THE EFFECT OF MALONATE ON TISSUE RESPIRATION*

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Malonate has long been regarded as a specific inhibitor for succinic dehydrogenase. The original evidence was obtained in Thunberg experiments with resting bacterial cultures (1, 2), and similar results were later obtained manometrically with brain and muscle tissue (3). The apparent specificity of this inhibition has led workers to use respiration experiments with malonate as a basis for theories of hydrogen transport (4, 5) and of carbohydrate breakdown—the "citric acid cycle" of Krebs and Johnson (6)—inasmuch as malonate inhibits the respiration of intact tissue as well as of enzyme preparations. However, analytical data of Weil-Malherbe (7) suggest that malonate may not act specifically on succinic dehydrogenase in intact tissue, even though its action on purified enzyme systems be highly specific. Moreover, Das (8) and Szent-Györgyi (9) have pointed out that fumarate and succinate differ quantitatively rather than qualitatively in their action on malonate-poisoned enzyme systems, and preliminary data of our own also seemed to indicate that malonate might not be a specific inhibitor (10). The experimental basis for the citric acid cycle has therefore been reexamined by a study of the respiration of the various component acids in the presence of malonate.

EXPERIMENTAL

All experiments were performed with minced pigeon breast muscle. The bird was decapitated, and the muscle removed as rapidly as possible, chilled on ice for 2 to 3 minutes, and minced

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in a chilled Latapie mincer. The mince was collected in a chilled Petri dish lined with filter paper moistened with saline solution. The tissue samples were weighed on cellophane on a torsion balance and the material dispersed in the appropriate solution contained in manometric flasks by means of a wire.

The oxygen consumption of the tissues was measured at 38° in the usual way in a standard Warburg apparatus. Unless otherwise stated, the medium used was a Ringer-phosphate buffer free of calcium, pH 7.4, in which muscle is known to be more sensitive to the dicarboxylic acids (11). The buffer was made up in twice normal concentration and diluted either with water or with supplementary solutions.

The following acids were studied for their effect upon muscle respiration in the presence and absence of malonate: citric, α-ketoglutaric, succinic, fumaric, and malic. In addition L(+)-glutamic acid was studied, since by oxidative deamination, it yields α-ketoglutaric acid (12, 13). The salts and acids were all commercial preparations except α-ketoglutaric acid. The solutions of the acids or their sodium salts were neutralized at concentrations of 0.2 M, and further dilutions then made from the neutral solution. Fresh solutions were made up at weekly intervals and stored at 0° when not in use. Solutions of α-ketoglutaric acid, however, were always made up just prior to their use.

The results of the manometric experiments were expressed in c.mm. of O₂ absorbed per mg. of tissue (dry weight) (11). Most experiments were run for 2 hours. In the analysis of results each experimental observation was compared with its own control, since the respiration of pigeon muscle varies from bird to bird. While for convenience, only the averages of many experiments have been tabulated, the results themselves were remarkably consistent. 0.001 or 0.005 M malonate invariably inhibited respiration as compared to the control, and the other acids invariably increased it. A similar consistency was observed when mixtures of acids were studied. Respiration was always better in fumarate plus malonate than in equimolar succinate plus malonate, which in turn was always better than citrate plus malonate.

1 We are indebted to M. A. Lipton for this preparation.
Results

While malonate inhibited muscle respiration in concentrations of 0.001 M or less, the most suitable concentration for a comparison of the sensitivity of the various acids to malonate was found to be 0.005 M. This concentration of malonate inhibited respiration about 70 per cent, and the various acids studied were markedly unequal in their ability to prevent this inhibition. Two levels of respiration were used for comparison, (a) the oxygen consumed by the unsupplemented control sample, and (b) that consumed by tissues supplemented with the various acids in concentrations of 0.001 or 0.005 M. This latter was designated "stabilized respiration." Over a 2 hour period glutamic acid increased respiration nearly 50 per cent; the other acids increased respiration about 30 per cent.

