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Effect of Inhibitors on Ammonia-, 2-Oxoglutarate-, and Oxaloacetate-Dependent O_2 Evolution in Illuminated Chloroplasts

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ABSTRACT

The evolution of O_2 in spinach chloroplasts in the presence of oxaloacetate (OAA) was inhibited by a wide range of dicarboxylates. In contrast, (ammonia, 2-oxoglutarate)-dependent O_2 evolution was stimulated by malate, succinate, fumarate, glutarate, maleiate, and L-tartrate although OAA has little effect. This increase in O_2 evolution was accompanied by a similar increase in ^{14}C incorporation from [5-^{14}C]oxoglutarate into amino acids which was sensitive to azaserine inhibition. Glutamate and aspartate inhibited (ammonia, 2-oxoglutarate)-dependent O_2 evolution, but this inhibition was relieved by the addition of succinate, malate, or fumarate. OAA-dependent O_2 evolution also was inhibited by glutamate and aspartate, but succinate, malate, or fumarate had little effect on this inhibition. Phthalonate and *n*-butyl malonate inhibited (ammonia, 2-oxoglutarate)-dependent O_2 evolution competitively with respect to 2-oxoglutarate and uncompetitively with respect to malate. Both these inhibitors inhibited OAA-dependent O_2 evolution competitively. This evidence suggests that different mechanisms might be involved in the transport of OAA, 2-oxoglutarate, and malate into the chloroplasts.

The chloroplast envelope forms an effective barrier to the free movement of metabolites into and out of the chloroplast. Transport of metabolites across this envelope involves specific carriers located in the inner membrane of the envelope (7, 11). During photosynthesis in C_3 plants, 2-OG[1] would be one of the major dicarboxylates transported into the chloroplast both for amino acid biosynthesis and for photorespiratory N recycling (21, 22). Uptake studies in isolated chloroplasts have shown that 2-OG and other dicarboxylates apparently share the same (dicarboxylate) carrier since the transport of one dicarboxylate is competitively inhibited by the other (12, 14). However, the transport of a dicarboxylate is more rapid when it is coupled to an exchange with another dicarboxylate.

Intact chloroplasts evolve O_2 in a light-dependent manner when supplied with NH_3 and 2-OG (2, 22). This (NH_3, 2-OG)-dependent O_2 evolution has been shown to be directly linked to ATP-dependent GS and reduced Fd-dependent GOGAT activity in the chloroplast (1, 2, 22). In a recent study, we have shown that (NH_3, 2-OG)-dependent O_2 evolution in intact chloroplasts is stimulated rather than inhibited by dicarboxylates (22). This increase in O_2 evolution is accompanied by a dramatic decrease in the affinity of (NH_3, 2-OG)-dependent O_2 evolution for 2-OG, presumably due to increased transport of 2-OG into the chloroplast in the presence of these dicarboxylates. This suggests that the transport of 2-OG into the chloroplast during (NH_3, 2-OG)-dependent O_2 evolution in the presence of a dicarboxylate could be intimately linked to a dicarboxylate exchange via the dicarboxylate carrier. Alternatively, a different carrier could be involved in 2-OG transport in the chloroplast during NH_3 assimilation.

In this study, we have attempted to resolve these alternatives by examining and comparing the effect of various inhibitors on (NH_3, 2-OG)-dependent O_2 evolution in spinach chloroplasts. The reduction of OAA in illuminated chloroplasts is coupled to O_2 evolution (3). Since OAA transport in chloroplasts has been assumed to be mediated by the dicarboxylate carrier (9, 12), OAA-dependent O_2 evolution in spinach chloroplasts is used as a control. The evidence in this study shows that 2-OG does not share the same transport system as OAA. Rather, a separate carrier is apparently involved in the transport of 2-OG into the chloroplast.

