INHIBITION OF SUCCINIC DEHYDROGENASE BY METHYLGLYOXAL*

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The reaction of the carbonyl group of methylglyoxal with —SH radicals of thiols has been the subject of numerous investigations (1-9). The reactivity of the keto aldehyde with —SH compounds suggested that a similar type of reaction may occur between methylglyoxal and —SH proteins. The physiological rôle of methylglyoxal as an inhibitor of —SH enzymes is of particular interest, since this keto aldehyde may be formed during carbohydrate metabolism (10-12). Mann and Quastel (13) have reported that other aldehydes have an inhibitory effect on brain metabolism. In this paper experiments are described which demonstrate that methylglyoxal can inhibit cell metabolism. The conditions of the inhibition and its possible significance in tissue metabolism are also discussed.

EXPERIMENTAL

It is well known that methylglyoxal is rapidly converted to lactic acid in crude tissue preparations (14, 15). Therefore, experimental conditions were sought under which the effect of methylglyoxal could be studied without the interference of the glyoxalase system. Schneider, Claude, and Hogeboom (16, 17) established that the succinate system of the rat liver is localized in the mitochondrial fraction. Since it was found in this laboratory that rat liver mitochondria in the presence of glutathione (GSH) do not convert methylglyoxal to lactic acid,1 this enzyme preparation was chosen for the inhibition studies.

Materials—An aqueous solution of methylglyoxal was prepared from dihydroxyacetone2 by the method of Neuberg et al. (18). The methylglyoxal concentration was determined according to Kuhn and Heckscher (19). The freshly prepared aqueous solution of methylglyoxal was neutral-

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1 Unpublished experiments.

2 Dihydroxyacetone, c.p., was obtained from the Delta Chemical Works, 23 West 60th Street, New York 23.
izd and diluted to appropriate concentrations immediately before being pipetted into the respirometer flasks. Rat liver mitochondria were prepared by a modified procedure of Schneider et al. (16, 17) as described by Kennedy and Lehninger (20). The freshly removed and chilled rat liver was ground up with 9 volumes of ice-cold 0.88 M sucrose. Large particles were removed by three successive centrifugations at 1500 × g on the Servall angle centrifuge (type SS-1A), which was operated in the cold room at 0–4°. The mitochondria were agglutinated by the addition of 1.5 M KCl to give a final KCl concentration of 0.15 M. The sedimentation of mitochondria was carried out at 5000 × g for 8 minutes, followed by three successive washings with 0.15 M KCl. During this procedure soluble —SH compounds were quantitatively removed, and all detectable (21) —SH radicals were found to be bound to the washed particles. The mitochondria were finally resuspended and homogenized in 0.15 M KCl to make 0.1 ml. of mitochondria suspension correspond to 100 to 120 mg. of total fresh liver weight.

Succinoxidase and cytochrome oxidase were tested manometrically, according to Schneider and Potter (22). Dehydrogenase activity was measured by the ferricyanide technique of Quastel and Wheatley (23).

**Results**

*Site of Action of Methylglyoxal on Succinate System of Rat Liver Mitochondria—* When a suspension of rat liver mitochondria was incubated with methylglyoxal, a progressively increasing inhibition of succinoxidase activity was observed. After a certain length of incubation complete loss of enzyme activity occurred. Results of such an experiment are shown in Table I. Succinate was added to the main compartment of the Warburg vessel containing the enzyme preparation simultaneously with methylglyoxal and after 15 or 30 minutes incubation of the mitochondria with the inhibitor.

