Acetoacetate and Glucose Uptake by Diaphragm and Skeletal Muscle from Control and Diabetic Rats*†

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Many workers have demonstrated that extrahepatic tissues utilize ketone bodies in the absence of insulin (1). However, it has been postulated that a decrease in peripheral utilization of ketone bodies is a contributory factor in diabetic ketosis (2). No careful comparison of peripheral utilization by muscle from diabetic and nondiabetic preparations has been reported using modern techniques. The present paper reports an investigation of the uptake of acetoacetate and glucose by diaphragm muscle from a series of control and diabetic rats. The diaphragm is a specialized muscle in constant motion and seems, in respect to oxidative metabolism (3-5), to occupy an intermediate position between constantly active heart muscle and intermittently active skeletal muscle. Accordingly, the uptake of acetoacetate and glucose by skeletal muscle fibers, obtained by modifying Shorr's technique (6), has also been studied. In these experiments both the diaphragm and fibers from diabetic rats took up less acetoacetate and glucose than the muscles from the controls.

EXPERIMENTAL

Female Sprague-Dawley rats weighing 180 to 220 gm. were used throughout these experiments. The rats were made diabetic by the subcutaneous injection of 7 to 8 mg. per 100 gm. of recrystallized alloxan monohydrate (10 per cent solution). No rats were used less than 4 weeks after alloxan administration because it has been shown (7) that some time is required for a stabilized diabetic condition to be established. The 20-hour fasting blood sugar levels of the diabetic animals were 270 mg. per 100 ml. or more. Rats for muscle preparations were given light Nembutal anesthesia (2.5 mg. per 100 gm.) and decapitated. The light anesthesia minimized muscle spasms and resultant changes in muscle components when the animals were decapitated. To obtain the muscle fiber groups from a rat, the animal was placed on his back, the hind legs skinned, the paws gently extended and the adductor muscles exposed. Groups of muscle fibers approximately 30 mm. long, weighing an average of 25 mg., were dissected free. It was possible to remove the diaphragm and obtain 2 gm. of skeletal muscle fibers in about 25 minutes. During dissection the diaphragm or muscle fibers, already removed from the animal, were soaked at room temperature in modified Krebs phosphate buffered medium (Ca++ 0.0006 M and Mg++ 0.0006 M). If uptakes were to be measured, the appropriate substrate was added to the soaking medium (150 mg. per cent of glucose and/or 3 to 6 mmoles per l. of acetoacetate). The uptakes were determined from the differences between the concentrations in the medium before and after incubation. Acetoacetate was prepared from ethyl acetocetoate by the method of Krebs and Eggleston (8) and a blank flask containing acetocetic acid plus medium was run with each experiment to eliminate error from spontaneous decomposition. Hemidiaphragms or 200 mg. aliquots of skeletal muscle fibers were blotted, placed in Warburg flasks in 3 ml. of medium and weighed. A similar aliquot of muscle was placed in an oven at 95° for dry weight determination. Potassium hydroxide was placed in the center well, the flasks gassed with 100 per cent oxygen and equilibrated for 15 minutes at 37° with a shaking rate of 70 cycles per minute.

Glucose was determined by the method of Somogyi (9) and ketone bodies by the methods of Lester and Greenberg (10) and Bessman and Anderson (11). Nitrogen was determined by digestion and vacuum distillation (12). The tissues were digested in KOH and a modification of Roe's anthrone method (13, 14) was used to estimate glycogen levels.

Results and Discussion

The average uptake of acetoacetate (as measured by the Lester and Greenberg (10) method for total ketone bodies) at a substrate level of 3 mmoles per l., by diaphragms from fasted rats, was 1.19 f 0.09 (12) mmoles per l. of acetoacetate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control</th>
<th>Alloxan diabetic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-hour fast: 3 mmoles per l. of acetoacetate</td>
<td>0.94 ± 0.05 (10)†</td>
<td>0.51 ± 0.12 (7)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>20-hour fast: 6 mmoles per l. of acetoacetate</td>
<td>1.19 ± 0.09 (12)</td>
<td>0.56 ± 0.13 (6)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fed: 3 mmoles per l. of acetoacetate</td>
<td>0.90 ± 0.02 (20)</td>
<td>0.72 ± 0.05 (14)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* Supported in part by research grant number A-213C4 and A-1157C from the National Institutes of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service.
† Presented in part at the Federation of the American Societies for Experimental Biology, March 1957 and 1958.

