The Pathway of myo-Inositol Degradation in Aerobacter aerogenes

DEHYDROGENATION AND DEHYDRATION*

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THOMAS BERMAN† AND BORIS MAGASANIK
From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

SUMMARY

The enzymes responsible for the first two steps in the degradation of myo-inositol by Aerobacter aerogenes were identified and isolated from extracts of a "constitutive" mutant, that is, a strain containing high levels of these enzymes even when grown in an inositol-free medium.

The enzyme catalyzing the initial step, the dehydrogenation of myo-inositol, is strongly inhibited by its product. It could be shown that the dehydrogenation involves the transfer of a hydrogen from carbon atom 2 of the inositol ring to nicotinamide adenine dinucleotide; the product is therefore 2-keto-myo-inositol. The second step is the dehydration of 2-keto-myo-inositol. The enzyme responsible for this reaction is unstable when exposed to oxygen, and requires glutathione and Co++ or Mn++ for maximal activity; its product was identified as D-2,3-diketo-4-deoxy-epi-inositol. This compound can be reduced by myo-inositol dehydrogenase in the presence of reduced nicotinamide adenine dinucleotide to a ketodeoxyinositol. However, this reaction does not appear to be involved in the degradation of myo-inositol.

Although it has long been known that Aerobacter aerogenes can utilize myo-inositol aerobically or anaerobically as a source of both carbon and energy, the complete biochemical sequence of the degradative pathway has not been elucidated. In plant tissues, d-glucuronic acid is formed after an oxidative cleavage between carbon atoms 1 and 6 of myo-inositol (1). Enzymes which effect a similar oxidation of myo-inositol to d-glucuronic acid have been isolated from yeast (2), and from rat kidney (3); a second enzyme which catalyzes the formation of 1-glucuronic acid may also be present in the latter organ (4).

The dissimilation of myo-inositol by A. aerogenes, however, follows a different pathway. Inositol dehydrogenase (myo-inositol: NAD oxidoreductase, EC 1.1.1.18), the presumed initial enzyme of the catabolic sequence, was demonstrated in extracts of myo-inositol-grown A. aerogenes and shown to be nicotinamide adenine dinucleotide-linked, inducible, and sensitive to catabolite repression (5). Only one report of a partial purification and characterization of this enzyme has been published (6). By analogy with the oxidation of myo-inositol in Acetobacter suboxydans (7), it was assumed that the product of the reaction with myo-inositol dehydrogenase in A. aerogenes is 2-keto-myo-inositol (myo-inosose-2; scyllo-inosose) (8). This point, however, has never been clearly proven. Indeed, Larner, Jackson, Graves, and Baumer (6) state (but 2-keto-myo-inositol could be detected when a partially purified enzyme preparation was used in a reaction with myo-inositol as substrate and NAD and potassium ferricyanide as hydrogen acceptors, although a different reducing compound could be found. Nevertheless, these investigators could show that the enzyme would oxidize NADH in the presence of 2-keto-myo-inositol. An independent study had shown, moreover, that the products of this reaction were NAD+ and myo-inositol (8).

Goldstone and Magasanik (9) had found that extracts of A. aerogenes, grown on myo-inositol, exhibited enzymatic activity towards 2-keto-myo-inositol. This was evidenced by an increase in absorbance of the reaction mixture at 261 mμ. When the extracts were treated with protamine sulfate, the enzymatic activity was retained in the supernatant. The reaction appeared to require no cofactors and only extracts of myo-inositol-induced bacteria were active. The authors suggested that the enzyme mediates the removal of water from the 2 carbon atoms vicinal to the keto group.

The present paper describes the partial purification of inositol dehydrogenase and ketoinositol dehydratase from extracts of A. aerogenes and the characterization of the products of the two enzymes. The further metabolism of the product of ketoinositol dehydratase is described in the succeeding paper (10).

EXPERIMENTAL PROCEDURE

Bacteria—The bacterial strains used in these studies were A. aerogenes 35, a capsulated strain originating from A. aerogenes 1033 (5, 11) and a mutant constitutive for at least the first three enzymes of myo-inositol dissimilation, C1, isolated in this laboratory by Dr. T. K. Sundaram. These bacteria were routinely
grown on a modified minimal Werkman medium supplemented as indicated (12).

A. suboxydans 621 was procured from the American Type Culture Collection, Washington, D. C.

Chemicals—The sources of various chemicals and reagents used were as follows: myo-inositol, 2-thiobarbituric acid, nicotinamide adenine nucleotides (NAD+ and NADP+), and the reduced forms of these nucleotides (NADH and NADPH) were obtained from Mann; n-inositol and glutathione, from Calbiochem; 2,3,5-triphenyltetrazolium-HCl from Dajac Laboratories, Philadelphia; and myo-inositol-2-3H from New England Nuclear Corporation.

2-Keto-myoinositol was prepared by the method of Posternak (13). L-2-Keto-epi-inositol and DL-2-keto-epi-inositol had been prepared previously (14). L-2-Deoxy-muco-inositol (d-quercitol, see Reference 15 for the nomenclature of inositol derivatives) was the gift of Dr. L. Anderson. Diaphorase (a partially purified extract from Clostridium kluyveri) was supplied by Worthington. Sephadex G-75 and G-200 resins were obtained from Pharmacia.