All of the acids studied stimulated respiration catalytically; that is, the extra oxygen consumed in the presence of small amounts of the acid was greater than the amount required completely to oxidize the added acid. Such data have already been reported for fumaric, malic, succinic (11), citric, and α-ketoglutaric acids (6, 14). We have been able to confirm the results of Krebs and Johnson with regard to the latter two acids, using Ringer-phosphate buffer, and in addition have observed catalysis with \( \ell \) (+)-glutamic acid. The amount of oxygen needed completely to oxidize 2 cc. of 0.0004 M glutamic acid is 80.6 c.mm. The extra oxygen consumed by 33.2 mg. (dry weight) of pigeon muscle in the presence of the acid was 249 c.mm. over a 2 hour period.

In Table I the various acids are listed in decreasing order of effectiveness in counteracting malonate inhibition. Fumarate and malate were most effective. In the presence of 0.005 M malonate, 0.005 M fumarate or malate restored respiration not only to the level of the unsupplemented control, but also to that of "stabilized respiration." When only half as much fumarate or malate was used, 0.0025 mole, the respiration was slightly below that of the control sample.

α-Ketoglutarate was nearly as effective as fumarate or malate. In equimolecular amounts of malonate and α-ketoglutarate, respiration exceeded that of the unsupplemented control, although it did not reach that of the "stabilized" samples.
Succinate was less effective than \( \alpha \)-ketoglutarate in compensating for malonate inhibition. In equimolecular amounts of succinate and malonate, respiration was slightly less than that of the unsupplemented control, but when twice as much succinate as malonate was used, respiration was restored to the "stabilized" level.

### Table I

*Respiration of Pigeon Breast Muscle in Presence of 0.005 Mole of Malonate and Various Stabilizing Supplements*

All data are expressed as c.mm. of \( \text{O}_2 \) absorbed per mg. of tissue (dry weight) in 2 hours.

<table>
<thead>
<tr>
<th>Acid supplements</th>
<th>Respiration in presence of supplement</th>
<th>Control respiration, unsupplemented</th>
<th>No. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Malonate</td>
<td>No malonate</td>
<td>Malonate</td>
</tr>
<tr>
<td>0.0025 M malic</td>
<td>11.7</td>
<td>14.3</td>
<td>3.8</td>
</tr>
<tr>
<td>0.005 &quot; &quot;</td>
<td>15.1</td>
<td>14.3</td>
<td>3.8</td>
</tr>
<tr>
<td>0.0025 &quot; fumaric</td>
<td>13.4</td>
<td>17.0</td>
<td>3.9</td>
</tr>
<tr>
<td>0.005 &quot; &quot;</td>
<td>18.6</td>
<td>18.7</td>
<td>4.3</td>
</tr>
<tr>
<td>0.01 &quot; &quot;</td>
<td>20.5</td>
<td>21.7</td>
<td>6.2</td>
</tr>
<tr>
<td>0.0025 &quot; ( \alpha )-ketoglutaric</td>
<td>12.4</td>
<td>18.3</td>
<td>3.8</td>
</tr>
<tr>
<td>0.005 &quot; &quot;</td>
<td>16.7</td>
<td>20.3</td>
<td>3.3</td>
</tr>
<tr>
<td>0.01 &quot; &quot;</td>
<td>15.6</td>
<td>18.3</td>
<td>3.5</td>
</tr>
<tr>
<td>0.005 &quot; succinic</td>
<td>13.3</td>
<td>19.8</td>
<td>4.4</td>
</tr>
<tr>
<td>0.01 &quot; &quot;</td>
<td>19.6</td>
<td>19.8</td>
<td>4.4</td>
</tr>
<tr>
<td>0.005 &quot; glutamic</td>
<td>10.4</td>
<td>24.4</td>
<td>3.9</td>
</tr>
<tr>
<td>0.01 &quot; &quot;</td>
<td>14.0</td>
<td>22.7</td>
<td>3.0</td>
</tr>
<tr>
<td>0.005 &quot; citric</td>
<td>8.3</td>
<td>19.8</td>
<td>4.4</td>
</tr>
<tr>
<td>0.01 &quot; &quot;</td>
<td>8.0</td>
<td>19.8</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Glutamic acid and citric acid were the least effective acids in countering malonate inhibition. These acids failed to restore respiration to the control level even in concentrations twice or 5 times\(^2\) that of the malonate used. When 0.001 M malonate, and corresponding amounts of the various supplementary acids were used, the differences observed between acids were less marked than in the presence of the higher level of malonate.