* Method of Lester and Greenberg (10).
† Standard error.
‡ Number of hemidiaphragms.
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Effect of fumarate (4 mmoles per 1.) on acetoacetate uptake (3 mmoles per 1.) by hemidiaphragms

<table>
<thead>
<tr>
<th>Series</th>
<th>No. of Rats</th>
<th>Change in acetoacetate uptake with fumarate</th>
<th>p for difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasted (20 hours)</td>
<td>5</td>
<td>+0.12 ± 0.03*</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Alloxan diabetic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasted (20 hours)</td>
<td>5</td>
<td>-0.06 ± 0.05</td>
<td>&gt;0.10</td>
</tr>
<tr>
<td>Fed</td>
<td>5</td>
<td>-0.03 ± 0.05</td>
<td>&gt;0.10</td>
</tr>
</tbody>
</table>

* Standard error.

Comparison of O₂ uptake (microliters per hour per mg. of wet weight) by hemidiaphragms from control and alloxan diabetic rats

<table>
<thead>
<tr>
<th>Time</th>
<th>Controls</th>
<th>Alloxan diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 minutes</td>
<td>1.13 ± 0.04* (22)†</td>
<td>1.00 ± 0.03 (16)</td>
</tr>
<tr>
<td>2nd hour</td>
<td>1.07 ± 0.03 (12)</td>
<td></td>
</tr>
<tr>
<td>+AA†</td>
<td>1.09 ± 0.05 (5)</td>
<td></td>
</tr>
<tr>
<td>+AA + F§</td>
<td>1.01 ± 0.04 (9)</td>
<td></td>
</tr>
</tbody>
</table>

* Standard error.
† Number of hemidiaphragms.
§ 3 mmoles per 1. of acetoacetic acid.
§ 4 mmoles per 1. of fumaric acid.

O₂ consumption by muscle fibers from fed rats

<table>
<thead>
<tr>
<th>Series</th>
<th>KP*</th>
<th>AA†</th>
<th>Glucose‡</th>
<th>Glucose plus insulin⁄‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diaphragm</td>
<td>1 hour</td>
<td>0.05 ± 0.04¶ (5)</td>
<td>1.10 ± 0.03 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 hours</td>
<td>1.00 ± 0.05 (5)</td>
<td>1.08 ± 0.03 (5)</td>
<td></td>
</tr>
<tr>
<td>Fibers</td>
<td>1 hour</td>
<td>0.72 ± 0.06 (4)</td>
<td>0.68 ± 0.03 (7)</td>
<td>0.74 ± 0.03 (8)</td>
</tr>
<tr>
<td></td>
<td>2 hours</td>
<td>0.52 ± 0.05 (5)</td>
<td>0.53 ± 0.02 (8)</td>
<td>0.60 ± 0.03 (6)</td>
</tr>
<tr>
<td>Alloxan diabetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diaphragm</td>
<td>1 hour</td>
<td>1.12 ± 0.08 (6)</td>
<td>1.00 ± 0.02 (6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 hours</td>
<td>1.00 ± 0.07 (6)</td>
<td>1.04 ± 0.03 (6)</td>
<td></td>
</tr>
<tr>
<td>Fibers</td>
<td>1 hour</td>
<td>0.59 ± 0.08 (5)</td>
<td>0.62 ± 0.03 (5)</td>
<td>0.60 ± 0.03 (5)</td>
</tr>
<tr>
<td></td>
<td>2 hours</td>
<td>0.47 ± 0.04 (5)</td>
<td>0.52 ± 0.02 (6)</td>
<td>0.48 ± 0.04 (5)</td>
</tr>
</tbody>
</table>

* Krebs phosphate medium.
† Acetoacetate, 4 mmoles per 1.
‡ Glucose, 150 mg. per 100 ml.
§ Glucose, 150 mg. per 100 ml. plus 1.4 units per ml. of insulin.
¶ Standard error.
‖ Number of animals.
the addition of glucose and insulin raised the acetoacetate uptake to a value 3 times the standard deviation or more. Further work, using C-labeled acetoacetate, is in progress to determine which pathways of acetoacetate metabolism in muscle are changed in the diabetic.

The uptake by the diaphragm was lower when measured as total ketone bodies instead of as acetoacetic acid, both in the control and diabetic series (Table V). A possible explanation could be that the diaphragm is converting 0.5 to 0.7 mg per gm. per hour of acetoacetic acid to beta-hydroxybutyric acid. There was probably some uptake of beta-hydroxybutyrate, as well as acetoacetate. Homodiaphragmas from 6 fed control rats showed an uptake of 0.56 mg per gm. per hour of total ketone bodies at substrate levels of 0.50 mmoles per l. of dl-beta-hydroxybutyrate (analyses by the method of Lester and Greenberg (10)).