MATERIALS AND METHODS

Intact chloroplasts were isolated from young spinach leaves grown in a glass-house under natural daylight (22). O_2 evolution was measured at 25°C with a Clark O_2 electrode (YSI 4004, Yellow Springs, OH). The standard assay medium (2.6 ml total volume) contained 0.33 M sorbitol, 50 mM Hepes-NaOH (pH 7.6), 0.5 mM K_2HPO_4/KH_2PO_4, 1 mM $MnCl_2$, 10 mM $MgCl_2$, 2 mM EDTA, 10 mM DL-glyceraldehyde, and catalase (600 units). Chloroplasts (50–100 μg Chl) and 0.5 mM NH_4Cl were added, and the above assay mixture was preilluminated (600 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 5 min. Preillumination was essential to eliminate endogenous O_2 evolution completely. For measurements of (NH_3, 2-OG)-dependent O_2 evolution in the light (600 $\mu E \cdot m^{-2} \cdot s^{-1}$), 2 mM 2-OG was added at the end of preillumination and O_2 evolution was monitored polarographically. For measurements of (NH_3, 2-OG)-dependent O_2 evolution in the presence of malate or succinate, the dicarboxylate (3 mM) was added at the start of preillumination and 2-OG (2 mM) added at the end of the preillumination period. For measurements of OAA-dependent O_2 evolution, 0.4 mM OAA was added at the end of preillumination.

Labeling studies (in duplicate) were carried out as described above for (NH_3, 2-OG)-dependent O_2 evolution with 2 mM 2-[5-^{14}C]OG (0.12 μCi/ μmol). O_2 evolution was determined and at the end of 6 min, 3 × 250-μl aliquot samples were removed into vials containing 2 ml of 8 N HCOOH and 15 μmol each of glutamate and glutamine. Samples were dried under an air-stream at room temperature and the basic fractions were separated on Dowex 50W [H^+] ion exchange resin. Radioactivity in the basic fractions was determined by liquid scintillation counting. Chloroplast intactness was determined by the ferricyanide reduction test (8). All acid compounds were adjusted to pH 7.0 with NaOH before used.

Phthalonic acid was prepared by Dr. Roger Summons, using published methods (20). The synthesized compound was homog-

[1] Abbreviations: 2-OG, 2-oxoglutarate; GS, glutamine synthetase; GOGAT, glutamate synthase; OAA, oxaloacetate.

enous as determined by TLC and MS. *n*-Butyl malonate was a gift from Dr. D. A. Day.

RESULTS

Table I shows the effect of organic acids and amino acids on OAA- and (NH_3, 2-OG)-dependent O_2 evolution in intact spinach chloroplasts. Except for acetate, maleiate, malonate, and asparagine, all the compounds examined are known to be transported by the dicarboxylate carrier (4, 10, 12). All the compounds examined inhibited OAA-dependent O_2 evolution, presumably by competitively inhibiting OAA transport into the chloroplasts. In contrast, the dicarboxylates malate, succinate, fumarate, glutarate, L-tartrate, and maleiate stimulated (NH_3, 2-OG)-dependent O_2 evolution. The amides glutamine and asparagine have only a small inhibitory effect on both OAA- and (NH_3, 2-OG)-dependent O_2 evolution.

Table II shows that the stimulation of (NH_3, 2-OG)-dependent O_2 evolution by malate and succinate is accompanied by a corresponding increase in ^{14}C-incorporation (from [5-^{14}C]OAA) into amino acids (presumably glutamate and glutamine [17]). The ratio of O_2 evolved to ^{14}C-labeled product formed is similar to the ratio

Table I. *Effect of Carboxylic and Amino Acids on OAA- and (NH_3, 2-OG)-Dependent O_2 Evolution in Spinach Chloroplasts*

For OAA reduction, standard assay medium also contained 0.5 mM NH_4Cl and 0.4 mM OAA; for (NH_3, 2-OG)-dependent O_2 evolution, 0.5 mM NH_4Cl and 2 mM 2-OG. Rates of O_2 evolution in control treatments of OAA- and (NH_3, 2-OG)-dependent O_2 evolution were 22.9 and 5.1 $\mu mol \cdot mg^{-1}$ Chl$\cdot h^{-1}$, respectively.

