Studies on Experimental Diabetes

II. CARBON DIOXIDE FIXATION*

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(Received for publication, June 25, 1962)

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 C14 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 CO2 fixation (3-6). Furthermore, under conditions where increased hepatic glucose production would be expected, e.g. prior treatment with cortisone, incorporation of C14 from both pyruvate-2-C14 and C14O2 into blood glucose is increased to a comparable extent (7).

The present studies report observations on C14O2 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 cortisol 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 intraperi-

tonal injection of 50 μg of C14-bicarbonate (specific activity 1 mc 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 an aliquot of the filtrate; then carrier glucose (10 mg) was added to 1 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 C14 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 Krali (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% O2-5% CO2. 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°, 5 μe of C14-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 C14 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 CO2 caused a 3-fold increase in glucose radioactivity over that observed in normal animals. However, if both C14 and anti-insulin serum were injected simultaneously, very little increase was observed. Cortisol administration also increases the C14O2 incorporation into glucose, as shown previously (7). That adrenal steroids are involved in increased CO2 fixation is also indicated by the reduced C14-glucose observed in adrenalectomized diabetic rats.

The incorporation of C14 from radioactive bicarbonate into tissue proteins in vivo is summarized in Table I. No marked
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I-

Normal
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Diabetic

I I I

Normal
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Adx Diabetic

0 30 60 90 120

0 30 60 90 120

FIG. 1. Radioactivity recovered in blood glucose, expressed as counts per minute per ml of blood, has been plotted as a function of time after injection of C\textsuperscript{14}-bicarbonate. Each curve represents the mean of four or more animals, and total spread of points is indicated.

Table I
Incorporation of C\textsuperscript{14} from radioactive C\textsuperscript{14}O\textsubscript{2} into tissue proteins

<table>
<thead>
<tr>
<th>Type of rat</th>
<th>Liver</th>
<th>Kidney</th>
<th>Diaphragm</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3710 ± 210 2730 ± 200 1746 ± 107</td>
<td>720 ± 120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alloxan diabetic</td>
<td>3327 ± 347 2103 ± 143 980 ± 120</td>
<td>360 ± 125</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal + anti-insulin serum</td>
<td>4130 ± 600 1820 ± 210 640 ± 200</td>
<td>1200 ± 240</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal + normal serum</td>
<td>3453 ± 513 2310 ± 490 1920 ± 280</td>
<td>1900 ± 260</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Differences in incorporation of CO\textsubscript{2} into tissue proteins were observed, except in the alloxan diabetic rats. In this case, C\textsuperscript{14} incorporation into muscle proteins was slightly reduced.

The studies on the incorporation of C\textsuperscript{14}O\textsubscript{2} 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 C\textsuperscript{14}O\textsubscript{2} into glucose by rat liver slices. However, 40 mM lactate added to the incubation medium increased C\textsuperscript{14} 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 C\textsuperscript{14}O\textsubscript{2} into glucose was increased in liver slices from alloxan diabetic rats. Increased CO\textsubscript{2} 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 C\textsuperscript{14}O\textsubscript{2} 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 C\textsuperscript{14}O\textsubscript{2} into pyruvate by liver slices from alloxan diabetic rats was increased by approximately the same extent as C\textsuperscript{14}-glucose; however, the amount of C\textsuperscript{14} recovered in pyruvate was only one-third that found in glucose.

No marked differences between normal and alloxan diabetic rats in incorporation of C\textsuperscript{14}O\textsubscript{2} 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 CO\textsubscript{2} fixation observed in intact rats. In insulin insufficiency, whether produced by alloxan or by prior treatment with anti-insulin serum, an increase in C\textsuperscript{14}-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 CO₂ fixation observed in the diabetic, since adrenalectomy of alloxan diabetic rats markedly reduces the amount of C¹⁴-glucose formed after CO₂ incorporation administration. Further evidence for the participation of adrenal steroids in the regulation of CO₂ fixation is that prior treatment of rats with cortisone (7) or cortisol will increase C¹⁴-glucose formation from CO₂.

When radioactive bicarbonate and anti-insulin serum were administered simultaneously to rats, very little increase in C¹⁴-glucose was observed. It is probable that most of the CO₂ fixation occurs within the first ½ hour after injection. Although anti-insulin serum results in a prompt increase in blood glucose (9, 13), it is possible that the full effects of insulin insufficiency are not obtained until several minutes after the serum is administered. Therefore, in a second series of experiments, anti-insulin serum was given to rats 30 minutes before the CO₂ was injected. In this case, a marked increase in C¹⁴-glucose was observed.