Enzyme Assays—myo-Inositol dehydrogenase was determined according to Levin and Magasanik (16). The reaction mixture contained, in a total volume of 1 ml, 20 μoles of sodium carbonate buffer, pH 10; 70 μoles of (NH₄)₂SO₄; 130 μoles of myo-inositol; and 0.7 μole of NAD⁺. The reaction, which was carried out at 25°, was initiated by adding 0.01 to 0.02 ml of enzyme (1 to 2 mg per ml of protein) and the increase of absorbance at 340 μ was followed.

Ketoinositol dehydratase was determined by following the ultraviolet absorbance of the product. The assay mixture contained, in a volume of 1 ml, 100 μoles of sodium acetate buffer, pH 6, and 1 μole of 2-keto-myoinositol. Upon addition of 0.01 ml of the enzyme (1 to 2 mg per ml of protein), the rate of increase of absorbance at 290 μ was measured at 25°.

Chemical Procedures—Proteins were determined by the adaptation by Lowry et al. (17) of the Folin phenol method, with bovine serum albumin (Armour) as standard. Formaldehyde was determined by the method of Nash (18), malondialdehyde and β-formyl pyruvate were determined by the thiobarbituric acid method (19, 20), and glyoxylate was determined as described by McFadden and Howes (21). Compounds were tested for the presence of 1,2-carbonyl groups by treatment with hydroxylamine and nickel acetate (22) and enolic functions were detected by the spot test described by Feigl (23).

Chromatography on Paper—The following solvent systems were used in the course of this investigation: Solvent 1, ethyl acetate-acetic acid-water, 3:1:3 (24); Solvent 2, ethanol-acetic acid-water, 8:2:2; Solvent 3, ethyl acetate-water-pyridine, 2:2:1 (24); Solvent 4, 1-butanol-ethylene glycol-water, 3:1:3 (25); and Solvent 5, 1-butanol-ethanol-water, 4:1:5 (26).

For location of myo-inositol and various intermediates of the catabolic pathway on paper chromatograms, the silver nitrate-acetone spray of Trevelyan, Prueck, and Harrison (27) was used. Reducing compounds were visualized as red spots by spraying the chromatograms with 0.5% 2,3,5-triphenyltetrazolium in 0.5 M NaOH (28).

RESULTS

Mutants—Initially, attempts were made to isolate mutant strains incapable of growing on myo-inositol, in the hope of finding mutants specifically blocked in one step of the catabolic pathway. However, the only mutants produced (with the use of nitrous acid, ethyl methanesulfonate, N-methyl-N-nitrosoguanidine, or 2-aminopurine as mutagens) were found to have extremely low or imperceptible levels of inositol dehydrogenase, of ketoinositol dehydratase, and of an enzyme catalyzing the subsequent reaction (10). A constitutive mutant, C1, isolated in our laboratory by Dr. T. K. Sundaram, possesses high levels of these enzymes when grown in an inositol-free medium containing L-histidine as the source of carbon. The specific activities of inositol dehydrogenase and of ketoinositol dehydratase in such an extract were 4.0 and 9.0 units per mg of protein, respectively, compared to 1.3 and 2.8 units per mg of protein for the activities of these enzymes in extracts of the wild strain growing on myo-inositol. When glucose or myo-inositol was added to the culture of strain C1 much lower enzyme levels were observed, the constitutive synthesis of these enzymes was apparently subject to catabolite repression.

Purification of myo-Inositol Dehydrogenase—The cell extracts were prepared from cultures of the constitutive mutant, C1, grown in minimal medium with 0.2% L-histidine as carbon source at 37°. All manipulations were carried out at 0-4°.

About 5 g (wet weight) of cells were suspended in 30 ml of 0.05 M potassium phosphate buffer, pH 7, containing 5 × 10⁻⁴ M glutathione (hereafter referred to as phosphate buffer). Batches of 5 ml of the suspension were sonically disrupted for 5 min in an MSE sonic disintegrator (Instrumentation Associates, New York). Combined disrupted extracts were then centrifuged at 38,000 rpm in a Spinco model L centrifuge for 90 min to remove cell debris. A solution of 2% protease sulfate was added dropwise to the supernatant to a final level of 10 mg of protamine sulfate per 100 mg of protein. A precipitate consisting largely of nucleic acid appeared and was removed by centrifugation. The supernatant was dialyzed for 16 hours against phosphate buffer and then fractionated by the addition of a saturated solution of ammonium sulfate. The precipitate which formed between 40 and 50% saturation of ammonium sulfate was collected by centrifugation. This precipitate was taken up in 6 ml of phosphate buffer and dialyzed for 16 hours against the same buffer. At this point the preparation still contained considerable ketoinositol dehydratase activity. The sample was then placed on a Sephadex G-200 column (2.5 × 30 cm) and eluted with phosphate buffer. Fractions of 1 ml were collected and assayed for both myo-inositol dehydrogenase and ketoinositol dehydratase. myo-Inositol dehydrogenase activity was found in tubes 46 to 50, and ketoinositol dehydratase activity in tubes 58 to 62. The tubes containing myo-inositol dehydrogenase activity only were pooled and concentrated, with the use of sucrose as described by Fling, Horowitz, and Heimann (29). The preparation was then dialyzed for 16 hours against phosphate buffer. Another fractionation was performed with saturated ammonium sulfate solution. The precipitate forming between 37 and 42% saturation was collected by centrifugation and was resuspended in phosphate buffer. The specific activity of myo-inositol dehydrogenase at this stage had increased 46-fold over the crude extract with a recovery of about 34% of the total enzyme units. No traces of ketoinositol dehydratase were discerned. Attempts to achieve further purification by the use of diethylaminoethyl cellulose columns, or by adsorption on calcium phosphate or alumina Cyt gels, were not successful. Table I shows the steps in the purification procedure.
The purified enzyme could be stored at -10° for several months without noticeable loss of activity.

