CHROMATOGRAPHIC DETERMINATION OF THE ACIDS OF THE CITRIC ACID CYCLE IN TISSUES*

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(Received for publication, July 2, 1951)

Many methods are to be found in the literature for the determination of several of the individual acids of the "citric acid cycle;" however, to date no method has been available for the simultaneous determination of all of the acids in a single sample of tissue. Isherwood (1) employed partition chromatography on silica gel to separate and determine subsequently a group of acids present in fruit, some of which are members of the tricarboxylic acid cycle. This work suggested to Marshall, Orten, and Smith (2) the possible applicability of partition chromatography to the determination of the acids present in animal tissues. These workers succeeded in separating and quantitatively determining fumaric acid in muscle, liver, kidney, brain, and blood by this procedure (2); during this investigation, several other acids of the citric acid cycle were located on the chromatogram (3). More recently, Marshall et al. (4–6) have also determined α-ketoglutaric, aconitic, malic, lactic, and succinic acids by the same procedure. Partition chromatography has also been employed by Marvel and Rands (7) for the determination of the chemical constitution of organic polymers.

In the present study, the work of Marshall and coworkers was extended and a method for the simultaneous determination of fumaric, α-ketoglutaric, oxalacetic, succinic, lactic, pyruvic, malic, cis-aconitic, isocitric, and citric acids was developed.

EXPERIMENTAL

Reagents—

4 per cent tertiary amyl alcohol-chloroform mixture; 4 ml. of tertiary amyl alcohol diluted to 100 ml. with chloroform.

10 per cent tertiary amyl alcohol; 10 ml. of tertiary amyl alcohol diluted to 100 ml. with chloroform.

* Aided by a grant from the Council on Pharmacy and Chemistry, American Medical Association. The data in this paper have been taken from the dissertation submitted by Charles E. Frohman for the degree of Doctor of Philosophy, Wayne University, 1951. A preliminary report was presented before the meeting of the American Society of Biological Chemists at Cleveland, May, 1951.

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12 per cent tertiary amyl alcohol; 12 ml. of tertiary amyl alcohol diluted to 100 ml. with chloroform.

Silica gel, prepared and standardized according to the method of Marshall, Orten, and Smith (2).

All the materials used in this investigation were "reagent grade."

Development of Method—The chromatographic column was prepared as follows: 0.15 ml. of concentrated sulfuric acid and 1 ml. of water were added to 3 gm. of silicic acid as prepared above. When the mixture had become smooth and pasty, 50 ml. of chloroform were added in 5 ml. portions with vigorous stirring between the additions. The resulting smooth, lump-free homogeneous slurry was poured into a glass tube 8 mm. in diameter and 75 cm. long. 200 ml. of chloroform were then passed through the silica gel in order to pack the column, after which it was ready for use. The silica gel was not used for more than one determination.

The acids to be investigated were first placed individually in the column to determine the order in which they were eluted. The purity of each acid had been established previously by its melting point and neutral equivalent. 1 ml. of a solution containing between 1 and 20 mg. per cent of the acid in 4 per cent tertiary amyl alcohol-chloroform was introduced into the column and, when the level of the solution had dropped to that of the silica gel, 50 ml. of chloroform were added. When the level had again reached that of the silica gel, 50 ml. of 4 per cent tertiary amyl alcohol-chloroform were introduced, followed in turn by 50 ml. each of 10 per cent and 12 per cent tertiary amyl alcohol-chloroform mixture. These amounts and concentrations of amyl alcohol in chloroform were found to separate and elute all of the acids under investigation. All eluates were collected in 2 ml. fractions with a mechanical fraction collector. 2 ml. of distilled water were added to each fraction which was then titrated with 0.05 N sodium hydroxide with a Rehberg micro burette and phenolphthalein as the indicator. After the individual acids were located, mixtures of the acids were separated on the column. A typical chromatogram of one of these mixtures is shown in Fig. 1. It is evident that a satisfactory separation of the various acids was obtained.

The positions of the acids in the chromatogram were further verified by other methods. The ultraviolet absorption spectra were employed to check the identity of cis-aconitic, fumaric, pyruvic, α-ketoglutaric, and oxalacetic acids (8). Chemical isolation was used to verify fumaric acid. In each case, the expected acid was identified as present in its established fraction of the chromatogram.