The distribution of C¹⁴ in glucose formed by hepatic tissue incubated with C¹⁴O₂ would be consistent with CO₂ fixation by pyruvate or propionate and subsequent conversion of the malate or succinate formed to phosphoenolpyruvate and glucose formation by the reversal of glycolysis (3). Further, the incorporation of pyruvate carbon into glucose most likely proceeds via oxaloacetate as an intermediate, rather than by direct reversal of glycolysis (4). Therefore, an increase in CO₂ fixation would be expected in diabetes to the extent that 3 carbon intermediates contribute to increased hepatic glucose formation. It is probable that an increase in CO₂ fixation in diabetic rats is due in part to an increase in protein catabolism, leading to an increased formation of metabolic intermediates capable of adding CO₂.


<table>
<thead>
<tr>
<th>Added substrate</th>
<th>No. of observations</th>
<th>Glucose</th>
<th>Pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µmoles/g</td>
<td>c.p.m./g</td>
</tr>
<tr>
<td>Normal control:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>94 ± 14</td>
<td>292 ± 34</td>
</tr>
<tr>
<td>Pyruvate, 40 mm</td>
<td>6</td>
<td>88 ± 13</td>
<td>502 ± 73</td>
</tr>
<tr>
<td>Lactate, 40 mm</td>
<td>6</td>
<td>70 ± 9</td>
<td>804 ± 76</td>
</tr>
<tr>
<td>Propionate, 40 mm</td>
<td>3</td>
<td>50 ± 8.3</td>
<td>150 ± 23</td>
</tr>
<tr>
<td>Alloxan diabetic:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>8</td>
<td>32 ± 4.7</td>
<td>383 ± 41</td>
</tr>
<tr>
<td>Pyruvate, 40 mm</td>
<td>6</td>
<td>33 ± 5.6</td>
<td>395 ± 73</td>
</tr>
<tr>
<td>Lactate, 40 mm</td>
<td>6</td>
<td>27 ± 7</td>
<td>464 ± 56</td>
</tr>
<tr>
<td>Propionate, 40 mm</td>
<td>6</td>
<td>16 ± 3</td>
<td>120 ± 11</td>
</tr>
<tr>
<td>Normal serum:*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, 5.5 mm</td>
<td>4</td>
<td>119 ± 8.0</td>
<td>150 ± 11</td>
</tr>
<tr>
<td>Propionate, 40 mm</td>
<td>4</td>
<td>87 ± 7.4</td>
<td>150 ± 22</td>
</tr>
<tr>
<td>Lactate, 40 mm</td>
<td>4</td>
<td>93 ± 12</td>
<td>650 ± 82</td>
</tr>
<tr>
<td>Anti-insulin serum:*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, 5.5 mm</td>
<td>4</td>
<td>58 ± 6.3</td>
<td>128 ± 12</td>
</tr>
<tr>
<td>Propionate, 40 mm</td>
<td>4</td>
<td>64 ± 7.0</td>
<td>470 ± 53</td>
</tr>
<tr>
<td>Lactate, 40 mm</td>
<td>4</td>
<td>70 ± 3.0</td>
<td>600 ± 47</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 C¹⁴-glucose. Although anti-insulin serum results in an increase in C¹⁴-glucose in vivo, no change in C¹⁴O₂ incorporation into glucose was obtained in liver slices from anti-insulin serum-treated rats. However, increased CO₂ fixation was observed in liver slices from alloxan diabetic rats as well as in the intact animal. It would appear that the increased C¹⁴-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 C¹⁴-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 C¹⁴-glucose formation from radioactive CO₂ in rat liver slices suggests that hydrogen atoms from the more reduced substrate may contribute to C¹⁴O₂ incorporation. Thus, CO₂ "fixation" and gluconeogenesis in hepatic tissue may be regulated in part by the availability of TPNH for malate formation from pyruvate and CO₂. The increase in CO₂ fixation observed with lactate might further involve the enzymatic transfer of hydrogen from DHN 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 CO₂ 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-C¹⁴ 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 Chakoff (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 C¹⁴O₂. 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**
