evidence for mechanisms which inhibit the fructose-bisphosphatase and sedoheptulosebisphosphatase when CO_2 is limiting, so that the intermediate pools in the Calvin cycle are not all converted to Ru(1,5)P_2. An accumulation of Fru(1,6)P_2 or Sedu(1,7)P_2 has not been found in spinach leaves [11,37] or wheat protoplasts [12] during photosynthesis in low CO_2, but this may reflect the differing relation between sucrose synthesis and the Calvin cycle in C_3 plants and maize, rather than the absence of an analogous regulation in C_3 plants. When photosynthesis is inhibited, sucrose synthesis is also inhibited and a central feature of this inhibition in C_3 plants is a decline in the triose phosphate concentration [10]. In maize, removal of triose phosphate for sucrose synthesis occurs mainly in the mesophyll [3]. Correspondingly, the triose phosphates concentration in the mesophyll decreases in CO_2-free conditions, but the concentration gradient between the cell types also decreases so that the triose phosphate concentration in the bundle sheath can remain unaltered or even rise when photosynthesis is inhibited (see Tables II and III). In these conditions, an inhibition of the stromal bisphosphatases can be revealed by an accumulation of Sedu(1,7)P_2 and Fru(1,6)P_2. In C_3 plants, however, sucrose is synthesised in the same cells as the Calvin cycle, so that any decrease of triose phosphates in the cytosol will lead to a decrease in the chloroplast stroma. Since Fru(1,6)P_2 and Sedu(1,7)P_2 are linked to triose phosphates by equilibrium reactions, they will also decrease. Thus, even if the fructose-bisphosphatase and sedoheptulosebisphosphatase were to be inhibited in such conditions, this could not be detected as their substrates cannot accumulate. The mechanism of this feedback control remains to be elucidated. It has recently been

shown that Fru-6-*P* is a very effective inhibitor of the stromal fructosebisphosphatase from spinach (Gardemann, A. and Heldt, H.W., unpublished data).

Concluding remarks

The results presented in this article demonstrate the feasibility of an intercellular transport system in C_4 plants in which diffusion in the symplasm plays a large role. The concentration gradients required can be established by the differing photochemical capacities of the mesophyll and bundle sheath cells, and the presence of specialised transport systems and enzymes and regulatory mechanisms in these cell types. Nevertheless, in view of the uncertainty over the structure of plasmodesmata and the fine organisation of the cytoplasm, the concept of diffusion should not be too strictly viewed as the physical events involved may be more complex than diffusion in a homogenous medium. Simultaneously, these results point to many regulatory features in C_4 plants which require further attention, and should provide a contrasting object to those found in C_3 plants. These include the balance between provision of ATP and NADPH by electron transport and their use in different reactions in the stroma, the coordination of turnover of the Calvin cycle with the rate of carboxylation of $Ru(1,5)P_2$, and the balance between fixation of CO_2 into phosphorylated intermediates and use of these intermediates for synthesis of sucrose or starch. Study of these regulatory phenomena should provide new insights into general aspects of the regulation of photosynthetic metabolism as well as increasing our understanding of C_4 plants.

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