The Pathway of myo-Inositol Degradation in Aerobacter aerogenes

RING SCISSION*

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The product of the first two reactions in the enzymatic degradation of myo-inositol by extracts of Aerobacter aerogenes, D-2,3-diketo-4-deoxy-epi-inositol, serves as the substrate for another enzyme also present in these extracts. The enzyme was partly purified and could be shown to catalyze, presumably after a preliminary isomerization, the hydrolytic cleavage of the inositol ring. The product of the reaction was isolated and identified by the study of its chemical and physical properties as a 4-deoxy-5-ketohexonic acid.

EXPERIMENTAL PROCEDURE

Materials—The bacterial strains, medium, and sources of chemicals were as previously noted (1). In addition, 2-keto-gluconic acid and 5-ketogluconic acid were obtained from General Biochemicals, myo-inositol-4C from Nuclear-Chicago, and 2,5-diphenyl-3-(4-styrylphenyl)tetrazolium chloride from Calbiochem. Dowex AG1-X8 and Dowex AG50-X8 (200 to 400 mesh) analytical grade resins were supplied by Bio-Rad Company.

The substrate for the assay of diketodeoxyinositol hydrolase, D-2,3-diketo-4-deoxy-epi-inositol, was prepared as described (1).

Chemical Determinations—In addition to the procedures previously noted (1), lactones were determined by the method of Hestrin (2) or, on paper, by the method of Abdel-Akher and Smith (3). A spray reagent active towards ketoses but not aldoses, consisting of a 1:1 mixture of 2,5-diphenyl-3-(4-styrylphenyl)tetrazolium chloride in ethanol and 0.1 N NaOH, was also used (4).

The solvent systems utilized for paper chromatography were: Solvent 1, ethyl acetate-acetic acid-water; Solvent 2: ethyl acetate-pyridine-water; and Solvent 3: ethyl acetate-2-propanol-water.

RESULTS

Diketodeoxyinositol Hydrolase

Enzymatic Degradation of myo-Inositol and of Its Metabolic Products—In order to discover whether D-2,3-diketo-4-deoxy-epi-inositol is an intermediate in the enzymatic degradation of myo-inositol the following experiment was carried out. Approximately 30 μmoles of myo-inositol, 2-keto-myo-inositol, or D-2,3-diketo-4-deoxy-epi-inositol were treated with 0.1 ml of a crude extract of A. aerogenes grown on myo-inositol (1) containing 15 mg of protein per ml, in 1 ml of 0.01 M potassium phosphate buffer, pH 7.0, in the presence and absence of 1 mM NAD+, NADP+, NADH, or NADPH. After a 3-hour period of incubation at room temperature the reaction mixtures were chilled and 0.1 ml of 50% trichloroacetic acid was added. The precipitated proteins were removed by centrifugation and 0.02-ml portions of the supernatants were subjected to descending chromatography on Whatman No. 3HR paper in Solvent 1 for 5 hours.

The two new compounds were eluted with water; the eluates were concentrated to a volume of 0.2 ml and were treated with 0.05 ml of 15% periodic acid and 0.05 ml of 1 M NaOH for 15 min at room temperature; excess periodate was then destroyed by the addition of 0.1 ml of 5 N H2SO4 and 0.2 ml of 1 N sodium arsenite. The samples were then tested for the presence of formaldehyde, malondialdehyde, and β-formylpyruvic acid.

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The compound with $R_f$ of 0.25 yielded malondialdehyde, but no formaldehyde or $\beta$-formylypyruvic acid, on treatment with periodate. Consequently, it appears to possess a methylene group situated between two secondary hydroxyl groups, and no primary hydroxyl group vicinal to a hydroxyl or keto group. These properties, as well as the observed $R_f$ value, are characteristic of the ketodeoxyinositol, the formation of which from $\alpha$-2,3-diketo-4-deoxy-epi-inositol and NADH catalyzed by myo-inositol dehydrogenase was described in the preceding paper (1).

The compound with $R_f$ of 0.4 yielded formaldehyde, but no malondialdehyde or $\beta$-formylypyruvic acid on treatment with periodate. This compound therefore appears to possess a primary hydroxyl group vicinal to a hydroxyl or a keto group; it is not a cyclic compound. Its formation from 2-keto-myo-inositol and from $\alpha$-2,3-diketo-4-deoxy-epi-inositol in the absence of an added hydrogen acceptor suggested that the crude cell extract contained, in addition to the inositol dehydrogenase and the ketoinositol dehydratase, a third enzyme capable of catalyzing the hydrolytic cleavage of the diketo compound.

