THE UTILIZATION IN VITRO OF C\textsuperscript{14}-LABELED ACETATE AND PYRUVATE BY DIAPHRAGM MUSCLE OF RAT\textdagger

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(Received for publication, June 10, 1949)

The usefulness of the rat diaphragm technique of Gemmill in the study of carbohydrate metabolism of muscle in vitro has been demonstrated by the experiments of Gemmill (1, 2), Stadie (3, 4), Krahl and Cori (5), Krahl and Park (6), and Verzar and Wenner (7, 8). The adaptation of this technique to experiments by use of C\textsuperscript{14}-labeled glucose was described in a previous paper (9). In those experiments, it was found that the addition of insulin increased the amount of glucose utilized, the amount of glycogen synthesized, and the amount of glucose carbon metabolized to carbon dioxide. The removal of the pituitary and adrenals also increased the rates of these reactions. Although most of the data could be explained by assuming that insulin and the hormones of the pituitary and adrenal glands affect the rate of the hexokinase reaction, other points of effect by these substances were not excluded. In the experiments reported here, the effect of insulin on the metabolism of C\textsuperscript{14}-labeled acetate and pyruvate was studied under varying experimental conditions. Other experiments comparing the utilization of labeled acetate and pyruvate in ventricle slices and diaphragm of the rat are reported elsewhere (10).

\textit{Materials and Methods}

The strain of rats and methods of adrenalectomy and of inducing diabetes were identical with those of the previous study (9). Rats were fasted 24 hours before being used in an experiment, and only those rats with a fasting blood glucose of 300 mg. per cent or more were included in the diabetic series.

Sodium acetate labeled with C\textsuperscript{14} in the carboxyl carbon (11) and potassium pyruvate labeled with C\textsuperscript{14} in the carbonyl carbon (12) were synthesized in this laboratory by Dr. Yale J. Topper. The radioactivities of these compounds were determined by their combustion to CO\textsubscript{2} and collection as BaCO\textsubscript{3}. The BaCO\textsubscript{3} was plated on stainless steel planchets and its radioactivity determined by an end window Geiger tube. The acetate had an activity of 3110 counts per minute per mg. when referred to an arbitrary

\textdagger This work was supported in part by a contract between Harvard University and the Office of Naval Research and the Atomic Energy Commission, and in part by a grant-in-aid from the Ella Sachs Plots Foundation.
standard of BaC$^{14}$O$_3$; the pyruvate had an activity of 1465 counts per minute per mg. when referred to the same standard.

The isolated hemidiaphragms were incubated in Warburg vessels in a medium of 0.04 M sodium phosphate, 0.005 M MgCl$_2$, 0.08 M NaCl, and either 0.01 M acetate or 0.01 M pyruvate. The initial pH of the medium, determined by glass electrode, was 6.8 and the pH after incubation was 6.8 ± 0.1. The center wells contained a piece of hard filter paper and 0.2 ml. of CO$_2$-free 5 per cent NaOH to absorb the CO$_2$ produced. A hemidiaphragm weighing about 100 mg. was placed in each vessel in 3.0 ml. of medium, 0.5 unit of insulin per ml. was added to one of each pair of flasks, and the vessels were incubated for 2 hours after an initial gassing with 100 per cent oxygen.

At the end of the incubation period, the CO$_2$ from the center well was precipitated as BaCO$_3$, plated, and counted with an end window Geiger tube. The diaphragm was removed and digested in boiling 30 per cent KOH; the glycogen was precipitated by alcohol, purified, hydrolyzed, and determined as glucose by the method of Nelson (13). Samples of the media were analyzed before and after incubation for pyruvate by the method of Friedemann and Haugen (14) and for lactate by the method of Barker and Summerson (15). Blood glucose determinations were made at the time of sacrifice by the method of Nelson (13).

**Results**

**Acetate Metabolism**—The amount of acetate oxidized to CO$_2$ was calculated from the ratio of the total activity in the respiratory CO$_2$ to the total initial activity of the acetate in the medium, multiplied by the initial concentration of acetate in the medium (Table I, Line 2). This calculation involves the assumption that the formation of radioactive CO$_2$ is a measure of the complete oxidation of acetate molecules, and that for each labeled (carboxyl) carbon appearing in the CO$_2$ 1 unlabeled ($\alpha$) carbon also appears.