\(^2\) This latter result should not be stressed, since citrate alone frequently inhibits respiration in concentrations above 0.02 M. However, at the critical concentration 0.01 M, citrate alone stimulated respiration.
However, citrate was again the least effective of all in counteracting malonate inhibition.

### TABLE II

**Effect of Di(Tri)-Carboxylic Acids and Malonate on Respiration of Supplemented Pigeon Breast Muscle**

All values are expressed as c.mm. of O₂ absorbed per mg. of tissue (dry weight) in 4 hours.

<table>
<thead>
<tr>
<th>Supplements</th>
<th>Respiration</th>
<th>Per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Malonate*</td>
<td>No malonate</td>
</tr>
<tr>
<td>0.005 M malate + muscle juice + insulin</td>
<td>30.3</td>
<td>41.9</td>
</tr>
<tr>
<td>0.01 &quot; &quot; + &quot; &quot; + &quot; + &quot; + &quot; + &quot; + &quot; + &quot; + &quot; + &quot; + &quot;</td>
<td>36.0</td>
<td>44.0</td>
</tr>
<tr>
<td>0.005 &quot; &quot; + &quot; &quot; + &quot; + &quot; + &quot; + &quot; + &quot; + &quot; + &quot; + &quot;</td>
<td>29.0†</td>
<td>30.1</td>
</tr>
<tr>
<td>0.005 &quot; α-ketoglutarate + muscle juice + insulin</td>
<td>24.0</td>
<td>31.2</td>
</tr>
<tr>
<td>0.01 M α-ketoglutarate + muscle juice + insulin</td>
<td>27.3</td>
<td>24.8†</td>
</tr>
<tr>
<td>0.001 M α-ketoglutarate + muscle juice + insulin</td>
<td>9.4†</td>
<td>11.2</td>
</tr>
<tr>
<td>0.005 M succinate + muscle juice + insulin</td>
<td>16.7</td>
<td>29.7</td>
</tr>
<tr>
<td>0.01 &quot; &quot; + &quot; &quot; + &quot; + &quot; + &quot; + &quot; + &quot; + &quot; + &quot; + &quot; + &quot;</td>
<td>29.1</td>
<td>34.0§</td>
</tr>
</tbody>
</table>
| 0.005 " " + " " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + " + 

* The malonate concentration is 0.005 M, except where 0.001 M was used, as indicated by †.
† 0.005 M α-ketoglutarate + muscle juice + insulin.
§ 0.005 M succinate + muscle juice + insulin.
|| 0.005 M citrate, etc.

The differences between the effects of the various acids persisted in the presence of other supplements, as for example, combinations of muscle juice (Kochsaft) (11), insulin, and cocarboxylase. The respiration of tissue thus highly fortified was
decreased 28 per cent or less in the presence of equimolecular amounts of malonate and fumarate, malonate and malate, or malonate and α-ketoglutarate (Table II). In the presence of equimolecular amounts of malonate and succinate “fortified” respiration was decreased 40 per cent but, as in “unfortified” tissue, respiration was increased when the amount of succinate was doubled. In fortified tissue 0.005 M malonate decreased respiration 70 per cent in the presence of equimolar citrate or glutamate. Since malonate inhibition ranged from 60 to 80 per cent in tissues variously fortified, it is doubtful whether the citrate or glutamate had exerted any protective action at all. Increased amounts of citrate failed to decrease the inhibition materially.

Malonate was also added to media in which tissues had been respiring for 30 minutes in the presence of the various acids, thus allowing time for the conversion of the acids into other active components as postulated in the citric acid cycle of Krebs and Johnson (6). Under these circumstances the effect of the in-
hibitor might have been modified. Actually, however, the same
differences between the various acids appeared as before (Table
III). Citrate was again the least effective acid in compensating
for malonate inhibition. When malonate was added first,
followed 30 minutes later by the various acids, fumarate and succi-
cinate increased the respiratory rate over that of the previous
period, whereas citrate failed to halt the rapid decline in the rate
of respiration (Table III).