Properties of myo-Inositol Dehydrogenase—The preferred hydrogen acceptor is NAD+*. When NADP+ was used in place of NAD+, the activity was only 5 to 10%. 2-Keto-myoinositol proved to be an inhibitor of myo-inositol dehydrogenase (see below). p-Hydroxymercuribenzoate had an inhibitory effect which could be relieved by glutathione or mercaptoethanol.

No-inositol, which can be metabolized slowly by A. aerogenes, also served as a substrate for myo-inositol dehydrogenase although the reaction rate was only about 10% of that with myo-inositol.

When 2-keto-myoinositol and NADH were incubated with the enzyme in 0.1 M phosphate buffer at pH 7, a decrease in the absorbance at 340 m\(\mu\) was observed, signifying the oxidation of the pyridine nucleotide and, presumably, the formation of myo-inositol.

myo-Inositol Dehydrogenase Action on \(\beta\)-2,3-Diketo-4-deoxy-epi-inositol—An oxidation of NADH was also noted when crude or partially purified preparations of myo-inositol dehydrogenase were incubated with \(\beta\)-2,3-diketo-4-deoxy-epi-inositol, the product of ketoinositol dehydratase (see below). In order to characterize the product of this reaction, 0.1 ml of a purified preparation of myo-inositol dehydrogenase containing 1 mg of protein per ml was added to 10 \(\mu\)moles of \(\beta\)-2,3-diketo-4-deoxy-epi-inositol (prepared as described later in this paper) and 10 \(\mu\)moles of NADH in 1 ml of 0.2 M potassium phosphate buffer, pH 7. The reaction vessel was gently shaken for 1 hour at room temperature, after which 0.1 ml of 70% perchloric acid was added and the sample was chilled in an ice bath.

Precipitated protein was removed by centrifugation and the pH was adjusted to 6.5 with 10 N KOH. The preparation was filtered to remove crystalline potassium perchlorate and then concentrated to 0.5 ml under reduced pressure. A 0.01-ml portion was spotted on Whatman No. 3MM paper and a descending chromatogram was developed for 5 hours in Solvent 1. When the paper was sprayed with silver nitrate reagent it was found that while a faint spot corresponding to the substrate (\(R_f\) 0.15) remained, a major new spot (\(R_f\) 0.25) and a secondary spot (\(R_f\) 0.4) had appeared on the paper. This latter compound was shown subsequently to be an open chain intermediate and was presumably formed by another enzyme still present in the purified enzyme preparation.

A sample of the first compound, \(R_f\) 0.25, was isolated by streaking 0.1-ml portions of the remaining preparation on sheets of Whatman No. 3MM paper and developing chromatograms as described above. The compound was located on the chromatograms by means of guide strips cut from the edges of the papers and was eluted with water. The eluates were combined and concentrated to 1 ml in a vacuum.

A small portion of the concentrate was again chromatographed in Solvent I: only a single compound capable of reducing the silver nitrate reagent was observed; its \(R_f\) was 0.25. Another portion of the concentrated eluate was found to reduce 2,3,5-triphenyltetrazolium readily at room temperature. A third portion, 0.2 ml, was treated with 0.05 ml of \(\Phi\) periodic acid and 0.05 ml of 1 N NaHCO\(_3\) for 15 min; the excess of periodate was destroyed by the addition of 0.1 ml of 5 \(\Phi\) HzSO\(_4\) and 0.2 ml of \(\Phi\) sodium arsenite. A portion of the mixture was treated with thiobarbituric acid. A colored compound was formed, which could be identified on the basis of its characteristic absorption spectrum with a maximum at 532 m\(\mu\) as the product of the reaction of malondialdehyde with thiobarbituric acid (19, 20). With the reported value of 13,000 for the molar extinction of the malondialdehyde derivative at 532 m\(\mu\) (19), the concentrated eluate from the paper was found to contain 4 \(\mu\)moles of the dialdehyde after periodate treatment. This value agrees with a rough estimation of the quantity of reducing material based on the intensity of the spot on the paper chromatogram produced by the treatment with silver nitrate reagent with 2-keto-myoinositol as a standard. The test of another portion of the periodate-treated paper eluate for formaldehyde gave a negative result.

