Previous our laboratory demonstrated that voluntary muscle fiber groups and diaphragms from depancreatized and alloxan-diabetic rats take up less acetoacetic acid than fiber groups and diaphragms from control animals (1, 2). The addition of glucose and insulin causes an increase in acetoacetic acid uptake by muscle fiber groups from both control and depancreatized rats. These experiments were performed on muscle from both fed rats and rats that had been deprived of food for 24 hours. It was concluded that the decrease in ketone body utilization by muscle from insulin-deficient animals is a contributing factor in diabetic ketosis. These conclusions have been further substantiated by Scow and Chernick (3), who reported that the utilization of both acetoacetic and \( \beta \)-hydroxybutyric acid is reduced in insulin-deficient rats. Shreeve (4) followed the conversion of acetate-\( ^{14} \)C to ketone bodies in diabetic patients and also presented evidence in support of improved utilization of \( \beta \)-hydroxybutyric acid. It has been demonstrated, by measuring patterns of enzyme activity, that the pathways by which the major portion of the ketone bodies are produced in liver differ in the ketosis associated with deprivation of food and that found in diabetic rats (5). The present paper reports an investigation of the effect of prolonged deprivation of food on the uptake of acetoacetic acid by diaphragms and skeletal muscle fiber groups of rats and the effect of insulin\(^*\) on the uptake of acetoacetate-\( ^{3} \)\(^{14} \)C\(^\#\) and its conversion to \( ^{14} \)CO\(_2\) by these muscles.

**EXPERIMENTAL PROCEDURE**

Female Sprague-Dawley rats, weighing from 180 to 200 g and fed Purina chow, were used. The animals were decapitated under Nembutal anesthesia (2.5 mg per 100 g). The light anesthesia minimized muscle spasms and resultant changes in muscle components following decapitation. The diaphragm was quickly excised. To obtain muscle fiber groups, the rat was placed on its back, the hind limbs were skinned, the hind paws were gently extended, and the adductor muscles were exposed. Groups of muscle fibers approximately 30 mm long, weighing an average of 20 to 30 mg, were dissected free. During dissection the diaphragm or fiber groups already removed from the animal were soaked in oxygenated, modified Krebs' glycylglycine- or bicarbonate-buffed medium (pH 7.4), 0.6 mM with respect to Ca\(^{++}\) and Mg\(^{++}\). This modified buffer has been in use in our laboratory for 15 years and proved satisfactory. Approximately 200-mg aliquots of mixed red and white muscle were placed in Warburg flasks containing 3 ml of medium. The diaphragm was divided into thirds, and each third was placed in a flask. The ventral third was used for the equilibration values and the two dorsal portions served as paired samples. The aliquots containing bicarbonate-buffed medium were flushed with \( 95\% \) \( O_2\)-\( 5\% \) \( CO_2 \), and those containing glycylglycine-buffed medium were flushed with oxygen. The flasks were equilibrated for 15 minutes and then incubated for 2 hours. It is necessary to pair the aliquots of muscle carefully for color, since it has been demonstrated that there are marked differences in the relative importance of various metabolic pathways of metabolism between predominantly red and predominantly white muscle fiber groups (6). Representative aliquots of muscle were dried to constant weight at 95\(^\circ\)C, and the nitrogen content was determined by digestion and vacuum distillation (7).

Lactate was determined by the method of Barker and Summers (8) on a copper lime filtrate (9), and the method of Roe (10) was used for glycogen analyses. Glucose was determined with glucose oxidase (Glucostat kit, Worthington Biochemical Corporation) on a Ba(OH)\(_2\)-ZnSO\(_4\) filtrate of the medium (11). Acetoacetic acid and \( \beta \)-hydroxybutyric acid in the blood and in the medium were determined chemically with the salicylaldehyde-acetone reaction on a Ba(OH)\(_2\)-ZnSO\(_4\) filtrate by the method of Besman and Anderson (12). Acetoacetic acid was converted to acetone with sulfuric acid. Sodium dichromate plus sulfuric acid was used to convert both acetooacetate and \( \beta \)-hydroxybutyrate to acetone. Alkaline salicylaldehyde was added, and the optical density was determined at 500 m\( \mu\). The value obtained by treatment with acid dichromate minus the value following sulfuric acid treatment was taken to represent \( \beta \)-hydroxybutyrate. In agreement with the results of

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\(^{\#}\) Details of this determination were obtained in a personal communication.
The recovery of acetoacetate and \(\beta\)-hydroxybutyrate was quantitative. The uptake of acetoacetate was determined chemically and was also calculated from the \(^{14}\)C data. A blank flasks containing acetoacetic acid plus medium was run with each experiment to eliminate error from spontaneous decomposition.

