Mutarotase from *Penicillium notatum*

I. PURIFICATION, ASSAY, AND GENERAL PROPERTIES OF THE ENZYME*

RONALD BENTLEY AND D. S. BHAT†

*From the Department of Biochemistry and Nutrition, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania*

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In a study of the mechanism of action of notatin, the flavin adenine dinucleotide-dependent glucose aerodehydrogenase of molds, Bentley and Neuberger (2) in 1949 obtained evidence suggesting that the enzyme preparation also catalyzed the mutarotation of glucose. Keilin and Hartree (3, 4) demonstrated that some notatin samples contained a second enzyme, named mutarotase, which was the most active catalyst known for the mutarotation of glucose. A partial separation from notatin was achieved by precipitation with ammonium sulfate. Levy and Cook (5) studied the specificity of mutarotase and found that the mutarotation of d-galactose was accelerated almost as much as that of d-glucose. In 1954, Keston (6) observed that mutarotase preparations could be obtained from animal sources; D-galactose, D-xylose, and L-arabinose were also substrates and the enzyme was inhibited by phlorizin. The present paper describes the assay and purification of highly active mutarotase from the culture fluids of *Penicillium notatum*.

EXPERIMENTAL

**Materials and Methods**

The strain of *Penicillium notatum*, F. D. 446, used in the original studies on notatin (7, 8), was obtained through the courtesy of Mr. C. E. Coulthard, Bacteriology Division, Boots Pure Drug Company Ltd., Nottingham, England. Subcultures were maintained on 2 ml slants of Czapek-Dox agar (9). We are indebted to Corn Products Sales Company for a supply of corn steep liquor.

β-D-Glucose was obtained from General Biochemicals, Inc., and 2-deoxy-d-glucose from Aldrich Chemical Company, Inc. The latter material had m.p. 147–148°, and [α]$_D^{20}$ = +33° (5 minutes), +46.7° (30 minutes) measured in water, c = 2; it was, therefore, the β anomer. All other sugar anomers had optical rotations and melting points in agreement with the literature values. The methods used in the determination of mutarotation coefficients were identical to those described in detail in the following paper (10). Protein was determined with the Folin-Ciocalteau reagent by the method of Lowry et al. (11).

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† Present address, Antibiotic Research Centre, Hindustan Antibiotics (Private), Ltd., Pimpri, India.

**RESULTS**

**Mutarotase Assay**—Keilin and Hartree (3) originally assayed mutarotase by a polarimetric method under anaerobic conditions to rule out changes in optical rotation caused by the conversion of glucose to δ-gluconolactone and gluconic acid in the presence of notatin. An automatic recording (5) and a photoelectric polarimeter (6) have also been used in mutarotase assay; apparently only the former has been used anaerobically with the aid of a specially constructed 4-dm flow cell. Anaerobic polarimetry was, however, found to be difficult with our crude preparations, owing to the deeply pigmented nature of the solutions, and to frothing during evaporation (12). Keilin and Hartree (4) devised a manometric assay, based on the oxidation of β-D-glucose, formed from α-D-glucose, with an excess of mutarotase-free notatin. This method was also inconvenient as a routine assay, because of the need to weigh accurately small amounts of α-D-glucose into Warburg vessels (12).

A chemical assay, based on the reactions shown below, was, therefore, devised; it is derived from the observation that at 0° and in solutions buffered with barium carbonate and barium bromide, the oxidation of β-glucose with bromine water is much more rapid than that of the α-anomer (13). The barium carbonate-barium bromide buffer was replaced with phosphate buffer at pH 5.7, and the method was particularly suitable for the assay of crude enzyme preparations.

\[
\begin{align*}
\text{α-D-Glucose} & \xrightarrow{\text{mutarotase}} \text{β-D-glucose} \\
\text{β-D-Glucose} + \text{Br}_2 & \rightarrow \text{δ-D-gluconolactone} + 2 \text{HBr} \\
\text{δ-D-Gluconolactone} + \text{H}_2\text{O} & \rightarrow \text{δ-gluconic acid}
\end{align*}
\]