These results suggest that the product of the hydrogenation of the diketodeoxinositol by NADH is a ketodeoxinositol in which the methylene group is located between secondary hydroxyl groups; apparently the keto group on carbon 3 of \(\beta\)-2,3 diketo 4 deoxy-epi-inositol has been reduced.

It was found that the ratio of the two enzymatic activities—dehydrogenation of myo-inositol by NADH and hydrogenation of the diketodeoxinositol by NADH—had a value of 0.2 at all stages of the purification procedure described in Table I. It is therefore likely that both reactions are catalyzed by the same enzyme.

Formation of 2-Keto-myoinositol by Inositol Dehydrogenase—Since a purified preparation of myo-inositol dehydrogenase, devoid of any significant ketoinositol dehydratase activity, was now available, the attempt was made to identify the product of the action of this enzyme on myo-inositol.

In order to ensure the stability of any ketoinositol formed, the reaction was carried out at pH 7 by providing an NAD+-generating system consisting of diaphorase, methylene blue, and oxygen. The reaction vessel contained, in a total volume of 6 ml, 48 \(\mu\)moles of potassium phosphate buffer, pH 7; 300 \(\mu\)moles of diaphorase (5 mg per ml), and 0.025 ml of purified myo-inositol dehydrogenase (1 mg of protein per ml). To initiate the reaction, 0.15 ml (1.5 \(\mu\)moles) of NAD was added and the flask was placed on a slowly moving rotary shaker at room temperature. Samples were removed at 0, 15, 30, 45, 60, and 90 min. At 90 min, 0.05 ml of a purified preparation of ketoinositol dehydratase (1.2 mg of protein per ml; see below) was added and the reaction was allowed to proceed for a further 10 min before a final portion was taken. The samples were treated with 0.1 ml of 50% trichloroacetic acid; the precipitate was removed by centrifugation and the trichloroacetic acid was removed by a four times repeated extraction with anhydrous ether. A 0.02-ml portion of each sample was then spotted on Whatman No. 1 paper for chromatography. The chromatogram was developed for 8 hours with

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifuged extract</td>
<td>30</td>
<td>3.3</td>
<td>6.1</td>
</tr>
<tr>
<td>Protamine supernatant</td>
<td>31</td>
<td>3.2</td>
<td>6.7</td>
</tr>
<tr>
<td>(NH(_4))(_2)SO(_4) precipitate I</td>
<td>6</td>
<td>1.8</td>
<td>14</td>
</tr>
<tr>
<td>Sephacryl eluate</td>
<td>5</td>
<td>1.6</td>
<td>93</td>
</tr>
<tr>
<td>(NH(_4))(_2)SO(_4) precipitate II</td>
<td>4</td>
<td>1.1</td>
<td>280</td>
</tr>
</tbody>
</table>

* One unit is defined as the ability to produce 1 \(\mu\)mole of NADH per min.

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Solvent 2. When the paper was sprayed with the 2,3,5-triphenyltetrazolium reagent, the appearance of a reducing compound having an \( R_f \) of 0.37, identical with that of 2-keto-myoinositol, could be clearly seen. By comparing the intensity of the spots with spots containing known amounts of 2-keto-myoinositol, it was estimated that at most 30 to 60 \( \mu \)moles of the enzymatic product had been formed in 45 min; a yield based on myo-inositol of approximately 15\%. It was also noted that this amount did not increase significantly after this time. The addition of the purified ketoinositol dehydratase at 90 min eliminated the spot at \( R_f \) 0.37 and gave rise to a new, more rapidly moving, reducing compound (\( R_f \) 0.73).

The first reducing compound formed in this reaction was also chromatographed in Solvent Systems 1, 3, and 4. In all cases this compound had an \( R_f \) identical with that of 2-keto-myoinositol but differing slightly from that of \( \mu \)-2-keto-\( \epsilon \)-myo-inositol.

In view of the low yield of presumed 2-keto-myoinositol formed it was performed the system described above, an experiment was carried out to study the effect of 2-keto-myoinositol on myo-inositol dehydrogenase activity. The rate of the reaction was measured at pH 7 by following the loss of absorbance at 550 nm of the hydrogen acceptor 2,6-dichlorophenolindophenol. The reaction mixture contained, in a volume of 1 ml, 10 \( \mu \)moles of potassium phosphate buffer, pH 7; 30 \( \mu \)moles of myo-inositol; 0.2 \( \mu \)mole of NAD\(^+\); 0.05 \( \mu \)mole of 2,6-dichlorophenolindophenol; 0.02 ml of diaphorase, 5 mg per ml; and 0.02 ml of myo-inositol dehydrogenase. Appropriate amounts of 2-keto-myoinositol were added to give final concentrations ranging from 1 to 5 \( \mu \)moles per ml. The reaction was started by adding myo-inositol dehydrogenase and the change in absorbance at 550 nm was followed in the Zeiss spectrophotometer. It was found that 2-keto-myoinositol caused 82\% and 94\% inhibition of the reaction at concentrations of 1 mm and 3 mm, respectively; the inhibition was relieved by the addition of ketoinositol dehydratase.

