Changes in Endogenous Substrates of Isolated Rabbit Heart Mitochondria during Storage*

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SUMMARY

Rabbit heart mitochondria were isolated in the presence of low concentrations (150 to 250 μM) of pyruvate-2- or -3-¹⁴C to label the endogenous substrates. Chromatographic analyses of protein-free filtrates of the mitochondria indicated that the principal labeled endogenous substrates were citrate, malate, glutamate, and alanine. During storage of the mitochondria under anoxic conditions at 0°, radioactive citrate disappeared while aspartate and succinate increased. Continual changes in the composition of the endogenous substrates occurred during storage. When air was supplied to the mitochondrial suspension, the changes were similar to, but more rapid than, those occurring under anoxic conditions. Under aerobic conditions, however, radioactive succinate did not appear. Changes in total citrate, alanine, glutamate, and aspartate paralleled the changes in their radioactivity. Although partially metabolized, the endogenous substrates were largely retained by the mitochondria during repeated washing. During storage, in addition to metabolism, partial loss of some substrates into the medium was evident. Possible relationships of the metabolism of the endogenous substrates to the metabolic properties of isolated mitochondria are discussed.

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rabbats as described previously (4). To label the endogenous amino and organic acids, a precursor substrate such as pyruvate-2-14C (150 to 250 μM) was added to the isolation medium prior to homogenization of the tissue mince. The homogenate was centrifuged at 200 × g (average) for 13 min, and the supernatant fluid containing the mitochondrial fraction was centrifuged at 5500 × g (average) for 13 min. The mitochondrial pellet was resuspended in isolation medium (without labeled substrate) and was recentrifuged at 5500 × g. This pellet was resuspended in 0.18 M KC1 so as to obtain a mitochondrial suspension containing from 20 to 30 mg of protein per ml.

Samples were deproteinized with 0.05 ml of 70% HClO4 per ml of sample and, after centrifugation to remove the precipitate, were stored at −20°. Immediately preceding an analysis, the protein-free filtrate was neutralized to pH 7.0 with 0.1 N KOH and, after removal of the KC1O4, was chromatographed on a Dowex 1-X8 column, 1 × 17 cm. The techniques used for the initial separations on Dowex 1 columns, for the resolution and identification of the amino acids on Dowex 50 columns, and of the organic acids on silicic acid columns were reported earlier (4). Saturated fatty acids other than acetate or butyrate were not removed from Dowex 1 by these procedures but could be detected by chromatography of another sample on a silicic acid column. Mitochondrial protein was determined by solubilization of a portion of the suspension with 0.05 ml of 10% sodium deoxycholate followed by use of a biuret procedure (14). Citrate was determined by the procedure of Natelson, Lugovoy, and Pincus (15) and amino acids were determined with a Beckman 120 B automatic amino acid analyzer (16).

**RESULTS**

**Labeling and Distribution of Radioactive Substrates in Cell Fractions**—When rabbit heart mitochondria were isolated in the presence of pyruvate-2-14C (150 to 250 μM), endogenous amino acids and Krebs cycle intermediates became radioactive. Table I shows the relative distribution of radioactivity at each stage of the isolation procedure. The homogenate fraction refers to a sample taken immediately after homogenization of the mince in a medium containing 150 μM pyruvate-3-14C. Alanine, glutamate, and lactate contained the highest percentages of the radioactivity in this fraction. The supernatant fraction refers to the material decanted from the mitochondrial pellet after the first centrifugation at 5000 × g. This fraction contained microsomal material, soluble portions of the cell, and the bulk of the isolation medium.

Radioactive alanine and citrate were associated predominantly with the mitochondrial fraction while succinate, malate, and lactate were associated with the soluble fraction. Glutamate-14C was more evenly distributed while aspartate-14C (see below) was not detected in these two fractions. Traces of radioactive acetate and β-hydroxybutyrate were found in the homogenate and supernatant fractions but not in the mitochondrial fraction. The supernatant fraction contained 41% of the total radioactivity as lactate, which indicated that the pyruvate was rapidly converted to lactate by lactate dehydrogenase associated with the soluble fraction of the cell. The labeling pattern and the distribution of the radioactive substrates in the mitochondrial fraction were remarkably consistent from one preparation to another. Changes in the composition and amount of mitochondrial endogenous substrates which occur during the isolation procedure cannot be determined.

