THE EFFICIENCY OF OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA FROM DIABETIC RATS*

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Considerable evidence has accumulated which indicates that insulin plays an important rôle in phosphate metabolism (1, 2). A number of workers have postulated that insulin has as its primary function the regulation of the efficiency of oxidative phosphorylation. In attempts to test this hypothesis, Goranson and Erulkar (3) observed that tissues from alloxan-diabetic rats appear to fix phosphate less efficiently than do those from normal animals, and Polis et al. (4) reported that insulin added in vitro increases phosphate fixation in aged preparations from normal rats. When P:O ratios are computed from their data, however, it is revealed that the former workers (3) obtained ratios between 0.03 and 0.2 and the latter (4) between 0.2 and 1.6. Since presently attainable P:O ratios are 2 to 100 times greater than these, the effects that were observed are difficult to interpret and have questionable relevance to the problem of insulin action. Judah and Williams-Ashman (5) were unable to detect significant alterations in P:O ratios when insulin was added in vitro to "cyclophorase" preparations from normal animals.

Methods are now available for the isolation of mitochondria of high phosphorylating ability (6). By the use of such mitochondria from liver, the P:O ratios for certain one- and two-step oxidative reactions have been measured for both normal and alloxan-diabetic rats. No differences which could be ascribed to insulin deficiency were detected.

EXPERIMENTAL

Production of Alloxan Diabetes—40 mg. of freshly dissolved alloxan per kilo of body weight were injected intravenously into adult (175 to 200 gm.)

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1 Moles of inorganic phosphate removed from the medium divided by gm. atoms of oxygen consumed.
albino rats, following a 48 hour fast as recommended by Kass and Waisbren (7). Studies were performed on two groups of animals; in the first series ("short term" diabetics), animals were sacrificed 6 to 10 days after alloxan injection, and in the second ("long term" diabetics) 3 weeks to 6 months after treatment with alloxan. The development of diabetes was noted by the occurrence of glucosuria, polyuria, polyphagia, polydipsia, and weight loss. Bilateral cataracts developed in many of the animals. No rat was considered diabetic unless its fasting blood sugar concentration, determined by the Reinecke procedure (8), was greater than 250 mg. per cent.

Determination of P:O Ratios—Rat liver mitochondria were prepared by a modification (9) of the procedure of Schneider (6). All other manometric and chemical procedures were essentially the same as those reported previously (10). In the "long term" diabetic series, the following minor modifications were instituted: Potassium phosphate and substrate additions were increased to 50 and 30 μmoles, respectively, 10 per cent trichloroacetic acid was used for deproteinization, and an 8 minute period of thermal equilibration in the water baths was employed. In some cases in which the adenosine triphosphate (ATP)-creatine transphosphorylase system replaced glucose-hexokinase as a means of trapping the high energy phosphate (permitting higher concentrations of ATP to exist in the reaction mixture (9)), the following were added per vessel: 150 μmoles of creatine and 1 mg. of ATP-creatine transphosphorylase.

The addition of 30 μmoles of malonate to the reaction mixtures made possible the study of the following one- and two-step oxidations (10):

(1) \( \alpha \)-Ketoglutarate \( \rightarrow \) succinate
(2) Pyruvate \( \rightarrow \) acetoacetate
(3) Caprylate \( \rightarrow \) acetoacetate
(4) Glutamate \( \rightarrow \) succinate

The inability of liver to metabolize significant amounts of acetoacetate made possible the study of the following one-step oxidation:

(5) \( \beta \)-Hydroxybutyrate \( \rightarrow \) acetoacetate

The oxidation of succinate was examined in the absence of the Krebs cycle inhibitors.

Materials—The reagents used were as described previously (10). Gluca-

gon-free insulin was generously donated by J. Lens, Organon Labora-
tories, Oss, Netherlands. Amorphous insulin was obtained from the Lilly Research Laboratories. ATP-creatine transphosphorylase was a highly
purified preparation from rabbit muscle made available by Dr. Kuby, Dr. Noda, and Dr. Lardy (11). Alloxan was purchased from the Eastman Kodak Company.

Results

"Short Term" Diabetic Mitochondria—Table I presents the results of experiments in which P:O ratios were compared, liver mitochondria being used from normal rats and from rats which had received alloxan injections 6 to 10 days prior to sacrifice. Most of the animals were studied 7 days after alloxan administration. While the average values are lower in the diabetic mitochondria with α-ketoglutarate and pyruvate as substrates, no consistent alterations in the P:O ratios were observed among the individual animals.

