DETERMINATION OF GLYOXALASE ACTIVITY*

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Dakin and Dudley (1) and Neuberg (2) discovered the enzyme glyoxalase in 1913, and showed that it could convert methylglyoxal to lactic acid. The usual assay for this enzyme is a manometric procedure (3). In 1928 Ariyama (4) described a method for the colorimetric determination of glyoxals involving the ability of these compounds to reduce Benedict's uric acid reagent (5) in an alkaline medium. Under his optimal conditions, Ariyama measured between 580 and 870 γ of glyoxal. By halving the volumes of the reagents he could detect as little as 60 γ of glyoxal. A modification of Ariyama's procedure, to be described herein, enables one to measure as little as 2 γ of methylglyoxal and also permits the determination of glyoxalase activity of tissues by examining the disappearance of methylglyoxal.

Materials and Methods

Reagents—
Methylglyoxal, Delta Chemical Works, 30 per cent in water.
Glutathione, Eastman Organic Chemicals, white label.
Arsenic acid, anhydride (arsenic pentoxide), Fisher, c.p.
Orthophosphoric acid, 85 per cent, Baker's Analyzed, c.p.
Sodium tungstate, Merck, reagent.
Sodium cyanide, Fisher, certified reagent, m in water.
Sodium carbonate, anhydrous, Fisher, certified reagent, m in water.
Colorimeter—Beckman quartz spectrophotometer, model DU, with photomultiplier attachment. Cuvettes, Beckman, Pyrex, No. 2097.

Rat Liver Extract—The extract was prepared from rat liver according to the method of Cohen (6), with the exception that only one-thirtieth volume of acetate buffer rather than one-third volume was used to precipitate the homogenate.1

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† Lederle Medical Faculty Award recipient.
‡ Medical Student Research Fellow of the National Foundation for Infantile Paralysis, Inc.
1 Personal communication.
The incubation mixtures contained 0.5 ml. of the enzyme extract (equivalent to an average of 0.52 mg. of N₂), 0.6 mg. of methylglyoxal in 0.5 ml. of distilled water, 0.05 mg. of reduced glutathione in 0.2 ml. of distilled water, and 0.3 ml. of 1.3 per cent NaHCO₃. The gas phase of the incubating vessels was saturated with 95 per cent N₂ and 5 per cent CO₂.

After incubation for various periods of time up to 30 minutes at 37°, 0.5 ml. of the mixture was added to 8.5 ml. of 0.083 N H₂SO₄. Then 1.0 ml. of 3 per cent Na₂WO₄·2H₂O was added. After centrifugation, the methylglyoxal remaining in an aliquot was determined colorimetrically by the procedure to be described below, and that amount subtracted from a similarly prepared sample, but precipitated immediately after addition of methylglyoxal to the extract.

**Dog Polymorphonuclear Leucocytes**—The leucocytes were obtained from dog blood by a process based on that described for human leucocytes (7). The dog was anesthetized with sodium pentobarbital (30 mg. per kilo intravenously). After cardiac puncture with a No. 13 gauge needle, blood was allowed to flow through Tygon tubing with an inner diameter of 6 mm. into a 250 ml. centrifuge bottle containing 5 per cent dextran² in saline and heparin sodium. The final concentration of dextran was 0.2 ml. per ml. of blood. The final concentration of heparin sodium was 0.04 mg. per ml. of blood. After the erythrocytes were allowed to settle, the supernatant plasma containing the leucocytes was centrifuged and treated as previously described (7). The vessels contained 0.5 ml. of the leucocyte suspension (equivalent to an average of 60,000,000 leucocytes per ml.), 0.6 mg. of methylglyoxal in 0.5 ml. of the buffered saline (7), and 0.5 ml. of buffer. The cells were incubated for various periods of time up to 30 minutes in air at 37°. It was not necessary to add exogenous glutathione since the leucocytes were intact cells; nor was it necessary to saturate the gas phase with nitrogen since the glyoxalase enzyme in leucocytes is very active under aerobic conditions. After the incubation period, the cells were precipitated by the modified Folin and Wu tungstic acid procedure (8), as described above, for the liver extract, and the amount of methylglyoxal utilized was calculated by subtracting the amount remaining in an aliquot from the amount in an aliquot of a cell sample precipitated immediately after mixing the cells and substrate.