The metabolism of acetate to CO$_2$ in diabetic diaphragm was much less than that in normal diaphragm (Table I, Line 2), 3.3 $\mu$M per gm. wet weight of diaphragm per hour in muscle from diabetic animals compared to 12.4 $\mu$M per gm. per hour in muscle from normal rats. This was not due to an over-all decrease in metabolism in the diabetic tissue, because the oxygen consumptions of the two types of tissue are comparable (Table I, Line 5). Rather, it is to be attributed to an actual decrease in the diabetic muscle of the percentage of the CO$_2$ carbons derived from acetate (Table I, Line 6). The percentage of CO$_2$ carbons derived from acetate carbons was calculated by dividing the specific activity (counts per minute per milli- mole of carbon) of the CO$_2$ by the specific activity (counts per minute per
millimole of carbon) of the acetate and multiplying by 100. This decrease in acetate metabolism in the diabetic was not relieved by the addition in vitro of insulin. The amount of acetate metabolized to carbon dioxide and the percentage of respiratory CO₂ derived from acetate in diaphragm from adrenalectomized rats was not significantly different from normal (Table I). Insulin had no effect on acetate metabolism in muscle from either normal or adrenalectomized rats. This is in striking contrast to its marked effect (when glucose is the substrate) on glucose utilization, glycogen formation, and the metabolism of glucose carbons to carbon dioxide (9).

There was no net increase in the amount of glycogen present in diaphragm incubated with acetate as the substrate. The amount of lactate

<table>
<thead>
<tr>
<th>Table I</th>
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</table>

| Effect of Insulin on Metabolism of Acetate by Diaphragm Muscle |

The figures given are the mean ± the standard error in micromoles per gm. per hour, except for respiratory CO₂ which is in per cent.

<table>
<thead>
<tr>
<th>Normal rat diaphragm</th>
<th>Diabetic rat diaphragm</th>
<th>Adrenalectomized rat diaphragm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without insulin</td>
<td>With insulin</td>
<td>Without insulin</td>
</tr>
<tr>
<td>1. No. of experiments</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>2. Acetate oxidized</td>
<td>12.4 ±0.53</td>
<td>12.9 ±0.60</td>
</tr>
<tr>
<td>3. Lactate produced</td>
<td>4.4</td>
<td>5.7</td>
</tr>
<tr>
<td>4. Glycogen made</td>
<td>±0.08 ±0.72</td>
<td>±0.47 ±1.01</td>
</tr>
<tr>
<td>5. Oxygen uptake</td>
<td>58.2 ±2.0</td>
<td>61.6 ±2.0</td>
</tr>
<tr>
<td>6. Respiratory CO₂ derived from acetate</td>
<td>42.2 ±2.1</td>
<td>43.6 ±2.9</td>
</tr>
</tbody>
</table>

produced by diaphragm muscle from adrenalectomized rats was less than half that produced by muscle from normal or diabetic animals (Table I, Line 3).

Pyruvate Metabolism—The metabolism of carbonyl-labeled pyruvate to CO₂ by muscle from diabetic rats was also significantly lower than that of normal diaphragm, but, in contrast to acetate metabolism, it was brought back to normal by the addition, in vitro, of insulin (Table II, Line 3). The amount of pyruvate metabolized to CO₂ was calculated by dividing the total activity in the respiratory CO₂ by the total activity of the pyruvate in the medium and multiplying by the initial concentration of pyruvate in the medium. This calculation involves the assumption that the formation of radioactive CO₂ is a measure of the complete oxidation of pyruvate
molecules, and that, for each labeled (carbonyl) carbon appearing in the CO$_2$, 2 unlabeled ones (a carboxyl and a methyl carbon) also appear as CO$_2$.

The total utilization of pyruvate, measured chemically as the disappearance of pyruvate from the medium, was also somewhat lower in diabetic diaphragm, and this was restored to normal by the addition in vitro of insulin (Table II, Line 2). In contrast, insulin caused no significant increase in either the total utilization of pyruvate or the metabolism of pyruvate to CO$_2$ in normal or adrenalectomized diaphragm (Table II). The percentage of the respiratory CO$_2$ derived from pyruvate was not de-

Table II

Effect of Insulin on Metabolism of Pyruvate by Diaphragm Muscle

The figures given are the mean ± the standard error in micromoles per gm. per hour, except for respiratory CO$_2$ which is in per cent.

