THE CONVERSION OF ALLOXAN TO ALLOXANIC ACID IN PLASMA

BY DAVID SELIGSON* AND HARRIET SELIGSON

(From the George S. Coz Medical Research Institute, University of Pennsylvania, Philadelphia, Pennsylvania)

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Since the discovery of alloxan diabetes in 1943 (1), several investigators have discussed the possibility that alloxan might be involved in some way in the pathogenesis of diabetes mellitus. Dunn et al. (1) considered this on the ground that alloxan was chemically related to uric acid. Lazarow (2) reviewed the possible relation of alloxan to diabetes and discussed the protective mechanism of the sulfhydryl compounds. Conn, Louis, and Johnston (3), having demonstrated that adrenocorticotropic hormone (ACTH) caused a fall in blood glutathione and temporary diabetes in human subjects, compared the action of ACTH to that of alloxan, which also lowers blood glutathione and causes glycosuria. The rapid disappearance of alloxan in plasma (4, 5) makes it necessary to determine quantitatively the end-products in investigating the relation of alloxan to diabetes mellitus. In this paper the decomposition products of alloxan have been studied as a contribution to the problem of the relationship of alloxan to diabetes mellitus.

The instability of alloxan has been studied (4–6) and the conversion of alloxan to salts of alloxanic acid in alkali has been described (7–9). Alloxanic acid has been characterized by the elementary analysis of its barium salt (8), of its methyl and ethyl esters (7), and by its oxidation to parabanic acid (7). The conversion of alloxan to alloxanic acid has also been followed by means of the hydrogen ion concentration of the reaction (9). Since these procedures are unsatisfactory for use in biological fluids, we have used hydrolytic and oxidative reactions combined with paper chromatography. With these methods, which have not hitherto been applied to this problem, the decomposition of alloxan in buffers and plasma has been studied.

Methods

The reaction of alloxan with alkali (7) has been employed for the preparation of the alloxanates used in this investigation (I and II). Hydrolysis of alloxan or alloxanate in hot alkali liberates the salt of oxomalonic acid (III) and this reaction was used for the preparation of oxomalonate.

* Postdoctorate Research Fellow of the National Institutes of Health, 1949–50.
CONVERSION OF ALLOXAN IN PLASMA

![Chemical structures](image)

(I) Alloxan monohydrate

(II) Alloxanic acid

(III) Oxomalonic acid hydrate

Determination of Oxomalonic Acid

Oxidation with Ceric Sulfate

Reagents—0.01 M disodium oxomalonate, 0.01 N ceric sulfate in 3 N H₂SO₄, 0.002 N ferrous ammonium sulfate in 3 N H₂SO₄, 18 N sulfuric acid, 0.005 M osmic acid in 0.1 N H₂SO₄, 0.005 M o-phenanthroline.

Procedure—1 ml. of 0.01 M oxomalonate is treated with a measured excess of 0.01 N ceric sulfate, 1 drop of osmic acid catalyst, and sulfuric acid to about 3 N. After standing 15 to 30 minutes, the excess ceric sulfate is titrated with standard ferrous ammonium sulfate with o-phenanthroline as the indicator. Oxidation may be performed without the catalyst by heating to 50° for 5 minutes or by standing at room temperature for 1 to 2 hours. The data in Table I show that the theoretical oxidation of oxomalonic occurs.

Oxidation to Liberate CO₂

Reagents—0.0006 M disodium oxomalonate, 0.4 N ceric sulfate in 3 N H₂SO₄ plus 1 drop osmic acid solution (above) per 2.5 ml. of ceric sulfate.

Procedure—0.5 ml. of oxomalonate is oxidized with 1 drop of the ceric sulfate reagent and the liberated CO₂ is received in Ba(OH)₂ and measured alkalimetrically by a microdiffusion method similar to that of Conway (10). Table I shows that the theoretical amount of CO₂ is obtained.