**Measurement of Methylglyoxal**—According to the modification developed in this laboratory, the sample containing methylglyoxal is added to a 13 X 75 mm. tube. Sufficient distilled water is added to bring the volume to 3.0 ml. Then, in order, 0.1 ml. of the arsenophosphotungstic acid reagent, prepared as described by Ariyama (4), 0.1 ml. of m NaCN, and 0.3 ml. of

² The dextran was a product of the Commercial Solvents Corporation, lot No. NA-649, and was generously contributed by Dr. Homer E. Stavely.
m Na₂CO₃ are added. The blank tube contains 3.0 ml. of distilled water in addition to the other reagents. After standing at room temperature (26-29°) for 60 minutes, the solutions are read at 705 mμ in the Beckman spectrophotometer.

Results

Factors Affecting Color Development

Absorption Spectrum—The blue color developed in this reaction has an absorption peak between 700 and 710 mμ.

Range of Methylglyoxal Concentrations—Between 2 and 16 μ of methylglyoxal may be conveniently determined by this method. The line of regression for the various concentrations of methylglyoxal, calculated according to the straight line formula \( y = a + bx \), was \( y = 0.006 + 0.047x \). The standard error of the estimate \((Sy)\) was 0.029 (9).

Recovery of Methylglyoxal from Biological Material—Quantities of methylglyoxal as low as 60 μ could be quantitatively recovered from rat liver homogenates or suspensions of dog leucocytes by using either trichloroacetic acid as the precipitant or the modified tungstic acid filtrates described above. However, only 44.6 per cent of the added methylglyoxal could be recovered from zinc hydroxide filtrates (10) of rat liver homogenates.

Sodium Carbonate—An excess of sodium carbonate yields precipitates, and the color is stable for a shorter period of time. With 0.5 ml. of the carbonate solution, color development was maximal within 30 to 60 minutes, but the color was stable for less than 1 hour.

Time—60 minutes are necessary for maximal color formation at room temperature, and the color so formed is stable up to 180 minutes.

Temperature—Heating does not increase the rate of color development. In fact, heating the tubes at 40° appreciably decreases the color intensity; nor did allowing the tubes to stand in the refrigerator (8-10°) enhance the rate or intensity of color development.

Reaction of Related Compounds with Reagents—Table I shows that certain compounds structurally related to methylglyoxal and certain other reducing agents react with the Benedict reagent. Both glyoxal and phenylglyoxal reduce the reagent. Also uric and ascorbic acids produce color in this system.

Glyoxalase Activity

Rat Liver Extract—It is apparent from Table II that this extract possesses a high degree of glyoxalase activity, as evidenced by the rapid disappearance of methylglyoxal.
### TABLE I

Color Produced by Certain Compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Optical density*</th>
<th>μmole†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylglyoxal</td>
<td>0.475</td>
<td>0.138</td>
</tr>
<tr>
<td>Glyoxal</td>
<td>0.629</td>
<td>0.172</td>
</tr>
<tr>
<td>Phenylglyoxal</td>
<td>0.398</td>
<td>0.075</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.176</td>
<td>0.057</td>
</tr>
<tr>
<td>Uric acid</td>
<td>0.328</td>
<td>0.059</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>0.018</td>
<td>0.111</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>0.013</td>
<td>0.115</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>0.052</td>
<td>0.333</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>0.017</td>
<td>0.227</td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>0.009</td>
<td>0.172</td>
</tr>
<tr>
<td>Glyceraldehyde</td>
<td>0.008</td>
<td>0.111</td>
</tr>
<tr>
<td>Glyoxalic acid</td>
<td>0.024</td>
<td>0.135</td>
</tr>
<tr>
<td>Diacetyl</td>
<td>0.013</td>
<td>0.116</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.020</td>
<td>0.056</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.014</td>
<td>0.056</td>
</tr>
<tr>
<td>Glutathione</td>
<td>0.007</td>
<td>0.032</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.043</td>
<td>0.082</td>
</tr>
</tbody>
</table>

* Each compound was tested for the intensity of color produced by 10 γ, and measured at 705 mμ under the same conditions as those described in the text.
† Number of micromoles represented by 10 γ of each compound.

