ISOLATION OF ASCORBIC ACID 2-SULFATE FROM SELECTED RAT ORGANS

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SUMMARY

Rats were injected subcutaneously with $^{35}$SO$_4^{2-}$ and [1-$^{14}$C]ascorbic acid. A compound was isolated from rat liver and spleen which had chromatographic properties identical to synthetic ascorbic acid 2-sulfate (AAS). This compound gave a positive methanolic FeCl$_3$ reaction, contained radioactive sulfur, and on hydrolysis it degraded to $^{35}$SO$_4^{2-}$ and [1-$^{14}$C]ascorbic acid. The isolated compound possessed identical $\lambda_{\text{max}}$ ultraviolet absorption to that of AAS and co-crystallized with standard AAS to constant specific activity. These data collectively indicate the isolated compound was AAS. Quantitation by ultraviolet techniques indicated the liver possessed a concentration of 369 $\mu$M AAS. Radiochemical and chromatographic evidence is also presented for the presence of AAS in rat urine and adrenal glands.

INTRODUCTION

Ascorbic acid 2-sulfate (AAS) has been synthesized and characterized by a number of investigators$^{1-3}$. It absorbs strongly in the ultraviolet, and is dianionic under physiological conditions. It gives a brick-red color when sprayed with methanolic FeCl$_3$ and it possesses cation-dependent chromatographic properties. We$^{2,4,5}$ as well as others$^1$ have proposed AAS as a possible intermediate in biological sulfation. The ability of AAS to act as an in vivo sulfation agent has been demonstrated$^6,7$. Mead and Finamore$^3$ reported the isolation of AAS from brine shrimp eggs. Baker et al.$^7$ have found AAS in human urine.

We now are reporting the isolation and identification of AAS in rat liver and spleen from rats that were injected subcutaneously with $^{35}$SO$_4^{2-}$ and [1-$^{14}$C]ascorbic acid. In addition, radiochemical and chromatographic evidence is presented for its presence in rat urine and adrenal glands. This is the first reported identification and quantification of AAS in tissues of vertebrates.

Abbreviation: AAS, ascorbic acid 2-sulfate.
MATERIALS AND METHODS

Reagents and equipment

All solvents were redistilled. [\textsuperscript{35}S]AAS was synthesized as previously reported. \textsuperscript{4} Samples were counted with a Tri-Carb liquid scintillation spectrophotometer (Model 526 Packard Instrument Co.). Thin-layer chromatograms were radiochemically scanned with an Actigraph strip recorder (Model 8735 Nuclear-Chicago Corp.). Carrier-free \textsuperscript{35}SO\textsubscript{4}\textsuperscript{2}\textsuperscript{−} and \textsuperscript{14}C ascorbic acid were purchased from New England Nuclear Corp.

Injection of rats

Male white rats (Wistar, approx. 150 g) were placed into three groups and injected subcutaneously with 0.25 ml of the following solutions per rat per day: Group I (20 rats) with 25 \textmu Ci \textsuperscript{35}SO\textsubscript{4}\textsuperscript{2}\textsuperscript{−} (carrier-free) plus 1.0 mg of ascorbic acid, Group II (5 rats) with 25 \textmu Ci \textsuperscript{35}SO\textsubscript{4}\textsuperscript{2}\textsuperscript{−} (carrier-free) plus 2 \textmu Ci \textsuperscript{14}C ascorbic acid (0.2 mg), and Group III (5 rats) with 2 \textmu Ci \textsuperscript{14}C ascorbic acid (0.2 mg).

The rats were watered and fed (Purina Chow) ad libitum for the entire length of the experiment, 4 days. All materials were dissolved in buffered saline (pH 7.4) prior to injection. The rats were sacrificed by cervical dislocation and the liver and spleen were immediately excised and frozen at \(-40^\circ\)C until analyzed.

An additional four rats were injected intracardially with 25 \textmu Ci \textsuperscript{35}SO\textsubscript{4}\textsuperscript{2}\textsuperscript{−} (carrier-free). The urine was collected and pooled. The rats were sacrificed after 40 h and the adrenal glands were immediately extracted.