Determination As 2,4-Dinitrophenylhydrazone

Reagents—0.0002 M disodium oxomalonate, 2,4-dinitrophenylhydrazine, 4 mg. per ml. of 6 N HCl, 1.5 N NaOH.
Procedure—Oxomalonate reacts readily with 2,4-dinitrophenylhydrazine, forming a yellow hydrazone which turns bright red in alkali. 1.0 ml. of oxomalonate is allowed to stand at room temperature with 0.25 ml. of the dinitrophenylhydrazine reagent for 1 hour. 10.0 ml. of 1.5 N NaOH are added and after 10 minutes the color is read in an Evelyn photoelectric colorimeter with a No. 490 filter. The conditions for this procedure were determined from the following data. The reaction between oxomalonate and 2,4-dinitrophenylhydrazine is complete in 60 minutes at 25°. Maximum production of the red color occurs when the hydrazine-hydrazone mixture is brought to a final concentration of NaOH of 1.3 to 1.6 N. The peak of optical density of the hydrazone is 455 μm, but the 490 Evelyn filter is used because it reduces to a negligible amount the interference by color due to the reagent. Under these conditions the colori-

**Table I**

Stoichiometry of Oxidation of Standard Solutions of Alloxan, Alloxanate, and Oxomalonate

<table>
<thead>
<tr>
<th>Compound</th>
<th>Theoretical</th>
<th>Observed*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ce(SO₄)₃ used</td>
<td>CO₂ liberated</td>
</tr>
<tr>
<td></td>
<td>equivalents per mole</td>
<td>moles per mole</td>
</tr>
<tr>
<td>Alloxan</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Alloxanate</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Oxomalonate</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

* Mean ± s.e.m., based on fifteen determinations.

The reaction between oxomalic acid and 2,4-dinitrophenylhydrazine follows Beer's law for the range from 0 to 0.075 M per ml. of final solution.

Reactions of Alloxanic Acid

Oxidation with Ceric Sulfate

Procedure—The methods described for oxomalic acid were used to measure ceric sulfate reduction by alloxanate and CO₂ liberation from it. The data shown in Table I demonstrate that the theoretical reactions are obtained.

Hydrolysis of Alloxanate

On alkaline hydrolysis alloxanate liberates oxomalic acid and the latter can be detected as described above. Hydrolysis at 15 pounds pressure in 0.05 to 1.0 N NaOH for 10 to 30 minutes gives stoichiometric liberation of the keto acid as measured against standard oxomalonate solutions. Acid hydrolysis releases oxomalonate, which partly decomposes in the reaction mixture.
Reaction with Diacetylmonoxime

Archibald (11) showed that yellow color is formed on heating diacetylmonoxime, a sulfuric-phosphoric acid mixture, and alloxanic acid. Fig. 1 shows that when this procedure is used, with the concentrations stated, the rate of color development of urea is almost 20 times that of alloxanate at 10 minutes. This reaction was used to determine whether or not urea or a substituted urea is formed during the breakdown of alloxan in plasma, since the formation of such ureas would lead to far greater color production than that of the known alloxanate concentration.

![Graph showing rate and amount of color formation on heating urea and alloxanic acid.](image)

**Fig. 1.** Rate and amount of color formation on heating urea (0.27 μM per ml.) and alloxanic acid (1.75 μM per ml.) with diacetylmonoxime in a sulfuric-phosphoric acid mixture. The measurements were made with the Evelyn colorimeter with Filter 440. O, urea; ●, alloxanic acid.

Paper Chromatography

Alloxanic acid can be qualitatively and quantitatively identified by isolating it on a partition paper chromatogram. On paper it is sufficiently acid to change brom phenol blue (0.04 per cent) from blue to yellow. When sprayed with a silver ammonia reagent (0.05 M AgNO₃ in 2 M NH₃OH) and exposed to light, the spot turns to a deep brown. The spot may be eluted and the eluate measured for ketones before and after alkaline hydrolysis, as described under oxomalonic acid. The difference is due to the presence of alloxanate. Ascending and descending chromatograms were used. In the former, butanol saturated with 1 N p-toluenesulfonic acid provided a solvent which isolated alloxanate (Rₚ 0.45). The method of Lugg and Overell (12), in which n-amyl alcohol is saturated with 5 N formic acid, gave low Rₚ values. However, when the solvent was allowed...
to run off the paper and the distance which alloxanic acid moved was compared to the distance moved by citric acid (an arbitrary standard), a ratio of alloxanic acid to citric acid of 0.30 was obtained. Oxomalonic acid was also identified by the same methods. It reduced silver to a deep brown color. Its mobility related to citric acid was 0.44 in the formic acid-amyl alcohol system.

Reactions of Alloxan

Oxidation with Ceric Sulfate

Alloxan in acid solution is in a high state of oxidation and does not reduce ceric sulfate or liberate CO₂ as does alloxanic acid (Table I). Dia- luric acid will reduce ceric sulfate to form alloxan (5) and consequently CO₂ is not liberated.

