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Pyruvate and Malate Transport and Oxidation in Corn Mitochondria[1]

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ABSTRACT

Pyruvate oxidation and swelling in pyruvate solutions by corn (*Zea mays*) mitochondria were inhibited by α-cyano-4-hydroxy-cinnamic acid, an inhibitor of pyruvate transport in animal mitochondria; however, there was no inhibition of pyruvate dehydrogenase activity, and malate and NADH oxidation were not affected. These results suggest the presence of a pyruvate$^-$-OH^- exchange transporter which supplies the mitochondrion with oxidizable substrate. Lactate appears to be transported also, but not dicarboxylate anions or inorganic phosphate. The rate of pyruvate transport was much slower than that of malate, however, and valinomycin was required to elicit appreciable swelling in potassium pyruvate.

Malate oxidation contributed significantly to respiration supported by pyruvate plus malate, and malate did not act solely as a "sparker" for pyruvate oxidation. NAD^+-malic enzyme activity was found in sonicated preparations, and comparison of O_2 consumption with CO_2 released from 1-^{14}C-pyruvate indicated that transported malate was being converted to pyruvate, particularly as the malate to pyruvate ratio increased. The results suggest that pyruvate transport becomes limiting under conditions of high energy demand, but that rapid malate transport makes up the difference, supplying pyruvate via malic enzyme and replenishing losses of tricarboxylic acid cycle intermediates.

It has been recently demonstrated that rat liver mitochondria possess a relatively specific pyruvate transporter in the inner membrane (8, 10, 19). While it seems definite that this carrier catalyzes pyruvate-hydroxyl exchange and is specifically inhibited by α-cyano-cinnamic acid and its derivatives (8), there is some confusion about its ability to exchange dicarboxylate ions for pyruvate (8, 19, 20, 22). The kinetics of pyruvate uptake suggests that the carrier may be important in the regulation of pyruvate metabolism, particularly gluconeogenesis (8).

Little is known about pyruvate entry into plant mitochondria although swelling studies have suggested that it differs from that of other carboxylic acids (13). Pyruvate oxidation by isolated mitochondria apparently is limited by availability of acetyl-CoA acceptor (23). On the other hand, pyruvate can be generated intramitochondrially from malate, via malic enzyme (2, 16, 18).

The present study sought to determine the means by which pyruvate is taken up by corn mitochondria and to what extent its oxidation, and that of malate, is governed by its transport. It is shown that pyruvate transport occurs via a mechanism similar to that in liver mitochondria, and that malate can act as the primary carbon source of the TCA[3] cycle. It is suggested that the relative contributions of malate and pyruvate, when both are supplied, to TCA cycle activity in the plant cell may be determined in part by the respective transport systems.

MATERIALS AND METHODS

Mitochondria were isolated from 3-day-old etiolated corn (*Zea mays*) shoots essentially as described by Hanson (11) except that TES buffer (pH 7.5) was used instead of KH_2PO_4 and 0.1% BSA was included in the grinding medium. Oxygen consumption was measured using a Clark O_2 electrode in a volume of 4.5 ml at 25 C as described previously (11). The standard reation medium consisted of 0.25 M sucrose, 10 mM TES buffer, 1 mM $MgSO_4$, and 0.1% BSA, adjusted to pH 7.2 with NaOH. Mitochondrial protein was approximately 1.5 mg/vessel.

Swelling was measured by following changes in absorbance at 520 nm in a Hitachi model 100-10 spectrophotometer at room temperature using cuvettes with a 1-cm light path.

Mitochondrial protein was estimated by the method of Lowry *et al.* (15) using BSA (fraction V) as the standard.

Pyruvate dehydrogenase activity in detergent-disrupted mitochondria was measured by following NADH production at 340 nm in a Hitachi spectrophotometer. The method was essentially that of Crompton and Laties (5) and the following assay medium was used: 20 mM KH_2PO_4 (pH 7.3), 0.5 mM EDTA, 1 mM $MgCl_2$, 1 mM $NaHSO_4$, 0.1 mM TPP, 0.15 mM CoA, 0.3 mM NAD^+, 1 mM KCN, 1 mg mitochondrial protein, and 0.03% Triton X-100. Pyruvate was added to start the reaction.