Complications due to dicarboxylic acid formation via CO2 fixation in these preparations were minimal since mitochondria isolated in the presence of HCO3− (480 μM) or pyruvate-1-14C (150 μM) contained insignificant amounts of radioactive organic or amino acids other than the lactate and alanine formed when the mitochondria were isolated in the presence of pyruvate-1-14C. In addition, mitochondria incubated in the presence of HCO3− and unlabeled pyruvate or HCO3− and pyruvate-1-14C with or without malonate did not incorporate radioactive into cycle intermediates. Radioactive fatty acids other than acetate were not detected when samples of the mitochondrial or supernatant fractions obtained from homogenates prepared in the presence of radioactive bicarbonate were chromatographed on silicic acid columns.1 Although saturated fatty acids of greater chain length than butyrate are retained on the Dowex 1 column, they are readily eluted from the silicic acid columns and should have been detected if present.

**Substrate Changes during Storage under Anoxic Conditions**—Fig. 1 depicts a typical experiment in which changes in the endogenous substrates labeled with radioactive carbon were measured during storage of rabbit heart mitochondria in the usual manner. The mitochondria were isolated in the presence of pyruvate-2-14C (150 μM). Changes in the radioactive substrates were followed over a 24-hour storage period. The mitochondrial suspension (24.4 mg of protein per ml) was kept at 0° without stirring except at the time the samples were withdrawn. Radioactive glutamate and malate increased initially, then gradually decreased and disappeared between 8 and 24 hours. Radioactive citrate decreased more rapidly than any other substrate and was absent after 8 hours. Lactate-14C remained essentially constant over the 24-hour period. As is the case with liver mitochondria (17), lactate dehydrogenase is essentially absent from these rabbit heart preparations.

Although separate was unlabeled at zero time, its radioactivity gradually increased and then remained essentially constant. As the radioactivity of glutamate decreased that of aspartate increased, which suggests that the glutamate-aspartate cycle de-

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1 The authors are grateful to Mr. Ronald Blackmore for conducting these experiments.
scribed by Krebs and Bellamy (18) was a predominant metabolic pathway. There was only a slight decrease in alanine-14C during the 24-hour storage period. Only traces of succinate-14C were present initially; the amount increased to a maximum at 4 hours and then decreased during the remainder of the anoxic storage period. Similar changes in the endogenous substrates of these mitochondrial preparations were consistently observed during storage under these conditions.

Substrate Changes during Storage under Aerobic Conditions—Fig. 2 illustrates the changes in the endogenous substrates occurring in mitochondrial suspensions kept relatively aerobic during storage by continuous agitation in a mechanical shaker with a relatively large surface area to depth ratio. As was observed in the anoxic storage experiments, radioactive glutamate and malate increased initially and then decreased (but more rapidly than under anoxic conditions) and were absent after 6 and 12 hours, respectively. Radioactive aspartate appeared more rapidly than was the case under anoxic conditions. Alanine-14C decreased rapidly over the first 2 hours and then decreased slowly for the remainder of the storage period. Under aerobic conditions, and in contrast to the anoxic conditions, radioactive succinate was not detected.

