Studies on Experimental Diabetes

II. CARBON DIOXIDE FIXATION*

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Experimental diabetes has been characterized as a metabolic state involving both overproduction and underutilization of glucose. Although these changes have been recognized for many years, the specific biochemical alterations resulting in increased hepatic glucose production have not been adequately described. The adrenal cortical steroids are known, however, to have an important role in the regulation of this process (1).

Previous studies from this laboratory have demonstrated that in alloxan diabetes there is an increase in the incorporation of CO\textsubscript{2} from labeled pyruvate and alanine into blood glucose (2). It would appear from our present knowledge of intermediary metabolism that the incorporation of labeled carbon atoms from 3 carbon glucogenic intermediates such as lactate, pyruvate, propionate, or alanine into hexose by hepatic tissue would require CO\textsubscript{2} fixation (3-6). Furthermore, under conditions where increased hepatic glucose production would be expected, e.g., prior treatment with cortisone, incorporation of CO\textsubscript{2} from both pyruvate-\textsuperscript{14}C and CO\textsubscript{2}O\textsubscript{2} into blood glucose is increased to a comparable extent (7).

The present studies report observations on CO\textsubscript{2}O\textsubscript{2} fixation in vivo and in vitro and the influence of insulin insufficiency induced by alloxan and anti-insulin serum on this process.

EXPERIMENTAL PROCEDURE

Animals—Male albino rats of the Wistar strain weighing between 100 and 150 g were used. They were fed ad libitum on Purina laboratory chow. Alloxan diabetes was produced by intravenous injection of alloxan monohydrate (40 mg per kg of body weight) in the manner described by Renold et al. (8). Adrenalectomized diabetics were produced by bilateral adrenalectomy of alloxan diabetic rats, and these animals were maintained on 0.9% NaCl for 4 or 5 days before use.

Anti-insulin serum was prepared as described previously (9). Acute insulin insufficiency was produced by the administration of 2 ml of anti-insulin serum, which would neutralize the activity of 2 to 2.5 units of beef insulin. This quantity of anti-insulin serum would increase the blood sugar of normal rats to 180 to 220 mg per 100 ml within 30 minutes.

Animals treated with cortisone received 5 mg of the steroid suspended in NaCl by subcutaneous injection twice a day for a period of 5 days.

Studies with Intact Rats—All animals received an intraperitoneal injection of 50 \mu{}g of CO\textsubscript{2}O\textsubscript{2}-bicarbonate (specific activity 1 me per mmole) in 1 ml of NaCl. Blood samples (0.2 ml) were collected from the severed end of the tail at 30-minute intervals for a period of 2 hours. Samples were deproteinized, and reducing sugar was determined on 2 ml of blood filtrate, and phenylglucosazone was formed for radioactivity assay (10).

At the end of the 2 hours, animals were stunned by a blow on the head and exsanguinated, and tissues were removed for analysis of CO\textsubscript{2}O\textsubscript{2} incorporation into protein. Tissue proteins were isolated by trichloroacetic acid precipitation, followed by extraction with hot trichloroacetic acid, performate digestion, and reprecipitation with trichloroacetic acid according to the method of Manchester and Krahl (11).

Studies with Liver Slices—Liver slices, prepared as described previously (12) from normal and other experimental animals, were incubated in a Ringer bicarbonate medium equilibrated with 95% O\textsubscript{2}-5% CO\textsubscript{2}. Unlabeled pyruvate, lactate, or propionate was added to the medium to give an initial concentration of 40 mm. Approximately 0.5 g of wet liver slices, weighed on a torsion balance, was incubated in 6 ml of medium. After 5 minutes of equilibration at 37\degree, 5 \mu{}g of CO\textsubscript{2}-bicarbonate were injected in each flask. After 90 minutes' incubation, tissues were analyzed for glycogen, and medium for glucose. When unlabeled pyruvate was initially present in the medium, the 2,4-dinitrophenylhydrazone was formed from final medium pyruvate isolated by absorption and elution from a Dowex 1 column.

Glucose, and glycogen after hydrolysis, were isolated as the phenylglucosazone for assay of radioactivity.

RESULTS

Studies on the incorporation of CO\textsubscript{2} from bicarbonate into blood glucose in intact rats are summarized in Fig. 1. A marked increase (7- to 8-fold) in the radioactivity of blood glucose was observed in alloxan diabetic as compared to control animals. Administration of 2 ml of anti-insulin serum ½ hour before the injection of radioactive CO\textsubscript{2} caused a 3-fold increase in glucose radioactivity over that observed in normal animals. However, if both CO\textsubscript{2} and anti-insulin serum were injected simultaneously, very little increase was observed. Cortisol administration also increases the CO\textsubscript{2}O\textsubscript{2} incorporation into glucose, as shown previously (7). That adrenal steroids are involved in increased CO\textsubscript{2} fixation is also indicated by the reduced CO\textsubscript{2}O\textsubscript{2}-glucose observed in adrenalectomized diabetic rats.