When succinate and methylglyoxal were added simultaneously, the onset of enzyme inhibition was retarded. Similar protective effect of succinate was reported by Mann and Quastel (13), Hopkins and Morgan (24), von Euler and Hellström (25), and Potter and Dubois (26) for other inhibitors of succinic dehydrogenase which react with —SH radicals of this enzyme. In order to test the validity of the assumption that methylglyoxal combines with the —SH radicals of succinic dehydrogenase and thereby inhibits its catalytic activity, —SH analyses (21) were performed on the contents of respirometer flasks containing the same constituents, except for succinate, as in the succinoxidase experiment. As can be seen in Fig. 1, a rapid decrease of —SH content of mitochondria occurred when incubated with methylglyoxal.
TABLE I
Effect of Time of Incubation on Inhibition of Succinoxidase by Methylglyoxal

The results are expressed in microliters of oxygen in 15 minutes.

<table>
<thead>
<tr>
<th>Time of manometer readings</th>
<th>Succinate and methylglyoxal added simultaneously</th>
<th>Succinate added after 15 min. incubation</th>
<th>Succinate added after 30 min. incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment</td>
<td>Control</td>
<td>Experiment</td>
</tr>
<tr>
<td>15</td>
<td>131</td>
<td>130</td>
<td>20</td>
</tr>
<tr>
<td>30</td>
<td>69</td>
<td>128</td>
<td>10</td>
</tr>
<tr>
<td>45</td>
<td>27</td>
<td>125</td>
<td>2</td>
</tr>
<tr>
<td>60</td>
<td>13</td>
<td>128</td>
<td>2</td>
</tr>
</tbody>
</table>

Each Warburg flask contained a suspension of rat liver mitochondria (corresponding to 100 mg. of fresh liver), cytochrome c, Ca++, Al++, phosphate buffer, and succinate in the concentrations given by Schneider and Potter (22), and 46 μM per ml. of methylglyoxal; 0.2 ml. of 20 per cent KOH in the center well, gas, air; temperature of bath 37°; total volume of reactants, 3 ml.; time of equilibration 5 minutes.

![Graph](http://www.jbc.org/)

**Fig. 1.** Effect of incubation with methylglyoxal on the —SH content of rat liver mitochondria. Each Warburg flask contained the same amount of enzyme, cytochrome c, Ca++, Al++, phosphate, and methylglyoxal as described in the legend of Table I, but no succinate. At 0, 5, 10, 15, and 25 minute intervals one experimental flask (containing methylglyoxal) and control flask were instantly analyzed for —SH groups, by carrying out the ferricyanide test of Anson (21) on 0.3 ml. samples.
Succinic dehydrogenase is the only known member of the succinoxidase system which requires —SH groups for its catalytic activity. The site of action of methylglyoxal on the succinate system was determined by comparing the rate of aerobic (22) and anaerobic (23) succinate oxidation in the presence of various concentrations of the inhibitor. When the percentage of inhibition of aerobic and anaerobic oxidation is plotted against the amount of inhibitor, the two series of experimental points (Fig. 2)

![Graph showing inhibition of succinic oxidase and dehydrogenase](Image)

**Fig. 2.** Inhibition of succinic oxidase and dehydrogenase. The aerobic and anaerobic oxidation of succinate by rat liver mitochondria was measured after 20 minutes incubation with the inhibitor (methylglyoxal). At the end of incubation in the aerobic systems (22) succinate was added from the side arm, and succinate + ferricyanide (0.1 ml. of 0.5 M succinate + 0.1 ml. of 20 per cent potassium ferricyanide, dissolved in 1.3 per cent NaHCO₃) in the anaerobic systems (23). For each set of aerobic and anaerobic test systems blanks were set up containing no succinate but varying amounts of methylglyoxal. Corrections for such blank values were carried out routinely in all measurements reported in this paper. Each flask contained, besides constituents of the test system, a suspension of mitochondria, corresponding to 100 mg. of fresh liver. The rate of succinate oxidation was determined by averaging three successive 10 minute manometer readings which did not deviate from the mean more than ±5 per cent. The reaction rates were linear in each case.

cannot be distinguished from each other, indicating that the percentage of inhibition is identical in both aerobic and anaerobic conditions. This observation strongly suggests that the anaerobic part of the succinate system is the limiting factor of the enzyme inhibition. This conclusion was also warranted by the finding that cytochrome oxidase (22) was unaffected by methylglyoxal in concentrations which inhibited the aerobic oxidation of succinate.