Reaction in Buffered Solutions

Alloxan was added to potassium acid phosphate solution to make a solution which was 0.2 M in phosphate and 0.01 M in alloxan. Water and standard sodium hydroxide were added to 5 ml. of the alloxan solution to double the volume and increase the pH of the solution over the range 5.5 to 8.0 by increments of 0.2 of a pH unit. At exactly 5 minutes after the addition of the alkali an aliquot was added to sulfuric acid, standard ceric sulfate, and osmic acid catalyst, and the excess ceric sulfate measured as described above. At the end of the reaction the pH of the buffer was determined with a glass electrode. The conversion of alloxan to alloxanic acid is measured by the amount of reducing agent formed in the various buffers. The fraction of alloxan that was converted to alloxanic acid as measured by ceric sulfate titration was confirmed by measurement of CO₂ liberation. Fig. 2 shows the per cent conversion of alloxan to alloxanic acid in buffers of varying pH in 5 minutes. Under these conditions the conversion is almost 50 per cent complete at pH 7.0 and 75 per cent complete at pH 8.0. Because this is a first order reaction, the data in Fig. 2 may be recalculated from the equation \( k = \frac{2.30}{t} \log C_0/C \), where \( k \) is the velocity constant, \( C_0 \) is the concentration of alloxan at the start of the reaction, and \( C \) is the concentration at time \( t \). In this way, the half life and the time to 99.5 per cent conversion under these conditions may be estimated. At pH 7.0 these are 5.2 and 40 minutes respectively, whereas at pH 8.0 they are 2.6 and 20 minutes respectively.

In phosphate buffer the rate of conversion of alloxan to alloxanate was measured by the production of alloxanic acid (Fig. 3, Curve 1). A solution of 0.1 M alloxan was diluted 1:10 with 0.1 M phosphate buffer, resulting in a reaction mixture of pH 7.35 at 25°. At measured time intervals,
samples of the reaction mixture were transferred to equal volumes of 5 N sulfuric acid and the samples measured for CO$_2$ produced by oxidation with ceric sulfate. The per cent of alloxan remaining plotted on a semi-logarithmic scale against time is linear, indicating that the conversion of alloxan to alloxanate is a first order reaction with a half life of 3.2 minutes under these conditions.

**Alkaline Hydrolysis**

Alloxan reacts with hot alkali to liberate oxomalonic acid, as does alloxan-

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![Graph 1](image1.png)

**Fig. 2.** Conversion of 0.01 M alloxan to alloxan acid in 0.2 M phosphate buffers of varying pH in 5 minutes at 25°C.

![Graph 2](image2.png)

**Fig. 3.** Rate of conversion of alloxan to alloxanate in buffer (Curve 1) and plasma (Curve 2). The per cent of alloxan remaining in the reaction mixture is plotted on a semi-logarithmic scale. See the text.
may first be converted to alloxanic acid before the hydrolysis to oxomalonic acid occurs.

Results

The foregoing methods were applied to the study of the fate of alloxan in plasma in the following way. A plasma control was prepared by adding 1 volume of water to 9 volumes of plasma. Plasma containing alloxan was prepared by employing 0.1 M alloxan instead of the water used in the control, which thereby produced a solution of plasma containing 10.0 \( \mu M \) of alloxan per ml. of plasma. A solution of alloxanate and plasma was prepared in the same manner, containing 10.0 \( \mu M \) of alloxanate per ml. of plasma.

### Table II

**Recovery from Plasma of Added Alloxan or Alloxanate**

<table>
<thead>
<tr>
<th>Sample</th>
<th>NH(_3)N</th>
<th>Urea N</th>
<th>Non-protein N</th>
<th>Substituted ureas*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per ml.</td>
<td>Added N</td>
<td>Per ml.</td>
<td>Added N</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>recovered</td>
<td></td>
<td>recovered</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.13 (\mu M)</td>
<td>9.0 (\mu M)</td>
<td>23.4 (\mu M)</td>
<td>0.115 (\mu M)</td>
</tr>
<tr>
<td>-=-+ alloxan†</td>
<td>0.38 (\mu M)</td>
<td>1.2 (\mu M)</td>
<td>1.0 (\mu M)</td>
<td>94 (\mu M)</td>
</tr>
<tr>
<td>-=-+ alloxanate†</td>
<td>41.7 (\mu M)</td>
<td>92 (\mu M)</td>
<td>0.308 (\mu M)</td>
<td>84 (\mu M)</td>
</tr>
</tbody>
</table>

* Expressed as alloxanate (see the text).
† Three aliquots of plasma were prepared from a single fresh sample. One of these was the blank; each of the others contained 10 \( \mu M \) per ml. of alloxan or alloxanate, or 20 \( \mu M \) of nitrogen.