Unlike the results in diaphragm, there was no difference in the uptake of acetoacetate acid and total ketone bodies by the adductor fibers. As compared to muscle from control rats, both diaphragm and fiber preparations from diabetic rats had a decreased uptake in terms of total ketone bodies. That the enzyme systems involved were not saturated with ketone bodies is indicated by the fact that the addition of glucose and insulin invariably increased ketone uptake in paired experiments. However, the number of animals involved in each series was not sufficient to warrant a statistical analysis.

To determine whether or not the difference between the control and diabetic series could be explained by the passage of ketone bodies from the muscle into the medium, ketone body concentrations were measured after incubation of muscle in medium to which no substrate had been added. As can be seen in Table VI, the leakage of ketone bodies out of muscle was insignificant.

These results on acetoacetate do not agree with those of Foster and Villee (18) on acetate. These workers demonstrated an increased acetate uptake by diaphragms from alloxan diabetic rats, although the amount of acetate oxidized to CO2 was decreased. However, acetate is not a physiological substrate for rat muscle, whereas acetoacetate is. Furthermore, acetoacetate acid is acylated by a thiol kinase which is specific for beta-keto acids and different from the thiol kinase that acylates acetate (19, 20). Therefore, it is not too surprising that acetate and acetoacetate uptakes are different.

There was no difference in the average dry weights (control 16.2 ± 0.3 per cent; diabetic 16.5 ± 1 per cent) or per cent of nitrogen (control 2.19 ± 0.09; diabetic 2.27 ± 0.13) for fibers from the two series.

Following incubation, there was no difference in the glycogen levels of fibers or diaphragm from control and diabetic rats without substrate (Table VII). The glycogen level was higher in the fibers than in the diaphragm. The addition of acetoacetic acid had no effect on glycogen concentration, whereas the addition of glucose raised the glycogen in both fibers and diaphragm.

When a comparison was made of glucose uptake by diaphragm and adductor fibers from the same fasted rats, the uptakes were similar (Table VIII), no adjustment being made for relative dry weights or nitrogen concentrations. The addition of insulin almost doubled the glucose uptake of the fibers. As expected, the glucose uptake by fibers from diabetic animals was lower than from control rats (Table IX). The facts that the fibers from alloxan diabetic rats showed the same metabolic defect as has been demonstrated in diaphragm and also showed increased glucose uptake in the presence of insulin, indicate that the fiber preparation is suitable for metabolic studies.

The circumstance that glucose uptake was similar in diaphragm and adductor fibers, whereas acetoacetic acid uptake was greater in the diaphragm than in the adductor fibers, could be explained in several ways. Acetoacetic acid thiol kinase might exist in larger quantities in diaphragm muscle. There is known to be a greater cyclophorase activity and mitochondrial content...
Acetoacetate and P-hydroxybutyrate (milligrams per gm. per hour of
Diaphragm
Adductor fibers
Glycogen levels (grams per 100 gm.) in muscle
Fibers
Diaphragm
Fibers

Glucose uptake (milligrams per gm. per hour) that escapes from diaphragm and adductor muscle
fibers when the muscle is incubated in Krebs phosphate medium for 1- or 2-hour periods

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Alloxan diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diaphragm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>0.01 ± 0.001* (5)</td>
<td>0.03 ± 0.005 (6)</td>
</tr>
<tr>
<td>β-hydroxybuty-</td>
<td>0.000 ± 0.002 (5)</td>
<td>0.07 ± 0.02 (6)</td>
</tr>
<tr>
<td>rate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average for 2-hour period</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>3.3 ± 0.33 (4)</td>
<td></td>
</tr>
<tr>
<td>β-hydroxybuty-</td>
<td>1.2 ± 0.40 (4)</td>
<td></td>
</tr>
<tr>
<td>rate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adductor fibers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>0.02 ± 0.001 (4)</td>
<td>0.03 ± 0.002 (6)</td>
</tr>
<tr>
<td>β-hydroxybuty-</td>
<td>0.005 ± 0.003 (4)</td>
<td>0.02 ± 0.005 (6)</td>
</tr>
<tr>
<td>rate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Standard error.
† Number of rats.

Table VIII
Glucose uptake (milligrams per gram per hour of wet weight) by
diaphragm and adductor muscle fibers of fasted female rats
over a 2-hour period
Krebs phosphate buffered medium with 150 mg. per 100 ml. of
glucose, pH 7.4 to 7.5, was used.

