Compartmentation of Organic Acids in Corn Roots
II. The Cytoplasmic Pool of Malic Acid

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Summary. The major conclusion drawn was that malate generated in corn roots during a 15-minute period of CO₂ fixation and malate introduced into the tissue during a similar period from the bathing medium share a common extramitochondrial compartment, the cytoplasmic pool. The utilization of these 2 forms of malate is normally much slower than that of malate generated in the mitochondria by the tricarboxylic acid cycle. By lowering the pH of the medium or treating the tissue with malonate or 2,4-dinitrophenol, similar increases in the rates of utilization of both forms of cytoplasmic malate were brought about. Changes in (A) the demand for acetyl acceptors in the mitochondria and (B) mitochondrial permeability were invoked to account for the increased utilization of the cytoplasmic malate under the various experimental treatments.

The differential labeling of 2 pools of malate in corn roots by a double labeling technique has been previously described (9). Further experiments bearing on the intracellular separation of the malate produced by CO₂ fixation and that produced by the tricarboxylic acid cycle and particularly the distinctive responses of these 2 pools to experimental treatments are described in the present paper. The effects of these treatments [exposure to media at pH 5.0 and pH 7.5, to a metabolic inhibitor (malonate) and to 2,4-dinitrophenol (DNP), an uncoupling agent] on the utilization of exogenously supplied malate were also examined. The results lead to the conclusion that both the malate produced during a 15-minute pulse of ¹⁴CO₂ and that taken up from an exogenous supply during a similar pulse are confined to the extra-mitochondrial cytoplasmic space.

Materials and Methods

Maize grains (var. Wf 9 × 38-11) were obtained from the Agricultural Alumni Seed Association, Lafayette, Indiana. Subapical root segments were prepared and the treatment, extraction, fractionation and radioactivity assays were performed as described earlier (9). The amounts of labeled material supplied to samples of roots (2.5 g) were as follows: Na-acetate-²H: 50 μc (0.1 μmole), Na-malate-³¹⁴C: 10 μc (28 μmoles), Na-bicarbonate-³¹⁴C: 10 μc (0.55 μmole).

The general procedure was to expose several samples of roots to the appropriate isotopically labeled metabolite in 0.01 M potassium phosphate, pH 7.5 for 15 minutes (the pulse). The roots were then rinsed and aerated in experimental solutions without the labeled metabolite for several hours (post pulse treatment). At intervals samples were killed and extracted, the malate was separated, and its isotopic content determined.

Results

Effects of Experimental Treatments on Behavior of the Malate Pools. Variation of External pH. As shown previously, the 2 pools of malate labeled during a 15-minute pulse of acetate-²H plus bicarbonate-¹⁴C behave very differently during a subsequent incubation at pH 7.5. Whereas the malate-²H (produced in the tricarboxylic acid cycle) is rapidly lost, there is virtually no utilization of the malate-¹⁴C (produced by CO₂ fixation). However, when the incubation after the pulse was carried out at pH 5.0, the malate-¹⁴C was utilized at an appreciable rate, while the malate-²H was lost at the same rate as at pH 7.5 (fig 1). This specific effect of varying the external pH on the utilization of malate-¹⁴C is no doubt due in part to the consequent change in the level of (bicarbonate + CO₂) in solution. At the higher pH the greater availability of CO₂ would be expected to maintain the malate produced by CO₂ fixation and thus prevent its net utilization. At pH 5.0 in this

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experiment loss of $^{14}$C apparently occurred through exchange reactions, in other experiments a small loss of total malate has been observed. An indirect effect of external pH leading to introduction of the malate-$^{14}$C into the tricarboxylic acid cycle is clearly not ruled out (see Discussion).

**Effects of Malonate.** The results observed when malonate (0.1 M) at pH 5.0 was added after the standard pulse of acetate-$^{3}$H plus bicarbonate-$^{14}$C are shown in figure 2. In comparison with the control 3 significant changes were brought about by the inhibitor during the post pulse incubation at pH 5.0. A) The rate of utilization of malate-$^{3}$H was slowed down, B) the rate of utilization of malate-$^{14}$C was increased and C) the total malate level decreased.

The net utilization of malate after the pulse equaled that produced from fixation of CO$_2$ from the alkaline medium during the pulse and the malate level returned to the normal control value (fig 3). The fact that the malate still contained $^{14}$C after this time suggests that some transfer to the vacuole may have occurred. It appears, then, that in addition to slowing the traffic through succinate in the tricarboxylic acid cycle (10), the inhibitor in effect induced a mixing of the malate-$^{14}$C and malate-$^{3}$H. An increased demand for acetyl acceptor would be expected in the presence of malonate. A transfer of malate-$^{14}$C into the cycle in response to this demand would result in a higher rate of oxidation of malate-$^{14}$C than occurred in the absence of the inhibitor and at the same time slow down the loss of malate-$^{3}$H by diluting this pool.