The assay procedure was as follows; 1 ml of mutarotase preparation and 3 ml of 0.1 M phosphate buffer, pH 5.7, in a 20-ml tube were cooled to 0°. One milliliter of an ice-cold, freshly prepared solution of α-D-glucose (3%) in 0.1 M phosphate buffer, pH 5.7, was added, and the tube was immediately transferred to a water bath at 20°. After incubation for 20 minutes, 2 ml of the incubation mixture were withdrawn with a precooled pipette, and were added to 10 ml of the oxidizing mixture in a stopped tube, cooled to 0° in an ice bath. The oxidizing mixture was 0.4 M phosphate buffer, pH 5.7, containing bromine (40% saturation); it was kept at 0°. After mixing, the solution was kept at 0° for exactly 8 minutes. The excess bromine was removed by addition of 1 ml of a mixture of corn oil and chloroform (1:1) followed by vigorous shaking. The
resulting emulsion was centrifuged and the clear supernatant containing unoxidized glucose was diluted (usually by a factor of 25) and assayed for glucose by the Nelson-Somogyi method (14). To allow for the spontaneous mutarotation a blank was carried out with 1 ml of a boiled sample of the enzyme preparation being assayed.

The oxidation rates of pure samples of \( \alpha \)- and \( \beta \)-d-glucose under the oxidation conditions just described are shown in Fig. 1. In the 8-minute oxidation period used for the mutarotase assay, 84% of \( \beta \)-glucose was oxidized, but only 14% of \( \alpha \)-glucose. In determining the formation of \( \beta \)-glucose, a correction was therefore applied to allow for the incomplete oxidation of \( \beta \)-glucose, and for the partial oxidation of the \( \alpha \)-glucose. The production of \( \beta \)-glucose by spontaneous mutarotation under these assay conditions amounted to between 105 and 110 μmoles. The enzyme concentration was so adjusted that the total \( \beta \)-glucose production in the sample tube was about 135 to 145 μmoles. The net production of \( \beta \)-glucose was calculated as production in sample minus production in the boiled control under identical conditions; mutarotase activity determined in this assay is expressed as μmoles net \( \beta \)-glucose produced per mg of protein per 20 minutes. Despite the high blanks from spontaneous mutarotation, consistent results were obtained.

Production of Mutarotase by \( P. \) notatum Cultures—The cultural conditions, developed by Coulthard et al. (7, 8) were modified by addition of corn steep liquor (approximately 60% of total solids), 20.0 g; water to 1 liter. Routinely, tap water was used in this medium since better growth was obtained than in control experiments on distilled water. The medium was adjusted to pH 6.2 and filtered prior to sterilization. Cultures were grown either in 900-ml Roux bottles containing 120 ml of medium, or in enamal trays (37 cm × 21 cm × 7.5 cm) covered with aluminum foil and containing 1100 ml of medium.

Under the above described cultural conditions, the initial pH of the medium declined to 3.0 by about the fourth day of growth, rising sharply on the fifth day to 7.9 and thereafter increasing slowly to a maximum of 9.2 (10 to 14 days). A final pH of at least 8.5 was desirable in view of the lability of notatin under alkaline conditions, and the observation that the subsequent purification was most satisfactory when this pH value had been reached. In preliminary experiments culture fluid samples were assayed for mutarotase activity after dialysis against several changes of distilled water at 4° for 40 hours. In the most active cultures, mutarotase activity (μmoles net \( \beta \)-glucose per mg of protein per 20 minutes) increased slowly from a value of 25 (fourth day) to a maximum of 35 (10th and 11th days) and thereafter declined slowly. Large scale cultures were usually harvested after 11 or 12 days growth.

Purification of Mutarotase

A purification of the enzyme was achieved by precipitation with aluminum sulfate and ammonium sulfate, treatment at pH 9.2 and 25° to ensure that any notatin withstanding the final pH of the medium was inactivated, treatment with Amberlite IR 45 to remove nucleic acid, and further precipitation with ammonium sulfate.

First Aluminum Sulfate Precipitation—The cooled (0°), filtered medium was treated gradually with stirring, with a 10% aluminum sulfate solution, using 1 ml for each 12.5 ml of filtrate. The pH during this addition was not allowed to fall below 4.5, and was finally adjusted to 5.8 by the addition of 0.1 N NaOH. The precipitate, collected by centrifugation, was extracted with ice-cold 0.2 M phosphate buffer, pH 5.8, with 1 ml of buffer per 10 ml of original filtrate. The brownish yellow extract, after dialysis against frequent changes of distilled water (0°) until no further pigment was removed, was lyophilized to half volume (Fraction A).