The acetoacetic acid-\(^{14}\)C uptake calculated by subtracting the activity in the flask after incubation from the original activity in the flask was less than the values obtained by chemical analysis. Therefore, it was postulated that acetoacetic acid was converted to some substance or substances that were then released back into the medium. Since Utter (13) has demonstrated that there is a significant amount of malic enzyme in rat muscle, it was assumed that the \(^{14}\)C label from acetoacetate-\(^{14}\)C might reach lactate via the conversion of labeled malate from the citric acid cycle to labeled pyruvate and lactate. In order to correct for this \(^{14}\)C-labeled compound produced from acetoacetate-\(^{14}\)C, the 2,4-dinitrophenylhydrazone of acetoacetate-\(^{3}\)W formed in a Ba(OH)\(_2\)-ZnSO\(_4\) filtrate of the medium (11) was extracted into CHCl\(_3\) and the aqueous phase was assayed for activity. Standard acetoacetate-\(^{3}\)W was treated with 2,4-dinitrophenylhydrazine and extracted to measure the non-extractable background. Standard lactate-\(^{14}\)C samples were extracted into CHCl\(_3\) and quantitative recovery of lactate-\(^{14}\)C was demonstrated.

On the basis of the specific activity of the acetoacetic acid-\(^{14}\)C originally in the flask, if the \(^{14}\)C material in the aqueous phase was lactate it would account for 5 to 17\% of the lactate produced. If total ketone bodies in blood were 13.9 \(\pm\) 2.4 mg per 100 ml, \(\approx\) 73\% \(\approx\) \(\beta\)-hydroxybutyrate, which is in agreement with values reported in the literature (5). Ketone body excretion in the urine was not followed, since the kidney threshold varies widely, even for small changes in blood levels.

**TABLE I**

<table>
<thead>
<tr>
<th>Rats</th>
<th>Fiber groups</th>
<th>Diaphragm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetoacetic acid</td>
<td>Glucose uptake</td>
</tr>
<tr>
<td></td>
<td>Uptake mg/g/h</td>
<td>Reduction*</td>
</tr>
<tr>
<td>Fed</td>
<td>0.43 (\pm) 0.02 (9)</td>
<td>0.04 (\pm) 0.008 (7)</td>
</tr>
<tr>
<td>Fasted</td>
<td>0.43 (\pm) 0.02 (11)</td>
<td>0.02 (\pm) 0.005 (7)</td>
</tr>
<tr>
<td>1 day*</td>
<td>0.47 (\pm) 0.02 (9)</td>
<td>0.03 (\pm) 0.009 (5)</td>
</tr>
<tr>
<td>2 days*</td>
<td>0.43 (\pm) 0.02 (9)</td>
<td>0.03 (\pm) 0.009 (5)</td>
</tr>
<tr>
<td>5 days</td>
<td>0.43 (\pm) 0.02 (9)</td>
<td>0.03 (\pm) 0.009 (5)</td>
</tr>
</tbody>
</table>

* Conversion of acetoacetic acid to \(\beta\)-hydroxybutyric acid.
+ Wet weight.
\(\approx\) Dry weight.
* Values for fed versus fasted series were greater than 0.10 in all instances.
* Total ketone bodies in blood were 12.1 \(\pm\) 1.2 mg per 100 ml.
* Total ketone bodies in blood were 13.9 \(\pm\) 2.4 mg per 100 ml, 73\% \(\beta\)-hydroxybutyric acid.

**RESULTS AND DISCUSSION**

It is well known that the concentration of ketone bodies in blood rises during starvation and in pathological states such as diabetes mellitus. It has been demonstrated that a decrease in hepatic production, contributes to the ketonemia of the diabetic preparation (1-4). Table I shows that fasting of rats for periods up to 5 days does not change the acetoacetic acid utilization by excised diaphragm muscle or voluntary skeletal muscle fiber groups. The substrate level of acetoacetate used in these experiments with muscle from fasted rats (4 mM) was the same as that used in the experiments with muscle from diabetic rats (1, 2). This substrate concentration, equivalent to 40 mg per 100 ml, was chosen as representing a moderate degree of ketonemia for rats. Harrison and Long (16) reported blood levels of 12 to 36 mg per 100 ml in fasted female rats, and Scow, Chernick, and Maurer (17) found levels of 250 mg per 100 ml in fasted, totally pancreatectomized rats. Fasting also had no effect on the conversion of acetoacetate to \(\beta\)-hydroxybutyric acid or on the uptake of glucose by diaphragm or muscle fiber groups. The fasted rats were mildly ketotic after 1 or 2 days of deprivation of food, with total ketone body levels in the blood of 12.1 \(\pm\) 1.2 (standard error) mg per 100 ml and 13.9 \(\pm\) 2.4 mg per 100 ml. About 73\% of the total ketone bodies were \(\beta\)-hydroxybutyrate, which is in agreement with values reported in the literature (5). Ketone body excretion in the urine was not followed, since the kidney threshold varies widely, even for
a single rat, and a rise or fall in ketonuria frequently does not parallel changes in ketonemia (18). Sauer (19) studied ketosis in non-diabetic guinea pigs (fed and fasted) following the injection of 14C-labeled acetate and acetoacetate. In agreement with the results reported in this paper, he found no evidence of impaired ketone body utilization in the non-diabetic guinea pig. Therefore, in view of the previously reported decrease in acetoacetate utilization by diabetic muscle, it seems that the patterns of enzyme activity must be different for muscle as well as liver in the ketosis of fasting as compared to the ketosis present in insulin deficiency.