Addition		O_2 Evolution	
		+OAA	+(NH_3, 2-OG)
	mM	*% control*	
2-OG	2	81	100
Malate	5	71	269
Succinate	5	48	324
Fumarate	5	67	319
Glutarate	10	32	282
L-Tartrate	10	37	237
Maleiate	10	45	148
Malonate	10		88
Phthalate	10	73	0
Acetate	5	71	69
Aspartate	5	51	59
Glutamate	5	28	10
Glutamine	5	89	92
Asparagine	5	88	74

Table II. *Relationship between O_2 Evolution and ^{14}C-Labeled Products Formed in the Basic (Amino Acid) Fraction of Chloroplast Extracts*

This action took place during 6 min of (NH_3, 2-OG)-dependent O_2 evolution in the light with 2-[5-^{14}C]OG in spinach chloroplasts (63 μg Chl; 85% intactness). Additions were: malate, succinate, 3 mM; OAA, 0.4 mM; azaserine, 1 mM.

Treatment	O_2 Evolution	Labeled Product Formed
	nmol	
Control, dark	0	0
Control	19	33
+Malate	79	139
+Succinate	68	116
+OAA	123	39
+OAA, Succinate	123	82
+Azaserine	0	0

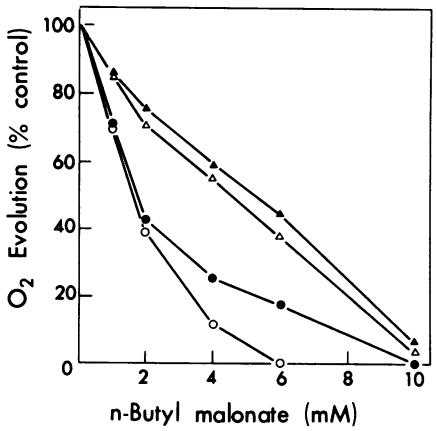

FIG. 1. Effect of *n*-butyl malonate on (NH_3, 2-OG)-dependent O_2 evolution in spinach chloroplasts (51 μg Chl; 80% intactness). Standard assays containing 2 mM 2-OG (○), 10 mM 2-OG (●), 2 mM 2-OG plus 3 mM malate (△) or 2 mM 2-OG plus 3 mM succinate (▲) were used. Rates of O_2 evolution in control treatments in the presence of 2 mM 2-OG, 10 mM 2-OG, 2mM 2-OG plus 3 mM malate, and 2 mM 2-OG plus 3 mM succinate were 3.8, 7.1, 11.7, and 11.8 $\mu mol \cdot mg^{-1}$ Chl$\cdot h^{-1}$, respectively.

expected when these activities are directly linked to GS/GOGAT activity in the chloroplast (20, 22). Azaserine, an inhibitor of GOGAT activity, inhibited both O_2 evolution and ^{14}C-incorporation, indicating that the above activities are indeed directly linked to GS/GOGAT activity in the chloroplast. In contrast to malate and succinate, OAA does not increase ^{14}C-incorporation in (NH_3, 2-OG)-dependent O_2 evolution and the observed increase in O_2 evolution is presumably due to the direct reduction of OAA. However, succinate stimulates ^{14}C-incorporation even when OAA is present. This indicates that, in contrast to the other dicarboxylates examined, OAA has little effect on (NH_3, 2-OG)-dependent O_2 evolution. The results in Tables I and II suggest that the transport of 2-OG and OAA into the chloroplast is unlikely to involve the same (dicarboxylate) carrier.

Figure 1 shows the effect of *n*-butyl malonate, an inhibitor of 2-OG and dicarboxylate transport in plant (6) and animal (19) mitochondria, on (NH_3, 2-OG)-dependent O_2 evolution in spinach chloroplasts. This activity is more sensitive to butyl malonate inhibition in the absence rather than in the presence of malate or succinate. However, the inhibition by butyl malonate is competitive both in the presence and in the absence of malate (Fig. 2).

Phthalonate resembles 2-OG structurally and has been found to be a potent inhibitor of 2-OG transport in rat liver mitochondria (16). In plant mitochondria, phthalonate inhibits OAA, citrate, and glutamate transport but has little effect on 2-OG transport (5). In all these studies, phthalonate does not appear to penetrate the inner membrane. Figure 3 shows that phthalonate inhibits (NH_3, 2-OG)-dependent O_2 evolution in spinach chloroplasts both in the absence and in the presence of malate in a competitive manner. Similarly, both phthalonate and butyl malonate also inhibit OAA-dependent O_2 evolution competitively (Fig. 4).