Pyruvate accumulation was measured by incubating 1 to 2 mg mitochondrial protein in 3 ml of standard reaction medium containing 0.4 μmol NADH, 20 μM rotenone, 2 mM arsenite, and 2 mM pyruvate. Other additions were made as given in Table V. The reaction was terminated by adding 0.1 mM CHCA and the mixture centrifuged at 30,000g for 2 min in a Sorvall RC-2 centrifuge at 2 C. The supernatant layer was decanted and the pellet rinsed with water and resuspended in 6% (v/v) cold perchloric acid. Precipitated protein was removed by centrifugation and the supernatant layer neutralized with KOH prior to assaying. Controls were run with 10 μM antimycin A in the medium to allow for passive penetration and intermembrane pyruvate. Pyruvate was determined as described by Bucher *et al.* (3) using lactate dehydrogenase.

Pyruvate oxidation was determined by measuring $^{14}CO_2$ evolution from 1-^{14}C-pyruvate. Mitochondria were incubated for 10 min at 25 C in 25-ml flasks containing 5 ml standard reaction medium (pH 6.8) to which had been added 1 mM ADP, 50 μM CoA, 50 μM TPP, 10 mM KH_2PO_4, 20 units hexokinase, 20 mM

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[3] Abbreviations: CHCA: α-cyano-4hydroxy-cinnamic acid; val: valinomycin; BM: *n*-butyl malonate; TCA: tricarboxylic acid; PEP: phosphoenolpyruvate; TPP: thiamin pyrophosphate.

glucose, and 1 mM 1-^{14}C-sodium pyruvate (0.01 μCi/mol). Varying amounts of unlabeled malate were also added. The flasks were fitted with serum stoppers and a small plastic well, containing 0.1 ml 20% (w/v) KOH and a filter paper wick, was suspended from the stopper by copper wire. The flasks were gently shaken in a water bath and the reaction was terminated by injecting perchloric acid (final concentration = 7%) into the mixture and the flasks were shaken for an additional 30 min. The wells were then removed, placed in Aquasol scintillation fluid (10 ml), and counted in a Beckman LS-230 scintillation counter. Radioactivity was determined using the channels-ratio method. In parallel experiments, O_2 uptake was measured polarographically under the same conditions.

Malic enzyme was assayed as described by Hatch and Kagawa (12). Sonicated mitochondria (about 1 mg) were added to 3 ml of a medium containing 10 mM TES buffer (pH 7), 0.2 mM EDTA, 5 mM dithiothreitol, 5 mM malate, 2 mM NAD^+, and 75 μM CoA. The reaction was started by adding 5 mM $MgCl_2$ and NADH production was measured spectrophotometrically at 340 mm; 10 μM antimycin A was added to prevent reoxidation of NADH.

1-^{14}C-Pyruvate (Na salt) was purchased from Amersham/Searle Corp. (Chicago, Ill.). Aquasol scintillation cocktail was a gift from New England Nuclear (Boston, Mass.). α-Cyano-4-hydroxy-cinnamic acid was purchased from Aldrich Chemical Co. (Milwaukee, Wis.), and dissolved in absolute ethanol. Other chemicals and enzymes were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS AND DISCUSSION

Effect of α-Cyano-4Hydroxy-Cinnamic Acid on Pyruvate Oxidation. When malate alone was added to isolated corn mitochondria, a slow rate of O_2 uptake was observed which could be markedly stimulated by adding pyruvate providing cofactors were also present (Fig. 1). However, when low concentrations of CHCA were added prior to pyruvate, the stimulation was prevented. CHCA did not prevent enhancement of respiration by glutamate (Fig. 1), and had little effect on NADH oxidation or pyruvate dehydrogenase (Table I). These results suggest that CHCA inhibition of malate plus pyruvate oxidation lies at the level of pyruvate entry to the matrix and suggest the existence of a specific pyruvate transporter, such as that shown to operate in liver mitochondria (8, 10, 19, 22).