Spectrophotometric Assay of Diketodeoxyinositol Hydrolase—The fact that the substrate of the enzyme, but not its product, has a strong extinction at 290 $\mu$m suggested a convenient spectrophotometric assay for the enzyme. The reaction was carried out at pH 6, because of the instability of the diketodeoxyinositol at higher pH values. Citrate was chosen as the buffer, when it was found, as will be described later, that phosphate inhibits the enzymatic reaction.

Sufficient $\alpha$-2,3-diketo-4-deoxy-epi-inositol is added to 0.02 M sodium citrate buffer, pH 6.35, in a total volume of 0.98 ml to give an absorbance of approximately 0.6 at 290 $\mu$m (approximately 0.1 mm). The slow decrease of the absorbance, approximately 0.007 per min, is measured for 1 min; then 0.02 ml of enzyme containing between 1 and 5 mg of protein per ml is added. For accurate measurements the decrease in absorbance after addition of the enzyme should be between 0.05 and 0.10 per min. The reaction proceeds linearly for 2 to 4 min and this initial rate of decrease, after correction for the nonenzymatic loss of absorbance, is taken as a measurement of enzyme activity.

This rate is proportional to the amount of enzyme added. A unit of enzyme is the amount that catalyzes the disappearance of 1 $\mu$mole of the substrate per min.

Control of Syntheses of Diketodeoxyinositol Hydrolase—In order to determine the conditions necessary for the synthesis of this enzyme, cells of the wild strain 1033, of the mutant unable to use myo-inositol as major source of carbon, and of the mutant constitutive for the first two enzymes of inositol metabolism, were grown in media containing 0.2% L-histidine with and without 0.2% myo-inositol. The results of this experiment revealed that, just as in the case of the first two enzymes, extracts of the constitutive mutant grown in the absence of inositol contained the highest level of this enzyme (specific activity, 0.15 unit per mg of protein); in the wild strain grown without inositol, and in the mutants unable to metabolize inositol, the enzyme could not be detected; in the wild strain grown with inositol the level was approximately one-third as high as in the constitutive mutant. It appears that all three enzymes are normally induced by myo-inositol and are affected in the same way by the mutations that have been observed so far. Because of the high level of diketo-deoxyinositol hydrolase in extracts of the constitutive mutant, strain C1, this organism, grown on histidine, was chosen for the purification of the enzyme.

Purification of Diketodeoxyinositol Hydrolase—About 5 g (wet weight) of A. aerogenes C1, grown in Werkman minimal medium (0.2% L-histidine), were suspended in 20 ml of 0.02 M potassium phosphate buffer, pH 7, containing $10^{-4}$ M glutathione (hereafter referred to as phosphate buffer), and the cells were disrupted by sonic vibration. All manipulations were carried out at 0-4°C, as described previously (1). The mixture was spun for 90 min in the Spinco model L centrifuge at 39,000 rpm and the supernatant was dialyzed overnight against phosphate buffer. A solution of 2% protamine sulfate was added slowly with stirring to a final concentration of 8 mg/ml of protein, the sample was centrifuged, and the precipitate was discarded. The supernatant was again dialyzed against phosphate buffer for 18 hours. The enzyme solution was then treated with saturated ammonium sulfate. The fraction precipitating between 37 and 47% saturation of ammonium sulfate was collected by centrifugation, redissolved in 3.8 ml of phosphate buffer, and dialyzed against the same buffer overnight. This preparation was placed on a Sephadex G-200 column (2.5 × 30 cm) and the adsorbed protein was eluted with phosphate buffer. Fractions of 1.0 ml were collected and tubes containing activity (Fractions 47 to 54) were pooled and concentrated with sucrose (6) to a volume of 3 ml. Following overnight dialysis against phosphate buffer, a second ammonium sulfate fractionation was carried out. The precipitate which formed between 40 and 50% saturation was found to contain most of the activity, and was taken up in 1.2 ml of phosphate buffer.

At this stage the specific activity of the enzyme had increased 24-fold over the crude extract, with a recovery of about 14% of the total enzyme units. No ketoinositol dehydratase could be detected, although there was still a considerable amount of myo-inositol dehydrogenase activity; the purification procedure had brought about a 2-fold increase in the specific activity of the diketodeoxyinositol hydrolase relative to that of myo-inositol dehydrogenase. An attempt to purify the enzyme further by use of a DEAE-cellulose column was unsuccessful.

TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Activity Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>unit $\times 10^2$</td>
</tr>
<tr>
<td>Centrifuged extract</td>
<td>8</td>
<td>2.2</td>
</tr>
<tr>
<td>Protamine supernatant</td>
<td>10</td>
<td>2.0</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$ precipitate I</td>
<td>18</td>
<td>0.73</td>
</tr>
<tr>
<td>Sephadex eluate</td>
<td>5.0</td>
<td>0.55</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$ precipitate II</td>
<td>7.5</td>
<td>0.30</td>
</tr>
</tbody>
</table>

* One unit is defined as the ability to hydrolyze 1 $\mu$mole of substrate per min.
It was also noted that the addition of phosphate ion slowed the reaction rate (Table II).

**Isolation of Product of Diketodeoxyinositol Hydrolase**—The preliminary experiments had shown that the same product could be obtained when crude cell extracts were incubated under suitable conditions with either myo-inositol or 2-keto-myo-inositol, or D-2,3-diketo-4-deoxy-epi-inositol. Since myo-inositol was readily available, a method was developed for generating this product from myo-inositol, with the use of a NAD⁺-regenerating system with diaphorase and methylene blue as intermediate hydrogen carriers.

To eliminate the possibility that more than one major product was being formed by the action of the crude extract on myo-inositol in this system, an experiment with uniformly labeled myo-inositol-14C was performed. The reaction was carried out in a small flask which contained 40 μmoles of myo-inositol, 5 μC of myo-inositol-14C, 2 μmoles of NAD⁺, 1 μmole of methylene blue, 0.3 ml of diaphorase (5 mg per ml), and 0.5 ml of crude extract (about 2 mg of protein) in a total volume of 5.0 ml of 0.05 m sodium citrate buffer, pH 6.35. The flask was gently shaken at room temperature. Aliquots of 0.8 ml were taken every 30 min and chilled on ice; then 0.5 ml of perchloric acid, 70.7%, was added to each sample and the precipitates were removed by centrifugation. From each aliquot, 0.02 ml was spotted on Whatman No. 3HR paper and a descending chromatogram was developed for 5 hours with Solvent 1. The dried paper was placed in contact with a sheet of Ansco "no-screen" x-ray film for 3 days. The spots were also located on the chromatogram with silver nitrate spray and compared with the radioautogram. It was noted that, as the amount of myo-inositol decreased with time, there was a concomitant increase in the reducing spot, RF 0.4, which formed the only reducing or radioactive major spot other than the substrate myo-inositol-14C.

A similar experiment with unlabeled myo-inositol and a higher level of methylene blue (10 μmoles per ml) revealed that the reaction yielded the same product whether oxygen was rigidly excluded or not. Finally, the reaction was carried out in a Warburg manometric apparatus; no CO₂ was released and the usual amount of product was formed.

In order to obtain sufficient product for identification the following procedure was carried out. A crude cell extract was prepared by treating in the MSE sonic disintegrator about 5 g (wet weight) of cells of strain C1 which had been grown in a minimal medium containing 0.3% L-histidine and had been suspended in 25 ml of 0.05 m potassium phosphate buffer, pH 7, containing 10⁻⁴ m glutathione. After centrifugation for 90 min at 39,000 rpm in the Spincor model L centrifuge, the supernatant was used for the preparation of the product.

The reaction mixture contained 8.5 μmoles of myo-inositol, 100 μmoles of NAD⁺, 20 μmoles of CoCl₂, 200 μmoles of methylene blue, and 3 ml of diaphorase, 5 mg per ml, in a total volume of 200 ml of 0.05 m sodium citrate buffer, pH 6.8. A 10-ml portion of the crude extract containing 2.5 mg of protein per ml was added to initiate the reaction. The solution was constantly stirred with a magnetic mixer and the pH was maintained between 6.5 and 6.8 by addition of N NaOH as required. After 2 hours, a further 5-ml portion of crude extract was added. When the reaction had proceeded for 10 hours, 2 ml of perchloric acid (70.7%) were added and the preparation was chilled. Precipitated protein was removed by centrifugation and filtration. The pH was adjusted to 6.8 with 10 N KOH and the sample was stored overnight at 0°C. About 100 mg of acid-washed Dareo G-60 were added to remove the cofactors and the methylene blue and the preparation was filtered and concentrated in a rotary evaporator to a volume of 15 ml. The syrupy solution was then placed on a Dowex 1 (Cl⁻) column (8.5 × 28 cm).