The change in the reaction rate caused by the addition of 2-keto-myoinositol is not due to an alteration of the equilibrium since the equilibrium of the reaction would have been pulled toward ketoinositol by the diaphorase-mediated oxidation of any NADH formed.

Thus, the inhibition of myoinositol dehydrogenase activity by 2-keto-myoinositol appeared to prevent the accumulation of amounts of the presumed intermediate sufficient for chemical characterization. However, the availability of myo-inositol-2-\(^3\)H suggested another way of identifying the reaction product unequivocally.

The first experiment was designed to examine the level of radioactivity of the ketoinositol formed from myo-inositol-2-\(^3\)H. Since it had not been found possible to achieve a clear separation of myo-inositol and 2-keto-myoinositol on paper chromatograms in a variety of solvent systems, the ketoinositol produced in the reaction was isolated in the form of its 2,4-dinitrophenylhydrazone.

The reaction mixture consisted of 60 \( \mu \)moles of myo-inositol; 0.01 ml of a solution of myo-inositol-2-\(^3\)H (approximately 1 \( \mu \)C); 1 \( \mu \)mole of NAD\(^+\); 0.06 \( \mu \)mole of methylene blue; 0.1 ml of diaphorase, 5 mg per ml; and 0.1 ml of myo-inositol dehydrogenase, 1 mg of protein per ml, in 1 ml of 0.1 m potassium phosphate buffer, pH 7. When the reaction had proceeded for 90 min, 0.1 ml of 50\% trichloroacetic acid was added, the precipitate was removed by centrifugation, and the supernatant was extracted four times with anhydrous ether. A 0.5-ml portion of 0.5\% 2,4-dinitrophenyldihydrazine in 2 N HCl was added to the aqueous phase of the ether-saturated sample. After 2 hours at room temperature, followed by 2 hours of chilling in an ice bath, the 2,4-dinitrophenyldihydrazone was extracted into 1 volume of ethyl acetate. The ethyl acetate phase was concentrated by evaporation to a volume of about 0.05 ml. This sample was then spotted on a sheet of Whatman No. 3MM paper and a descending chromatogram was developed in Solvent \( \alpha \). The 2,4-dinitrophenyldihydrazone appeared as a discrete yellow spot. This spot, which moved with the same \( R_f \) as a sample of genuine 2,4-dinitrophenyldihydrazone of 2-keto-myoinositol, was cut out, eluted with 1 ml of 1 N NaOH in ethanol, and assayed for radioactivity. The activity of the total aqueous phase remaining after ethyl acetate extraction was likewise determined.

It was found that the 2,4-dinitrophenyldihydrazone had no significant radioactivity (220 cpm, compared to 93,320 cpm in the aqueous phase).

In order to follow the fate of the tritium in the inositol dehydrogenase reaction, an experiment was devised to show the predicted transfer of the \(^3\)H from the substrate, myo-inositol, to the NADH formed during the reaction. The experiment was carried out in a quartz cuvette which contained the following: myo-inositol, 66 \( \mu \)moles; myo-inositol-2-\(^3\)H, 50 \( \mu \)C; NAD\(^+\), 2.5 \( \mu \)moles; and myo-inositol dehydrogenase (1 mg of protein per ml), 0.01 ml, in 1 ml of 0.07 m sodium carbonate buffer, pH 9.8. A control with 2.5 \( \mu \)moles of NADH in addition to the other ingredients, but containing no enzyme, was also prepared. The course of the reaction in the first cuvette was followed spectrophotometrically by the increase of absorbance at 340 nm. After the absorbance had reached a constant reading of about 1.6 another 0.01-ml portion of enzyme was added until a final absorbance of about 3 had been reached. Portions of 0.025 ml from both the test and control mixtures were then placed on strips of Whatman No. 1 paper and subjected to electrophoresis at 1500 volts for 2 hours in 0.01 m potassium phosphate buffer, pH 8. The papers were dried and the NADH spots, which were located by their ultraviolet absorbance, were cut out and eluted with 0.8 ml of 0.2 m Tris-HCl buffer, pH 8.9. The absorbance of the eluates at 340 nm was noted and their radioactivity was determined. It was found that the enzymatically formed NADH had 20 times as much radioactivity as the NADH from the control. The micromolar radioactivity of the enzymatically formed NADH, 1.7 \( \times \) 10\(^6\) cpm, was 0.88 times that of the myo-inositol-2-\(^3\)H used in the experiment.

These results show clearly that 2-keto-myoinositol is the product of the reaction of myo-inositol with NAD\(^+\) catalyzed by inositol dehydrogenase.
The purified enzyme (1.2 mg of protein per ml) was added in 0.05-ml portions to 0.45-ml samples of Tris-HCl buffer, pH 9, containing 0.1 mM glutathione and CoCl₂, MnCl₂, and EDTA in the indicated concentration. The samples were kept at 0° for 20 min. Substrate was then added and the enzyme assay was performed in the usual fashion.