Withholding food from the rat does not change the acetoacetate uptake by excised muscle. Therefore, the following experiments on the effect of insulin on acetoacetic acid metabolism were performed on muscle taken from rats fasted for 20 hours. In agreement with previous work at the higher substrate level of 4 mM (2), the addition of insulin to the medium increased the uptake of acetoacetate-3-14C by the voluntary

### TABLE II

**Acetoacetic acid-3-14C uptake and conversion to CO2 by voluntary skeletal muscle fiber groups**

The muscle was incubated for 2 hours in glycylglycine-buffered Krebs medium, pH 7.4, plus 0.7 mM acetoacetate-3-14C (3.2 μC per flask). The rats were fasted for 18 hours. Values are means ± standard error of the mean in terms of wet weight. Numbers in parentheses denote the number of animals.

<table>
<thead>
<tr>
<th>Additions to fiber group</th>
<th>Acetoacetic acid-14C Uptake</th>
<th>Lactic acid production</th>
<th>14CO2 production</th>
<th>QO2 1 hour</th>
<th>2 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/g/hr</td>
<td>mg/g/hr</td>
<td>c.p.m./mg/hr</td>
<td>μl/mg/hr</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.21 ± 0.02 (9)</td>
<td>0.38 ± 0.04 (9)</td>
<td>81 ± 6 (9)</td>
<td>0.64 ± 0.06 (6)</td>
<td>0.67 ± 0.04 (6)</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.23 ± 0.01 (7)</td>
<td>0.78 ± 0.04 (7)</td>
<td>85 ± 8 (7)</td>
<td>0.61 ± 0.06 (9)</td>
<td>0.58 ± 0.03 (9)</td>
</tr>
<tr>
<td>p Value</td>
<td>&gt;0.10</td>
<td>&lt;0.01</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.25 ± 0.01 (9)</td>
<td>0.41 ± 0.03 (9)</td>
<td>93 ± 6 (9)</td>
<td>0.65 ± 0.02 (6)</td>
<td>0.62 ± 0.02 (6)</td>
</tr>
<tr>
<td>p Value</td>
<td>&lt;0.05</td>
<td>&gt;0.10</td>
<td>&lt;0.01</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
</tr>
<tr>
<td>Glucose and insulin</td>
<td>0.29 ± 0.02 (7)</td>
<td>0.06 ± 0.01 (4)</td>
<td>1.00 ± 0.07 (7)</td>
<td>1.01 ± 0.07 (8)</td>
<td>0.63 ± 0.02 (9)</td>
</tr>
<tr>
<td>p Value</td>
<td>&lt;0.02</td>
<td>&gt;0.10</td>
<td>&lt;0.01</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
</tr>
</tbody>
</table>

* Dry weight, 17.6 ± 1.2%; total nitrogen on the basis of dry weight, 13.5 ± 0.8 g per 100 g.
* Disappearance of 14C from the medium, corrected for the 14C produced from the acetoacetic acid-14C by the muscle and returned to the medium. The acetoacetic acid uptakes calculated from the 14C data are the same as those obtained by chemical analysis.
* Conversion of acetoacetic acid to β-hydroxybutyric acid.
* Lactate in the medium after 135 minutes of incubation minus that in the medium after 15 minutes of incubation.
* Crystalline insulin, glucagon-free (Lilly), 0.1 unit per ml.
* Increase with addition of insulin + 11.6 ± 2.3 (standard error) c.p.m. per mg per hour.
* Increase with addition of insulin + 15.9 ± 2.3 (standard error) c.p.m. per mg per hour.

### TABLE III

**Acetoacetic acid-3-14C uptake and conversion to CO2 by diaphragm muscle**

The muscle was incubated for 2 hours in glycylglycine-buffered Krebs medium, pH 7.4, plus glucose, 150 mg per ml, and 0.7 mM acetoacetate-3-14C (3.2 μC per flask). The rats were fasted for 18 hours. Values are means ± standard error of the mean and are in terms of wet weight. Numbers in parentheses denote number of animals.