*Conditions of Enzyme Inhibition*—Since the inhibitory effect of methyl-
glyoxal was apparently due to its carbonyl group, it could be assumed that substances which react with this radical protect —SH enzymes against methylglyoxal inhibition. In agreement with this assumption it was found that, when rat liver mitochondria were incubated with methylglyoxal in the presence of semicarbazide, the inhibitory effect of methylglyoxal was greatly reduced. From a physiological viewpoint the most important compound which is known to react with methylglyoxal is GSH, the coenzyme of the glyoxalase system (2, 3, 14, 15, 27, 28). It is evident that GSH together with glyoxalase plays an important rôle in the

TABLE II

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme with and without semicarbazide HCl (0.01 M)</td>
<td>0</td>
</tr>
<tr>
<td>“ + methylglyoxal (46 μM per ml.)</td>
<td>76-80</td>
</tr>
<tr>
<td>“ + semicarbazide HCl (0.01 M) + methylglyoxal</td>
<td>37</td>
</tr>
<tr>
<td>Enzyme + 1 mg. GSH + methylglyoxal</td>
<td>59</td>
</tr>
<tr>
<td>“ + 3 “ “ + “</td>
<td>21</td>
</tr>
<tr>
<td>“ + 5 “ “ + “</td>
<td>0</td>
</tr>
<tr>
<td>“ + 1 “ “ + 0.2 ml. liver extract + methylglyoxal</td>
<td>0</td>
</tr>
<tr>
<td>Enzyme + 0.2 ml. liver extract + methylglyoxal</td>
<td>52</td>
</tr>
</tbody>
</table>

Freshly prepared rat liver mitochondria (corresponding to 120 mg. of fresh liver) were incubated anaerobically for 20 minutes with and without the reactants listed in the table. Succinic dehydrogenase activity was measured by tipping in 0.1 ml. of 0.5 M Na succinate and 0.1 ml. of 0.2 per cent potassium ferricyanide from the side arms, and CO₂ formation was measured in 5 minute intervals for 30 minutes. Correction was made for ferricyanide reduction by GSH, semicarbazide, and liver extract. GSH alone had no effect on the freshly prepared enzyme. Final volume of reactants, 3 ml.; NaHCO₃ concentration 0.025 M; gas phase, 95 per cent N₂ + 5 per cent CO₂; temperature 37°.

progressive system of tissues against methylglyoxal inhibition. Therefore the protective rôle of GSH was investigated separately and also in the presence of the glyoxalase enzyme system. It was found that the magnitude of inhibition of succinic dehydrogenase of mitochondria was a function of the GSH concentration in the absence of the glyoxalase enzyme system. The protective effect of GSH, similar to that of semicarbazide, did not change with time. The protective effect of GSH was markedly potentiated by liver extract. The liver extract was prepared by homogenizing 1 part of liver (by weight) with 2 parts of ice-cold 0.15 M KCl, then centrifuging for 30 minutes at 25,000 × g in the cold room. The supernatant had no
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succinic dehydrogenase activity, but contained an active glyoxalase system. Calculated from the CO₂ evolved in the course of 20 minutes incubation, approximately one-half to two-thirds of the methylglyoxal was converted to lactic acid if GSH and liver extract were present simultaneously. The glyoxalase reaction came to an almost complete standstill after 20 minutes, a finding similar to that reported by Hopkins and Morgan (27). The partial protection by liver extract alone against methylglyoxal inhibition can be explained by the —SH (GSH, —SH protein, amino acid) content of the liver extract. Results of these experiments are summarized in

**Table III**

*Effect of Malonate and Incubation with GSH on Reversibility of Inhibition of Succinic Dehydrogenase by Methylglyoxal*