The samples were allowed to stand open to the air for 1 hour at room temperature. The whole plasma was measured for free ammonia, urea, and CO\(_2\) (Tables II and III). Substituted ureas were measured by treating 2.0 ml. of plasma with urease to remove urea, deproteinizing with 12 ml. of 3.3 per cent trichloroacetic acid, and carrying out the diacetylmonoxime reaction on 4 ml. of the filtrate. The results are listed in Table II as optical density from the reaction and are compared to a standard alloxanate solution. The three plasma mixtures were deproteinized with 4 volumes of 5 per cent trichloroacetic acid. The filtrates were analyzed for non-protein nitrogen (Table II), CO\(_2\) liberated by ceric sulfate oxidation, and ceric sulfate reduced (Table III). The trichloroacetic acid filtrates were made weakly alkaline with NaOH and hydrolyzed under pressure. The liberation of CO\(_2\) after oxidation, ceric sulfate reduction, and ketone formation were measured on the hydrolyzed specimens (Table IV). The plasma filtrates contained some ketones which were increased slightly.
after hydrolysis (Table IV). The nature of these plasma ketones has not been determined.

**Table III**

*Oxidation of Filtrates of Plasma to Which Alloxan or Alloxanate Was Added*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Free CO₂ per ml plasma</th>
<th>CO₂ from oxidation of filtrate</th>
<th>Ce(SO₄)²⁻ reduced by filtrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>µM per cent</td>
<td>µM per cent</td>
</tr>
<tr>
<td>Plasma</td>
<td>19.0</td>
<td>3.28</td>
<td>3.75</td>
</tr>
<tr>
<td>&quot; + alloxan</td>
<td>16.2</td>
<td>12.50</td>
<td>92</td>
</tr>
<tr>
<td>&quot; + alloxanate</td>
<td>12.84</td>
<td>12.84</td>
<td>91</td>
</tr>
</tbody>
</table>

* Trichloroacetic acid filtrates, prepared 1:5, 1 hour after the addition of the compounds tested.
† Alloxanate is oxidized by 2 equivalents of Ce(SO₄)²⁻ and on complete oxidation liberates 1 µM of CO₂. Therefore, 10 µM of alloxan per ml. of plasma, if converted to alloxanate, are equivalent to 20 microequivalents of Ce(SO₄)²⁻ or 10 µM of CO₂.
‡ The mixture contains 10.0 µM of alloxan or alloxanate per ml. of plasma.

**Table IV**

*Oxidation of Hydrolysates of Filtrates of Plasma to Which Alloxan or Alloxanate Was Added*

<table>
<thead>
<tr>
<th>Sample</th>
<th>CO₂ from oxidation</th>
<th>Ce(SO₄)²⁻ reduced</th>
<th>Ketones liberated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per ml. plasma</td>
<td>Oxomalonate recovered†</td>
<td>Per ml. plasma</td>
</tr>
<tr>
<td></td>
<td>µM</td>
<td>per cent</td>
<td>µg. per cent</td>
</tr>
<tr>
<td>Plasma</td>
<td>11.5</td>
<td>6</td>
<td>15.0</td>
</tr>
<tr>
<td>&quot; + alloxan</td>
<td>30.1</td>
<td>62</td>
<td>42.6</td>
</tr>
<tr>
<td>&quot; + alloxanate</td>
<td>30.5</td>
<td>63</td>
<td>42.1</td>
</tr>
</tbody>
</table>

* Trichloroacetic acid filtrates of plasma, prepared 1:5, were hydrolyzed in dilute alkali and determinations made on the hydrolysates.
† The assumption is made that alloxanate is hydrolyzed to oxomalonate, which in turn is oxidized by 4 equivalents of Ce(SO₄)²⁻ and on complete oxidation liberates 3 moles of CO₂.
‡ Contained 10.0 µM of alloxan or alloxanate, which after hydrolysis is equivalent to 30 µM of CO₂ or 40 microequivalents of Ce(SO₄)²⁻.