Labeling of Endogenous Substrates with Uniformly Labeled Malate-14C—Fig. 3 illustrates changes in substrates occurring during storage of a mitochondrial suspension isolated in the presence of a low concentration of uniformly labeled malate-14C (35 mCi). The changes which occurred during storage under anoxic conditions at 0° were similar to those observed with mitochondria which had been labeled with pyruvate-14C. Citrate decreased rapidly while malate decreased much more slowly. Aspartate-14C, absent at zero time, increased rapidly and remained at a nearly constant amount for the first 12 hours. Over the period from 24 to 48 hours, the increase in aspartate radioactivity was roughly proportional to the decrease in radioactivity of the glutamate. As was the case when the mitochondria were isolated in the presence of pyruvate-14C, only traces of radioactive succinate were present immediately following isolation. Succinate-14C increased during the first 12 hours of storage and then gradually decreased. Only a trace of alanine-14C was present and lactate-14C was absent. These compounds would be expected under these conditions only after a decarboxylation of oxalacetate to pyruvate followed by transamination with glutamate to form alanine or followed by a reduction to form lactate. The essential absence of lactate dehydrogenase explains the total absence of lactate under these conditions. Only a small fraction of the radioactivity added to the medium prior to isolation of the mitochondria appeared as malate. Of the approximately 2.0 × 10^7 cpm added to the isolation medium, only 3.8 × 10^4 cpm appeared as malate in the isolated mitochondria.
Changes in Total Amount and Radioactivity of Glutamate, Aspartate, Alanine, and Citrate—Numerous free amino acids were detected in protein-free filtrates of rabbit heart mitochondrial suspensions. Although the free amino acids of rabbit heart mitochondria and rat liver mitochondria (8) differ somewhat in nature and in their relative proportions, the amounts of the individual free amino acids are of the same order of magnitude (1 to 15 μmoles per g of mitochondrial protein). Table II lists the amounts of glutamate, aspartate, and alanine, the only amino acids which incorporated significant amounts of radioactivity when rabbit heart mitochondria were isolated in the presence of pyruvate-2- or -3-14C. The total amount of glutamate and its radioactivity decreased while the total amount of aspartate and its radioactivity increased during storage. While the total amount of alanine did not change substantially over the 8-hour storage period, its radioactivity decreased during the initial 4 hours, then remained relatively constant during the final 4 hours of the storage period. The specific activity of each of these amino acids decreased during storage, possibly due to dilution following synthesis from unlabelled precursors or to the presence of two compartments with differing specific activities.

Total citrate, as well as its radioactivity, was also determined during storage of several mitochondrial suspensions. Different preparations of freshly isolated rabbit heart mitochondria contained between 3.0 and 5.0 μmoles of citrate per g of mitochondrial protein. In a typical experiment, the mitochondria immediately after isolation in the presence of pyruvate-2-14C (specific activity, 5.6 μC per μmole) contained 3.5 μmoles of citrate per g of mitochondrial protein, with a specific activity of 2.62 × 10^6 counts per μmole. After 4 hours of storage (mitochondrial concentration, 18.3 mg of protein per ml), no citrate was detected by chemical means and only a trace of radioactivity was present. These data indicate that the change in total citrate paralleled the change in its radioactivity.

Retention of Endogenous Substrates during Repeated Washing of Mitochondria—Isolated mitochondria are usually washed one or more times to remove soluble extraneous substances. The effect of repeated washing on the endogenous substrates is illustrated in Fig. 4.

The mitochondrial fraction containing labeled endogenous substrates was washed repeatedly with the isolation medium described previously (4). The data denoted MIT. 1 in Fig. 4 depict the individual 14C-labeled substrates contained in 1 ml of the original mitochondrial suspension (protein concentration, 20.0 mg per ml). The suspension was then centrifuged at 5500 × g for 10 min, and 1 ml of the resulting supernatant solution was deproteinized and analyzed for 14C-substrates. This sample is represented in Fig. 4 as Medium 1. With a small Dounce homogenizer with a loosely fitting pestle, the mitochondrial pellet from this centrifugation was resuspended in a volume of medium 1 ml less than that used for the preceding mitochondrial concentration was 28.6 mg of protein per ml. All other procedures were as noted in Fig. 1.

![Graph](https://via.placeholder.com/150)

**Table II**

<table>
<thead>
<tr>
<th>Storage time</th>
<th>Aspartate</th>
<th>Glutamate</th>
<th>Alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hrs</td>
<td>1.1 μmol</td>
<td>0.32 μmol</td>
<td>0.37 μmol</td>
</tr>
<tr>
<td>4 hrs</td>
<td>2.4 μmol</td>
<td>1.8 × 10^4</td>
<td>7.7 × 10^4</td>
</tr>
<tr>
<td>8 hrs</td>
<td>4.1 μmol</td>
<td>1.9 × 10^4</td>
<td>4.0 × 10^4</td>
</tr>
</tbody>
</table>

a Specific activity, 5.6 μC per μmole.

b Micromoles per g of mitochondrial protein.

c Counts per min per g of mitochondrial protein.

d Counts per min per μmole.
drial suspension in order to maintain a constant protein concentration of the mitochondrial suspension for each successive washing.