The kinetic parameters of these inhibitors on OAA- and (NH_3, 2-OG)-dependent O_2 evolution are summarized in Table III. As was observed previously (22), the $K_{1/2}$ (2-OG) value for (NH_3, 2-OG)-dependent O_2 evolution in the presence of malate is more than an order of magnitude less than that determined in the absence of malate. The apparent K_i (butyl malonate) values determined for both OAA- and (NH_3, 2-OG)-dependent O_2 evolution are similar. But the apparent K_i (phthalonate) values for (NH_3, 2-OG)-dependent O_2 evolution are 5- to 7-fold greater than that observed for OAA-dependent O_2 evolution.

Figure 5 shows that OAA-dependent O_2 evolution is strongly inhibited by succinate, glutamate, and aspartate. The inhibition

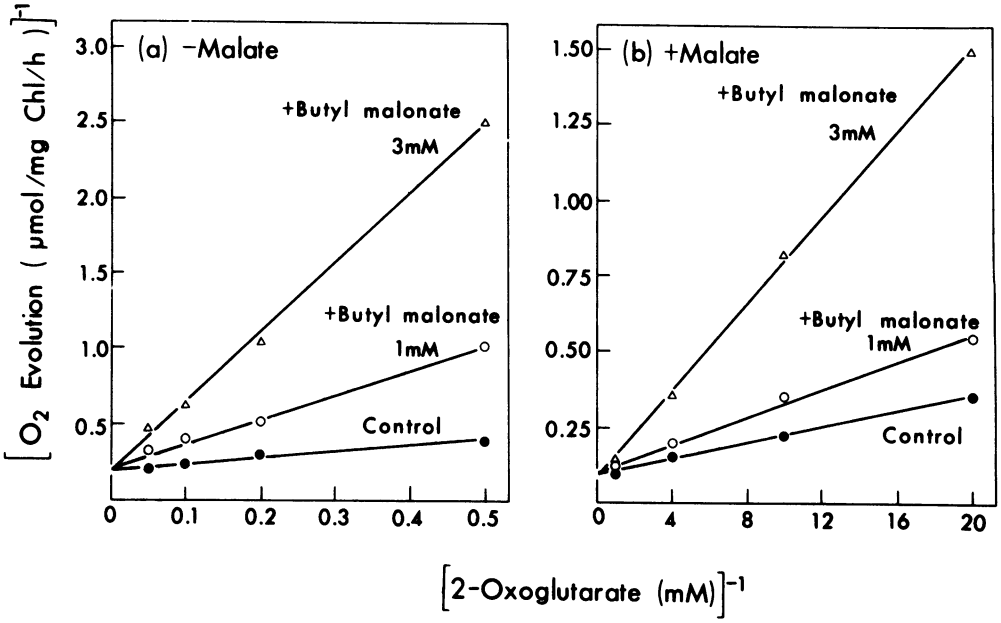

FIG. 2. Lineweaver-Burk plot of (NH_3, 2-OG)-dependent O_2 evolution with *n*-butyl malonate in the absence (a) and presence (b) of malate (3 mM) in spinach chloroplasts (50 µg Chl; 85% intactness).