Treatment at pH 9.2—Fraction A at 0° was adjusted to pH 9.2 by careful, dropwise addition, initially of 1 N, finally of 0.1 N NaOH. The solution was then brought to 25°, allowed to stand for 20 minutes at this temperature, cooled to 0°, and adjusted to pH 4.2 with dilute phosphoric acid. After standing overnight at 0°, an inactive precipitate was removed by centrifugation. The supernatant solution was referred to as Fraction B.

Ammonium Sulfate Precipitation—Fraction B, diluted to a protein content of 1.5 mg per ml was treated with stirring, with solid ammonium sulfate at 0°, using 22 g per 100 ml. After an inactive precipitate had been removed by centrifugation, the supernatant was dialyzed for three days against frequent changes of distilled water at 0° (Fraction C).

Second Aluminum Sulfate Precipitation—The dialyzed Fraction C was treated with 1 ml of 10% aluminum sulfate per 15 ml of solution, as described previously. The centrifuged precipitate was reextracted with 1 ml of 0.2 M phosphate buffer for each 5 ml of Fraction C. The extract was dialyzed until salt free, lyophilized, and dissolved in water to give a solution with final protein concentration of 3 mg per ml (Fraction D).

Treatment with Amberlite IR 45—Fraction D, adjusted to pH 7.0 with 0.1 N NaOH was added to a column of Amberlite IR 45 (2 cm diameter), with a 1.1 cm length of resin column per ml of Fraction D. The resin had been previously treated with 4% NaOH, and then with 4% acetic acid, followed by washing.
with water. After the original extract had moved down on to the column, elution was continued with water. A first fraction, equal to the original volume of Fraction D was collected and discarded. A second fraction (2.5 times the original volume) was collected, dialyzed, lyophilized, and redissolved in water to a protein concentration of 1 mg per ml (Fraction E).

Second Ammonium Sulfate Precipitation—Fraction E, cooled in ice, was stirred and treated with solid ammonium sulfate using 42 g per 100 ml. After an inactive precipitate had been removed by centrifugation, a further addition of 14 g of ammonium sulfate per 100 ml of original Fraction E, gave a precipitate containing most of the mutarotase activity. After centrifugation and solution in water, it was dialyzed until salt free.

The results obtained on fractionation of a typical batch of 8350 ml of culture filtrates are shown in Table I. An approximately 15-fold purification was achieved. The absorption spectra of preparations of Fraction D showed a broad plateau in the region 250 to 280 mp; after treatment with Amberlite IR 45, a sharp minimum at 250 mp and a sharp maximum at 275 mp were observed, presumably as a result of the removal of nucleic acid. In view of the maximum at 275 mp, freedom from nucleic acid was judged from the ratio of absorbancies at 275 and 290 mp, rather than from the ratio at 250 and 260 mp as is the usual practice (15). The highest ratio observed in Fraction F was 1.25; a value of 1.25 for Rzu/2m with enolase corresponds to 1.5% of nucleic acid. Our mutarotase preparations therefore contain at most, only small amounts of nucleic acid.

Properties of Mutarotase Fraction F

Aqueous solutions of Fraction F had a pronounced amber color. The absorption spectrum in the visible region was essentially the same at pH 3.5, 6.2, and 7.8. There was an absorption maximum in the region 360 to 380 mp, a second less well defined at 400 mp, and an inflexion between 440 and 480 mp. This absorption spectrum was not changed by the addition of substrate glucose. On denaturation by heat, or by treatment with acid, the solution (after removal of the precipitated protein) gave an absorption spectrum declining as an essentially smooth curve. In alkaline solution, a shallow broad maximum was exhibited in the region from 350 to 400 mp; this was unchanged on reacidification. Treatment with potassium borohydride led to a bleaching of the solution and the disappearance of the absorption maximum at 370 mp. Although the absorption spectra of mutarotase preparations were similar to the spectrum of succinic dehydrogenase (16) attempts to liberate possible flavins by tryptic digestion (17) gave inconclusive results.

Typical preparations of Fraction F showed a net production between 200 and 260 umoles of d-glucose calculated per mg protein per 20 minutes, and the most active fraction formed 280 umoles of glucose. The preparations had no glucose oxidase or catalase activity. The mutarotase activity was unaffected by Mn++, Mg++, and Hg++ at concentrations of 5 x 10^{-4} M, but the same level of Hg++ abolished the activity completely.