<table>
<thead>
<tr>
<th></th>
<th>Normal rat diaphragm</th>
<th>Diabetic rat diaphragm</th>
<th>Adrenalectomized rat diaphragm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without insulin</td>
<td>With insulin</td>
<td>Without insulin</td>
</tr>
<tr>
<td>1. No. of experiments</td>
<td>10</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>2. Total pyruvate utilization</td>
<td>42.4</td>
<td>44.3</td>
<td>32.1</td>
</tr>
<tr>
<td></td>
<td>±2.1</td>
<td>±2.5</td>
<td>±3.8</td>
</tr>
<tr>
<td>3. Pyruvate oxidized</td>
<td>8.5</td>
<td>8.8</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>±0.38</td>
<td>±0.50</td>
<td>±0.60</td>
</tr>
<tr>
<td>4. Glycogen made</td>
<td>1.23</td>
<td>0.87</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>±0.10</td>
<td>±0.30</td>
<td>±0.98</td>
</tr>
<tr>
<td>5. Lactate produced</td>
<td>18.5</td>
<td>17.7</td>
<td>22.1</td>
</tr>
<tr>
<td></td>
<td>±2.7</td>
<td>±1.8</td>
<td>±2.8</td>
</tr>
<tr>
<td>6. Oxygen uptake</td>
<td>63.2</td>
<td>65.0</td>
<td>48.4</td>
</tr>
<tr>
<td></td>
<td>±3.0</td>
<td>±3.3</td>
<td>±4.3</td>
</tr>
<tr>
<td>7. Respiratory CO$_2$ derived from pyruvate</td>
<td>34.8</td>
<td>35.1</td>
<td>35.4</td>
</tr>
<tr>
<td></td>
<td>±1.4</td>
<td>±1.6</td>
<td>±3.2</td>
</tr>
</tbody>
</table>

creased in the diabetic muscle (Table II, Line 7), in contrast to the marked decrease in the percentage of respiratory CO$_2$ derived from acetate in diaphragms of diabetic animals.

Although the over-all utilization of pyruvate was not increased in the muscle from adrenalectomized animals (Table II, Line 2), the rate of metabolism of pyruvate to CO$_2$ was increased significantly (Table II, Line 3), and hence the fraction of the pyruvate utilized that is metabolized to CO$_2$ was increased. In normal and diabetic diaphragms, approximately 20 per cent of the pyruvate disappearing from the medium was metabolized to CO$_2$, but in muscle from adrenalectomized rats 30 per cent of the pyruvate disappearing from the medium was metabolized to CO$_2$. The amount
of lactate produced by adrenalectomized muscle was significantly less than
that produced by normal or diabetic muscle when pyruvate was the
substrate.

The amount of glycogen produced by diaphragm muscle incubated with
pyruvate as a substrate was slight and variable (Table II, Line 4). There
were no significant differences in this respect between normal, diabetic,
or adrenalectomized muscle, or between muscle incubated with or without
insulin.

**Table III**

Effect of Added Dicarboxylic and Tricarboxylic Acids on Acetate Metabolism in Muscle

The figures given are the mean ± the standard error.

<table>
<thead>
<tr>
<th>Outlet area</th>
<th>Acetate oxidized, µM per gm. per hr.</th>
<th>Oxygen uptake, µM per gm. per hr.</th>
<th>Respiratory CO2 from acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without addition</td>
<td>With addition</td>
<td>Without addition</td>
</tr>
</tbody>
</table>

| Normal muscle |                                      |                                   |                             |                             |
|---------------|--------------------------------------|-----------------------------------|-----------------------------|
| 1. Aconitate, 20 mM per liter | 8 | 10.4 | 11.8 | 66.2 | 72.4 | 34.1 | 32.8 |
|                | ±0.74 | ±0.89 | +2.3 | ±3.0 | ±3.0 | ±2.4 |
| 2. α-Ketoglutarate, 20 mM per liter | 4 | 8.2 | 10.6 | 66.0 | 73.8 | 29.4 | 30.3 |
|                | ±0.88 | ±0.80 | ±1.6 | ±2.4 | ±1.8 | ±3.3 |
| 3. Oxalacetate, 10 mM per liter | 4 | 8.5 | 9.3 | 61.0 | 69.4 | 31.8 | 13.0 |
|                | ±0.82 | ±0.87 | ±2.2 | ±2.9 | ±2.4 | ±1.9 |