After an initial washing with 3 liters of distilled water, 0.01 N HCl was used to elute the reducing compound; 10-ml fractions were collected and small portions of the fractions were tested for the presence of the compound by their reaction in a porcine spot plate with 2 drops of 0.6% 2,3,5-triphenyltetrazolium-HCl in 0.5 N NaOH. The contents of the tubes giving a positive reaction, Fractions 100 to 130, were pooled, the pH was carefully brought to 6.5 with 1 N NaOH, and the solution was concentrated by rotary evaporation in a vacuum to about 20 ml. The slightly yellow liquid was then passed through a Millipore filter over which had been placed a thin layer of acid-washed charcoal (Dareo G-60) and acid-washed Celite (1:2). Paper chromatography of the resulting clear solution, in Solvent 1, showed a single reducing spot, RF 0.4, and a gray spot of RF 0.1 corresponding to sodium chloride. In some preparations other trace impurities were also seen on the paper.

In order to eliminate these impurities the sample was concentrated to 5 ml, placed on an LKB 3500 A Chromatex pressurized paper chromatography column (LKB Instruments, Inc.), and eluted with 85.5% ethanol. It was important to wash this column thoroughly with approximately 1 liter of the same solvent prior to applying the sample. Fractions of 10 ml were collected and assayed as before with 2,3,5-triphenyltetrazolium. The contents of the tubes containing the compound were combined and concentrated by rotary evaporation in a vacuum to about 2 ml. A flocculent white precipitate was found in the concentrate which appeared to be a contaminant from the column. About 8 ml of distilled water were added to the preparation, which was then filtered again through a Millipore filter overlaid with Dareo G-90 and Celite. A small aliquot, 0.01 ml, of the clear solution was chromatographed in Solvent 1 and showed only a single reducing spot of RF 0.4.

Chemical procedures devised for characterization of the compound were carried out on this preparation. The concentration of the compound in aqueous solutions was determined by the

**Table II**

<table>
<thead>
<tr>
<th>Molarity</th>
<th>Activity</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Citrate buffer</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>Molarity</td>
<td>μg/min</td>
<td>μg/min</td>
</tr>
<tr>
<td>0.01</td>
<td>0.16</td>
<td>0.10</td>
</tr>
<tr>
<td>0.05</td>
<td>0.16</td>
<td>0.08</td>
</tr>
<tr>
<td>0.10</td>
<td>0.15</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*Effect of phosphate on diketodeoxyinositol hydrolase*

The salts used for the buffers were sodium citrate, sodium phosphate, and a mixture of the two, at pH 6.35 and the indicated molarity. The same results were obtained when potassium phosphate was used in place of the sodium salt. Sufficient D-2,3-diketo-4-deoxy-epi-inositol was added to the buffers to give the indicated molarity and an absorbance of 0.6 at 290 mp in a total volume of 0.98 ml. After measuring the nonenzymatic breakdown of the substrate, 0.02 ml of a partially purified enzyme extract containing 0.1 mg of protein was added and the change in absorbance due to enzymatic activity, ΔAₘₘ/s per min, was determined.
method of Fairbridge, Willis, and Booth (7) with the use of 2,3,5 triphenyltetrazolium and with glucose as standard; when crystalline material became available the method was found to give correct values.

Characterization of Product of Diketodeoxyinositol Hydrolase

Preliminary Tests—The reducing material in the purified eluate of the paper column was subjected to electrophoresis on paper in sodium citrate or acetate buffers with pH varying from 3.8 to 6.5; it was found to migrate toward the anode at the same rate as 2-ketoglucconate and 5-ketoglucconate. It gave colorimetric tests characteristic of ketohexoses (4), and of enols (8). It did not give colorimetric reactions characteristic of aldehydes, 1,2-diketones, α-keto acids, or lactones.

A 2-ml sample containing approximately 40 μmoles of the compound was treated with 2 ml of 0.1 m periodic acid and 2 ml of 0.5 M NaHCO₃ at room temperature in the dark for 20 min; this treatment resulted in the reduction of 2.9 moles of periodate and in the production of 0.95 mole of formaldehyde per mole of the compound. Similar experiments were carried out with unbuffered periodic acid; in this case 2.2 moles of periodic acid were reduced and 1.0 mole of formaldehyde and 0.86 mole of glyoxylic acid were produced per mole of the compound. Neither malondialdehyde nor β-formylpyruvic acid could be detected among the products of the periodate oxidations.