Isolation of AAS

The livers and spleens (approx. 40 g per sample, wet weight) were homogenized for 30 min (Virtis Homogenizer) in 100 ml of chloroform-methanol (2:1, v/v) to which 30 ml of water had been added. The cellular debris was removed by centrifugation. The organic-aqueous extract was placed in a separatory funnel and KCl (20\%) was added to effect the complete separation of the emulsion. The aqueous phase was separated, evaporated to dryness under vacuum, and brought back to minimal volume with distilled water. Sufficient cold absolute ethanol was added to produce a solution of 80\% ethanol to effect deproteinization. The protein was removed by centrifugation and the resulting ethanolic solution was concentrated under vacuum to a small volume and used for subsequent chromatographic analyses.

The adrenal glands and urine of rats injected intracardially with 25 \textmu Ci \textsuperscript{35}SO\textsubscript{4}\textsuperscript{2}\textsuperscript{−} (carrier-free) were extracted as previously described. The aqueous extracts were examined for the presence of [\textsuperscript{35}S]AAS by thin-layer chromatography and paper chromatography.

Chromatography

Thin-layer chromatography was performed with 0.5-mm thick Silica gel (Supelcosil 12B, Supelco, Inc.) and with Eastman Chromagram Sheet (Distillation Products, Inc.). Two solvent systems were used with thin-layer chromatography: chloroform-methanol-water-acetic acid in two combinations (65:50:15:1, by vol.) and (65:50:15:2, by vol.) Two solvent systems were also used with paper chromatography: phenol-water (100:40, w/w); \textsuperscript{1}butanol-propionic acid-water (10:5:7, v/v/v) utilizing Whatman No. 4 paper. AAS was visualized on thin-layer chromatography and paper chro-
matography by a FeCl₃ spray (1% in methanol). This spray is specific for 3-substituted ascorbic acids. Visualization was also effected on thin-layer chromatography with a light H₂SO₄ char.

RESULTS

The liver and spleen extracts from Group I, II and III rats were chromatographed in two thin-layer solvent systems and two paper chromatographic solvent systems (see experimental). A brick-red colored band which possessed Rₚ values identical to that of standard AAS (Table I) was observed in each chromatogram when it was sprayed with 1% methanolic FeCl₃. Analysis of the chromatograms with a one-dimensional radioactive scanner and X-ray film indicated the positive FeCl₃ area of the chromatograms possessed radioactivity and co-chromatographed with standard [³⁵S]AAS. Chromatograms of the extracts of Group II and III rats also possessed a weak radioactive area corresponding to [¹⁴C]ascorbic acid.

TABLE I

<table>
<thead>
<tr>
<th>Extract (group)</th>
<th>Thin-layer chromatography</th>
<th>Paper chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Solvent I</td>
<td>Solvent II</td>
</tr>
<tr>
<td>Liver (I)</td>
<td>0.14</td>
<td>0.40</td>
</tr>
<tr>
<td>Spleen (I)</td>
<td>0.13</td>
<td>0.39</td>
</tr>
<tr>
<td>Liver (II)</td>
<td>0.15</td>
<td>0.38</td>
</tr>
<tr>
<td>Spleen (II)</td>
<td>0.15</td>
<td>0.39</td>
</tr>
<tr>
<td>Adrenals</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Synthetic AAS(K⁺)</td>
<td>0.14</td>
<td>0.39–0.40</td>
</tr>
</tbody>
</table>

Liver and spleen extracts of Groups I, II and III rats were separated on preparative thin-layer chromatography using solvent system chloroform–methanol–water–acetic acid (65:50:15:1, by vol.). The outer edges of the chromatograms were sprayed with methanolic FeCl₃ to locate the AAS. The unsprayed silicic acid portion of the chromatogram corresponding to the Rₚ of the weakly positive brick-red colored band, was removed and eluted with distilled water and this eluate was used for subsequent analysis.