The data shown in Figures 2 and 3 support these ideas. Figure 2 depicts the effect of varying concentrations of CHCA on the pyruvate stimulation of malate oxidation; 50% inhibition was achieved with 1.8 μM CHCA. The inhibition of malate and pyruvate oxidation was noncompetitive with respect to pyruvate (Fig. 3); and the *Km* for pyruvate was 0.53 mM. On the other hand, the apparent *Km* of pyruvate dehydrogenase was found to be 0.21 mM (see also ref. 5). These results suggest that the kinetics of pyruvate oxidation reflects that of the transporter rather than the dehydrogenase, and that the transporter may play a role in the regulation of pyruvate oxidation.

Swelling Studies and Pyruvate Uptake. Swelling was measured in a variety of K^+ salts of monocarboxylic acids (Fig. 4). Very little passive swelling in 10 mM K^+ salt was observed (Fig. 4A). Respiration-driven swelling in pyruvate and lactate solutions was slight unless valinomycin was present (Fig. 4, B and C). On the other hand swelling in acetate and propionate, which cross the inner membrane as undissociated acids (4), was much more pronounced (Fig. 4, D and E), suggesting that pyruvate and lactate enter the mitochondria in a different fashion than the other monocarboxylic acids.

The NADH-driven swellng in pyruvate and valinomycin was severely inhibited by CHCA, and was also inhibited by mersalyl (Fig. 5A), indicating that the pyruvate carrier is −SH-sensitive,

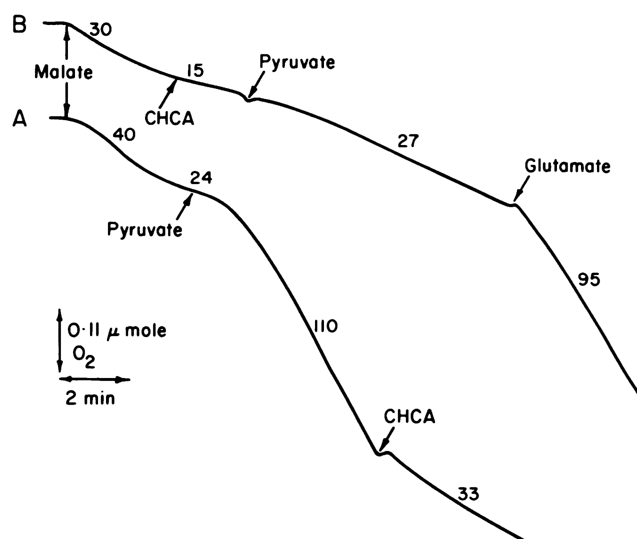

FIG. 1. Effect of α-cyano-4hydroxy-cinnamic acid on pyruvate oxidation. Mitochondria (1.2 mg protein) were added to 4.5 ml of standard reaction medium to which had been added: 5 mM KH_2PO_4, 50 μM CoA, 0.1 mM TPP, and 4.5 μmol ADP. Where indicated, 2 mM malate, 20 mM pyruvate, 20 μM CHCA or 6 mM glutamate were added. Oxygen uptake is expressed as nmol $O_2/min^{-1} \cdot$ mg protein^{-1}.

Table I. Effect of α-cyano-4-hydroxy-cinnamic acid on corn mitochondria

Oxygen uptake rates shown are state 3 rates; CHCA was added during state 3. When pyruvate was used 50 μM CoA and 0.1 mM TPP were included in the reaction medium. Pyruvate dehydrogenase was assayed as described in Materials and Methods; CHCA, when present, was added to the medium.

Substrate	Expt	Oxygen Uptake: Control	+10 μM CHCA	+50 μM CHCA
		nmoles·min^{-1}·mg protein^{-1}		
NADH	1	212	212	217
	2	183	183	185
5 mM malate + 5 mM glutamate	1	150	145	140
	2	127	127	123
2 mM malate + 5 mM pyruvate	1	125	22	17
	2	98	17	11.5
pyruvate dehydrogenase	1	75	73.5	--
	2	42.5	42.5	42.5