**Effects of DNP.** The addition of $10^{-5}$ M DNP increased the O$_2$ uptake of the roots by about 50%. When DNP was added after the standard pulse, it was observed that the rate of utilization of malate-$^{3}$H was increased (fig 4, compare with fig 1). At the same time a rapid utilization of both the total titratable malate produced during the pulse and of the malate-$^{14}$C was induced. As with malonate, one effect of DNP was apparently to make possible the transfer of malate-$^{14}$C into the cycle. Such a transfer might result from the increased rate of turnover in the cycle or to an effect on permeability brought about by the uncoupling action of DNP. However the fact that the oxidation rate of the malate-$^{14}$C, though strikingly increased, still did not equal the increased rate of oxidation of malate-$^{3}$H shows that the DNP effect was not simply due to a complete mixing of the 2 pools. Effects on a third (vacuolar?) compartment may have been induced.
to the utilization of malate-1,4-\(^{14}\)C (produced by CO\(_2\) fixation) in the cycle, that of malate-3-\(^{14}\)C would lead to a resynthesis of labeled malate if it escaped sequestration in pools of other acids in the cycle and drainage to other constituents. The fact that the curves for \(^{14}\)C disappearance from the 2 forms of malate-\(^{14}\)C (i.e. net utilization) are so similar indicates that the amount of resynthesis of malate-\(^{14}\)C from the pulse of malate-3-\(^{14}\)C was insignificant in these experiments.

Utilization of Exogenous Malate-\(^{14}\)C. The rapid responses of that part of the total malate labeled by \(^{14}\)CO\(_2\) to the external treatments described above speak against the vacuole as its intracellular location and argue for a cytoplasmic site with normally limited access to the mitochondria. It was therefore surmised that the same limitations might apply to the access of externally added malate.

Preliminary experiments in which a pulse of malate-3-\(^{14}\)C was supplied showed that it was utilized at a much slower rate than malate generated in the tricarboxylic acid cycle under similar conditions from either acetate-\(^{3}\)H or acetate-\(^{1-14}\)C. A series of experiments was then performed in which the effects of the same treatments (pH, malonate and DNP) which had been shown to affect the utilization of the malate produced by CO\(_2\) fixation were examined on the utilization of exogenous malate. This was introduced into the tissue during a 15-minute pulse at pH 7.5. At the end of the exposure, the pulsing medium was removed and after rapidly washing the roots they were transferred to the appropriate solutions and the behavior of the labeled malate was followed.

The results are shown in figure 5. It is clear that the response of exogenously supplied malate to the 4 treatments is quite different from that of malate-\(^{3}\)H shown in the previous experiments and in fact reflects almost exactly that of malate produced by \(^{14}\)CO\(_2\) fixation. The values summarized in table I allow a direct comparison of the behavior of these 2 forms of malate. It is recognized that, in contrast

Discussion

The results described bear on 4 separate questions: A) The effect of pH of the medium on the rate of oxidation of exogenously supplied substrates, B) the probable location of the malate produced by CO\(_2\) fixation, C) the nature of the stimulatory effect of DNP and D) the permeability of mitochondria to malate in vivo.

Effect of pH. It has been known for many years that a low external pH, by decreasing ionization facilitates the entry of weak acids into cells and tissues (14). It is now apparent that a low external pH promotes not only the immediate entry of the acid (malate) but also its accessibility to the enzymatic machinery of the mitochondria. At least 2 barriers separate the exogenous organic acid from the oxidative enzymes: one is the external cell membrane and the second the mitochondrial membrane. The pulse technique eliminates considerations of uptake into the cytoplasm since only the rate of utilization of the substrate already in the tissue at the end of the pulse is measured. Under these conditions the rate of utilization is determined by the physical barrier separating the cytoplasmic substrate (taken up from the medium) and the activity of the oxidative enzymes. There is no indication that the concentration of hydrogen ions within the cytoplasm is af-
fectected by the pH of the bathing medium. We therefore believe that the observed effect of pH is an indirect one, possibly due to an exchange of H+ ions in the medium with certain cations within the extramitochondrial space or in the mitochondrion itself (4).

Location of the Dark Fixation Pool. If one assumes that the location of the malate-3H pool produced in the tricarboxylic cycle is in the mitochondria then the pool of malate-14C produced by 14CO2 fixation must be extramitochondrial. One can visualize, in general terms, 3 extramitochondrial sites for this malate-14C: A) the vacuole, B) a cytoplasmic organelle other than the mitochondria, C) the nonparticulate, soluble cytoplasm. The permeability properties of the tonoplast towards inorganic ions (3) indicate that this membrane constitutes a very firm barrier, and the contents of the vacuole are lost to the cytoplasm at a very slow rate, at least in cells of *Vitellaria obtusa* (11). It therefore seems unlikely that the rapid utilization of the malate produced by CO2 fixation which was brought about by the inhibitors involved a mass transfer of material from the vacuole. Moreover, as we have shown (fig 3) the bulk of the malate present in the tissue before the bicarbonate pulse remains unaffected by the inhibitors. This suggests that the pool of malate produced by CO2 fixation is a discrete one and does not represent vacuolar malate.