| Diabetic muscle |                                      |                                   |                             |                             |
|-----------------|--------------------------------------|-----------------------------------|-----------------------------|
| 4. Aconitate, 20 mM per liter | 4 | 4.9 | 11.1 | 50.4 | 61.2 | 26.3 | 30.3 |
|                | ±0.71 | ±0.77 | ±3.2 | ±2.5 | ±2.2 | ±2.5 |
| 5. α-Ketoglutarate, 20 mM per liter | 2 | 5.1 | 7.3 | 69.6 | 74.6 | 22.2 | 21.6 |
|                | ±0.80 | ±0.91 | ±4.3 | ±3.6 | ±1.7 | ±1.9 |
| 6. Oxalacetate, 5 mM per liter | 5 | 4.2 | 8.6 | 52.6 | 65.0 | 21.7 | 16.2 |
|                | ±0.84 | ±0.72 | ±2.3 | ±2.9 | ±2.0 | ±1.4 |

The effects of the addition of certain Krebs tricarboxylic acid cycle
intermediates on the metabolism of labeled acetate and pyruvate were
also studied. Both aconitate and α-ketoglutarate (20 mM per liter) pro-
duced slight increases in the amount of acetate metabolized to CO2 by
normal muscle, but since they also increased the over-all metabolism (as
measured by oxygen consumption), there was no effect on the percentage
of the respiratory CO2 derived from the labeled acetate (Table III). The
addition of aconitate to the incubation medium increased markedly the
amount of acetate metabolized to CO2 by diabetic diaphragm and brought
the value up to the normal level (Table III, Line 4). As in normal muscle, the addition of aconitate increased the oxygen consumption of muscle from diabetic rats, and hence the increase in the percentage of respiratory CO₂ derived from acetate was slight. In contrast, added α-ketoglutarate had less effect on acetate metabolism in diabetic diaphragm.

Added aconitate (20 mM per liter) produced a marked increase in the amount of pyruvate utilized (from 49 to 60 μM per gm. per hour) (Table IV). There was also observed an increase in the amount of pyruvate metabolized to CO₂ (from 9 to 16 μM per gm. per hour), and in the percentage of respiratory CO₂ derived from pyruvate. In contrast, α-ketoglutarate produced only a slight, statistically insignificant increase in the amount of pyruvate metabolized to CO₂ and no change in the percentage of respiratory CO₂ derived from pyruvate. Neither aconitate nor α-ketoglutarate increased the amount of lactate produced with either pyruvate or acetate as the substrate.

Oxalacetate had no effect on the amount of acetate or pyruvate metabolized to carbon dioxide in normal muscle (Tables III and IV) but, be-

### Table IV

**Effect of Added Dicarboxylic and Tricarboxylic Acids on Pyruvate Metabolism in Muscle**

The figures given are the mean ± the standard error.

<table>
<thead>
<tr>
<th>No. of experiments</th>
<th>Total pyruvate utilized, μM per gm. per hr.</th>
<th>Pyruvate oxidized, μM per gm. per hr.</th>
<th>Oxygen uptake, μM per gm. per hr.</th>
<th>Respiratory CO₂ from pyruvate (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without addition</td>
<td>With addition</td>
<td>Without addition</td>
<td>With addition</td>
</tr>
<tr>
<td><strong>Normal muscle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Aconitate, 20 mM per liter</td>
<td>4</td>
<td>49.0 ± 2.6</td>
<td>60.3 ± 4.9</td>
<td>9.1 ± 0.68</td>
</tr>
<tr>
<td>2. α-Ketoglutarate, 20 mM per liter</td>
<td>4</td>
<td>46.6 ± 2.2</td>
<td>40.4 ± 2.8</td>
<td>9.3 ± 0.76</td>
</tr>
<tr>
<td>3. Succinate, 10 mM per liter</td>
<td>2</td>
<td>39.1 ± 3.1</td>
<td>7.3 ± 2.8</td>
<td>7.3 ± 0.66</td>
</tr>
<tr>
<td>4. Oxalacetate, 5 mM per liter</td>
<td>4</td>
<td>49.9 ± 3.3</td>
<td>8.5 ± 2.8</td>
<td>8.5 ± 0.81</td>
</tr>
<tr>
<td><strong>Diabetic muscle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Aconitate, 20 mM per liter</td>
<td>4</td>
<td>43.9 ± 4.6</td>
<td>55.2 ± 4.5</td>
<td>6.8 ± 1.3</td>
</tr>
<tr>
<td>6. Oxalacetate, 5 mM per liter</td>
<td>3</td>
<td>24.4 ± 3.1</td>
<td>3.1 ± 2.8</td>
<td>3.1 ± 0.52</td>
</tr>
</tbody>
</table>
cause it was rapidly decarboxylated and produced a large amount of carbon
dioxide, diluting that from the metabolism of the acetate and pyruvate,
the percentage of the respiratory CO₂ derived from acetate and pyruvate
was markedly decreased in these experiments. Oxalacetate increased the
amount of acetate, but not of pyruvate, metabolized to carbon dioxide
by diabetic diaphragm.