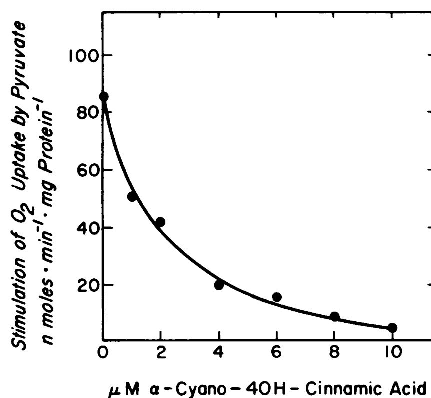

FIG. 2. Inhibition of pyruvate stimulation of respiration by α-cyano-4hydroxy-cinnamic acid. O_2 uptake was measured as in Figure 1B. Respiration was started with 2 mM malate; when a steady rate was obtained, variable concentrations of CHCA were added, and then 10 mM pyruvate. The rate of O_2 uptake before addition of pyruvate was subtracted from that after pyruvate was added, and this value was plotted against CHCA concentration.

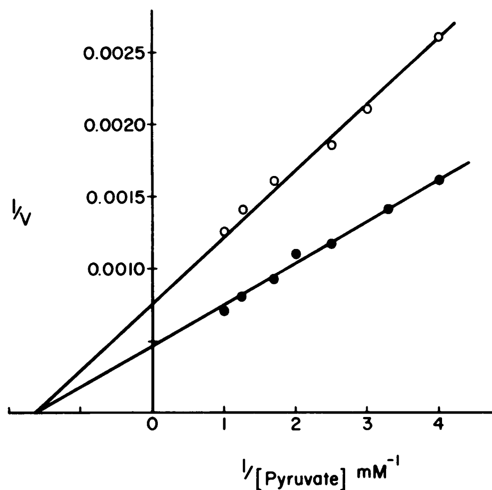

FIG. 3. Effect of α-cyano-4hydroxy-cinnamic acid on the kinetics of pyruvate oxidation. O_2 uptake with 2 mM malate and variable pyruvate as substrate was measured as described in Figure 1, except that 1.5 μM CHCA was included in the medium when used. ●——●; control; ○——○; plus 1.5 μM CHCA.

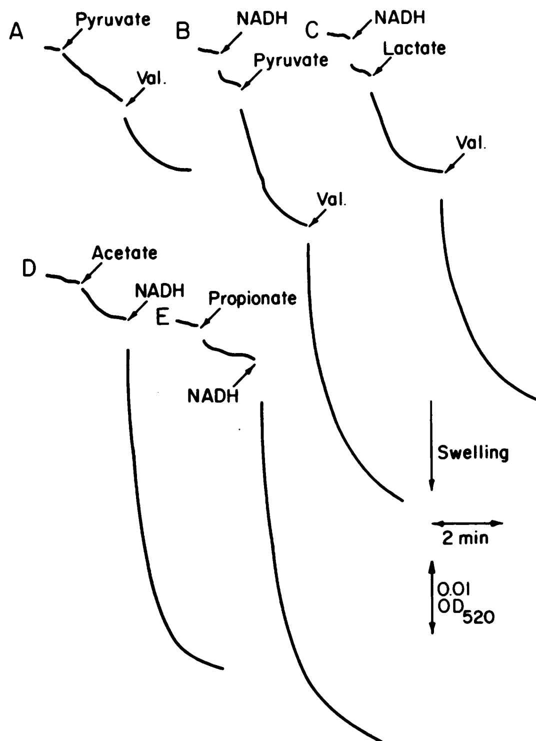

FIG. 4. Swelling in monocarboxylate salts. Mitochondria (1.6 mg) was suspended in 2.9 ml of standard reaction medium containing 10 μM rotenone, and swelling was monitored as a decrease in A at 520 nm. The following additions were made as indicated: 10 mM K^+ pyruvate, lactate, propionate or acetate, 1.5 μmol NADH, 0.3 μg valinomycin.

as demonstrated for liver mitochondria (8, 9). Acetate and propionate swelling was insensitive to these inhibitors. Measurements of pyruvate accumulated support the swelling studies, both CHCA and mersalyl being inhibitory (Table II). However, pyruvate uptake was not altered by the presence of Pi and malonate (Table II), and this fact together with the observation that malate and glutamate oxidation was not inhibited by CHCA (Table I) indicate independence of the pyruvate and dicarboxylate transport systems. Lactate swelling was inhibited by CHCA (Fig. 5B) and lactate reduced pyruvate uptake (Table II), suggesting that both anions enter on the same carrier. The similarity of the corn system to that found in rat liver (8) suggests that the carrier exchanges pyruvate for hydroxyl ions.