With the method of administering alloxan used in these experiments, usually one-third to one-half of the animals succumbed between the 3rd and 10th day after treatment. The administration of 4 units per day of protamine zinc insulin did not improve the percentage of survival. This suggested that damage to tissues other than the pancreas is severe during this time interval and is consistent with earlier reports of the severe nephrotoxic and mild hepatotoxic action of this compound in rats (12, 13). A number of workers have reported the irregular occurrence of histological evidence of mild degenerative changes in liver parenchymal cells in the 1st week after alloxan treatment (12–15). This raised the question whether the occasional lowering of P:O ratios which we observed with "short term" diabetics may have been due to a direct toxic effect of alloxan.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>No. of animal pairs</th>
<th>P:O, * normals</th>
<th>P:O, * diabetic</th>
<th>P:O of diabetic animals as per cent of P:O of paired control animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>10</td>
<td>2.6 (2.1-2.9)</td>
<td>2.0 (1.0-3.0)</td>
<td>104, 99, 95, 76, 76, 72, 71, 69, 51, 39</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>8</td>
<td>3.8 (3.3-4.1)</td>
<td>3.1 (2.3-4.2)</td>
<td>102, 98, 93, 91, 86, 77, 68, 57</td>
</tr>
<tr>
<td>Succinate</td>
<td>4</td>
<td>1.5 (1.2-1.6)</td>
<td>1.6 (1.2-2.0)</td>
<td>125, 117, 107, 92</td>
</tr>
</tbody>
</table>

* P:O ratios are listed as average values, with the range of the P:O ratios indicated by the figures in parentheses.
† In each experiment a diabetic and a control rat were studied simultaneously. The figures indicate the P:O ratios of the individual diabetic animals with their paired control animals.
Table II

P:O Ratios of Normal and "Long Term" Diabetic Rat Liver Mitochondria

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Phosphate acceptor</th>
<th>Preparation</th>
<th>No. of animals</th>
<th>P:O, average</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>Glucose</td>
<td>Normal</td>
<td>7</td>
<td>2.4</td>
<td>2.0 - 2.8</td>
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<tr>
<td></td>
<td></td>
<td>Diabetic</td>
<td>7</td>
<td>2.1</td>
<td>1.7 - 2.4</td>
</tr>
<tr>
<td></td>
<td>Creatine</td>
<td>Normal</td>
<td>4</td>
<td>2.0</td>
<td>1.8 - 2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diabetic</td>
<td>5</td>
<td>1.9</td>
<td>1.5 - 2.2</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>Glucose</td>
<td>Normal</td>
<td>10</td>
<td>3.1</td>
<td>2.75 - 3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diabetic</td>
<td>7</td>
<td>3.3</td>
<td>3.1 - 3.7</td>
</tr>
<tr>
<td></td>
<td>Creatine</td>
<td>Normal</td>
<td>1</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diabetic</td>
<td>2</td>
<td>2.4</td>
<td>2.3 - 2.5</td>
</tr>
</tbody>
</table>

*"Glucose" indicates the addition of glucose and hexokinase, and "creatine" the addition of creatine and ATP-creatine transphosphorylase, as described in the text.

Table III

Addition of Insulin in Vitro to Rat Liver Mitochondria*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Substrate</th>
<th>Phosphate acceptor†</th>
<th>Preparation</th>
<th>Insulin‡</th>
<th>P</th>
<th>O</th>
<th>P:O</th>
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<tbody>
<tr>
<td>1</td>
<td>α-Ketoglutarate</td>
<td>Glucose</td>
<td>Normal</td>
<td>γ</td>
<td>17.4</td>
<td>5.5</td>
<td>3.2</td>
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<td>″</td>
<td>20</td>
<td>17.1</td>
<td>4.6</td>
<td>3.7</td>
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<td>2</td>
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<td></td>
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<td>21.2</td>
<td>0.8</td>
<td>3.1</td>
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<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19.0</td>
<td>5.6</td>
<td>3.4</td>
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<td>4</td>
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<td></td>
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<td>19.2</td>
<td>5.9</td>
<td>3.3</td>
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<tr>
<td>5</td>
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<td></td>
<td></td>
<td>19.3</td>
<td>5.7</td>
<td>3.4</td>
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<td></td>
<td></td>
<td>16.0</td>
<td>6.5</td>
<td>2.5</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16.5</td>
<td>6.4</td>
<td>2.6</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12.3</td>
<td>5.6</td>
<td>2.2</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>12.5</td>
<td>6.2</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*"Long term" diabetic animals were used in Experiments 3, 4, and 5.
† "Glucose" indicates the addition of glucose and hexokinase, and "creatine" indicates the addition of creatine and ATP-creatine transphosphorylase, as described in the text.
‡ Glucagon-free insulin (Experiments 1, 2, and 3), amorphous insulin (Experiments 4 and 5).