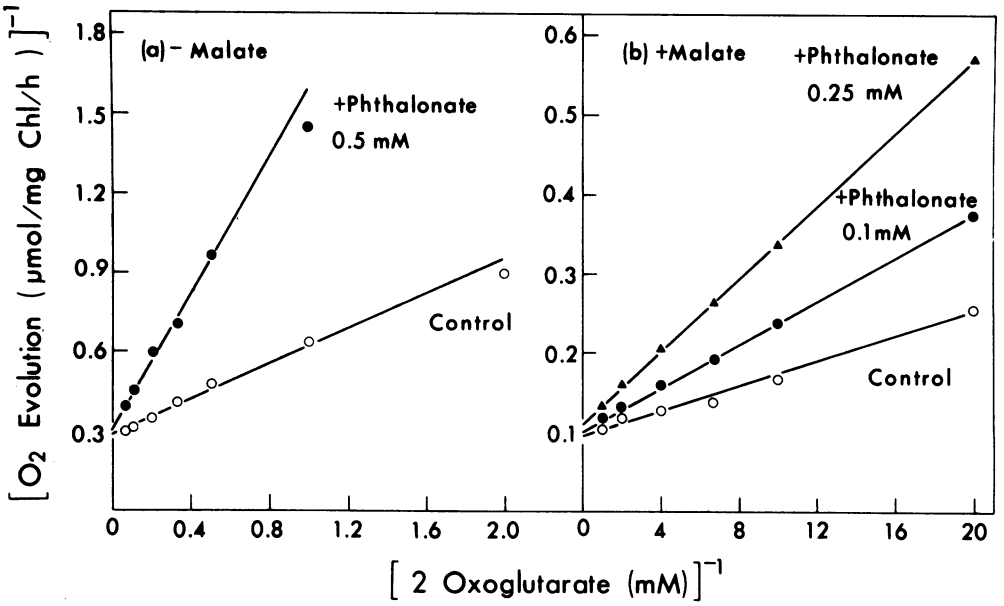

FIG. 3. Lineweaver-Burk plot of (NH_3, 2-OG)-dependent O_2 evolution with phthalonate in the absence (a) and presence (b) of malate (3 mM) in spinach chloroplasts (54 µg Chl; 87% intactness).

of O_2 evolution by glutamate (Fig. 4, trace B) and aspartate (Fig. 5, trace C) does not appear to be affected by the addition of succinate. Glutamate and aspartate also inhibit (NH_3, 2-OG)-dependent O_2 evolution (Fig. 6). But in contrast to the results in Figure 5, (NH_3, 2-OG)-dependent O_2 evolution is restored by the addition of succinate. Malate and fumarate are found to have similar effect as succinate. OAA, on the other hand, does not relieve the inhibition of (NH_3, 2-OG)-dependent O_2 evolution by glutamate (Fig. 7).

DISCUSSION

Uptake studies (4, 10, 12) suggest that the transport of OAA, succinate, malate, fumarate, and 2-OG into the chloroplast is mediated by the same dicarboxylate carrier. Under these conditions, the effect of an inhibitor on the transport of each of these dicarboxylates would be expected to be identical. The inhibition of OAA-dependent O_2 evolution by other dicarboxylates (Table I) does implicate the involvement of such a dicarboxylate carrier for OAA transport during OAA reduction in the chloroplast. But the evidence presented in the present study suggests that the transport of these dicarboxylates is apparently more complicated than is indicated by such uptake studies (4, 10, 12).

Our study shows that malate, succinate, fumarate, and other dicarboxylates stimulated (NH_3, 2-OG)-dependent O_2 evolution and NH_3 assimilation, whereas OAA had little effect (Tables I and II). This is surprising and it seems that the malate formed during OAA reduction apparently had little effect on NH_3 and 2-OG assimilation. The reason for this is not known but it might be related to the ATP and NADPH requirements in the chloroplast during (NH_3, 2-OG)-dependent O_2 evolution in the presence of

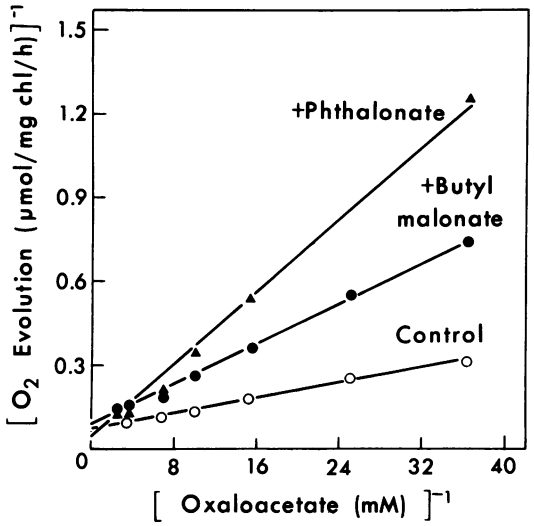

FIG. 4. Lineweaver-Burk plot of OAA-dependent O_2 evolution in spinach chloroplasts (53 μg Chl; 85% intactness) with phthalonate (0.25 mM) and *n*-butyl malonate (1 mM).