DISCUSSION

These experiments indicate that, in alloxan diabetes of the rat, the over-
all utilization of pyruvate and its metabolism to CO₂ by diaphragm muscle
are interfered with, and that this condition can be remedied by the addition
in vitro of insulin. This suggests that insulin is involved in the metabolism
of pyruvate to CO₂. The experiments comparing metabolism in cardiac
and skeletal muscle of the rat (10), in which slightly different experimental
techniques were used, also showed decreases in the metabolism of acetate
and pyruvate to CO₂ in diabetic as compared to normal diaphragm. These
investigators also found that the addition of insulin in vitro would increase
in diabetic but not in normal diaphragm the amount of pyruvate but not
of acetate metabolized to carbon dioxide. These findings are in agreement
with the experiments of Shorr (16) who found that heart muscle slices from
depancreatized diabetic dogs showed a decreased utilization of pyruvate
and lactate, and with the results of Rice and Evans (17) who demonstrated
an effect of insulin or pyruvate utilization by pigeon breast muscle mince.
An effect of insulin in increasing the incorporation of pyruvate into lipide
by rat diaphragm muscle has also been observed (18, 19). Further evidence
that insulin may be involved in the metabolism of pyruvate is supplied by
the experiments of Charalampous and Hegsted (20) who found that acety-
lation, as measured by the acetylation of injected p-aminobenzoic acid,
was decreased in alloxan-diabetic rats and brought back to normal by the
injection of insulin. Charalampous and Hegsted also found that acetyla-
tion in the diabetic was increased by the injection of adenosine triphos-
phate, acetyl phosphate, and certain di- and tricarboxylic acids, of which
malate was most effective. None of the materials effective in diabetic
rats had any effect on acetylation when injected in normal rats. This
suggests that insulin is involved in the metabolism of pyruvate in the
Krebs cycle, since the injection of Krebs cycle components, but not of
lactate or pyruvate, increased the amount of acetylation in diabetic
animals.

Our experiments show that alloxan diabetes of the rat is also character-
ized by a marked reduction in the metabolism of acetate to carbon dioxide.
This reduction is unchanged when insulin is added in vitro. The experi-
ments on diabetic diaphragm could be accounted for by interference with
the condensation of acetate and oxalacetate in the Krebs cycle. Since
the addition of insulin to diabetic diaphragm did not restore the metabolism
of acetate to normal, it is probable that insulin deficiency per se was not
responsible for the diminished acetate metabolism.

We wish to express our thanks to Dr. A. K. Solomon of the Biophysical
Laboratory for performing the C¹⁴ analyses, and to Miss Kathleen White-
house and Mrs. Vivien White for their technical assistance.

SUMMARY
1. The metabolism of acetate and pyruvate to carbon dioxide, the
synthesis of glycogen, the accumulation of lactic acid, and the disappear-
ance of pyruvate have been measured in vitro in diaphragm muscle isolated
from normal, diabetic, and adrenalectomized rats.

2. The amount of acetate metabolized to carbon dioxide is much less
in muscle from diabetic rats than in muscle from normal rats. This de-
crease is not restored by the addition of insulin. The metabolism of
acetate to carbon dioxide in muscle from adrenalectomized rats was at
the normal level and was unaffected by insulin.

3. The amount of pyruvate metabolized to carbon dioxide was also less
in muscle from diabetic animals than in normal muscle, but this was re-
stored to normal by the addition in vitro of insulin. The total utilization
of pyruvate was also decreased in diabetic muscle and brought back to
normal when insulin was added. Insulin caused no significant increase
in either the total utilization of pyruvate or its metabolism to carbon dioxide
in muscle from normal or adrenalectomized rats.

4. Adrenalectomy produced an increase in the fraction of the total
pyruvate utilized that is metabolized to carbon dioxide and a decrease
in the accumulation of lactate.

5. The effects of the addition of certain intermediates in the Krebs
tricarboxylic acid cycle on the metabolism of acetate and pyruvate by
diaphragm muscle from normal and diabetic rats were also studied.

BIBLIOGRAPHY
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