**TABLE II**

Purification of ketoinositol dehydratase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Activity</th>
<th>Specific activity</th>
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<tbody>
<tr>
<td>Centrifuged extract</td>
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<td>14 units/mg protein</td>
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<tr>
<td>Protamine supernatant</td>
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<td>9.9</td>
<td>18 units/mg protein</td>
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<tr>
<td>(NH₄)₂SO₄ precipitate I</td>
<td>5 ml</td>
<td>3.5</td>
<td>58 units/mg protein</td>
</tr>
<tr>
<td>Sephadex eluate</td>
<td>10 ml</td>
<td>2.2</td>
<td>170 units/mg protein</td>
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<tr>
<td>(NH₄)₂SO₄ precipitate II</td>
<td>5 ml</td>
<td>1.6</td>
<td>270 units/mg protein</td>
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</tbody>
</table>

* One unit is defined as the ability to produce 1 μmole of diketone (e at 260 μμ = 6000) per min.

**TABLE III**

Activation of ketoinositol dehydratase

<table>
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<tr>
<th>Metal ion concentration</th>
<th>EDTA concentration</th>
<th>Co++</th>
<th>Mn++</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.30</td>
<td>0.38</td>
</tr>
<tr>
<td>0</td>
<td>10⁻⁴</td>
<td>0.34</td>
<td>0.38</td>
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<tr>
<td>5 X 10⁻⁴</td>
<td>0</td>
<td>0.92</td>
<td>0.78</td>
</tr>
<tr>
<td>5 X 10⁻⁴</td>
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<tr>
<td>5 X 10⁻⁴</td>
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<td>0.86</td>
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</table>

* Increase in absorbance per min.

A thick suspension of cells (approximately 4 g, wet weight) in 20 ml of 0.025 M Tris-HCl buffer, pH 9, containing 5 X 10⁻⁴ M glutathione and 10⁻⁴ M Co++ was disrupted in the MSE sonic disintegrator, as described earlier. The sample was then spun at 38,000 rpm for 90 min in a Spinco model L centrifuge and the pellet was discarded. A solution of 5% protamine sulfate was added to the supernatant with stirring under nitrogen to give a final concentration of 17 mg of protamine sulfate per 100 mg of pellet. After 1 hour, the preparation was centrifuged to remove the precipitate. Solid ammonium sulfate was added to the supernatant to a concentration of 70%. After 2 hours, the precipitate was collected by centrifugation and extracted with 5 ml of 0.01 M sodium acetate buffer, pH 7, containing 10⁻⁴ M Co++. The sample was again centrifuged and the supernatant was placed on a Sephadex G-75 column (2.5 X 30 mm). This column had been equilibrated with 0.05 M Tris-HCl buffer, pH 8, containing 10⁻⁴ M Co++. The absorbed protein was eluted with the same buffer and fractions containing 3 ml of eluate were collected. The tubes with enzymatic activity (Fractions 34 to 39) were pooled and concentrated to 10 ml with the use of aseptize (27).

A further fractionation was performed by collecting the precipitate formed between 40 and 50% saturation of ammonium sulfate. This precipitate was dissolved in 5 ml of 0.05 M Tris-HCl buffer, pH 9, containing 5 X 10⁻⁴ M glutathione and 10⁻⁴ M Co++. At this point the enzyme was considerably more stable than in the crude extract and could be stored under nitrogen at -10° for approximately 1 month without losing more than 50% of its activity. Further attempts at purification were not successful; they included DEAE-cellulose column elution, adsorption with calcium phosphate and alumina Cγ gels, and fractionation with cold acetone. The specific activity of the purified enzyme was increased 19-fold in comparison with the crude extract, and the preparation was devoid of any diketodeoxyinositol hydrolase (8) or inositol dehydrogenase activity. About 15% of the total activity was recovered. An outline of the purification steps is given in Table II.

Properties of Ketoinositol Dehydratase—Stability and activation: The ketoinositol dehydratase activity, in crude extracts of A. aerogenes, was unstable. A minimum loss of activity was found when the enzyme was maintained under an atmosphere of nitrogen at pH 9 in the presence of 5 X 10⁻⁴ M glutathione and either 10⁻⁴ M Co++ or Mn++. A number of supplements were tested for their effects on the purified enzyme. Mn++ and Co++ were found to increase the rate of the reaction; whereas Mn++ was effective at lower concentrations, the greatest increase (about 4-fold over the control) was given by Co++ at a concentration of 5 X 10⁻⁴ M. The inhibition caused by ethylenediaminetetraacetic acid could be alleviated by either Mn++ or Co++ (Table III). The enzyme was also inhibited by p-hydroxymercuribenzoate; on addition of glutathione or mercaptoethanol a considerable portion of the original activity was regained.

pH optimum: The activity peak for ketoinositol dehydratase appeared to be at about pH 6 to 6.2. However, since the product of the enzymatic reaction itself decayed rapidly at higher pH values, it was impossible to ascertain the true optimum for the enzyme.

Michaelis constant: A Lineweaver-Burk (30) plot for keto-
inositol dehydratase gave a K₉ value for 2-keto-myo-inositol of 1.5 X 10⁻⁴ M.