Table III. *Kinetic Parameters of Substrate and Inhibitor Affinity for OAA Reduction and (NH_3, 2-OG)-Dependent O_2 Evolution in Spinach Chloroplasts*

Values are the average of two experiments done in triplicates. Standard assay conditions were used.

Kinetic Parameter	OAA Reduction	(NH_3, 2-OG)-Dependent O_2 Evolution	
		−Malate	+Malate
K_m(OAA)	35 μM		
K_m(2-OG)		2.6 mM	100 μM
K_i (Butyl malonate)	0.90 mM	0.50 mM	0.81 mM
K_i (Phthalonate)	30 μM	0.21 mM	0.15 mM

OAA. Furthermore, glutamate and aspartate inhibit both (NH_3, 2-OG)-dependent and OAA-dependent O_2 evolution, but only the activity of (NH_3, 2-OG)-dependent O_2 evolution was restored by the addition of succinate, malate, or fumarate (Figs. 5 and 6).

These results could not be explained simply on the basis of a modified exchange shuttle involving only a single (the dicarboxylate) carrier (4, 10, 18) nor a modified exchange shuttle involving only a single carrier for all these dicarboxylates (9, 22). Rather, the evidence suggests that different carriers are likely to be involved in the transport of OAA and 2-OG. Thus, it seems that, like plant mitochondria (5, 6), chloroplasts may also contain specific carriers for OAA and 2-OG. The presence of a carrier specific for the transport of 2-OG is consistent with the importance of 2-OG transport for photorespiratory NH_3 reassimilation during photorespiration (21, 22).

Using the silicone layering centrifugation technique, Lehner and Heldt (14) have reported that *n*-butyl malonate did not have any effect on the transport of dicarboxylates via the dicarboxylate carrier. Using the same technique, we have found that *n*-butyl malonate and phthalonate also did not appear to have any effect on the exchange (efflux) of 2-OG, succinate, malate, and glutamate with other dicarboxylates (data not shown). The reasons for the discrepancies between these findings and the evidence in Figures 2, 3, and 4 are not known but might be related to the different experimental conditions involved. With the silicone layering technique, a dicarboxylate anion could presumably be transported by more than one carrier if different carriers with overlapping specificities and affinities are present in the chloroplast inner envelope. Consequently, the inhibition of one carrier by a specific inhibitor

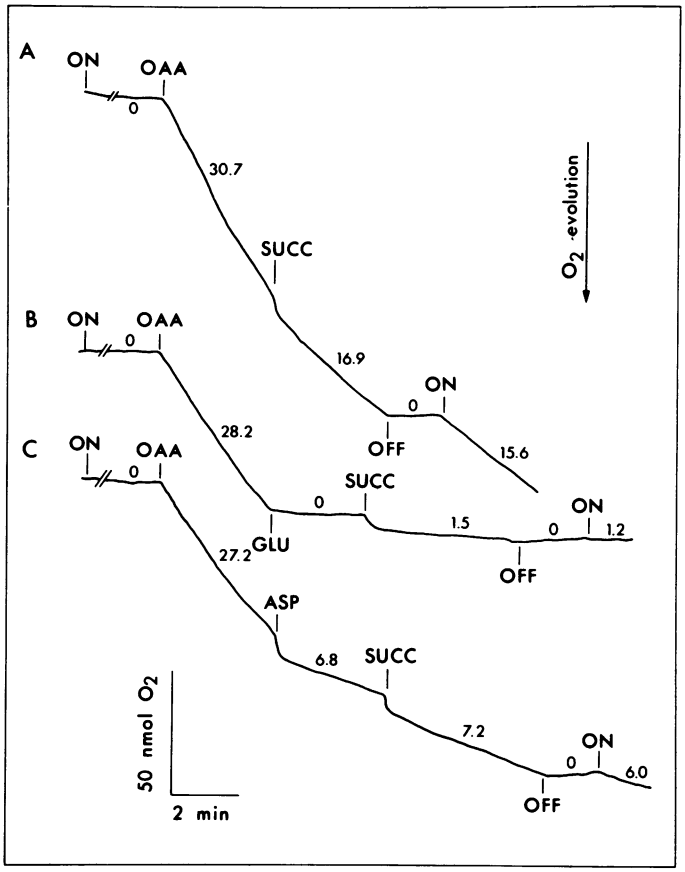

FIG. 5. Effect of glutamate on OAA- and (NH_3, 2-OG)-dependent O_2 evolution in spinach chloroplasts (49 μg Chl; 80% intactness). Standard assay conditions were used. Additions were: 2-OG, 2 mM; OAA, 1 mM; succinate, 3 mM.

