Acetoacetic Acid Metabolism by Skeletal Muscle Fibers from Control and Diabetic Rats*

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Previously we demonstrated that both diaphragm and adductor fibers from alloxan diabetic rats take up less acetoacetic acid than diaphragm and fibers from control rats (1). To date it has not been possible to show an influence of insulin on ketone utilization by muscle (2).

The present paper extends the previous work on acetoacetic acid uptake to pancreatectomized rats, which show a decrease in uptake similar to that found in alloxan diabetes, and also reports on the effect of glucose and of glucose and insulin on the acetoacetic acid uptake of skeletal muscle fibers from control and pancreatectomized rats. In agreement with present concepts of intermediary metabolism, insulin in the presence of glucose increased acetoacetic acid uptake by skeletal muscle fibers from either control or pancreatectomized rats. Furthermore, the amount of C14O2 formed from acetoacetate-C14 was decreased in fibers from diabetic as compared to control rats.

**EXPERIMENTAL PROCEDURE**

Methods

Female Sprague-Dawley rats, weighing 180 to 220 g and fed on Purina chow, were used in the experiment on acetoacetic acid uptake. Food was taken from the rats at 9:00 a.m. At 4:00 p.m. each rat was given 6 ml of evaporated milk by stomach tube and then fasted overnight. In view of the general toxic effects of alloxan, partially pancreatectomized rats were used in most of the experiments. The series of rats made diabetic by ingestion of alloxan showed blood sugar levels over 200 mg/l00 ml by the seventh postoperative day. The fasted rats were fed ground Purina chow containing 2 g of pancreatin per 100 g of chow. The animals used in the C14 experiments were male Sprague-Dawley rats weighing about 200 g. Rats to be made diabetic were fasted 48 hours and given 6 mg of alloxan per 100 g of body weight intramuscularly. All the rats had 24-hour fasting blood sugars of at least 180 mg/l00 ml 3 weeks after alloxan. These animals were used 3 hours after their last meal.

Adductor fiber groups were prepared (1) and incubated in either Krebs phosphate or Krebs bicarbonate buffered medium (pH 7.4), 0.6 mM with respect to both Ca++ and Mg++. Representative aliquots of muscle fibers from each experiment were dried to constant weight at 95°. The average percentage dry weights of the skeletal muscle fibers from control and pancreatectomized rats were the same, 17.9 and 17.8, respectively. The total nitrogen in grams per 100 g on the basis of dry weight was also the same for the two series; see Tables I and II.

The method of Barker and Summerson was used for lactate analyses (3), the method of Somogyi (4) for glucose, and the method of Bessman and Anderson (5) for ketone bodies (expressed as acetoacetic acid). Acetoacetic acid was prepared from ethyl acetoacetate by the procedures of Krebs and Eggleston (6). A flask containing acetoacetate plus medium was run as a blank with each experiment to correct for the error caused by spontaneous decarboxylation.

C14O2 activities were determined on the KOH solution from the center well of the Warburg flasks as infinitely thick BaCO3 plates. Conversion of acetoacetate to CO2 was calculated as the per cent of the added acetoacetate-C14 activity in each flask appearing as C14O2 after 1-hour incubation.

**RESULTS AND DISCUSSION**

Four mM per liter of acetoacetate (equivalent to 40 mg/100 ml of blood) represents a moderate degree of ketonemia for rats. Harrison and Long (7) reported blood levels of 12 to 36 mg/100 ml in fasted female rats, and 27 to 285 mg/100 ml in fasted, phlorizinized, male rats, whereas Scow et al. (8) found levels of 250 mg/100 ml in fasted, totally pancreatectomized rats.

The acetoacetic acid uptake (0.51 ± 0.03 mg per gram per hour) at 4 mM per liter substrate level by fibers from fasted control rats (Table I) was larger than the uptake (0.37 ± 0.04 mg per gram per hour) previously reported for fibers from fed control rats at this substrate level (1). The acetoacetic acid uptake also seemed larger in bicarbonate than in phosphate buffered medium (Tables I and II). However, these differences in uptake may not be real since the series were not run simultaneously.