Recoveries were made from mixtures of the acids, both in pure solutions and after addition to tissues. The results of the latter studies are shown in Table I. The data show that satisfactory recoveries from tissues were
obtained for all the acids. Equally good recoveries were obtained from pure solutions.

I. Acetic acid
2. Fumaric acid
3. \(\alpha\)-Ketoglutaric acid
4. Oxalacetic acid
5. Succinic acid
6. Lactic acid
7. Pyruvic acid
8. Malic acid
9. Aconitic acid (cis)
10. Iso-citric acid
11. Citric acid

![Typical chromatogram of a mixture of acids in pure solution](image)

**Table I**

<table>
<thead>
<tr>
<th>Acid</th>
<th>Added</th>
<th>Found</th>
<th>Per cent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fumaric</td>
<td>87</td>
<td>85</td>
<td>98</td>
</tr>
<tr>
<td>Oxalacetic</td>
<td>64</td>
<td>58</td>
<td>98</td>
</tr>
<tr>
<td>(\alpha)-Ketoglutaric</td>
<td>92</td>
<td>90</td>
<td>98</td>
</tr>
<tr>
<td>Succinic</td>
<td>71</td>
<td>69</td>
<td>97</td>
</tr>
<tr>
<td>Lactic</td>
<td>120</td>
<td>114</td>
<td>95</td>
</tr>
<tr>
<td>Pyruvic</td>
<td>67</td>
<td>63</td>
<td>94</td>
</tr>
<tr>
<td>Malic</td>
<td>82</td>
<td>80</td>
<td>98</td>
</tr>
<tr>
<td>cis-Aconitic</td>
<td>48</td>
<td>45</td>
<td>94</td>
</tr>
<tr>
<td>Isocitric</td>
<td>64</td>
<td>62</td>
<td>97</td>
</tr>
<tr>
<td>Citric</td>
<td>56</td>
<td>58</td>
<td>104</td>
</tr>
</tbody>
</table>

The range of effectiveness of the above procedure varies from approximately 1 to 15 mg. of total acids per sample. These values, however, would obviously depend upon the amounts of the individual acids present and their ease of resolution.

**Determination of Levels of Acids in Tissues of Normal Fasted Rats**—Male albino rats of the Sprague-Dawley strain weighing from 200 to 300 gm.
were used throughout the investigation. The tissues of ten rats were pooled for each determination. The rats were fed a purified basal diet consisting of 20 per cent casein, 30 per cent sucrose, 41 per cent glucose, 4 per cent cottonseed oil, 4 per cent salt mixture (9), and an adequate vitamin mixture. 24 hours before the rats were sacrificed, food was withheld. At the time of sacrifice, each animal was given 0.3 ml. of 0.1 per cent nembutal by subcutaneous injection. When the rat was sufficiently anesthetized, as much blood as possible was removed by aortal puncture. The blood was immediately heparinized, pooled with the blood of the other rats, and frozen with solid carbon dioxide. The skull of the rat was then

**Table II**

*Average Concentrations and Standard Deviations* of Organic Acids in Tissues of Normal Rats Fasted for 24 Hours

The values are expressed in mg. per 100 gm. of tissue.

<table>
<thead>
<tr>
<th>Acid</th>
<th>Brain</th>
<th>Liver</th>
<th>Kidney</th>
<th>Muscle</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg. per cent</td>
<td>mg. per cent</td>
<td>mg. per cent</td>
<td>mg. per cent</td>
<td>mg. per cent</td>
</tr>
<tr>
<td>Fumaric</td>
<td>13.9 ± 1.2</td>
<td>8.5 ± 0.2</td>
<td>7.1 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>α-Ketoglutaric</td>
<td>19.0 ± 1.2</td>
<td>2.8 ± 0.1</td>
<td>2.5 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Oxalacetic</td>
<td>8.5 ± 0.2</td>
<td>0.85 ± 0.2</td>
<td>0.75 ± 0.2</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Succinic</td>
<td>4.0 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Lactic</td>
<td>121.0 ± 10.0</td>
<td>12.1 ± 0.3</td>
<td>10.8 ± 0.2</td>
<td>8.3 ± 0.1</td>
<td>13.6 ± 0.2</td>
</tr>
<tr>
<td>Pyruvic</td>
<td>17.2 ± 0.6</td>
<td>2.9 ± 0.2</td>
<td>2.45 ± 0.2</td>
<td>2.2 ± 0.1</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Malic</td>
<td>3.2 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>cis-Aconitic</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Isocitric</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Citric</td>
<td>5.3 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>&lt;0.1</td>
<td>1.4 ± 0.1</td>
</tr>
</tbody>
</table>