might not significantly inhibit the continued transport of a dicarboxylate anion. In contrast, during steady state assimilation of NH_3 in isolated chloroplasts in the light, the transport of metabolites involved is presumably more complex and seems also to be dependent on the maintenance of a membrane potential across the chloroplast inner envelope (22). Under these conditions, the inhibition of phthalonate and *n*-butyl malonate observed seems to suggest that the transport of either 2-OG or OAA is specific and cannot be mediated by other carriers. To a certain extent, this is similar to the situation in plant mitochondria where OAA transport in actively respiring organelles was mediated apparently by a specific carrier (5), even though it could also participate in exchanges on the dicarboxylate and 2-OG carriers (6). It is likely that the differences observed between the uptake and the exchange processes of these dicarboxylates in these organelles might also be linked to differences in the affinity and kinetics of the two processes involved.

High levels of aspartate and glutamate are present in the spinach leaves used in this study, and in chloroplasts isolated from spinach leaves (14). The values determined for aspartate and glutamate are 235 to 273 and 366 to 532 nmol/mg Chl, respectively, in chloroplasts (14) and 5 to 8 and 10 to 16 μmol/g fresh weight, respectively, in leaves. However, large inhibition of (NH_3, 2-OG)-dependent O_2 evolution by these amino acids (Figs. 6 and 7) is observed in the absence but not in the presence of malate, succinate, or fumarate. Thus, if the levels of these amino acids in the cytosol are similar to those found in chloroplasts and leaves, it is conceivable that the transport of 2-OG into the chloroplast for the assimilation of NH_3, *in vivo* during photosynthesis/photorespiration at ambient atmospheric conditions would be inhibited unless

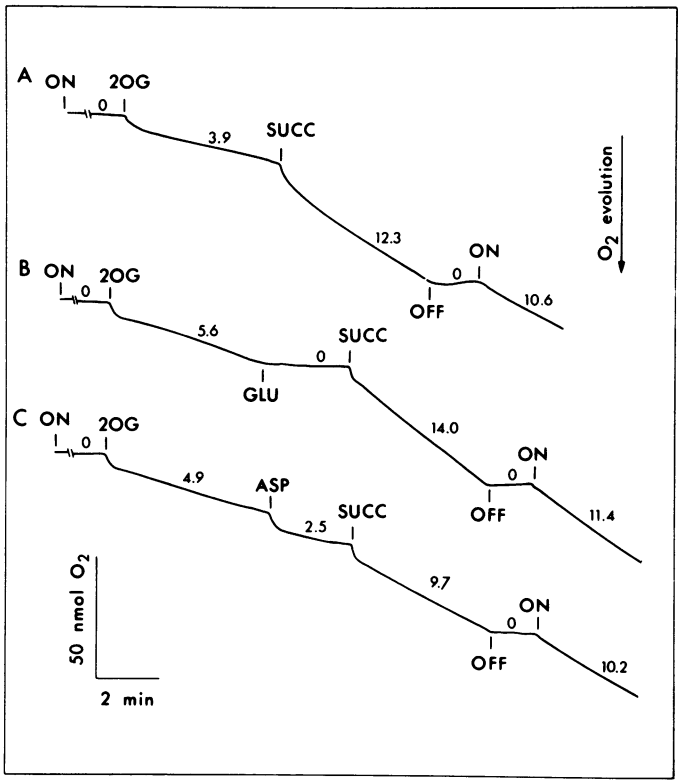

FIG. 6. Effect of succinate, glutamate, and aspartate on OAA-dependent O_2 evolution in spinach chloroplasts (44 μg Chl; 74% intactness). Additions were: succinate, 3 mM; glutamate and aspartate, 10 mM; and OAA, 0.4 mM.