Specificity: The activities of purified preparations of keto-
inositol dehydratase were compared when 2-keto-myo-inositol or L-2-keto-epi-inositol was used as substrate. The assay was carried out in the usual manner with 1 μmole per ml of either 2-keto-myo-inositol or L-2-keto-epi-inositol. It was found that the rate of the reaction was 5 to 7 times faster with 2-keto-myo-inositol than with L-2-keto-epi-inositol.

Identification of Product of Ketoinositol Dehydratase—The product was prepared by incubating 50 μmoles of 2-keto-myo
inositol in 5 ml of 0.01 M sodium acetate buffer, pH 6, with 0.04 ml of purified ketoinositol dehydratase containing 1.2 μg of protein per ml. The reaction was followed spectrophotometrically at 280 μμ by diluting 0.01-m1 portions into 0.99 ml of water. After 40 min the absorbance of the diluted samples reached a maximum of 0.6. The pH was then adjusted to 1.8 by the addition of HCl; the sample was filtered to remove precipitated protein, and was stored at -10°. Chromatography of a portion of the preparation in Solvent 2 showed that at least 90% of the substrate, 2-keto-myo-inositol, Rp 0.37, had been converted into a ultraviolet-absorbing compound with an Rp of 0.52. Unfortunately, this compound proved to be unstable and all attempts...
at crystallization were unsuccessful. The breakdown of the compound was considerably attenuated at low pH and at \(-10^\circ\). The spectrum of the compound at pH 1.8 shows a sharp peak at 290 \(\mu\)m. The molar extinction coefficient, calculated on the assumption that for every mole of 2-keto-myo-inositol that has disappeared 1 mole of the product has accumulated, was found to be approximately 6000.

The solution containing the compound reduced Fehling's solution and 2,3,5-triphenyltetrazolium in the cold, and gave positive tests for 1,2-diketones and for enols (22, 23). An aliquot of the solution was treated with periodic acid for 15 min; the excess of periodic acid was then destroyed by the addition of \(\times H_2SO_4\) and \(\times\) sodium arsenite. A portion of the mixture was treated with thiobarbituric acid. The colored compound formed had the characteristic absorption spectrum (with a maximum at 549 \(\mu\)m) of the product of the reaction of \(\beta\)-formylpyruvic acid with thiobarbituric acid. On the basis of the molar extinction values reported for this compound (19, 20), approximately 0.5 to 0.7 mole of \(\beta\)-formylpyruvic acid had been produced by periodate oxidation from every mole of the ultraviolet-absorbing compound. Neither malondialdehyde nor formaldehyde could be detected as products of the periodate oxidation.

The ultraviolet extinction and the results of the chemical tests described in the preceding paragraph suggested that the compound was the enol of an \(\alpha\)-diketodeoxyinositol. For further characterization the corresponding bisphenylhydrazone was prepared in the following manner: 180 mg of 2-keto-myo-inositol were dissolved in 6 ml of 0.01 \(\mu\)m sodium acetate buffer, pH 6, and 0.5 ml of purified ketoinositol dehydratase was added. A further addition of 0.1 ml of enzyme was made 10 min after the start of the reaction. At 25 min, 3 ml of phenylhydrazine reagent (0.5 g of phenylhydrazine-HCl and 0.8 g of sodium acetate in 5 ml of water) were added and the flask was scraped and chilled. A flocy reddish yellow precipitate appeared almost immediately. This was collected and recrystallized twice from 60% aqueous ethanol, and once from ethanol alone. The final product was in the form of greenish yellow, needlelike crystals and melted with decomposition at 195-199\(^\circ\). It had the following elemental composition:

\[
\text{C}_{10}\text{H}_{10}\text{O}_2\text{N}_4 \quad \text{(bisphenylhydrazone of diketodeoxyinositol)}
\]

**Calculated:** C 63.5, H 5.9, N 16.5

**Found:** C 63.2, H 5.8, N 16.5

The ultraviolet and visible light absorption spectrum of the compound was characteristic for osazones (31) and identical with the spectrum of the bisphenylhydrazone of the \(\alpha\)-diketodeoxyinositol obtained by Magasanik and Chargaff as the oxidation product of 1,2-deoxy-muco-inositol by \(A\). suboxydans (32). When the bisphenylhydrazone was treated with periodic acid, as described by Magasanik and Chargaff (32), it was found that 2.04 moles of periodic acid were reduced per mole of the bisphenylhydrazone.

These observations established clearly that the compound formed enzymatically from 2-keto-myo-inositol is the enol of an \(\alpha\)-diketodeoxyinositol in which the methylene group is vicinal to one of the keto groups.

The complete identification of the product was achieved by comparison with \(\nu\)-2,3-diketo-4-deoxy-\(\alpha\)-epi-inositol. This compound was produced as described by Magasanik and Chargaff by the oxidation of 1,2-deoxy-muco-inositol with resting cells of \(A\). suboxydans (32). The solution in which the bacterial oxidation had taken place was found to contain a compound indistinguishable in its physical properties (ultraviolet absorption, migration on paper in Solvents 1 and 2) and its chemical properties (reduction of Fehling's solution and of 2,3,5-triphenyltetrazolium) from the product of the action of the Aerobacter enzyme on 2-keto-myo-inositol. The bisphenylhydrazone prepared from the oxidation product 1,2-deoxy-muco-inositol and the bisphenylhydrazone prepared from the enzymatic product of 2-keto-myo-inositol had identical melting points, 195-199\(^\circ\), and KBr pellets of the compounds had identical infrared spectra.