A comparison of pyruvate- and malate-induced swelling is shown in Figure 6 (see also ref. 6). In the absence of valinomycin, NADH-driven swelling in pyruvate was much less extensive than that in malate, and the initial rate was slower. While Pi increased the rate and extent of malate swelling, it had little effect on that in pyruvate (Fig. 6). (Since the mitochondria were preswollen in 1 mM Pi prior to malate or pyruvate addition, the increased swelling was not due to an osmotic effect of the Pi accumulated; see ref. 6.) Initial swelling in malate was 3- to 4-fold faster (depending on presence of Pi) than that in pyruvate in the absence of valinomycin; even when valinomycin was present, malate swelling was faster (Table III).

A rough estimate of the rate of pyruvate transport can be made from steady-state accumulation (Table II), and the initial NADH-energized swelling rate (Fig. 4). This is about 21 nmol/ $min^{-1} \cdot mg\ protein^{-1}$, which is adequate to support an oxidation rate of 52.5 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$. This is almost certainly an underestimate due to leakage of pyruvate during reisolation of the mitochondria, but nonetheless, the above rate is similar to that obtained with liver mitochondria (8), which also have a dicarboxylate transport system which is much more active than that for pyruvate (8).

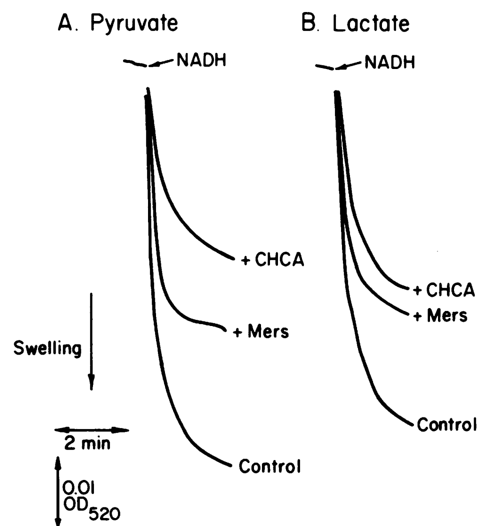

FIG. 5. Effect of mersalyl and α-cyano-4hydroxy-cinnamic acid on swelling in potassium pyruvate and lactate. Mitochondria (1.3 mg) were added to 2.9 ml standard reaction medium which also contained 10 μM rotenone, 0.25 μg valinomycin, and either 10 mM K^+ pyruvate (A) or 10 mM K^+ lactate (B). Swelling was monitored as described under "Materials and Methods"; when present, 50 μM CHCA and 25 μM mersalyl were included in the reaction medium. Swelling was initiated by adding 1.5 μmol NADH.

Table II. Pyruvate accumulation by corn mitochondria.

Pyruvate accumulated by the mitochondria was measured as described in Materials and Methods. An antimycin A 'blank' value of 21.4 was obtained and this was subtracted from the other values. An average of 5 experiments is shown.

Substrate	pyruvate content
	nmoles·mg protein^{-1}
2 mM pyruvate	10.3 (± 3.4)
2 mM pyruvate + 50 μM CHCA	0.4 (± 0.2)
2 mM pyruvate + 25 μM mersalyl	4.0 (± 1.4)
2 mM pyruvate + 2 mM Pi + 2 mM malonate	9.1 (± 1.2)
2 mM pyruvate + 3 mM lactate	5.3 (± 1.9)