In the ultracentrifuge, the preparations showed a major, slow moving component, together with a very small amount of a fast moving material. (See Fig. 2). A further purification was achieved by centrifugation of Fraction F in the preparative ultracentrifuge at 100,000 x g for 20 hours. The contents of the tube were separated into an upper and lower layer. The upper layer contained most of the mutarotase activity and this material was essentially free of the fast moving component as indicated by the ultracentrifuge patterns shown in Fig. 2. This solution still had a pronounced yellow to amber color. Assuming a partial specific volume of 0.75 and a reasonably spherical shape (frictional ratio of 1.15) as well as a small concentration dependence of the sedimentation constant, the molecular weight was estimated to be 70,000 with a lower limit of 60,000.

The relationship between glucose concentration and enzyme activity at a fixed level of mutarotase was investigated. As shown in Fig. 3 the net production of d-glucose increased with increased substrate concentration, up to a level of about 4% glucose. Thereafter, the activity began to decrease. The relationship between the net production of d-glucose and the amount of enzyme protein was also investigated at a fixed glucose concentration of 1%. As shown in Table II, no direct proportionality was observed. These relationships will be discussed later.

The work of Levy and Cook (5) with the low level of mutarotase activity present in some notatin samples, and that of Keston with animal mutarotase (6) indicated that only d-glucose approached d-glucose as a mutarotase substrate. It

![Table I: Fractionation of mutarotase from P. notatum](https://www.jbc.org/)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total protein</th>
<th>Net d-glucose per mg protein per 20 minutes</th>
<th>Total activity: net d-glucose</th>
<th>Rm/ru</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture fluid</td>
<td>8350</td>
<td>6095</td>
<td>16</td>
<td>97,500</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>500</td>
<td>775</td>
<td>50</td>
<td>38,750</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>220</td>
<td>397</td>
<td>66</td>
<td>26,200</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>88</td>
<td>266</td>
<td>85</td>
<td>22,600</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>133</td>
<td>133</td>
<td>150</td>
<td>19,950</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>50</td>
<td>75</td>
<td>250</td>
<td>18,750</td>
<td></td>
</tr>
</tbody>
</table>

\( a \) \text{R}_{275/260} \text{is the ratio of optical densities at 275 and 260 nm. See text.}

![Fig. 2. Ultracentrifuge patterns of mutarotase preparations.](https://www.jbc.org/)
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Figu. 3. The effect of substrate concentration on the production of excess β-glucose by mutarotase. The standard mutarotase assay method was used, with varying concentrations of α-D-glucose. Each tube contained 1 ml of a Fraction F mutarotase preparation (125 µg per ml).

TABLE II

Relationship between protein content and mutarotase activity

These determinations were carried out under the standard assay conditions described in the text, with a Fraction A preparation of relatively low activity.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Net β-glucose formed</th>
<th>Net β-glucose per mg protein per 20 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg</td>
<td>µmoles</td>
<td>µmoles</td>
</tr>
<tr>
<td>466</td>
<td>21.5</td>
<td>46.1</td>
</tr>
<tr>
<td>500</td>
<td>23.5</td>
<td>43.5</td>
</tr>
<tr>
<td>960</td>
<td>42.0</td>
<td>43.7</td>
</tr>
<tr>
<td>1400</td>
<td>57.0</td>
<td>40.7</td>
</tr>
</tbody>
</table>

The results shown in Table III, led to the recognition of two new substrates for mutarotase, namely cellobiose and the heptose, D-glycero-D-galactoheptopyranose.