The incorporation of CO\textsubscript{2} from radioactive bicarbonate into tissue proteins in vivo is summarized in Table I. No marked
differences in incorporation of CO₂ into tissue proteins were observed, except in the alloxan diabetic rats. In this case, C¹⁴ incorporation into muscle proteins was slightly reduced.

The studies on the incorporation of C⁴O₂ in liver slices are summarized in Table II. Addition of pyruvate or propionate, 40 mM, to the incubation medium did not significantly increase the incorporation of C⁴O₂ into glucose by rat liver slices. However, 40 mM lactate added to the incubation medium increased C¹⁴ incorporation from 8,250 c.p.m. per g in the absence of added substrate to 23,600 c.p.m. per g in the presence of lactate.

The incorporation of C⁴O₂ into glucose was increased in liver slices from alloxan diabetic rats. Increased CO₂ fixation in the diabetic was evident in the absence of added substrate or in the presence of added pyruvate, lactate or propionate. Such an increase was not observed in livers from animals treated with anti-insulin serum. No significant differences in C⁴O₂ incorporation into glucose were observed between animals previously treated with normal guinea pig serum and those given anti-insulin serum.

Pyruvate isolated from the incubation medium in experiments in which unlabeled pyruvate had previously been added was found to have appreciable radioactivity. The incorporation of C⁴O₂ into pyruvate by liver slices from alloxan diabetic rats was increased to approximately the same extent as C¹⁴-glucose; however, the amount of C¹⁴ recovered in pyruvate was only one-third that found in glucose.

No marked differences between normal and alloxan diabetic rats in incorporation of C⁴O₂ into liver glycogen were observed in vitro. Less glycogen was found in livers from anti-insulin serum-treated animals than in livers from normal serum-treated rats.

DISCUSSION

Both insulin and the adrenal steroids appear to be involved in the regulation of CO₂ fixation observed in intact rats. In insulin insufficiency, whether produced by alloxan or by prior treatment with anti-insulin serum, an increase in C¹⁴-glucose was observed in the blood after administration of radioactive
bicarbonate. Absence of insulin, per se, does not appear to be responsible for the increased CO$_2$ fixation observed in the diabetic, since adrenalectomy of alloxan diabetic rats markedly reduces the amount of C$^14$-glucose formed after CO$_2$ administration. Further evidence for the participation of adrenal steroids in the regulation of CO$_2$ fixation is that prior treatment of rats with cortisone (7) or cortisol will increase C$^4$-glucose formation from CO$_2$. When radioactive bicarbonate and anti-insulin serum were administered simultaneously to rats, very little increase in C$^14$-glucose was observed. It is probable that most of the C$^14$0$_2$ was incorporated into glycogen and glucose by rat liver slices.

**Table II**

<table>
<thead>
<tr>
<th>Added substrate</th>
<th>No. of observations</th>
<th>Glycogen</th>
<th>Glucose</th>
<th>Pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\mu$moles/g</td>
<td>c.p.m./g</td>
<td>$\mu$moles/g</td>
</tr>
<tr>
<td>Normal control:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>94 ± 14</td>
<td>292 ± 34</td>
<td>83 ± 3.7</td>
</tr>
<tr>
<td>Pyruvate, 40 mm</td>
<td>6</td>
<td>88 ± 13</td>
<td>502 ± 73</td>
<td>94 ± 6.1</td>
</tr>
<tr>
<td>Lactate, 40 mm</td>
<td>6</td>
<td>70 ± 9</td>
<td>804 ± 76</td>
<td>88 ± 5.1</td>
</tr>
<tr>
<td>Propionate, 40 mm</td>
<td>3</td>
<td>50 ± 8.3</td>
<td>150 ± 23</td>
<td>114 ± 7.7</td>
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<tr>
<td>Alloxan diabetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>8</td>
<td>32 ± 4.7</td>
<td>383 ± 41</td>
<td>66 ± 6.1</td>
</tr>
<tr>
<td>Pyruvate, 40 mm</td>
<td>6</td>
<td>33 ± 5.6</td>
<td>395 ± 73</td>
<td>82 ± 5.6</td>
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<tr>
<td>Lactate, 40 mm</td>
<td>6</td>
<td>27 ± 7</td>
<td>641 ± 56</td>
<td>64 ± 8.8</td>
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<tr>
<td>Propionate, 40 mm</td>
<td>6</td>
<td>16 ± 3</td>
<td>120 ± 11</td>
<td>35 ± 2.7</td>
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<tr>
<td>Glucose, 5.5 mm</td>
<td>4</td>
<td>119 ± 8.0</td>
<td>150 ± 11</td>
<td>131 ± 7.5</td>
</tr>
<tr>
<td>Propionate, 40 mm</td>
<td>4</td>
<td>87 ± 7.4</td>
<td>150 ± 11</td>
<td>91 ± 2.1</td>
</tr>
<tr>
<td>Lactate, 40 mm</td>
<td>4</td>
<td>93 ± 12</td>
<td>650 ± 82</td>
<td>105 ± 7.1</td>
</tr>
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<td>Anti-insulin serum:*</td>
<td>4</td>
<td>58 ± 6.3</td>
<td>128 ± 12</td>
<td>137 ± 3.4</td>
</tr>
<tr>
<td>Glucose, 5.5 mm</td>
<td>4</td>
<td>64 ± 7.0</td>
<td>470 ± 53</td>
<td>83 ± 5.1</td>
</tr>
<tr>
<td>Propionate, 40 mm</td>
<td>4</td>
<td>70 ± 3.0</td>
<td>600 ± 47</td>
<td>110 ± 3.6</td>
</tr>
</tbody>
</table>