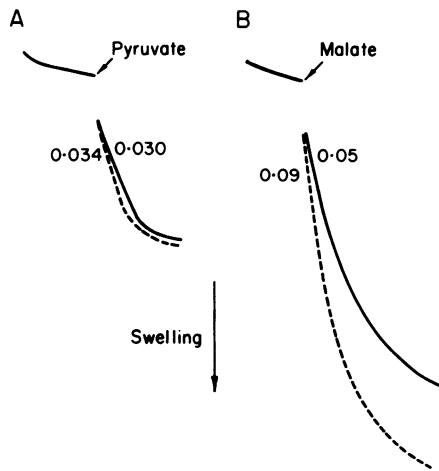

FIG. 6. Swelling in potassium malate and pyruvate. Mitochondria (1.4 mg protein) were added to 2.9 ml standard reaction medium containing 30 μM rotenone and swelling measured as described under "Materials and Methods." Swelling was driven by NADH, as shown in Figure 4, B and C. Phosphate (1 mM), when used, was added immediately after NADH and 15 mM K^+ pyruvate or malate added when Pi-induced swelling was completed (see ref. 6). Numbers on the curves are intial swelling rates in $\Delta A_{520} \cdot min^{-1}$. Solid lines: controls; dashed lines: prior addition of Pi.

Table III. Swelling rates of corn mitochondria in pyruvate and malate solutions.

Swelling was measured as described in Figure 6. When used, valinomycin (0.25 µg) was included in the medium. Approximately 1 mg mitochondrial protein was used per assay. Potassium salts of pyruvate and malate were used.

Substrate	Expt	Initial rate of swelling	
		control	+ valinomycin
		$\Delta OD_{520} \cdot min^{-1}$	
10 mM malate	1	0.027	0.094
	2	0.028	0.088
10 mM malate + 1 mM Pi	1	0.056	0.154
10 mM pyruvate	1	0.009	0.066
	2	0.01	0.048

Malate *versus* Pyruvate Oxidation. Since malate is transported faster than pyruvate, it may contribute much respiratory NADH both in being oxidized to oxaloacetate and to pyruvate. When butyl malonate was added to inhibit malate transport under conditions where "sparker" pools should have been established (Fig. 7A) malate plus pyruvate respiration declined by approximately 32%; subsequent addition of CHCA inhibited further. Butyl malonate inhibited malate plus glutamate oxidation more severely (by approximately 63%) while CHCA had no effect (Fig. 7B). Obviously, sustaining maximum pyruvate plus malate oxidation involves consumption of exogenous malate and requires continuous malate transport; that is, malate does not act solely as a sparker. The more rapid rate of malate plus glutamate oxidation suggests a more rapid rate of oxaloacetate turnover than when pyruvate is supplied. Hence, the rate-limiting step in malate plus pyruvate oxidation is probably pyruvate transport or oxidation to acetyl-CoA.

The rate of malate oxidation can be affected by the pH of the medium also, as noted previously by Macrae (16). Cofactors necessary for the oxidation of pyruvate (derived via malic enzyme) are effective in promoting O_2 uptake at pH 6.8 (Fig. 8B), but not at pH 7.2 unless pyruvate is supplied (Fig. 8A). Macrae (16) ascribed this effect of pH to malic enzyme activity which was found to have optimum activity at pH 6.8. However, lowering the pH may also affect substrate penetration since swelling of corn mitochondria in response to substrate (and Pi) anions is greater at lower pH values (11; Bertagnolli and Hanson, unpublished results).

In order to be certain that malic enzyme was present, determinations were made of malic enzyme activity in sonicated corn mitochondria. Rates varying from 120 to 175 nmol NADH/$min^{-1} \cdot mg\ protein^{-1}$ were found. This compares favorably with the range of activities reported by Macrae (17).

Table IV shows the effect of increasing malate concentration on the $^{14}CO_2$ released from 1-^{14}C-pyruvate. Assuming no signifi-