Fig. 4 shows that mitochondrial 14C-citrate decreased with successive washings. Metabolism of citrate probably accounts for this loss since the decrease in the amount in the mitochondria was greater than the amount found in the supernatant fluid. The large decrease in radioactivity of mitochondrial alanine probably also resulted from metabolism since only small amounts of 14C-alanine appeared in the medium. Glutamate-14C was readily retained by the mitochondria; only the initial washing fluid contained a detectable amount of this acid. Lactate-14C, on the other hand, was readily removed by the washing procedure. Only small amounts of radioactive succinate appeared in the wash liquids, and the activity of mitochondrial succinate remained relatively constant. An appreciable fraction of the malate was removed with each washing. An initial decrease in mitochondrial malate was followed by a definite and continued increase, which suggested a continued production, possibly from citrate and alanine.

It should be emphasized that Fig. 4 compares the amount of the radioactive substrates found in 1 ml of the supernatant solution with the amount of radioactive substrates contained within the mitochondria represented by 20.0 mg of protein. If the amount of intramitochondrial water is comparable with that reported by O'Brien and Brierly (19) for bovine heart mitochondria (2.0 μl of water per mg of protein), and if it is assumed that the substrates are uniformly distributed in the intramitochondrial water, an indication of the relative concentrations of 14C-labeled substrates in the mitochondria and supernatant solution is obtained. Each mitochondrial sample represents 40 μl of intramitochondrial water, while each supernatant solution or wash represents 1 ml of water. To obtain the 14C-substrate concentration differences between the mitochondria and the supernatant solution, the difference in the amount of a particular 14C-labeled substrate in the two samples must be increased 25-fold. The resulting concentration difference may be several hundred-fold depending on the substrate. It is apparent that endogenous substrates were retained to a remarkable degree by the mitochondria during five successive washing operations.

Compartmentation of Labeled Endogenous Substrates during Storage—The effect of an 8-hour anoxic storage at 0° on the compartmentation of the 14C-labeled endogenous substrates was investigated. Samples were withdrawn from the storage vessel at the times noted in Fig. 5. A control sample was analyzed for labeled substrates. A second sample was centrifuged immediately to separate the mitochondria from the medium. Both the pellet and the supernatant fractions were deproteinized and

![Fig. 4](image-url)

**Fig. 4.** The effect of repeated washing of mitochondria on the distribution of the endogenous substrates between the mitochondrial pellet and supernatant solution. Mitochondrial concentration was 20 mg of protein per ml. MIT. 1, original mitochondrial suspension. MEDIUM 1, supernatant solution following centrifugation (3500 X g for 10 min) of the original mitochondrial suspension. MIT. 2, resuspension of the pellet from the previous centrifugation in isolation medium (free of pyruvate-14C) 1 ml less in volume than that of the original mitochondrial suspension. Other operations are noted in the text. ---, 14C-labeled substrates present in 1 ml of the mitochondrial suspension; -- --, 14C-labeled substrates in 1 ml of the wash solution after removal of the mitochondria by centrifugation.

![Fig. 5](image-url)

**Fig. 5.** The distribution of endogenous substrates between the mitochondria and KCl solution during storage without stirring. Mitochondrial concentration was 20.0 mg of protein per ml. Samples were centrifuged to separate the mitochondria. The pellet and supernatant solution were analyzed as noted in the text. ---, 14C-labeled substrates in the mitochondria; -- --, 14C-labeled substrates in the supernatant solution.
mitochondrial suspension. Sample fuge d to separate the pellet and supernatant fractions prior to analysis. Concentration of the original mitochondrial suspension was 23.9 mg of protein per ml.

During the initial period of storage, 14C-malate increased in the mitochondrial pellet and then remained constant in the supernatant fractions during the later portion of the storage period. 14C-Aspartate was formed in the supernatant during the later portion of the storage period. Approximately equal amounts appeared in the pellet and supernatant solution. This observation confirms the results of the experiment shown in Fig. 4 and the observations of Schneider et al. (5), who reported the impermeability of the mitochondrial membrane to citrate. Initially, 14C-lactate was eveny distributed between the pellet and supernatant solution during the later stages of the storage period.

14C-Citrate rapidly disappeared from the particles and did not appear in the supernatant solution. This observation confirms the results of the experiment shown in Fig. 4 and the observations of Schneider et al. (5), who reported the impermeability of the mitochondrial membrane to citrate. Initially, 14C-lactate was evenly distributed between the pellet and supernatant solution, then increased in the supernatant solution during the later stages of the storage period.