* The animals were treated with normal or anti-insulin guinea pig serum 1½ hours before death.

It was found that livers from anti-insulin serum-treated and alloxan diabetic rats formed different amounts of C$^14$-glucose. Although anti-insulin serum resulted in an increase in C$^14$-glucose in vivo, no change in C$^14$O$_2$ incorporation into glucose was observed in liver slices from anti-insulin serum-treated rats. However, increased CO$_2$ fixation was observed in liver slices from alloxan diabetic rats as well as in the intact animal. It would appear that the increased C$^14$-glucose formation observed in the alloxan diabetic is not due to the presence of endogenous substrates. Of the unlabeled substrates added, only lactate resulted in an increase in C$^14$-glucose formation, and this increase was observed in liver slices from diabetic as well as normal rats.

That added lactate, but not pyruvate, will increase C$^14$-glucose formation from radioactive CO$_2$ in rat liver slices suggests that hydrogen atoms from the more reduced substrate may contribute to C$^14$O$_2$ incorporation. Thus, CO$_2$ "fixation" and gluconeogenesis in hepatic tissue may be regulated in part by the availability of TPNH for malate formation from pyruvate and CO$_2$. The increase in CO$_2$ fixation observed with lactate might further involve the enzymatic transfer of hydrogen from DH$^2$P to TPN. Such a mechanism has been suggested as a possible point of action of adrenal cortical steroids in the regulation of gluconeogenesis (14).

It is tempting to speculate that the observed increase in CO$_2$ fixation in diabetic liver may be due in part to a diversion of TPNH, formerly used for fatty acid synthesis, for malate formation from pyruvate. In addition, reduced coenzyme formed by fatty acid oxidation and increased ketone body formation in the diabetic may contribute to reductive carboxylation of pyruvate. Previous experiments (15) have demonstrated that a decrease in pyruvate-2-C$^14$ incorporation into fatty acids occurs at the same time that an increased incorporation of pyruvate carbon into glucose is observed in the induction of experimental diabetes. The idea that reduced coenzyme is necessary for gluconeogenesis is not new. Fitch and Chaikoff (16) have suggested that TPNH produced by glucose oxidation via the pentose phos-
phosphate pathway may be used to drive the pyruvate to malate reaction and thus contribute to increased glucose formation in the diabetic liver.

Studies on the incorporation of radioactivity from CO₂ into protein show no marked difference in various tissues examined. Only a small decrease was observed in muscle and diaphragm proteins from alloxan diabetic rats. These observations are in agreement with previous studies (2).

**SUMMARY**

Carbon dioxide fixation has been studied both in vivo and in vitro in experimental animals with various treatments. Alloxan diabetic rats showed a 7- to 8-fold increase in incorporation of radioactive CO₂ into glucose in vivo and a 2- to 3-fold increase in vitro. Anti-insulin serum- and cortisol-treated rats also exhibited an increase in C¹⁴-glucose formation from C¹³O₂. CO₂ fixation in vivo occurs in the following decreasing order: alloxan diabetic, anti-insulin serum-treated, cortisol, normal unfed, adrenalectomized diabetic, normal with normal serum, and normal. Increased CO₂ fixation was also observed in liver slices from alloxan diabetic rats both in the absence of added substrate or in the presence of added pyruvate, lactate, or propionate. Alloxan diabetic rats showed a 50% decrease in the incorporation of C¹⁴ from bicarbonate into muscle proteins, with no alterations in liver or kidney proteins.

**REFERENCES**

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