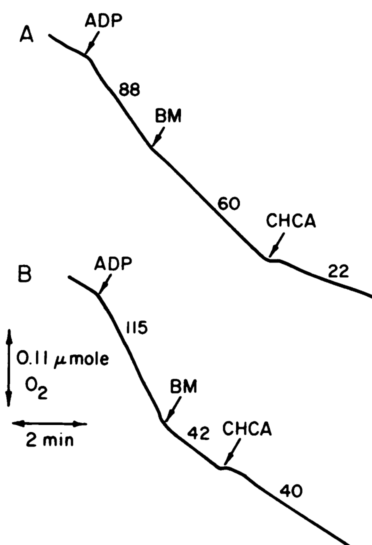

FIG. 7. Effects of transport inhibitors on malate and pyruvate oxidation. Mitochondria (1.4 mg protein) were added to 4.5 ml standard reaction medium (pH 7.2) which also included 5 mM KH_2PO_4, 50 μM CoA, 0.1 mM TPP, and either 5 mM pyruvate (A) or 5 mM glutamate (B). Malate (1 mM) was added to initiate O_2 uptake and 5 μmol ADP, 10 mM butylmalonate, and 50 μM CHCA were added as indicated. O_2 uptake is expressed as $nmol/min^{-1} \cdot mg\ protein^{-1}$.

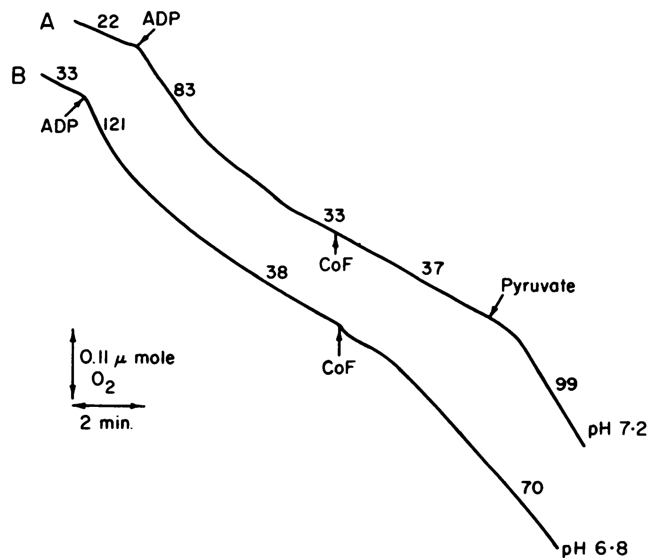

FIG. 8. Effect of pH and cofactors on malate oxidation. O_2 uptake was measured as described in Figure 7 except that no pyruvate or glutamate was present in the medium, and 5 mM malate was added to initiate O_2 uptake. Subsequently, 5 μmol ADP, cofactors (CoF; 50 μM CoA + 0.1 mM TPP) and 5 mM pyruvate were added as indicated. The pH of the medium (pH 7.2 in A and pH 6.8 in B) was adjusted prior to assaying. O_2 uptake is expressed as $nmol/min^{-1} \cdot mg\ protein^{-1}$.

Table IV. Pyruvate decarboxylation by corn mitochondria.

$^{14}CO_2$ evolution and oxygen uptake were measured as described in Materials and Methods. Each experiment was run for 10 min and 2 mg mitochondrial protein was used. The results shown are averages from 3 separate determinations.

Substrate	$^{14}CO_2$ produced*	O_2 consumed observed	O_2 consumed expected†	O_2 consumed difference	malate√ oxidized
			nmoles		
1 mM pyruvate	--	0	0	0	0
+0.2 mM malate	199	638	498	140	47
+0.5 mM malate	306	1058	765	293	98
+1 mM malate	366	1300	915	385	128
+2 mM malate	393	1503	983	520	174
+4 mM malate	403	1880	1008	872	291

*Equals 1-^{14}C-pyruvate oxidatively decarboxylated to produce acetyl CoA.
†Calculated from $C_3H_4O_3 + 2.5\ O_2 \rightarrow 3CO_2 + 2H_2O$.
√Calculated from $C_4H_6O_5 + 3\ O_2 \rightarrow 4CO_2 + 3H_2O$.

cant accumulation of acetyl-CoA, the amount of transported pyruvate entering the TCA cycle is proportional to $^{14}CO_2$ released. If malate is serving as a source of "cold" pyruvate via malic enzyme, in addition to serving as a source of oxaloacetic acid, there will be extra O_2 consumption not accounted for by $^{14}CO_2$ released from transported pyruvate.