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Before incubation with GSH</th>
<th>After 35 min. incubation with GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme activity*</td>
<td>Per cent inhibition</td>
</tr>
<tr>
<td>Control (no methylglyoxal, no malonate)</td>
<td>560</td>
<td>0</td>
</tr>
<tr>
<td>Enzyme + methylglyoxal</td>
<td>38</td>
<td>95</td>
</tr>
<tr>
<td>&quot; + malonate + methylglyoxal</td>
<td>264</td>
<td>33</td>
</tr>
<tr>
<td>&quot; + &quot;</td>
<td>353</td>
<td>37</td>
</tr>
</tbody>
</table>

Mitochondria (equivalent to 800 mg. of fresh liver) were incubated for 15 minutes with malonate (1.0 ml. of 0.5 M), methylglyoxal (208 μM), and malonate + methylglyoxal. Distilled water was substituted for malonate and methylglyoxal in the controls. Volume of reactants, 3 ml. After incubation mitochondria were sedimented at 9000 × g and washed thrice with 5 ml. of 0.15 M KCl. The mitochondria were then resuspended in 3 ml. of 0.15 M KCl, and in 0.5 ml. samples enzyme activity was tested immediately and after 35 minutes incubation with 5 mg. of GSH under anaerobic conditions. The test system was the same as that described in the legend to Table II.

* Enzyme activity is expressed in terms of CO₂ evolved (corrected values) due to anaerobic succinate oxidation by a 0.5 ml. suspension of mitochondria (equivalent to 133 mg. of fresh liver).

Table II where the inhibitory effect of methylglyoxal is demonstrated on comparable amounts of enzyme under various experimental conditions.

In further experiments the reversibility of the inhibition of succinic dehydrogenase by methylglyoxal was investigated. The inhibitory effect of methylglyoxal was compared to the effect of malonate, which is a well known competitive inhibitor of succinic dehydrogenase (29). Equal amounts of rat liver mitochondria were incubated for 15 minutes in the presence of malonate, methylglyoxal, and malonate + methylglyoxal (methylglyoxal was added after malonate). The amount of malonate present (1 ml. of 0.5 M malonate in 3 ml. final volume of reactants) completely inhibited succinic dehydrogenase of the mitochondria. After 15 min-
utes incubation at 37°, the mitochondria were quantitatively sedimented by centrifugation at 9000 x g for 20 minutes at 0°, repeatedly washed with portions of 0.15 M KCl, and then resuspended and rehomogenized in a final volume of 3 ml. Succinic dehydrogenase activity was determined by the ferricyanide technique (23) immediately after this procedure and also after anaerobic incubation of the mitochondria with 5 mg. of GSH. The results are shown in Table III. During the procedure of incubation and repeated washings without inhibitor, about 50 per cent of the succinic dehydrogenase was inactivated which could be completely reactivated by anaerobic incubation with GSH. The reversible inactivation of succinic dehydrogenase is readily explained by the reversible oxidation of —SH groups by molecular oxygen (30-32), which occurred in the course of handling the mitochondria. It is of interest that repeated washings and incubation with GSH did not reverse the inhibition of succinic dehydrogenase by methylglyoxal (95 per cent inhibition before, 92 per cent after incubation with GSH). Attempts to reverse the enzyme inhibition by incubation of the inhibited mitochondria with liver extract (0.2 ml. of 0.15 M KCl extract of rat liver) in addition to GSH were also unsuccessful. Malonate had a protective effect against methylglyoxal. The enzyme inhibition due to malonate alone could be completely reversed by repeated washings and anaerobic incubation with GSH.