The acetoacetate uptake by fibers from control rats incubated in bicarbonate buffered medium decreased only 16% between the first and second hours (± 0.06 ± 0.02 mg per gram, n = 5, 1 mM per liter of acetoacetate). This per cent decrease in uptake was of the same magnitude as that previously reported for fibers from either control or diabetic rats incubated in phosphate buffered medium (4 mM per liter of acetoacetate) (1).

In support of the postulate that an insulin-deficient state causes a decrease in the peripheral utilization of ketone bodies,
<table>
<thead>
<tr>
<th>Substrate ControP</th>
<th>Pancreatectomized</th>
<th>p — Control versus diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>0.51 ± 0.03^6 (6)^c</td>
<td>0.19 ± 0.04 (6)^c</td>
</tr>
<tr>
<td>Plus glucose**</td>
<td>0.50 ± 0.04 (6)</td>
<td>0.22 ± 0.04 (6)</td>
</tr>
<tr>
<td>p^ for glucose addition</td>
<td>&gt;.10</td>
<td>&gt;.10</td>
</tr>
<tr>
<td>Plus glucose and insulin^f</td>
<td>0.65 ± 0.04 (6)</td>
<td>0.44 ± 0.04 (9)</td>
</tr>
<tr>
<td>p^ for insulin addition</td>
<td>&lt;.02</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Plus glucose, insulin, and glucagon^g</td>
<td>0.70 ± 0.03 (6)</td>
<td>0.53 ± 0.06 (9)</td>
</tr>
<tr>
<td>p^ for glucagon addition</td>
<td>&gt;.10</td>
<td>&gt;.10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate ControP</th>
<th>Pancreatectomized</th>
<th>p — Control versus diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>0.49 ± 0.02 (6)^c</td>
<td>0.18 ± 0.07 (5)</td>
</tr>
<tr>
<td>Plus glucose**</td>
<td>0.50 ± 0.05 (6)</td>
<td>0.22 ± 0.05 (5)</td>
</tr>
<tr>
<td>p^ for glucose addition</td>
<td>&gt;.10</td>
<td>&gt;.10</td>
</tr>
<tr>
<td>Plus glucose and insulin^f</td>
<td>0.61 ± 0.05 (6)</td>
<td>0.44 ± 0.07 (9)</td>
</tr>
<tr>
<td>p^ for insulin addition</td>
<td>&lt;.02</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Plus glucose, insulin, and glucagon^g</td>
<td>0.68 ± 0.08 (4)</td>
<td>0.46 ± 0.07 (9)</td>
</tr>
</tbody>
</table>

* Total nitrogen, grams per 100 g, on basis of dry weight 13.44 ± 0.17 for controls and 13.54 ± 0.14 for pancreatectomized.
^ Standard error.
^ Number of experiments.
** 150 mg of glucose per 100 ml.
^ p value calculated on basis of paired observations.
^f 0.47 units per ml of crystalline insulin, glucagon-free (Lilly).
^g 20 μg/ml of crystalline insulin-free glucagon (Lilly).

The data (Table I) demonstrate that fibers from pancreatectomized rats incubated in Krebs phosphate medium with or without glucose took up less than half as much acetacetate as did fibers from control rats. Previous work with fibers from alloxan diabetic rats at the same substrate level showed a similar decrease in uptake (1).

As shown in Table II even with substrate levels as low as 1 mM per liter of acetacetate, there was a decrease of over 50% in uptake by fibers from diabetic rats. Furthermore, it was possible to demonstrate low acetacetate uptakes by fibers from diabetic rats in both phosphate and bicarbonate buffered media (Tables I and II). Since muscle comprises 40% of the body weight and liver only about 5%, the 50 to 60% reduction in uptake of acetacetate by muscle from diabetic rats would have an effect on ketonemia comparable to the 4.8-fold increase in production of ketone bodies reported by Stadie et al. (9) for liver slices from diabetic cats.