<table>
<thead>
<tr>
<th></th>
<th>No. of rats</th>
<th>Glucose uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diaphragm</td>
<td>6</td>
<td>2.49 ± 0.17*</td>
</tr>
<tr>
<td>Fibers</td>
<td>14</td>
<td>2.01 ± 0.12</td>
</tr>
</tbody>
</table>

* Standard error.

Table IX
Glucose uptake (milligrams per gm. per hour) by adductor muscle
fibers from fed female rats in Krebs phosphate buffer medium
plus 180 mg. per 100 ml. of glucose (pH 7.4 to 7.5)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Alloxan diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>First hour</td>
<td>1.50, 1.50, 1.27</td>
<td>0.88 ± 0.14* (5)</td>
</tr>
<tr>
<td>Average of 2-hour period</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No insulin</td>
<td>1.22± ± 0.06 (9)</td>
<td>0.65 ± 0.09 (6)</td>
</tr>
<tr>
<td>Plus insulin†</td>
<td>2.43± ± 0.17 (7)</td>
<td>0.29, 0.60, 2.13§</td>
</tr>
</tbody>
</table>

* Standard error.
† p for control with and without insulin < 0.005.
‡ 1.4 units per ml.
§ Each value represents at least a 3-fold increase in utilization as compared to the utilization without insulin.

Table X
Creatine phosphate levels (milligrams per 100 gm. P) in striated
muscle of fed female rats

<table>
<thead>
<tr>
<th></th>
<th>Heart</th>
<th>Diaphragm</th>
<th>Skeletal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13.3 ± 0.07* (17)†</td>
<td>32.2 ± 1.2 (4)</td>
<td>75.1 ± 0.77 (15)</td>
</tr>
</tbody>
</table>

* Standard error.
† Number of rats.

(3-5) in diaphragm than in skeletal muscle. According to
Perry (4), the relative amounts of creatine phosphate reserves
and glycolytic activity are lower in constantly active muscle than
in intermittently active muscle. The relative values found in
our laboratory for creatine phosphate places diaphragm muscle
between heart and gastrocnemius (Table X). Therefore, there
seems to be a lower ratio of glycolytic enzyme activity to tri-
carboxylic acid cycle enzyme activity in diaphragm than in
skeletal muscle. If much of the acetoacetic acid that disap-
ppeared were being oxidized by way of the tricarboxylic acid cycle,
one might expect more acetoacetic acid to be utilized by the
diaphragm than the adductor fibers. Furthermore, the rat diaphragm is covered on both sides by complex membranes, the
peritoneum and the pleura, which might differ in permeability
and metabolism from the muscle cells. This mesothelial cover-
ing seems to be characterized by numerous microvilli (21, 22),
the purpose of which is as yet speculative. However, whether
their function is secretory, absorptive, or merely to increase cell
surface, it seems quite possible that they may have permeability
characteristics of their own, different from those of the cell
membrane of the underlying muscle. Below the microvilli lies
a layer of ovoid mesothelial cells, then a double basal membrane,
layer of finely fibrillar material and finally, the organized
collagenous tissue. The muscle fiber groups have no similar
nonmuscular coverings. However, it is possible that the muscle
fiber preparation has a predominant number of cut fibers in
which permeability is no longer a limiting factor as far as glucose
metabolism is concerned (23). If this were true, then the higher
glucose uptake by the fiber in the presence of insulin could not be
explained in terms of an increase in permeability.

Since these data suggest that this fiber preparation may be
different from the diaphragm in the relative importance of the
metabolic pathways concerned with glucose and ketone body...
metabolism, we intend to investigate the effect of hormones other than insulin on fiber metabolism. Some of the discrepancies between results in vivo and in vitro may be due to the use of diaphragm muscle as typical of skeletal muscle.

SUMMARY

1. A technique of preparing viable adductor muscle fiber groups from the rat for use in comparison with the diaphragm in a Warburg apparatus has been developed.

2. Both diaphragm and adductor fibers from alloxan diabetic rats take up less acetoacetic acid than diaphragm and fibers from control rats.

3. The adductor muscle fibers from control rats take up approximately the same amount of glucose as the diaphragm, but one quarter as much acetoacetic acid as the diaphragm.

4. The adductor muscle fibers from diabetic rats take up less glucose than fibers from control rats. The addition of insulin increases glucose uptake by adductor muscle fibers.

REFERENCES

Acetoacetate and Glucose Uptake by Diaphragm and Skeletal Muscle from Control and Diabetic Rats
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