### TABLE II

Glyoxalase Activity of Rat Liver Extract and Dog Leucocytes

<table>
<thead>
<tr>
<th></th>
<th>Per cent added methylglyoxal utilized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min.</td>
</tr>
<tr>
<td>Rat liver extract*</td>
<td>26.4</td>
</tr>
<tr>
<td>Polymorphonuclear leucocytes (dog)†</td>
<td>39.1</td>
</tr>
</tbody>
</table>

* The tubes contained 0.5 ml. of enzyme extract (equivalent to an average of 0.52 mg. of N₂), 0.6 mg. of methylglyoxal, and 0.05 mg. of reduced glutathione. The samples were incubated at 37°C; pH 6.8.
† The tubes contained an average of 30,000,000 leucocytes and 0.6 mg. of methylglyoxal. The samples were incubated at 37°C; pH 7.2. Bird et al. (11) present a conversion factor which states that 1,000,000 leucocytes are equivalent to an average of 21.7 γ of N₂.

Dog Polymorphonuclear Leucocytes—Table II also indicates that leucocytes too have an active glyoxalase system on the basis of their rapid utilization of methylglyoxal.
DISCUSSION

This report describes a microadaptation of a standard calorimetric analysis for the general group of glyoxals. Specifically the variations allow for the determination of microgram quantities of methylglyoxal. The availability of such a method to measure the disappearance of such small amounts of methylglyoxal provides a precise method for the study of glyoxalase activity in various tissues, circumventing the problem of CO₂ retention encountered in the usual manometric assay for this enzyme. Also it is possible by this method to substantiate manometric results with a simple colorimetric method.

Analysis of glyoxalase from two different sources is presented in this study. Both an extract of rat liver and a suspension of leucocytes from dog blood exhibited a high degree of enzyme activity when this colorimetric analysis to measure substrate disappearance was used. Calculating enzyme activity on the basis of nitrogen content shows that the rat liver extract utilized approximately 2.2 mg. of methylglyoxal per mg. of N₂ per hour. By the factor of Bird et al. (11), which states that 1,000,000 leucocytes contain an average of 21.7 γ of N₂, it can be calculated that in this system the dog leucocytes utilized approximately 1.7 mg. of methylglyoxal per mg. of N₂ per hour. The slightly lower activity of the leucocyte preparation might possibly be explained on the basis of the more alkaline pH in this incubation mixture (Table II), since the pH optimum of the rat liver glyoxalase seems to be between pH 6.6 and 7.0 (12). However, in view of the approximation relating cell count to N₂ content in the leucocyte preparation, it is evident that these two sources of glyoxalase display essentially the same degree of enzyme activity. The apparently high activity of the enzyme from both sources is in agreement with Cohen's original work with the extract (6) and with the previously reported existence of an active glyoxalase system in human leucocytes (13).

Because of the rapid rate of conversion of methylglyoxal to lactic acid by glyoxalase, this method is not immediately applicable to an investigation in which it is desired to demonstrate biosynthesis of methylglyoxal. However, it is conceivable that the availability of such a method as this for substrate analysis might aid in the elucidation of the rôle of methylglyoxal and glyoxalase in metabolism.

SUMMARY

1. The determination of glyoxalase activity in an extract of rat liver and a suspension of leucocytes from dog blood by studying the disappearance of methylglyoxal colorimetrically is described.
2. The two enzyme sources exhibit essentially the same degree of activity.

3. The simple colorimetric analysis employed in this study is a microadaptation of an earlier method, the ability of methylglyoxal to reduce Benedict's uric acid reagent being employed. With this variation, quantities as low as 2 μg of methylglyoxal may be determined conveniently.

4. Certain factors involved in the colorimetric procedure are outlined. The absorption maximum lies between 700 and 710 μm. An excess of alkali produces a precipitate. Room temperature is the optimal temperature because heating the samples decreases the maximal intensity of color produced. 1 hour is necessary for the maximal color development, which remains stable for an additional 2 hours. Certain other compounds which produce color in the system are mentioned.

BIBLIOGRAPHY

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