On the basis of this preliminary evidence the compound appeared to be a keto-oxo-hexadecanionic acid. For further characterization derivatives of the compound were prepared.

Products of Borohydride Reduction—The keto group of the compound was reduced to a hydroxyl group by treatment with sodium borohydride. To 0.4 mmole of the reducing compound in 8 ml of 0.2 M sodium acetate buffer, pH 6.5, was added 1 m NaBH₄ in 0.02-ml portions with rapid stirring at room temperature until, in the course of 1 hour, the pH had risen to 7.5. The mixture was stirred for an additional 2 hours, when the test of a small portion with 2,3,5-triphenyltetrazolium failed to show the presence of reducing material. The mixture was concentrated to 1 ml in a vacuum, and was then passed through a Dowex 50 (H⁺) column (2.5 X 22 cm). The material present in the mixture capable of reducing silver nitrate was retained on the column, but could be eluted with a small volume of water. The eluate was concentrated in a vacuum to a viscous syrup. In order to eliminate the borate from the preparation, six 2-ml portions of methanol were added; the mixture was again evaporated in a vacuum to a syrupy residue, which was finally dissolved in 0.3 ml of H₂O.

Small portions of the solution were chromatographed for 4 hours in Solvent 1 or for 6 hours in Solvent 2. In both cases two compounds capable of reducing silver nitrate could be detected. The Rf values of these compounds were 0.85 and 0.63 in the first solvent, and 0.89 and 0.84 in the second solvent. Both compounds could be identified as lactones by their reaction on paper with hydroxylamine and FeCl₃ (2).

The lactones were isolated by streaking 0.1-ml portions of the mixture on sheets of Whatman No. 3HR paper and developing a descending chromatogram in Solvent 1 for 4 hours. Narrow guide strips cut from the edges of the papers were sprayed with silver nitrate reagent and the appropriate areas of the large sheets were then eluted with about 5 ml of water. The samples of two lactones were concentrated by evaporation in a vacuum to volumes of 1 ml. When checked by chromatography in Solvent 1 single spots (lactone A, Rf 0.85; lactone B, Rf 0.63) were obtained from each preparation.

The lactones could be converted to acids by treatment with alkali. To 0.5-ml portions of the lactones 0.1 M NaOH was added until the pH reached 8. The volumes were brought to 1 ml with water, and the solutions were kept in a water bath at 90° for 30 min. Chromatography on paper in Solvent 1 revealed that each lactone had been converted by the treatment with alkali to a compound with an Rf value of 0.26; this compound reduced silver nitrate, reacted as an acid when sprayed with a solution of bromoresol green, but no longer reacted as a lactone in the hydroxylamine-FeCl₃ test. Acidification of the solutions containing the acids of Rf 0.26 brought about regeneration of the lactones; in each case only the lactone which had given rise to the acid was produced.

These results indicate that the reduction of the keto group of the reducing compound by borohydride gives rise to two lactones which are presumably stereoisomers with regard to the hydroxyl group produced by the reduction of the keto group.

The acids derived from the lactones were subjected to oxidation by periodate; 0.5 ml of 0.1 M periodic acid and 0.2 ml of 0.5 M NaHCO₃ were added to 0.2 ml of the solutions containing the acids, and the mixtures were kept at room temperature in the dark for 10 min. After destruction of the excess of periodate by the addition of 0.05 ml of 10 M H₂SO₄ and 0.3 ml of M sodium arsenite, the mixtures were found to contain malondialdehyde, formaldehyde, and glyoxylic acid. This result identifies the acids produced by the reduction of the reducing compound as 6 carbon acids, substituted with hydroxyl groups in positions 2, 3, 5, and 6 (Compound II, Scheme 1). Together with the earlier observations, this finding indicates that the reducing compound carries its keto group either on carbon 3 or on carbon 5.