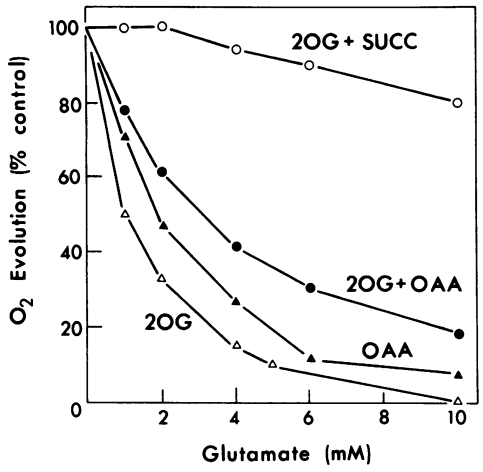

FIG. 7. Effect of succinate, glutamate, and aspartate on (NH_3, 2-OG)-dependent O_2 evolution. Additions were: 2-OG, 2 mM; succinate, 3 mM; and glutamate and aspartate, 10 mM. Rates of O_2 evolution in the control treatments in the presence of 2-OG, 2-OG plus OAA, 2-OG plus succinate, and OAA were 3.8, 36.8, 10.2, and 41.7 $\mu mol \cdot mg^{-1}$, respectively.

it also involves the participation of a dicarboxylate (presumably malate).

Glutamate is the final product of GS/GOGAT activities in the chloroplast. The increase in and accumulation of glutamate during (NH_3, 2-OG)-dependent O_2 evolution in the presence of malate (data not shown) indicates that the glutamate formed during NH_3 assimilation is predominantly exported out of the chloroplast. Thus, it seems likely that a glutamate/dicarboxylate and/or a 2-OG/dicarboxylate exchange would take place during (NH_3, 2-OG)-dependent O_2 evolution in isolated chloroplasts in the presence of the dicarboxylates malate, succinate, or fumarate. A glutamate/dicarboxylate exchange would presumably help to prevent the build-up and removal of glutamate formed inside the chloroplast. However, compared to other dicarboxylates, the uptake of glutamate in counter exchange with succinate and aspartate, as well as its unidirectional uptake is relatively slow (14). Furthermore, the rate of the efflux of [U-^{14}C]glutamate in the presence of either 2-OG or OAA is 4- to 10-fold greater than that in the presence of either glutamate, malate, or succinate (Table IV). This evidence suggests that the exchange of glutamate with either malate or succinate is relatively slow, whereas there would be a more rapid efflux of glutamate during NH_3 assimilation if it is linked to an exchange with 2-OG. However, a glutamate/dicarboxylate exchange could still occur during (NH_3, 2-OG)-dependent O_2 evolution in the presence of malate or succinate, but it seems unlikely to be responsible for the stimulation of (NH_3, 2-OG)-dependent O_2 evolution observed in the presence of these dicarboxylates. On the other hand, a 2-OG/dicarboxylate exchange would greatly facilitate the entry of 2-OG into the chloroplast (10, 14) and stimulate ((NH_3, 2-OG)-dependent O_2 evolution (22). In such an exchange, malate would presumably enter the chloroplast and then exchange for 2-OG via the 2-OG carrier (see earlier discussion). This exchange mechanism is presumably similar to those involved in the observed stimulation of 2-OG transport by malate or malonate (19) and of aspartate transport by glutamate (13) in rat liver mitochondria.

Table IV. *Efflux of [U-^{14}C]Glutamate during Counter Exchange with Other Dicarboxylates*

Chloroplasts (15 μg Chl) were preincubated with 10 mM [U-^{14}C]glutamate (0.37 Ci/mol) at 0°C in the dark for 20 min, and the counter exchange with dicarboxylates (1 mM) was carried out at 4°C as described (14).

Dicarboxylate Added	Efflux of [U-^{14}C]Glutamate
	nmol/mg Chl·30 s
Glutamate	4.3
Malate	10.1
Succinate	4.5
OAA	45.9
2-OG	41.6

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