In order to determine whether the two bisphenylhydrazones are stereoisomers or identical, their optical rotations were determined on 7-mg samples dissolved in 1.5 ml of pyridine-ethanol (1:1) in a Rudolph No. 80 polarimeter. Values were obtained for the compounds derived from 1,2-deoxy-muco-inositol and 2-keto-myo-inositol of \([\alpha]_{\text{D}}^{20} \pm 54.8^\circ\) and \([\alpha]_{\text{D}}^{20} \pm 51.3^\circ\), respectively. Magasanik and Chargaff (32) reported for the former compound \([\alpha]_{\text{D}}^{20} \pm 62^\circ\).

It is therefore evident that the enzymatic product of 2-keto-myo-inositol is identical with the oxidation product of 1,2-deoxy-muco-inositol, which has been identified as \(\nu\)-2,3-diketo-4-deoxy-\(\alpha\)-epi inositol. The enzyme present in the extract of \(A\). aerogenes is apparently a ketoinositol dehydratase and catalyzes the following reaction.

**Reaction 2**

\[
\text{OH} \quad \text{OH} \quad \text{OH} \quad \text{OH} \quad \text{OH} \quad \text{OH} + \text{H}_2\text{O}
\]

**DISCUSSION**

It has been previously reported that intact cells of capsulated strains of \(A\). aerogenes secrete upon contact with myo-inositol the ability to oxidize myo-inositol, 2-keto-myo-inositol, and \(\nu\)-1,2-diketo-myo-inositol rapidly to \(\text{CO}_2\) and water (25). Other inositoles or ketoinositoles are metabolized by these induced cells at a much slower rate, or not at all. The cells acquire upon contact with myo-inositol also the ability to convert myo-inositol, 2-keto-myo-inositol, and \(\nu\)-1,2-diketo-myo-inositol anaerobically to mixtures of \(\text{CO}_2\), acetate, ethanol, and lactate (33).

The results of the experiments described in this paper show that growth on myo-inositol induces \(A\). aerogenes to form an enzyme that converts myo-inositol to 2-keto-myo-inositol (Reaction 1), and another enzyme that converts 2-keto-myo-inositol to \(\nu\)-2,3-diketo-4-deoxy-\(\alpha\)-epi inositol (Reaction 2). In the following paper it will be shown that myo-inositol induces yet a third enzyme, one that converts the diketodeoxyinositol nonoxidatively to an open chain compound (10). The three enzymes are produced in a "constitutive" mutant of \(A\). aerogenes even in the absence of myo-inositol. The essential role of these enzymes in inositol metabolism is supported by the observation that mutants lacking the three enzymes are unable to grow on myo-inositol.
The degradation of myo-inositol in *A. aerogenes* begins with the transfer of hydrogen to NAD$^+$ from carbon atom 2 (see Reaction 1) and is followed by the removal of water from carbon atoms 3 and 4 (see Reaction 2). The first enzymatic attack is directed against the unique carbon in myo-inositol that carries a hydroxyl group in an axial position (14). The second enzyme is highly stereospecific, in that it chooses for dehydration the pair of carbon atoms 3 and 4 of 2-keto-myo-inositol over the enantiomeric pair of carbon atoms 1 and 6. The observation that 2-keto-myoinositol is the product of one of the enzymes induced by myo-inositol and the substrate of another explains perfectly well the finding that cells capable of degrading myo-inositol are also capable of degrading 2-keto-myoinositol. The ability of inositol-grown cells to metabolize dehydrating myo-inositol are also capable of degrading 2-keto-myoinositol over the enantiomeric pair of carbon atoms 1 and 6. The ketoinositol produced in Reaction 4 may then be dehydrated by the ketoinositol dehydratase present in the inositol-grown cells. In this manner the two enzymes, inositol 7-dehydrogenase and ketoinositol dehydratase, could together accomplish the conversion of L-1,2-diketo myo-inositol to D-2,3-diketo-4-deoxy-epi-inositol (Reaction 3) suggests that this enzyme may also be capable of reducing L-1,2-diketo-myoinositol (Reaction 4).

The ketoinositol produced in Reaction 4 may then be dehydrated by the ketoinositol dehydratase present in the inositol-grown cells. In this manner the two enzymes, inositol dehydrogenase and ketoinositol dehydratase, could together accomplish the conversion of L-1,2-diketo myo-inositol to D-2,3-diketo-4-deoxy-epi-inositol.

It is of some interest that inositol dehydrogenase can in the presence of NADH reduce 2-keto-myoinositol (Reaction 1) and the ketoo group on carbon 3 in D-2,3-diketo-epi-inositol (Reaction 3), but apparently cannot reduce the keto group on carbon 3 in D-2,3-diketo-4-deoxy-epi-inositol or the ketoinositol formed in Reaction 3. The inability to reduce these keto groups may be associated with the lack of a hydroxyl group in the position meta to the keto group.

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