<table>
<thead>
<tr>
<th>Additions to diaphragm</th>
<th>Acetoacetic acid-14C Uptake</th>
<th>Lactic acid production</th>
<th>14CO2 production</th>
<th>QO2 1 hour</th>
<th>2 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/g/hr</td>
<td>mg/g/hr</td>
<td>c.p.m./mg/hr</td>
<td>μl/mg/hr</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.43 ± 0.04 (8)</td>
<td>0.13 ± 0.01 (6)</td>
<td>0.92 ± 0.06 (6)</td>
<td>1.03 ± 0.05 (8)</td>
<td>1.05 ± 0.06 (8)</td>
</tr>
<tr>
<td>p Value</td>
<td>&gt;0.10</td>
<td>&lt;0.01</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
</tr>
<tr>
<td>Glucose and insulin</td>
<td>0.44 ± 0.04 (8)</td>
<td>0.14, 0.09, 0.13 (3)</td>
<td>1.40 ± 0.15 (6)</td>
<td>1.51 ± 0.21 (6)</td>
<td>0.98 ± 0.07 (8)</td>
</tr>
<tr>
<td>p Value</td>
<td>&gt;0.10</td>
<td>&lt;0.01</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
</tr>
</tbody>
</table>

* Dry weight, 20.7 ± 1.6%; total nitrogen on the basis of dry weight, 12.7 ± 0.4 g per 100 g.
* Disappearance of 14C from the medium corrected for the 14C produced from the acetoacetic acid-14C by the muscle and returned to the medium. The acetoacetic acid uptakes calculated from the 14C data are the same as those obtained by chemical analysis.
* Conversion of acetoacetic acid to β-hydroxybutyric acid.
* Lactate in the medium after 135 minutes of incubation minus that in the medium after 15 minutes of incubation.
* Crystalline insulin, glucagon-free (Lilly), 0.1 unit per ml.
* Increase with addition of insulin + 11.6 ± 2.3 (standard error) c.p.m. per mg per hour.
* Increase with addition of insulin + 15.9 ± 2.3 (standard error) c.p.m. per mg per hour.
muscle fiber groups in both the presence and absence of glucose (Table II). Similarly, the production of 14CO2 (counts per minute per mg of muscle, wet weight) from acetocetate-3-14C was increased by the addition of insulin both with and without glucose. The Q02 values are included in Tables II and III to indicate the viability of muscle fiber groups and diaphragm in glycylglycine-buffered medium. These Qo2 values are very similar to those previously reported for these muscle preparations incubated in Krebs phosphate buffered medium (1). In the absence of glucose in the medium, addition of insulin caused no change in production of lactate. When glucose was present in the medium, insulin caused an increase in lactate production in addition to increasing acetocetate uptake. Previously, it has been demonstrated that the percentage of original acetocetate-3-14C activity in the medium appearing as 14CO2 is decreased with muscle fiber groups from insulin-deficient as compared to control rats (2). This decrease in CO2 production probably reflected, at least in part, a decrease in the utilization of acetocetate acid. Uptake of the 14C-labeled acetocetate was determined in the present experiments, and the percentage of 14C uptake appearing in the CO2 fraction was compared in the presence and absence of insulin. With no glucose in the medium, the 14C data indicate that of the acetocetate-14C utilized, 68 ± 4% of the 14C label appeared in 14CO2 with no added insulin, and 73 ± 4% appeared in 14CO2 with added insulin. With glucose in the medium (150 mg per 100 ml), the respective values oxidized to 14CO2 were 70 ± 4 and 75 ± 5%. The p values calculated on the basis of paired samples are greater than 0.10 for the addition of insulin both with and without glucose in the medium.

No effect of insulin on acetocetate-3-14C uptake or 14CO2 production by diaphragm muscle was noted under the conditions of these experiments (Table III). The percentage of the acetocetate-14C uptake appearing in 14CO2 was again similar with and without insulin (65 ± 7 and 65 ± 3, respectively). Previous work in this laboratory (1) has shown a decrease in acetocetic acid uptake by diaphragm muscle from diabetic as compared to control rats (Krebs-phosphate-buffered medium, pH 7.4).

Addition of insulin to the medium increases uptake of acetocetate-3-14C by voluntary skeletal muscle fiber groups but not by diaphragm muscle. This increase in acetocetate-14C uptake can be demonstrated with and without glucose in the medium. The production of 14CO2 (counts per minute per mg of muscle, wet weight) from labeled acetocetate by voluntary muscle fiber groups, but not by diaphragm muscle, is also increased by the addition of insulin to the medium. However, under the present experimental conditions, insulin had no effect on the percentage of 14C label from the acetocetate-14C uptake appearing as 14CO2.

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Clarissa H. Beatty, Ruth D. Peterson, Rose Mary Bocek and Edward S. West


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