DISCUSSION

The differences in the activity of the various acids in the
presence of malonate were apparently not due to differences in
their rates of penetration into the cell, since in the absence of
malonate the various acids all stimulated respiration to approxi-
mately the same degree. Such differences as were observed
could not be correlated with malonate sensitivity, although a
survey of a large variety of tissues supplemented in various ways
indicated that citrate catalysis was less frequent than fumarate
catalysis (10). Glutamate, however, was the most effective acid
of all in stimulating respiration, although, like citrate, its effect
was completely nullified by malonate.

Two explanations might be advanced for the unequal behavior
of the various acids in the presence of malonate. The first in-
volves the assumption that malonate acts specifically on succinic
dehydrogenase. Since the amounts of fumarate and succinate
normally found in muscle are small, their effect would be com-
pletely wiped out by added malonate, and respiration would then
depend upon the rapidity with which the various acids added
could restore these substances to a concentration sufficient to
function in hydrogen transport. The ineffectiveness of citrate
(and glutamate) in restoring the respiration of malonate-poisoned
tissue would therefore suggest a slow or inefficient conversion of
citrate to substances necessary in respiration, such as succinate-
fumarate. The failure of citrate to restore respiration even when
added 30 minutes prior to the malonate only emphasizes this
inefficient conversion. However, if citrate is converted to other
substances only slowly, or with difficulty, this would exclude it as
a member of an essential respiration cycle, since a basic prerequisite
for such a cycle is the ready interconversion of its components.

But citrate unquestionably catalyzes respiration in the absence
of malonate (6, 10, 14). This catalysis, however, might not be
due to the citrate itself, but rather to catalytic substances formed
from citrate in the presence of tissue, such as the C₄ acids of
Szent-Györgyi. This idea has been expressed by Elliott and
Elliott (15) and Szent-Györgyi (9). It is given some support by
the fact that with malonate, glutamate acts much like citrate.
Glutamate has not been postulated as a member of the citric acid
cycle, but is known to yield α-ketoglutarate on contact with
respiring tissue.

A somewhat different interpretation of our results involves
the suggestion of Weil-Malherbe (7) that malonate is not a
specific inhibitor of succinic dehydrogenase, but that it also
inhibits other systems in intact tissue. Whereas increased
amounts of succinate completely compensated for malonate
inhibition, extra citrate or glutamate failed to do so, suggesting a
greater, or more permanent malonate sensitivity of the citrate
and glutamate systems than succinic dehydrogenase itself.
Isocitric dehydrogenase, however, is not sensitive to malonate
(16).

If non-specific inhibition of malonate be assumed, it is still
difficult to reconcile our results with the theory of a citric acid
cycle essential for respiration. The inhibition of respiration by
malonate in the presence of citrate could be attributed to an
interference with the degradation of citrate, an essential reaction
in the citric acid cycle. But if this be true, neither fumarate,
malate, α-ketoglutarate, nor succinate should have been able to
restore respiration in the presence of malonate, since they can
hardly be concerned with citrate degradation. Hence, it is
possible that whereas part of the citric acid cycle may be essential,
the entire cycle as such is not; and furthermore, that the component
parts of the cycle can break down in other ways than those
postulated. Thus α-ketoglutarate is said to be intermediate
between citrate and succinate: citrate → α-ketoglutarate → succ-
cinate (6, 17, 18). One might therefore expect its respiratory activity to be like that of citrate or succinate, or somewhat between the
two. In the presence of malonate, however, α-ketoglutarate was
definitely more effective in restoring respiration than succinate,
and very much more effective than citrate. The experiments of
Hallman and Simola (19) likewise suggest other pathways of
C. A. Baumann and F. J. Stare

degradation. α-Ketoglutarate incubated with muscle yielded much more citrate than did any of the other members of the cycle.

SUMMARY

1. The respiration of pigeon breast muscle inhibited by malonate was effectively restored by the addition of fumarate, malate, or α-ketoglutarate. Succinate also restored the respiration, but relatively more was needed. Citrate and glutamate completely failed to restore respiration when 0.005 M malonate was used, and were inferior to the other acids in the presence of lower amounts of malonate.

2. Glutamic acid, like citric, α-ketoglutaric, and the C₄ acids, stimulated respiration catalytically.

3. An intact citric acid cycle does not appear to be essential for the respiration of muscle. The same conclusion is reached whether malonate is regarded as a general inhibitor, or as a specific inhibitor for succinic dehydrogenase.

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