The rates of utilization of exogenous malate-14C under the experimental treatments described are so similar to those of malate-14C produced by CO2 fixation (table I) that the conclusion that they share a common pool seems inescapable. It is most unlikely that the exogenous malate is concentrated in a cellular organelle during the short pulse and we therefore believe that both malate produced by CO2 fixation and exogenous malate are mixed in the nonparticulate volume of the cytoplasm, a compartment now designated cytoplasmic.

The Nature of the Stimulatory Effect of DNP. The effect of DNP on the rate of oxidation of the cytoplasmic pool of malic acid suggests that the stimulations of O2 uptake brought about by DNP might have at least 2 bases: A) uncoupling of oxidative phosphorylation; B) increased availability of acetyl-CoA acceptors from extramitochondrial malate. A similar effect of DNP on the rates of oxidation of exogenous 14C respiratory substrates has been observed in avocado tissue slices (8). In cell free systems, in which the permeability properties of isolated mitochondria are presumably far different from those in vivo, uncoupling might be the only factor responsible for increased O2 uptake, although a strict distinction between uncoupling and permeability increase may no longer be possible (12). The consequences of applying DNP to intact tissues are complex and the stimulation of O2 uptake may not be due exclusively to removal of the limitation of phosphate acceptors. The argument that no limitation of acetyl acceptors exists in a tissue because exogenous malate fails to stimulate respiration is weakened by our observations that the introduction of cytoplasmic malate into the cycle is very limited under the experimental conditions generally employed (phosphate buffer pH 5-7).

Permeability of Mitochondria in vivo. The different rates of disappearance of cytoplasmic malate (that produced by dark fixation or added exogenously) and malate generated in the cycle (from acetate-3H) indicate that these 2 pools are separated. What constitutes the barrier between them? There seem to be 3 possibilities: A) The impermeability of one (or both) mitochondrial membranes to the cytoplasmic pool; B) an organizational barrier. The first is a common and self-explanatory case. The second is based on numerous observations, mainly in animal cells. If multi-enzyme systems are attached to membranes as suggested (1) and if intermediates of the cycle are asymmetrically oxidized (7) there could be a functional preference of the enzymatic machinery for substrate molecules coming from the neighboring enzyme over those from the surrounding space. Metabolic inhibitors might have an effect on the physical arrangement of the multi-enzyme system making the individual enzymes more available to the cytoplasmic pool.

Several papers during the past 10 years indicate that the natural state of mitochondria is one tightly closed to most external substrates and coenzymes. We may recall the work of Bendall and de Duve (2) showing how experimental treatments increasing

### Table 1. Utilization of 2 Forms of Labeled Cytoplasmic Malate under Different Conditions

<table>
<thead>
<tr>
<th>Pulse</th>
<th>Incubation</th>
<th>Malate-14C utilization, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicarbonate-14C</td>
<td>Potassium phosphate pH 7.5</td>
<td>0-12</td>
</tr>
<tr>
<td>Malate-3-14C</td>
<td>Potassium phosphate pH 7.5</td>
<td>5-11</td>
</tr>
<tr>
<td>Bicarbonate-14C</td>
<td>Potassium phosphate pH 5.0</td>
<td>35-45</td>
</tr>
<tr>
<td>Malate-3-14C</td>
<td>Potassium phosphate pH 5.0</td>
<td>40-50</td>
</tr>
<tr>
<td>Bicarbonate-14C</td>
<td>0.1 mM Na-malonate</td>
<td>50-73</td>
</tr>
<tr>
<td>Malate-3-14C</td>
<td>0.1 mM Na-malonate</td>
<td>66-70</td>
</tr>
<tr>
<td>Bicarbonate-14C</td>
<td>10^{-3} M DNP</td>
<td>85-93</td>
</tr>
<tr>
<td>Malate-3-14C</td>
<td>10^{-4} M DNP</td>
<td>87-97</td>
</tr>
</tbody>
</table>

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permeability led to 20-fold increases in the apparent activities of mitochondrial malate and glutamate dehydrogenases, and that of Simon (13) on cytochrome oxidase in plant mitochondria. Lester et al. (6) showed that specially prepared, tightly coupled mitochondria were unable to oxidize added isocitrate, although they were capable of carrying out the complete tricarboxylic acid cycle with acetate as substrate. Only when the mitochondria were experimentally damaged (5, 13) did the capacity to oxidize exogenous isocitrate appear.

These observations support the view expressed in this paper that the mitochondrial membrane (or some inherent organizational characteristic) constitutes the physical barrier separating the mitochondrial and the cytoplasmic pools of malic acid.

The simultaneous comparison of the rates of oxidation of endogenous (3H-labeled) and exogenous (14C-labeled) pools of malic acid under different experimental conditions permits, in effect, the demonstration of changes in mitochondrial “permeability” in vivo. The quotes intend to express the imprecision of our understanding of this property. The essential fact is that the transfer between the cytoplasmic pool of malate and the oxidative machinery of the cycle is normally limited and that certain experimental conditions increase the degree of the transfer.

Acknowledgment

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Literature Cited