Osazones—In order to determine the position of the keto group, the reducing compound was converted to its osazone in the following manner: 5 ml of phenylhydrazine reagent (0.5 g of phenylhydrazine hydrochloride and 0.8 g of sodium acetate in 5 ml of H₂O) were added with stirring to 5 ml of a solution containing 1 mmole of the compound. The heavy walled centrifuge tube containing the mixture was placed in a boiling water bath. After approximately 10 min, when the yellow solution had become slightly cloudy, the tube was removed and permitted to cool to room temperature. The walls of the tube were scratched with a glass rod and a heavy yellow precipitate appeared. After further cooling for 2 hours in an ice bath, the precipitate was collected by centrifugation and washed once with cold distilled water. The osazone was then dissolved in 2.5 ml of ethanol and filtered through a thin layer of Darco G-60. Upon addition of 50% aqueous ethanol the osazone precipitated. A second crystallization from 50% aqueous ethanol was carried out, after which the material was collected and dried over P₂O₅. The yield, 220 mg of small rod-shaped yellow crystals, was 65% of the theoretical.

Osazones of 2-ketoglucconic acid and 5-ketoglucconic acid were similarly prepared for comparison.

The osazone of the reducing compound melted with decomposition at 113°.

C₁₆H₂₅O₃N₄ (osazone of deoxyketohexonic acid)

Calculated: C 60.7, H 5.7, N 15.7
Found: C 59.9, H 5.8, N 15.8

The ultraviolet visible light spectrum of the osazone is shown...
**Scheme 1.** Reactions of the product (I) of the enzymatic reaction with borohydride and with phenylhydrazine, and oxidation of this compound and its derivatives with periodic acid.

**Fig. 1.** Ultraviolet and visible light absorption spectra of the osazones of 2-ketogluconic acid (---), 5-ketogluconic acid (---), and of Compound III (see Scheme 1) (-----). The osazones were dissolved in ethyl alcohol, and the spectra were determined in a Cary recording spectrophotometer.

Together with the spectra of the osazones of 2-ketogluconic acid and of 5-ketogluconic acid in Fig. 1. It is apparent that the spectra of the osazones of the reducing compound and of 5-ketogluconic acid are very similar and differ markedly from the spectrum of the osazone of 2-ketogluconic acid. It is likely, therefore, that the keto group of the reducing compound is on carbon 5 (Compound I, Scheme 1). Conclusive evidence for this view was provided by the study of the oxidation of the osazone of the compound by periodic acid.

A 1.5-mg sample of the osazone (5 µmoles) was dissolved in 2 ml of 66% aqueous ethanol and treated with 0.2 ml of 0.2 M periodic acid in the dark at room temperature for 1 hour. A flocculent precipitate that appeared soon after addition of the periodic acid was removed by filtration, and the filtrate was assayed for residual periodic acid, glyoxylate, and formaldehyde. It was found that 1.05 moles of periodic acid had been consumed and 0.87 mole of glyoxylate had been formed per mole of the osazone; no formaldehyde was found.

The osazone of a 4-deoxy-5-ketohexoaldonic acid (Compound III, Scheme 1) should yield, upon oxidation by periodic acid, in addition to glyoxylic acid, the 3,4-bisphenylhydrazone of 1,3,4-trioxo-n-butane (Compound IV, Scheme 1). This bisphenylhydrazone was produced in the following way. To 100 mg of the osazone, dissolved in 40 ml of 66% aqueous ethanol, 1.6 ml of 0.6 M periodic acid were added. A flocculent red precipitate appeared immediately. After 20 min at room temperature, the precipitate was collected and washed with 5 ml of cold 66% aqueous ethanol and 20 ml of cold distilled water. The material was recrystallized once more from 66% ethanol, washed as before, and dried over P2O5. A 42% yield of yellow, needle-like crystals was obtained.

The isolated compound melted at 177-178° and had the following composition:
Compound IV was compared with the bisphenylhydrazone of mesoxaldehyde, prepared by oxidation of glucosazone with periodic acid (9). The two compounds had identical ultraviolet spectra (see Reference 9), but different infrared spectra (Fig. 2). The spectrum of Compound IV possesses an absorption band at a frequency of 2920 to 2940 cm\(^{-1}\), which is not found in the spectrum of the other bisphenylhydrazone; this band in the region of aliphatic C—H stretching agrees with the presence of the methylene carbon in Compound IV, and its absence in the bisphenylhydrazone of mesoxaldehyde.

On the basis of these findings it is possible to identify the compound formed enzymatically from \(\alpha\)-2,3-diketo-4-deoxy-epi-inositol as a 4-deoxy-5-ketohexaaldonic acid (Compound I, Scheme 1).