DISCUSSION

With the use of the mutarotase-containing notatin, Keilin and Hartree (4) observed that the mutarotation coefficient for a fixed amount of enzyme decreased with increasing glucose concentration and slowly approached the value observed in buffer alone. The latter value is, of course, independent of glucose concentration. They attributed this result to an increase in inhibition of the enzyme by glucose itself. By measuring the actual net β-glucose produced at different glucose concentrations, we observed that there was an increased production of β-glucose even at constant enzyme concentration until a glucose concentration of about 4% was reached (see Fig. 3). This result is not inconsistent with that of Keilin and Hartree. If their results are recalculated in the more informative form of net β-glucose production, for example in 10 minutes, a curve rising to a maximum at about 4% glucose and thereafter declining, is obtained. It is obvious that beyond about 4% glucose concentration, the net β-glucose production steadily declines and at higher values there is only an immeasurably small difference between the spontaneous and enzyme catalyzed reactions. It is also apparent that any inhibition by glucose does not take place at concentrations less than 4%, both from the results of Keilin and Hartree and from those reported here. The enzyme never becomes saturated with substrate in the sense that a constant rate (amount of net β-glucose formed in a definite time interval) is reached and is not changed by further increases in substrate concentration. It follows from these observations that assay of mutarotase under conditions of zero order kinetics is never possible, and a definition of a mutarotase unit presents some problems. Kei-
lin and Hartree (1) demonstrated a linear increase in mutarotation coefficient with increased amount of enzyme at constant glucose concentration. If these results are recalculated as actual production of net $\beta$-glucose in a given period, the relationship is no longer a linear one. Keston (20) has used a mutarotase unit based on the time required for half mutarotation with 0.84% $\alpha$-$D$-glucose in 0.025 M phosphate buffer at pH 7.1 and 24°. A unit is that quantity for which $1/t_{\text{mutarotase}} = 1/t_{\text{sample}} - 1/t_{\text{spontaneous}} = 1$. Again, however, the relationship between the number of units and the net $\beta$-glucose production may be shown to be nonlinear.

As pointed out earlier, enzyme assay by polarimetry was not desirable in the purification of the $P$. notatum mutarotase. Instead, the net $\beta$-glucose production was determined under defined conditions, with a boiled sample of the enzyme to define the spontaneous production. In these assays, a number of dilutions were used and the activity was determined from that dilution yielding a net $\beta$-glucose production in the range of 30 to 35 pmoles. The best preparations we have examined gave about 280 pmol per net $\beta$-glucose over a 20 minute period, calculated back to the basis of 1 mg of protein. Again, because of the nonlinear relationship between excess $\beta$-glucose production and quantity of enzyme, this calculated figure is not representative of the true activity of that amount of enzyme. However, since these assays were all calculated from that enzyme dilution giving the arbitrarily selected yield of 30 to 35 pmol, it was possible to compare the enzyme activity at the various stages of fractionation.

Keilin and Hartree's notatin Z preparations, shown on ultracentrifugation (21), a main sedimenting component (notatin) of 25 pg per ml gave a coefficient of 21.3 X 10⁻³ (decimal logarithms, min⁻¹, 20°) or 17.3 X 10⁻³ (natural logarithms) or 15.3% of mutarotase, a figure which agrees well with the earlier estimate. The acceleration, relative to glucose, observed with a number of mutarotase substrates has been compared for the crude notatin sample, strongly suggesting that a single enzyme is responsible for all of the observed activities. There are marked differences, however, between the mold enzyme and the preparations of Keston (6). Although the relative acceleration with $L$-arabinose is the same, the animal preparations are reported to have higher relative accelerations with $D$-galactose and $D$-xylose.

**SUMMARY**

1. Mutarotase production by *Pencillium notatum* grown on Czapek-Dox medium containing 2% corn steep liquor has been studied. Enzyme activity is determined by a direct measurement of the net amount of $\beta$-glucose formed from $\alpha$-glucose in a definite time interval. The method is based on the more rapid oxidation of $\beta$- than $\alpha$-glucose in buffered aqueous solutions of bromine at 0°. Other assay methods have been discussed.

2. The enzyme was purified by precipitations with aluminum sulfate and ammonium sulfate, inactivation of glucose oxidase by treatment with alkali at pH 9.2, and a removal of nucleic acid.
acid by treatment with Amberlite IR 45. Further purification by the use of the preparative ultracentrifuge yields preparations showing only one peak which migrates slowly in the analytical ultracentrifuge.

3. The enzyme is a protein with a molecular weight of about 70,000. Activity is lost in the presence of Hg++, but Mn++ and Mg++ are without effect. No coenzyme requirement has been established. The activity at a fixed enzyme concentration increases with increasing glucose concentration up to a level of about 4%, and thereafter declines.

4. Two new substrates for mutarotase are β-cellobiose and the hoptose, β-D-glycero-D-galactoheptopyranose. 2-Deoxy-D-glucose is not a substrate. Evidence is presented that a single enzyme catalyzes all of the known mutarotations.

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