14C-Labeled succinate and glutamate increased initially, then decreased. Both substrates were retained within the mitochondrial pellet; only small amounts of either of these substrates were found in the supernatant solution. Initially, 14C-alanine diffused from the mitochondrial pellet and then remained constant in the supernatant fraction during the later portions of the storage period. Diffusion of 14C-alanine into the supernatant solution was reflected in the decrease in 14C-alanine detected in the pellet. Thus, it is evident that during storage the mitochondria retained certain substrates to a marked extent, while others were less readily retained and diffused into the medium.

### TABLE III

Comparison of radioactivity of endogenous substrates of mitochondrial suspension with distribution of substrates between mitochondrial pellet and supernatant fractions

<table>
<thead>
<tr>
<th>Storage time</th>
<th>Sample</th>
<th>Alanine</th>
<th>Glutamate</th>
<th>Aspartate</th>
<th>Succinate</th>
<th>Malate</th>
<th>Citrate</th>
<th>Lactate</th>
<th>Total counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hrs</td>
<td>I</td>
<td>18.1</td>
<td>5.6</td>
<td>0</td>
<td>1.7</td>
<td>3.7</td>
<td>7.4</td>
<td>3.1</td>
<td>39.5</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>15.9</td>
<td>6.7</td>
<td>0</td>
<td>2.6</td>
<td>3.4</td>
<td>6.7</td>
<td>3.7</td>
<td>32.9</td>
</tr>
<tr>
<td>1 hrs</td>
<td>I</td>
<td>15.4</td>
<td>7.1</td>
<td>0.6</td>
<td>1.0</td>
<td>6.4</td>
<td>3.8</td>
<td>3.8</td>
<td>38.1</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>18.1</td>
<td>7.8</td>
<td>1.3</td>
<td>6.3</td>
<td>1.7</td>
<td>3.3</td>
<td>3.9</td>
<td>30.9</td>
</tr>
<tr>
<td>2 hrs</td>
<td>I</td>
<td>17.1</td>
<td>6.6</td>
<td>1.2</td>
<td>3.8</td>
<td>5.1</td>
<td>2.1</td>
<td>3.0</td>
<td>30.4</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>16.2</td>
<td>7.4</td>
<td>1.0</td>
<td>6.3</td>
<td>3.2</td>
<td>3.5</td>
<td>37.5</td>
<td></td>
</tr>
<tr>
<td>4 hrs</td>
<td>I</td>
<td>16.8</td>
<td>5.9</td>
<td>2.0</td>
<td>5.2</td>
<td>3.5</td>
<td>0.5</td>
<td>4.0</td>
<td>37.8</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>17.8</td>
<td>6.7</td>
<td>1.2</td>
<td>7.6</td>
<td>2.1</td>
<td>4.1</td>
<td>4.1</td>
<td>39.6</td>
</tr>
<tr>
<td>8 hrs</td>
<td>I</td>
<td>17.9</td>
<td>2.5</td>
<td>2.8</td>
<td>1.3</td>
<td>2.2</td>
<td>0.2</td>
<td>4.2</td>
<td>31.1</td>
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<tr>
<td></td>
<td>II</td>
<td>14.3</td>
<td>1.7</td>
<td>2.2</td>
<td>0.9</td>
<td>1.2</td>
<td>3.5</td>
<td>23.8</td>
<td></td>
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</tbody>
</table>

* Values given are in counts per min $\times 10^{-4}$.
tissue by Goldberg, Passonneau, and Lawry (24), but have not
been reported previously for isolated mitochondria. The ac-
cumulation of succinate under anoxic conditions may be a result of the
lack of anaerobic dismutation reactions of succinate and of a
decrease in the phosphate potential \((\text{ATP}/\text{ADP} \times \text{Pi})\) (25) com-
patible with the demonstrated stimulatory effect of ATP on suc-
cinate oxidation (26–28).

In these studies, intramitochondrial citrate was observed to be
the substrate most readily metabolized. Extramitochondrial
citrate has been shown to be poorly metabolized by these as well as
by other mitochondrial suspensions (29, 30). Although exogenously added citrate (1 to 10 \(\mu\)M) penetrates the mito-
chondrial membrane (30, 31), inaccessibility to a metabolically
active compartment of the mitochondrion may prevent its
metabolism.