Crystalline Sodium Salt of Compound I—Several earlier attempts to crystallize Compound I had proven unsuccessful, apparently because of the instability of the compound in the presence of trace impurities. Towards the end of this investigation, however, when a clear, concentrated solution of the compound was lyophilized to dryness, a homogeneous crystalline preparation was obtained. The colorless, granular crystals, which were extremely hygroscopic, melted without decomposition at 67–68°. The optical rotation of the compound dissolved in water was: \([\alpha]_{D}^{25} +5.53°\). The crystals were stored in a desiccator over P,O\(_5\) at 0°.

The following data, obtained with this material, indicate that the crystals were those of the sodium salt of Compound I.

\[\text{C}_{22}\text{H}_{21}\text{O}_{7}\text{Na}.\text{H}_{2}\text{O} \] (hydrated sodium salt of Compound I, Scheme 1)

Calculated: C 33.0, H 5.1, Na 10.5

Found: C 33.1, H 5.2, Na 10.3

The infrared spectrum of a KBr pellet of the crystalline compound (Fig. 3) showed absorbance at frequencies of 1580 to 1588 cm\(^{-1}\) and 1400 to 1405 cm\(^{-1}\); these bands coincide closely with those reported for the carboxyl function of an organic sodium salt (10). Absorbances at frequencies of 1725 cm\(^{-1}\) and 1405 to 1410 cm\(^{-1}\) could be ascribed to carbonyl stretching vibrations and to the bending vibration of a methylene group adjacent to a carbonyl group, respectively. A very broad unresolved peak at a frequency of 3600 to 3400 cm\(^{-1}\) may be attributed to hydroxyl stretching vibrations.

**DISCUSSION**

It has been shown in the preceding paper that cells of \(A.\ aerogenes\) contain after growth in the presence of myo-inositol, and in the case of a constitutive mutant even after growth in the absence of myo-inositol, two enzymes that together catalyze the conversion of myo-inositol to \(\alpha\)-2,3-diketo-4-deoxy-epi-inositol. It has now been shown that these cells contain in addition an enzyme system that can bring about the cleavage of the cyclohexane ring of the diketodeoxyinositol. The product of this cleavage could be identified as an optically active 4-deoxy-5-ketohexaaldonic acid.

In order to understand the nature of the enzymatic conversion of the cyclic compound to the open chain compound, we consider the fact that carbon atoms 2, 3, 4, and 5 of the latter carry the same substituents as carbon atoms 6, 5, 4, and 3 of the former. If we assume that these 4 carbon atoms have remained unchanged in the course of the enzymatic reaction, it is likely that the carboxyl group of the product (carbon 1) is derived from carbon 1 of the substrate, and that the primary hydroxyl group of the product (carbon 6) is derived from carbon 2 of the substrate. The cleavage of the ring should therefore have occurred between carbon atoms 1 and 2 of the diketodeoxyinositol, and was pre-
sumably preceded by the isomerization of these carbons (Formula 1).

If we assume that neither the isomerization nor the hydrolysis brought about epimerization at carbon atom 5 or 6 of the diketo-deoxyinositol, the product of the enzymatic hydrolysis can be tentatively identified as 4-deoxy 5-keto-3-hexonic acid.

Although it is possible that a single protein is responsible for the isomerization of the α-diketone to the β-diketone and for the hydrolytic cleavage of the β-diketone, it is likely that our partially purified extracts contained separate enzymes catalyzing these reactions. The purification was not carried far enough to distinguish between these alternatives.

The isomerization reaction is similar to the well known isomerization reactions encountered in the metabolism of hexoses and pentoses (see Reference 11). The hydrolytic reaction is, as far as we know, the first example of a nonoxidative enzymatic cleavage of a cyclohexane ring. However, the enzymatic cleavage of a β-diketone leading to the production of a carboxylic acid has been described: thus 2,4-diketovaleric acid is cleaved by an enzyme found in animal tissues to acetic acid and pyruvic acid (12).

The rapid enzymatic conversion of myo-inositol in the presence of NAD+ and an NAD+-regenerating system to a deoxyketohexonic acid suggests strongly that the acid is an intermediate in the complete degradation of myo-inositol. Preliminary experiments suggest that extracts of inositol-grown A. aerogenes can convert the deoxyketohexonic acid in the presence of ATP to another compound capable of reducing silver nitrate. The study of this reaction should reveal the as yet unknown later steps in the catabolism of myo-inositol.

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