The addition of 150 mg/100 ml of glucose to the medium had no effect on acetacetate uptake, but the addition of glucose plus insulin increased uptake in both the control and diabetic series (Table I). Even in the presence of an excess of exogenous

^ Lilly crystalline insulin free of glucagon, courtesy of Dr. C. W. Pettinga.
insulin, the acetoacetate uptake by fibers from diabetics was less than that of fibers from controls. Previously we found that insulin raised the glucose uptake of fibers from depancreatized rats so that the final uptakes in the presence of insulin were similar for control and diabetic muscle (10). The addition of glucagon to glucose- and insulin-treated fibers did not change the uptake or $\beta$-hydroxybutyrate that escaped from the fibers in Krebs bicarbonate buffered medium without substrate was insignificant. In preliminary experiments, the conversion of acetoacetate-4-C14 to C14O2 was investigated by fibers and diaphragms from diabetic and diabetic muscle. The experiments were done in Krebs bicarbonate buffered medium without substrate was insignificant and was not responsible for the different uptakes of the control and diabetic series. In a previous paper (1) the uptake of acetoacetate by diaphragm muscle was lower when determined as total ketone bodies than when determined as acetoacetate. No difference was observed in either the control or diabetic series when uptake of fibers, measured as total ketone bodies, was compared to uptake measured as acetoacetate (Table I).

To determine whether or not the decrease in acetoacetate uptake was accompanied by a decrease in acetoacetate oxidation, the conversion of acetoacetate-3-C14 to C14O2 was investigated in control and diabetic rat muscle. The experiments were done at a substrate level of 0.15 mM per liter of acetoacetate (1.5 mg/100 ml) which is approximately the concentration of blood ketones of normal nonketotic rats. When fibers and diaphragms from diabetic rats are compared with the respective control series (Table III), decreases are observed in the conversion of acetoacetate-3-C14 to C14O2 in the diabetic animals. These results constitute evidence that less acetoacetate was being metabolized by the tricarboxylic acid cycle in the diabetic preparation. However, a larger acetate pool in the diabetic series would dilute the C14 label and also cause a decrease in CO2 production. In preliminary experiments, the conversion of a tracer dose of acetate-1-C14 (0.42 mc per liter) to C14O2 by fibers and diaphragm from diabetic as compared to control animals was similar (24 ± 1 and 26 ± 2% of the initial activity in the flask appearing as C14O2 for the control series of 10 fibers and 10 diaphragms, respectively, and 27 ± 2 and 25 ± 1% of the original activity for the diabetic series of 6 fibers and 6 diaphragms), indicating that probably the acetate pool was not increased in these diabetic muscle preparations. This finding is in keeping with the observations of Elwood et al. (12) who found that even liver slices of diabetic rats showed no apparent increase in the size of the "acetate pool."

This laboratory has demonstrated previously that the energy reserve is low in muscle tissue from diabetic rats (13, 14). Since energy is required for the activation of acetoacetic acid via the $\beta$-keto thio kinase or thiophorase enzymes, it would be reasonable to expect a decrease in acetoacetic acid activation by diabetic muscle. Evidence is available (15) that insulin can stimulate the incorporation of C14-labeled acetate into muscle protein by a mechanism distinct from an effect on transport. However, at present there is no direct evidence on the possible site or sites of insulin activity that is not metabolic in nature.

### Table III

<table>
<thead>
<tr>
<th>Muscle</th>
<th>No. of rats</th>
<th>Control</th>
<th>Alloxan diabetic</th>
<th>$p$ = Control versus diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diaphragm</td>
<td>8</td>
<td>18.6 ± 1.1*</td>
<td>10.2 ± 1.4</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Adductor fibers</td>
<td>8</td>
<td>14.6 ± 1.2</td>
<td>8.9 ± 0.9</td>
<td>&lt;.01</td>
</tr>
</tbody>
</table>

* Standard error.

### Table IV

<table>
<thead>
<tr>
<th>Substrate</th>
<th>N</th>
<th>Controls</th>
<th>N</th>
<th>Depancreatized</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6</td>
<td>0.32 ± 0.081</td>
<td>7</td>
<td>0.42 ± 0.04</td>
</tr>
<tr>
<td>Plus 4 mM/liter of acetoacetate acid</td>
<td>6</td>
<td>0.57 ± 0.14</td>
<td>7</td>
<td>0.60 ± 0.07</td>
</tr>
<tr>
<td>$p$ for addition of acetoacetate acid</td>
<td>&lt;.05</td>
<td>&lt;.005</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Lactate production is defined as the lactate in the medium after 135 minutes of incubation minus that in the medium after 15 minutes of incubation.