* Standard deviation = \( \sqrt{\frac{\sum d^2}{n}} \).
C. E. FROHMAN, J. M. ORTEN, AND A. H. SMITH

any volatile acids originally present in the sample as well as the added hydrochloric acid. 2 ml. of 10 per cent tertiary amyl alcohol-chloroform mixture were then added to the residue and allowed to stand with occasional shaking for 1 hour. This solution was washed into the column with chloroform and the acids were separated and determined as described in the previous section. The average levels and standard deviations of the acids in the brain, kidney, liver, muscle, and blood of these animals are shown in Table II. The values given are averages of four separate determinations, each in duplicate, with the pooled tissues of ten rats for each run.

The levels of the acids were also determined in the tissues of the animals fasted for 72 hours. The results of these determinations are shown in Table III. These values are averages of two separate determinations, again in duplicate, on pooled tissues. It can be seen that the extra period of fasting caused no significant change in the levels of the determined acids.

**DISCUSSION**

The values for the concentrations of the various acids in the tissues studied agree in general with those found in the literature obtained by determinations of the acids individually. The values for pyruvate and lactate also agree closely with those obtained in this laboratory by standard colorimetric methods.

The levels of the acids in brain found in this study are higher than those in other tissue. However, it is believed that the values, at least for lactic
and pyruvic acids, do not represent the concentrations present in vivo but are elevated due to traumatic changes induced during the removal of the brain. Indeed, Stone has shown that pyruvate and lactate levels in brain rise over 800 per cent within 5 minutes after the spinal cord is severed (10).

One of the difficulties encountered in determining the order in which the acids emerged from the column was presented by oxalacetic acid. This acid exists in three forms, cis and trans enol forms and a keto form. Since by passing through the keto form, the trans and cis isomers are easily interchangeable, both forms can exist in the column. The trans form of the acid leaves the column much sooner than does the cis form. As the trans form is eluted, however, the equilibrium between the trans and cis forms is disturbed, thus causing the slow conversion of all cis-oxalacetic acid to the trans form. The result of this would be a chromatogram consisting of a peak representing trans-oxalacetic acid, followed by a somewhat lower plateau representing the oxalacetic acid converted in the column from cis to trans. Since the succinic acid fraction appears in the same region in the chromatogram, the value obtained obviously represents a summation of these two acids. Attempts to remove oxalacetic acid from the tissues and determine it separately were unsuccessful because they invariably resulted in destruction of some other acid present in tissues. However, recoveries of 90 per cent were obtained with oxalacetic acid despite the incomplete separation. Since the concentration of oxalacetic acid in tissues is extremely low, the remaining 10 per cent affected the chromatogram of succinic acid insignificantly. The problem of oxalacetic acid in this type of determination is not completely solved. Large amounts of oxalacetic acid would invalidate this portion of a chromatogram.

During the study, it was found that a racemic mixture of malic acid left the column in a fraction different from that in which either L- or D-malic acid appeared. This observation demonstrated the importance of preventing the racemization of optically active isomers in a study of this type.

Another point of caution should be raised with reference to the importance of the pH of the silica gel; variations in its pH may change markedly the position of an acid in the chromatogram, a point which emphasizes the necessity of maintaining a close control of the pH of the system.

**SUMMARY**

A procedure for the separation and simultaneous quantitative determination of fumaric, α-ketoglutaric, oxalacetic, succinic, lactic, pyruvic,
malic, cis-aconitic, isocitric, and citric acids in animal tissues by partition chromatography on silica gel is presented.

The method has been employed satisfactorily for determining amounts of total acids varying from approximately 1 to 15 mg. per sample of tissues. The levels of the above acids were determined in brain, liver, kidney, muscle, and blood of normal rats which had been fasted for 24 and for 72 hours, respectively. No significant differences were found in the concentrations of the acids in the tissues of the rats of the two groups.

BIBLIOGRAPHY

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