DISCUSSION

Evidence presented in the experimental part of this paper strongly suggests that the inhibitory effect of methylglyoxal on succinic dehydrogenase is due to its reaction with the enzyme protein. It was found in preliminary experiments that other —SH enzymes (malic-, glutamic-, triosedehydrogenases, hexokinase, and adenosinetriphosphatase) present in rat tissue homogenates or extracts are also inhibited by methylglyoxal, similarly to succinic dehydrogenase. The conditions of enzyme inhibition, as exemplified by the succinate system of rat liver mitochondria, are of particular interest. The GSH-glyoxalase system of the cell can completely protect —SH enzymes against the inhibitory effect of methylglyoxal. Once the reaction of the keto aldehyde with succinic dehydrogenase occurred, it was impossible to reverse the inhibition under our experimental conditions.

A tentative explanation of the apparently irreversible inhibitory effect of methylglyoxal can be offered. It is known that the rest of the thiol molecule plays an important role in determining the nature of the methylglyoxal-thiol complex. Schubert (8) has shown that, while GSH combines with methylglyoxal to form a labile addition compound, thiols like cysteine and thiourea, which have free amino radicals in the vicinity of their —SH groups, react with the carbonyl group of methylglyoxal with a loss of H₂O.
to form a more stable condensation product. Such a condensation compound of cysteine and methylglyoxal has the property of changing its color to brown (8, 33) during incubation.

A darkening of the mitochondria could be observed upon incubation with methylglyoxal. It is, therefore, conceivable that methylglyoxal combines with —SH enzymes to form a stable condensation compound, since the location of the enzyme —SH group might be of the cysteine type. The stoichiometric relationship between methylglyoxal binding of enzyme —SH groups and decrease of enzymatic activity must be determined with a crystalline —SH enzyme. The protective effect of GSH in the absence of the glyoxalase enzyme system can readily be understood on the basis that GSH combines with the keto aldehyde to form a labile addition compound and thereby competitively inhibits the reaction of methylglyoxal with the —SH groups of the enzyme protein.

It is evident that in the presence of GSH and glyoxalase methylglyoxal is rapidly detoxified. The first step of the enzymatic conversion of methylglyoxal to lactic acid is, according to Jowett and Quastel (3), the reversible combination of methylglyoxal and GSH. Yamazoye (9), and recently Racker (28), stated that in the presence of glyoxalase a different type of methylglyoxal-GSH complex is formed compared to the labile complex described by Schubert (8). Hopkins and Morgan (27) separated glyoxalase into two protein fractions. Racker (28) recently reported that the combination of GSH and methylglyoxal is catalyzed by a protein fraction of glyoxalase and the conversion to lactic acid is also brought about by a separate enzyme. It is possible that both enzyme fractions of the glyoxalase system participate in the inactivation of methylglyoxal, first by its combination with GSH, then its conversion to lactic acid.

The physiological rôle of glyoxalase in muscle tissue was suggested by Meyerhof (34), who pointed out that this enzyme system may eliminate the spontaneously formed methylglyoxal. The importance of this mechanism is underlined by the results described in this paper. Whenever the formation of methylglyoxal is increased, or its detoxification impaired, the inactivation of —SH enzymes by methylglyoxal may occur. Further studies are now being carried out to determine the occurrence and rôle of methylglyoxal in abnormal cell metabolism.

SUMMARY

1. Upon incubation with methylglyoxal an inhibition of the aerobic and anaerobic oxidation of succinate by rat liver mitochondria occurs. The inhibition of succinic dehydrogenase by methylglyoxal was shown to coincide with the disappearance of —SH groups of the mitochondrion particles.

2. Succinate, malonate, semicarbazide, and GSH have a protective
effect against the inhibitory effect of methylglyoxal. Semicarbazide and GSH protect the enzyme by complex formation with methylglyoxal.

3. Rat liver extract, containing the glyoxalase enzyme system, potentiates the protective effect of GSH.

4. The inhibition of succinic dehydrogenase by methylglyoxal could not be reversed by repeated washings and anaerobic incubation of the inhibited enzyme with GSH, even in the presence of glyoxalase.

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