PREPARATION OF TISSUE EXTRACTS FOR THE DETERMINATION OF MALIC ACID*

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Hummel's (1) fluorometric determination of malic acid requires the precipitation of calcium malate by alcohol as a means of fractionation from biological materials. Fructose-1,6-diphosphate, glucose-6-phosphate, or glycogen if present must be removed. Below is described a method for preparing extracts of tissues which applies the principles of chromatography on silica gel. Osazone formation, precipitation of malate, and preliminary hydrolysis for the removal of glycogen are thereby eliminated, since interfering carbohydrates do not appear with malic acid in the chromatographic zone selected. When the same chromatographic column that is employed for the preparation of the malate for analysis separates simultaneously other acids of the Krebs cycle (2), the determination has enhanced utility.

Procedure

Samples of tissue ranging from 0.5 to 5 gm. were minced in acetone containing 3 ml. of 10 N sulfuric acid per liter of solvent, and the suspensions were made to 100 ml. in volume with the solvent. After standing for 2 to 3 hours, the mixture was filtered through Whatman paper No. 1 and aliquots representing 0.5 to 2 gm. of tissue were placed in beakers. These aliquots were dried in moving air and the residues were mixed with 1 ml. of 0.5 N sulfuric acid and 1 gm. of silica gel prepared as previously described (3). To the mixture were then added 15 ml. of 50 per cent (volume per volume) butanol in chloroform solution, and the suspension was poured into a vertically supported glass tube 10 mm. in diameter, having a cotton plug at a constricted bottom. When the suspension settled to a continuous mass, 35 ml. of the 50 per cent butanol-chloroform mixture were added to the top of the column and the entire effluent was collected in a 50 ml. beaker placed in a path of moving air so that evaporation continued throughout the extraction. Following the evaporation of the solvent, the contents were dissolved in 0.75 ml. of 30 per cent (volume per volume) tertiary amyl alcohol-chloroform and transferred to a 1 ml. volumetric flask.

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the washings completing the volume. One-half of this sample was placed on a silica gel column (2) formed from 1 gm. of silica gel suspended in chloroform. Glass tubing having a diameter of 6 mm. was used and the silica gel columns had an average height of 20 cm. 30 per cent tertiary amyl alcohol-chloroform was the solvent added. The first 15 ml. of the effluent were discarded and the next 30 ml. were collected in a vessel calibrated for this volume. When 30 ml. had collected, the contents were mixed and 10 ml. aliquots were evaporated in air. The orcinol-sulfuric acid mixture was added directly to the dry residues, and the procedure from this point followed that of Hummel (1).

**Calibration**

Although previous studies (2, 3) had indicated that chromatography on silica gel in general is applicable to the determination of malic acid, the recovery of malate from the assigned portion of the effluent was examined.

<p>| TABLE I |
|---|---|---|---|---|
| <strong>Recovery of Malate from Effluent</strong> |
| <strong>Values in micrograms.</strong> |</p>
<table>
<thead>
<tr>
<th>No. of chromatograms</th>
<th>Malate taken</th>
<th>Average malate found</th>
<th>Standard deviation (for measurement)</th>
<th>Average recovery per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>9.5</td>
<td>9.2</td>
<td>±0.3</td>
<td>97</td>
</tr>
</tbody>
</table>

The data shown in Table I indicate that the effluent collected (16th to the 45th ml.) contains over 95 per cent of the malate added to the column.

**Application to Tissues**

In applying the procedure to tissues, it was first determined whether the malate zone of the effluent contained materials which would interfere with the fluorometric method of analysis. Samples of tissues were chromatographed by obtaining 1 ml. fractions of the effluent instead of collecting the entire acid zone without fractionation. The fractions were mechanically collected with the Technicon (4) collector, dried in air, and analyzed fluorometrically for malic acid. The symmetry of the curves resulting when the observed fluorescence is plotted against the fraction number served as an index of the homogeneity of the effluent band. The symmetry of such curves was examined by plotting the cumulative observed fluorescence, treated as frequencies, and expressed as cumulative per cent (5), against the fraction number on arithmetic probability paper.

Data for a typical experiment in kidney, comparing observed and theoretical points on the curve, appear in Fig. 1. These data indicate that
Fig. 1. A comparison of the observed chromatographic curve for malic acid in rat kidney (solid line) with the normal distribution curve (dotted line (5)). The observed fluorescence readings from alternate fractions are treated as frequencies and expressed as cumulative per cent (abscissa). The fraction number (ordinate) numerically equals ml. of effluent. The sample weighed 0.93 gm., and the concentration of malic acid found, expressed as mg. per cent of wet tissue, was 1.5.

Table II
Concentration of Malic Acid in Tissues of Rat and Mouse

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Rat* (5 per group)</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Standard deviation</td>
</tr>
<tr>
<td></td>
<td>mg. per cent</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.36 ±0.08</td>
<td>4</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.88 ±0.26</td>
<td>3</td>
</tr>
<tr>
<td>Brain</td>
<td>1.26 ±0.21</td>
<td>4</td>
</tr>
</tbody>
</table>

The rats were males of the Long-Evans strain, weighing approximately 150 gm., fasted 6 hours. The mice were males, C3H strain, weighing approximately 20 gm., fasted 6 hours.

* All determinations were in duplicate.

the experimental curve approached the normal distribution curve (5) through the greatest number of points.

In this experiment, the observed concentration of malate in the tissue...
was 1.50 mg. per cent, which compared with the 1.45 mg. per cent obtained on an aliquot of the same tissue analyzed without fractionation of the malate zone.

RESULTS AND DISCUSSION

In Table II are indicated measurements of malic acid by this procedure in several tissues of the rat and mouse. In both species the malate content of the kidney is greatest. Unlike that of fumarate (2), the malate concentration of the brain is less than that of the kidney. All mouse tissues tested showed a higher concentration of malic acid than corresponding tissues of the rat. The foregoing combination of the chromatographic and fluorometric procedure couples the specificity of the former with the sensitivity of the latter. In the analysis for Fig. 1 the weight of the tissue sample was less than 1 gm.

SUMMARY

A procedure is described for the preparation of extracts of certain tissues for the fluorometric determination of malic acid. The application of the method to kidney, liver, and brain of the fasted rat and mouse showed that the highest concentration occurred in kidney. Concentrations of malate in the tissues of the mouse were higher than those in the corresponding tissues of the rat.

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