† Standard error.

### Table V

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control (6)*</th>
<th>Depancreatized (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hour</td>
<td>2 hours</td>
</tr>
<tr>
<td>No additions</td>
<td>0.73 ± 0.01</td>
<td>0.54 ± 0.02</td>
</tr>
<tr>
<td>Plus acetoacetate-4 mM/liter</td>
<td>0.72 ± 0.06</td>
<td>0.54 ± 0.05</td>
</tr>
</tbody>
</table>

* Number of rats.

† Standard error.

### Table VI

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control (6)*</th>
<th>Pancreatotomized (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 mM/liter of acetoacetate</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>* 150 mg of glucose per 100 ml</td>
<td>0.35 ± 0.05</td>
<td>0.20 ± 0.06</td>
</tr>
<tr>
<td>p for Acetoacetate versus glucose</td>
<td>&lt;.05</td>
<td>&lt;.01</td>
</tr>
<tr>
<td></td>
<td>4 mM/liter of acetoacetate and 150 mg of glucose per 100 ml</td>
<td>0.35 ± 0.05</td>
</tr>
<tr>
<td>p for Glucose versus glucose plus acetoacetate</td>
<td>&gt;.10</td>
<td>&gt;.10</td>
</tr>
</tbody>
</table>

* Number of rats.

† Statistical analysis on basis of paired observations.
action of insulin on acetoacetate metabolism. The data presented in this paper do not differentiate between direct and indirect effects of insulin on acetoacetate acid uptake. However, the fact that insulin in the absence of substrate (glucose) has no effect on the acetoacetate acid uptake (1) points to an indirect rather than a direct effect of insulin.

The increased production of lactate after the addition of acetoacetate to the medium (Table IV) is in agreement with data reported by Ottaway and Sarkar (16) for perfused rat heart and rat diaphragm in vitro. The concentration of acetoacetate used in the experiments reported in this paper represents a moderate degree of ketosis for the rat (7, 8) and is considerably lower than the concentration (0.01 M) used by Ottaway and Sarkar (16). It is interesting to note that the effect of acetoacetate on lactate production also was present with fibers from pancreatectomized rats (Table IV) although acetoacetic acid uptake by these fibers was greatly reduced. Further experiments with the use of acetoacetate-C14 and lactate-C14 are planned. The addition of acetoacetate to the medium had no effect on oxygen consumption (Table V).

In agreement with previous work in this laboratory (10) no difference was observed in the glycogen levels of the control and diabetic series (Table VI) at the end of the incubation period. Glucose (150 mg/100 ml) maintained the average glycogen levels at higher values than did 4 mM per liter of acetoacetate (40 mg/100 ml). No additional effect on glycogen values was observed when glucose and acetoacetate were used together as substrates. In view of the observations by Villar-Palasi and Larner (17) that UDPG-glycogen transglucosylase is insulin-sensitive, no explanation is offered for the fact that although the fibers from diabetic rats utilized less glucose (10) and less acetoacetate, these diabetic fibers were equally capable of maintaining glycogen levels as compared to the control series.

SUMMARY

1. Adductor muscle fibers from depancreatized rats took up less acetoacetate than fibers from control animals. This was true in phosphate and bicarbonate buffered media. Since muscle comprises about 40% of the body weight and liver only 5%, the reduction in uptake of acetoacetate by diabetic muscle seems to have an effect on ketonemia comparable to that caused by the increase in production of ketone bodies reported for diabetic liver slices.

2. The addition of glucose had no effect on the uptake of acetoacetate, but the addition of glucose and insulin caused an increase in uptake by fibers from both control and diabetic rats.

3. The per cent of original acetoacetate-3-C14 activity in each flask appearing in C14O2 was decreased with fibers and diaphragm from alloxan diabetic rats as compared to the control series.

4. The addition of acetoacetate to the medium caused an increase in lactate production by muscle fibers from both control and diabetic preparations.

REFERENCES