The fact that intramitochondrial citrate was rapidly metabo-
lized and was not eluted during washing nor during storage and
the fact that extramitochondrial citrate is a poor substrate for
mitochondria suggest that the metabolism of citrate is poorly
understood.

Changes in the total amounts of citrate, alanine, aspartate,
and glutamate paralleled the changes in the radioactivity of these
substrates. The observation that nonradioactive aspartate was
present in the mitochondria immediately after isolation (Figs. 1, 2, and 3) indicates that radioactive aspartate is
not formed during the isolation procedures.

Utilization of aspartate by the following series of reactions,
with Reaction 2 proceeding only from left to right, could account
for these observations.

\[
\begin{align*}
\text{Pyruvate + glutamate} & \rightarrow \text{alanine + } \alpha\text{-ketoglutarate} \quad (1) \\
\text{Aspartate + } \alpha\text{-ketoglutarate} & \rightarrow \text{oxalacetate + glutamate} \quad (2) \\
\text{Pyruvate } & \rightarrow \text{acetyl-CoA + } \text{CO}_2 \quad (3) \\
\text{Oxalacetate + acetyl-CoA } & \rightarrow \text{citrate} \quad (4)
\end{align*}
\]

The absence of a source of acetyl-CoA following isolation of
the mitochondria could lead to an increase in both total and
radioactive aspartate paralleling the decreases in radioactive
citrate, glutamate, malate, and alanine during anoxic storage.

The oxidation of \(^{14}\text{C}\)-labeled endogenous lipids by isolated
diffused rat heart has been reported (32). The immediate
precursor of the \(^{14}\text{CO}_2\) and the extent of oxidation of any endo-
genous mitochondrial lipids cannot be identified from the data
reported. Long chain fatty acids have been shown to be readily
oxidized by rabbit heart mitochondria under the conditions of
our experiments.\(^a\) Also, it has been reported that, in the absence of added carnitine, mitochondria from rat heart oxidized only
short and medium chain fatty acids (10).

While it has been proposed that fatty acids may be endogenous
substrates of isolated mitochondria (9–11) several observations
suggest that they are unlikely to be responsible for the endo-
genous respiration of the preparations described in this and the
accompanying paper (12, 22). Only traces of radioactive
aspartate accumulated when acetyl-CoA was available. Follow-
ing exhaustion of exogenous sources of acetyl-CoA, there
was an increase in the radioactivity of aspartate and a decrease
in that of glutamate. Depletion of the endogenous respiration is
accompanied by a depletion of the oxidizable endogenous
Krebs cycle substrates (12). The presence of serum albumin in
the isolation medium may also tend to avoid contamination of
the mitochondrial preparations by fatty acids and other lipids.
In other studies,\(^a\) a low endogenous respiratory activity was
paralleled by a low mitochondrial content of Krebs cycle sub-
strates.

Mitochondrial retention of labeled endogenous substrates
during repeated washing may indicate that the mitochondrial
membrane is impermeable to most of the intramitochondrially
generated substrates. Alternately, an apparent impermeability
may arise from the binding of these substrates by the mito-
chondrion in the form of enzyme-substrate or enzyme-product
complexes as suggested by Jones and Gutfried (33–35). Re-
peated washing of mitochondria in an attempt to deplete them
of endogenous substrates may be of little value since any positive
contribution of substrate depletion may be offset by a decreased
stability of the mitochondrial preparations.

Compartmentation of endogenous substrates indicated by the
experiment shown in Fig. 5 further supports these conclusions.
This experiment indicates that endogenous substrates, e.g. succinate, glutamate, and citrate, are avidly
retained by mitochondria while other substrates, e.g. alanine, aspartate, and lactate, are retained less readily and diffused into
the extramitochondrial compartment. Malate concentration
remained essentially constant in the extramitochondrial com-
partment while decreasing in the intramitochondrial space. The
\(^{14}\text{C}-\text{malate utilization curves illustrated in Figs. 1 and 2 indicate}
that an initial increase in mitochondrial malate was followed by a
rapid and then a more gradual decrease. This observation
ensemble with the compartmentation shown in Fig 5 suggests
that metabolism of malate following prolonged storage may be
dependent upon its re-entry into the mitochondrion.
An investigation of the relationships between mitochondrial per-
meability, compartmentation, and metabolism may offer more
critical insight into the dynamic nature of the tricarboxylic acid
cycle.

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