on liver cells rather than to insulin deficiency. Bailey, Bailey, and Hagan (15) have reported that, while mild fatty metamorphosis occurred in rab-

2 In recent publications, M. U. Dianzani (16) has reported that mitochondria from fatty livers resulting from carbon tetrachloride or phosphorylated oil administration display markedly lowered P:O ratios and Dury (17) observed chemical alterations, including marked increases in the neutral fat content of livers from rats which had received alloxan injections from 4 to 72 hours prior to analysis.
bit liver in the first few days after alloxan treatment, no histological liver abnormalities could be detected after 2 to 5 months. Therefore, the examination of animals with long standing alloxan diabetes was indicated.

"Long Term" Diabetic Mitochondria—The effects of long standing diabetes (alloxan treatment from 3 weeks to 6 months prior to study) upon the P:O ratios of liver mitochondria are shown in Table II. No significant deviation from the ratios determined in normal rats was observed, either with the glucose-hexokinase or the creatine-ATP creatine transphosphorylase system used as the phosphate trap.

Addition of Insulin in Vitro—In view of the observations of Polis et al. (4) and Goranson and Erulkar (3) that insulin added in vitro increases the efficiency of oxidative phosphorylation, it was considered of interest to determine whether insulin could so affect preparations capable of high phosphorylative ability. As can be seen in Table III, the addition of insulin to normal or diabetic mitochondria caused no significant alteration of the P:O ratio. This is in agreement with the findings of Judah and Williams-Ashman (5) on preparations from normal animals.

Other Substrates—In addition to the substrates described above, the following were examined in a limited number of experiments: β-hydroxobutyrate, caprylate, and glutamate. No appreciable differences between normal and diabetic liver mitochondria were observed.

DISCUSSION

Liver was chosen as the tissue for study in these experiments because its metabolism is profoundly influenced by the diabetic state, and also because mitochondria may be isolated from this tissue in a readily reproducible manner and have been studied extensively in this laboratory (9, 10). The P:O ratios determined in these experiments are in satisfactory agreement with those reported earlier for normal animals (10).

While these studies have failed to detect any impairment arising from diabetes in the metabolic machinery by which mitochondria carry out tightly coupled oxidative phosphorylation, they have not ruled out possible effects on oxidative phosphorylation resulting from the interaction of mitochondria with other intracellular components. The examination of such interactions is beset with many uncertainties at both the technical and interpretive levels. For example, it has been observed by Ashmore et al. (18) and independently in our laboratory (19) that the glucose-6-phosphatase activity of alloxan-diabetic rat livers is markedly increased. In liver this enzyme is found exclusively in the microsomal fraction (20). The inclusion of liver microsomes in a P:O ratio determination reaction mixture would cause the release of inorganic phosphate from the hexokinase-produced glucose-6-phosphate resulting in an apparent P:O ratio which is lower than the true value. This effect would be much greater.
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with diabetic than with normal liver microsomes and therefore an even lower apparent P:O ratio would be obtained.

The authors wish to express their appreciation to Professor H. A. Lardy for advice and helpful criticism.

SUMMARY

1. P:O ratios for several substrates have been compared for normal and alloxan-diabetic rat liver mitochondria.

2. With "short term" diabetic rats (6 to 10 days after alloxan), no consistent alterations in P:O ratios were observed, although the average ratios were lower than those of the control rats. The liver mitochondria from an occasional animal displayed a marked lowering of the P:O ratio. This was possibly the result, not of insulin deficiency, but of the irregularly occurring mild hepatotoxic action of alloxan.

3. In long standing (3 weeks to 6 months) alloxan diabetes, no alteration in the efficiency of oxidative phosphorylation of liver mitochondria was detected.

4. Addition of insulin in vitro did not significantly affect the P:O ratios of normal or diabetic rat liver mitochondria.

BIBLIOGRAPHY

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