The results indicate that negligible amounts of urea and ammonia were liberated when alloxan was added to plasma (Table II). On the other hand, since alloxanate is formed from alloxan, free CO₂ was lost from the plasma. Table II shows that 92 to 94 per cent of alloxan nitrogen and
alloxanic acid nitrogen was recovered in the filtrate. The diacetylmonoxime reaction on a trichloroacetic acid filtrate of the urea-free plasma samples showed that the plasma containing alloxan gave the same color as the plasma containing alloxamic acid. This indicates that no monosubstituted urea is formed by the decomposition of alloxan in plasma, since, as shown in Fig. 1, urea gave considerably more color than alloxanate. The product of the decomposition of alloxan also followed an oxidative and hydrolytic pattern similar to that of alloxamic acid in the trichloroacetic acid filtrates. Therefore, the conversion of alloxan to alloxamic acid was at least 90 per cent complete in plasma at room temperature for 1 hour. The reaction may be complete, since pure alloxanate also was recovered from plasma to the extent of 90 per cent.

On alkaline hydrolysis, the plasma filtrates yielded 60 to 70 per cent of the added alloxan or alloxamic acid as oxomalonic acid. When pure oxomalonic acid was added to plasma and put through the same reactions, essentially complete recovery was obtained in the unhydrolyzed filtrates, but only 50 to 60 per cent was recovered in the hydrolyzed filtrates. This indicates that plasma filtrates contain materials which when heated in alkali destroy oxomalonic acid.

Qualitatively the breakdown product of alloxan in plasma was established by paper chromatography. The trichloroacetic acid filtrate was extracted with ether three times to remove the trichloroacetic acid. The filtrate was concentrated by evaporation and applied to filter paper. The alloxanic acid was moved as described above and identified by its ability to reduce silver. It was extracted from paper, hydrolyzed, and measured as the 2,4-dinitrophenylhydrazone of oxomalonic acid. In this way the conversion of alloxan to alloxamic acid was established qualitatively. The rate of conversion of alloxan to alloxamic acid in plasma was measured as described above for alloxan in buffers. A solution of 0.1 M alloxan was diluted with 9 parts of fresh plasma. Samples of this reaction mixture (temperature 25°C; pH 7.35) were added at measured time intervals to 5 N sulfuric acid to stop the reaction. The CO₂ liberated by oxidation with ceric sulfate was measured in each sample. The CO₂ of the same plasma not containing alloxan subtracted from the above provides the analytical data for establishing the conversion of alloxan to alloxamic acid in plasma. The data so obtained are shown in Fig. 3, Curve 2. The conversion in plasma is a first order reaction as in buffers, although the rate is slightly slower.

**DISCUSSION**

The results indicate that alloxan in plasma and neutral buffers is converted to alloxamic acid under the conditions employed. The change is
stoichiometric and complete in the buffers and probably so in plasma. In plasma 90 per cent of alloxan was recovered as alloxanic acid. No enzyme system is needed, for the reaction is catalyzed by hydroxyl ions, and, as Fig. 2 shows, is rapid. The almost complete conversion of alloxan to alloxanic acid in plasma leaves little alloxan available in blood for detection by chemical methods. However, the relatively stable alloxanic acid is available for determination, and the presence of alloxanic acid in biological material should be good evidence of the previous presence of alloxan. Therefore, a study of alloxanic acid should aid in determining whether or not alloxan is related to uric acid metabolism and human diabetes.

The results presented here indicate that alloxan added to plasma under the conditions described is nearly all converted to alloxanic acid. This does not mean that alloxanic acid is the only product formed under all conditions. Other workers (2, 5) have shown that alloxan can react with certain constituents of plasma to form reaction products other than alloxanic acid. The reaction of smaller amounts of alloxan or the reaction of alloxan with tissues (cells) instead of plasma might well differ from that observed in our experiments. Nevertheless, it seems that the study of alloxan metabolism by means of the conversion to alloxanic acid should be pursued as one method of attacking this complex problem.

SUMMARY

The nature of the decomposition of alloxan in plasma and buffered solutions has been examined. Alloxan is converted to alloxanic acid under the conditions employed. This reaction is complete or nearly so, is more rapid with increasing alkalinity, and is a rapid first order reaction at room temperature. The conversion of alloxan to alloxanic acid has been studied by the following procedures which have not previously been applied to this reaction: (a) Oxidimetry with ceric sulfate, which distinguishes between alloxan, alloxanic acid, and oxomalonic acid. Alkaline hydrolysis results in the stoichiometric formation of oxomalonic acid from alloxan or alloxanic acid. (b) The colorimetric determination, by a method described herein, of oxomalonic acid.

If alloxan plays any part in diabetes mellitus, its rôle might be further elucidated by the study of its more stable conversion product, alloxanic acid.

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David Seligson and Harriet Seligson


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