Oxidation of transported pyruvate tends to saturate above 1 mM malate, but O_2 consumption does not (Table IV). Since oxidation via the TCA cycle requires acetyl-CoA production, malate must have been oxidized via malic enzyme and pyruvic dehydrogenase. At 4 mM malate, 42% of the pyruvate being consumed is derived from malate (provided there is no loss of TCA cycle intermediates from the matrix). This figure agrees reasonably well with those estimated from blocking malate or pyruvate transport (Fig. 7). Clearly, the relative contributions made by direct pyruvate transport and by malic enzyme will be a function of substrate concentrations, but the data also suggest that pyruvate transport rates can become limiting.

GENERAL DISCUSSION

The results obtained demonstrate the operation in corn mitochondria of a relatively specific pyruvate-transporting system, which apparently catalyzes the exchange of pyruvate for hydroxyl ions. Lactate also appears to be transported by this carrier which, apart from this feature, resembles that found in liver mitochondria, particularly with respect to CHCA inhibition. Halestrap (9) has suggested that CHCA attacks sulfhydryl groups on the carrier, and the inhibition by mersalyl observed here supports this. The specificity and potency of CHCA make it a potentially useful inhibitor for *in vivo* studies.

Plant cells are furnished with sugars which are converted in the cytoplasm to PEP, and from PEP produce two respiratory substrates, pyruvate and malate (Fig. 9). Malate appears to be ubiquitous in plants and is often accumulated in large amounts; the requisite enzymes (PEP carboxylase, PEP carboxykinase, and malic dehydrogenase) are commonly found (7, 21); dark CO_2 fixation is proving to have a central role in many aspects of plant metabolism (1, 7). Lips and Beevers (14) have reported dark fixation of CO_2 into malate and turnover of this malate by the TCA cycle under conditions which stimulate respiration (*e.g.* uncoupling). The role of intramitochondrial malic enzyme in utilizing vacuolar malate during repression of glycolysis and in intermediary metabolism has been discussed elsewhere (12, 18). Hence, it is important to recognize that two sources of respiratory substrate are furnished to plant mitochondria, not just pyruvate, as is commonly taught.

Isolated corn mitochondria require a continuous supply of both pyruvate and malate for maximum oxidation rates (Fig. 7; Tables I and IV). This was not expected, because respiratory-

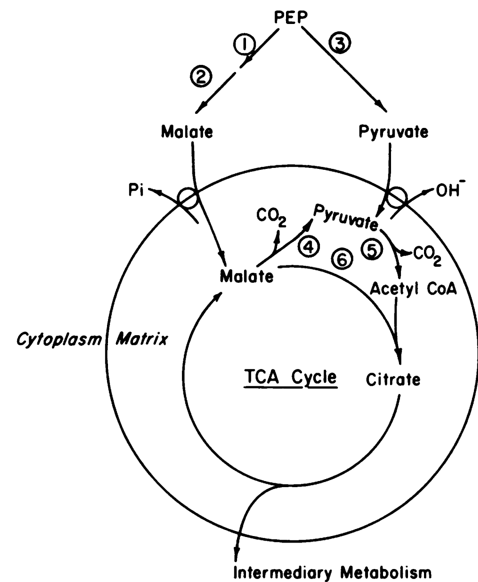

FIG. 9. Respiratory substrate supply, transport, and metabolism in corn mitocondria. 1: PEP carboxylase; 2: NADP-malate dehydrogenase; 3: pyruvate kinase; 4: NAD-malic enzyme; 5: pyruvate dehydrogenase; 6: NAD-malic dehydrogenase.

driven malate uptake is very rapid (6), and sparker levels of TCA cycle intermediates should be abundant after a minute or two. There are two possible explanations. First, the rates of pyruvate transport are not very large (16-33% of malate transport rates, Table III), and appear to be inadequate to sustain maximum respiration; malate transport and oxidation via malic enzyme will supplement here (Table IV). Second, during respiration, the mitochondria may be exporting TCA cycle intermediates which would normally be utilized in intermediary metabolism of the cell. These can be replaced by malate uptake but not by pyruvate. Depending on metabolic demands, it is possible that dark CO_2 fixation diverts much PEP from pyruvate to malate formation, with malate entering the mitochondria and filling two roles—replenishing the TCA cycle pool, and furnishing part of the respiratory carbon (see Fig. 9).

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