Synthetic AAS (550 mg) was added to a portion of the above eluate from Group I rats (3250 dpm ³⁵S) and the resulting solution evaporated to dryness. The AAS was recrystallized in absolute methanol. The specific activity was 5.3 dpm/mg following the initial crystallization and remained constant throughout three additional recrystallizations. Similar results were obtained in a duplicate experiment.

Aliquots of the above eluate from Groups I and II were hydrolyzed in 0.1 M HCl for 3 h at 60 °C. The hydrolysate was then re-chromatographed. The hydrolysate in Group I showed disappearance of a radioactive spot corresponding to standard AAS, with accumulation of ³⁵SO₄²⁻ at the origin. Chromatograms of Group II hydrolysates showed the disappearance of the radioactive area corresponding to AAS and the accumulation of ³⁵SO₄²⁻ at the origin and a spot corresponding to [¹⁴C]ascorbic acid.

The FeCl₃-positive band, obtained from known aliquots of the liver extracts from Group I rats, was eluted from paper chromatograms and analyzed by ultraviolet spectroscopy. The isolated band possessed a λmax of 233 nm at pH 2.0 and of 254 nm at pH 7.0, identical to that of AAS. A liver concentration of 369 μM AAS was determined using the values ε₂₃₄ nm = 16000 at pH 7.0 and ε₂₅₄ nm = 12800 at pH 2.0. This concentration of AAS may vary with age of the rats and is probably a minimal value since no data was obtained on the accuracy of the quantitative technique. Also Group I rats were injected with 1 mg ascorbic acid per day which may affect the amount of AAS in the liver.

Chromatograms (thin-layer and paper) of the aqueous extracts of the adrenal glands and urine of rats injected intracardially with 25 μCi ³⁵SO₄²⁻ showed the presence of a FeCl₃-positive spot possessing ³⁵S labeling and exhibiting chromatographic properties identical to that of AAS (Table I).

**DISCUSSION**

AAS is a dianion under physiological conditions and possesses cation-dependent chromatographic properties⁴. The original organ extracts contain a mixture of physiological cations and thin-layer chromatography of this solution gave a poorly defined positive FeCl₃ area (Rᵢ₀ 0.15–0.40 in chloroform–methanol–water–acetic acid; 65:50:15:1, by vol.). However, a KCl solution was added to the original extract to effect the complete separation of the emulsion. This KCl solution masks any physiological cations and subsequent thin-layer chromatography gave a sharply defined positive FeCl₃ area.

The radiolabeled compound isolated from rat liver and spleen by chromatographic techniques had identical chromatographic properties to the potassium salt of synthetic AAS. It gave a FeCl₃-positive reaction, and on hydrolysis degraded to ³⁵SO₄²⁻ and ¹⁴C ascorbic acid (Group II). The isolated compound possessed λmax ultraviolet absorption identical to AAS at pH 2.0 and pH 7.0. Recrystallization to constant specific activity indicated a homogeneous radiolabeled mixture of isolated ³⁵S₂AAS and synthetic AAS. These data, collectively, indicate the radiolabeled isolated compound from rat liver and spleen to be ascorbic acid 2-sulfate. In addition, radiochemical and chromatographic evidence is also presented for the presence of AAS in rat urine and adrenal glands.

Rats have the ability to synthesize ascorbic acid in their livers. The ascorbic acid concentration in rat liver has been reported to be 40.2 mg/100 g tissue and varies with age and weight⁹. We are now reporting an AAS liver concentration of 369 μM or 12.3 mg/100 g tissue. The concentration of AAS may depend upon the concentration of the ascorbic acid pool. Thus one would expect to find significantly lower AAS tissue concentrations in the non-ascorbic acid synthesizing animal.

Additional investigations are underway for the purpose of isolating and quantifying AAS in other rat organs as well as human organs.

**ACKNOWLEDGMENT**

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L-Ascorbic acid 2-sulfate has previously been reported as L-ascorbic acid 3-sulfate. However, recent X-ray studies have proven the structure to be L-ascorbic acid 2-sulfate. (B.W. McClalland and J. B. Einstein, Winter Meeting of the American Crystallographic Assoc.).

REFERENCES