The Anomeric Specificity of Yeast Galactokinase[1]

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ABSTRACT

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In order to investigate the specificity of yeast galactokinase toward the α and β anomers of galactose, a chromatographic system has been developed for separating and identifying α- and β-galactose-1-phosphates. The purified enzyme was incubated with β-galactose, and the phosphorylated product isolated and identified as α-Gal-1-P. Optical rotation studies show no mutarotase activity in the enzyme preparation; however, other components of the incubation mixture are shown to accelerate the mutarotation of β-galactose. Thus, while it cannot be stated conclusively that β-galactose cannot be phosphorylated by this enzyme, the absence of any β-Gal-1-P in the product confirms that α-galactose is the preferred substrate, and that the previously observed reaction of purified galactokinase with β-galactose probably consists of nonenzymatic mutarotation, followed by phosphorylation of the resulting α-galactose.

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Solutions of galactose at mutarotation equilibrium contain a mixture of approximately one-quarter α-galactopyranose, three-quarters β-galactopyranose, and smaller amounts of several other forms. The product of the reaction of yeast galactokinase with such a galactose solution has been shown to be the α anomer of galactose 1-phosphate (α-Gal-1-P) (1, 2). Because galactokinase catalyzes the phosphorylation of carbon-1, the specificity of the enzyme toward each of the anomers of the pyranose form had been determined (3). It was found that the enzyme reacted immediately, and at a rapid rate, with α-galactose, but that it also reacted with β-galactose at an appreciable rate after a short lag period.

Among the many sugar kinases, only a few catalyze the phosphorylation of carbon-1 (4). Little is known about the specificity or mechanisms of these enzymes, in their reactions with sugars which are in rapid mutarotation equilibrium between several forms. The present studies are an attempt to determine the specificity of galactokinase and the effect of mutarotation upon the reaction.

EXPERIMENTAL

Materials.- Galactose was obtained from Pfanstiehl Laboratories or from Sigma Chemical Company. ATP was a Pabst Laboratories product, and galactose-6-phosphate was purchased from Nutritional Biochemical Corporation. α-Galactose and β-galactose were prepared by the fractional crystallization procedures of Hudson and Yanofsky (5).

α-D-Gal-1-P was synthesized by the method of Hansen et al. (6), with some details taken from Krahl and Cori (7). β-D-Gal-1-P was synthesized according to Reithel (8) and Putnam (9).

Methods.- Total hexose was determined by the cysteine-sulfuric acid method of Diedrich and Anderson (10). Solutions were read in a Klett colorimeter at 420 mμ. The method was found to be useful in the range of 0.025 to 0.3 μmole. The Nelson method was used for determination of reducing sugars (11). Inorganic, acid-labile, and total phosphate were determined by the Fiske and SubbaRow method, as described by Leloir and Cardini (12). Optical rotation was determined in a Bellingham and Stanley polarimeter, using the 546.1 mμ mercury line source.

Galactokinase isolation.- The enzyme was prepared from Saccharomyces fragilis, strain C-106, by the procedure previously described (3), with the following modifications. The first extract of the air-dried yeast was heated by swirling in a water bath at 50° for 10 minutes. After rapid chilling and centrifugation, the supernatant solution was taken through the ammonium sulfate fractionation and pH 5 precipitation steps. The calcium phosphate gel and bentonite steps were omitted because they were too variable. Fractionation on a DEAE-cellulose column was accomplished with a linear gradient from 0.005 M ammonium phosphate, pH 7, to 0.005 M ammonium phosphate-0.5 M KCl,

pH 4.5. In some cases a column of DEAE-Sephadex was used instead, with a linear gradient from 0.01 M ammonium phosphate, pH 6.5, to 0.01 M ammonium phosphate-1.0 M KCl, pH 4.5. There was no detectable difference in the galactokinase prepared by these two column methods. Fractions from the column were assayed by the spectrophotometric procedure. The fractions which contained the peak of galactokinase activity were combined, and solid ammonium sulfate added to 80% saturation. The protein which precipitated after 48 hours was centrifuged down, resuspended in a minimum amount of 0.05 M ammonium phosphate buffer, pH 7.0, and frozen in 0.5 ml. portions. The enzyme deteriorates upon repeated freezing and thawing, or when frozen in dilute solution. Immediately before use, the amount of enzyme needed was desalted by being passed through a small column of Sephadex G-25, with 0.05 M ammonium phosphate, pH 7.0, as eluant. The eluate contained approximately 2 mg. of protein per ml., and catalyzed the phosphorylation of 5 to 6 μmoles of galactose/min./ml.

Galactokinase assay.- In the presence of added pyruvate kinase and lactate dehydrogenase, the production of ADP in the galactokinase reaction results in oxidation of DPNH, which is recorded spectrophotometrically (3). For each assay, the following is added to a cuvette of approximately 1.5 ml. capacity: 0.3 ml. of potassium phosphate buffer, 0.16 M, pH 7.0; 0.1 ml. each of 0.8 M KCl; 0.02 M EDTA; 0.1 M magnesium sulfate; ATP,

3 mg./ml.; phosphoenolpyruvate, 3.8 mg./ml.; lactate dehydrogenase (containing pyruvate kinase), 0.2 mg./ml.; DPNH, 0.7 mg./ml. The contents are mixed, and enzyme (usually 0.01 ml.) and galactose are added. The cuvette is mixed by inversion, and immediately placed in the spectrophotometer to follow the rate of change in absorbance at 340 mμ. For routine assays, the galactose is 0.1 ml. of a 3 mg./ml. solution. When galactose anomers are tested, a weighed, dry sample is added to the cuvette. Figure 1 illustrates the rapid reaction of α-galactose, as followed by this assay, and the lag in the reaction of β-galactose.

ION EXCHANGE SEPARATION OF α- AND β-GALACTOSE 1-PHOSPHATES

Many systems have been described for the separation of phosphate esters of different sugars (10, 13-16), and Mowery (17) has separated α and β glycosides on starch columns. There were no reports of the resolution of the α and β forms of sugar 1-phosphates, however. (After this work was completed, Ray and Roscelli (18) reported the separation of α- and β-glucose 1,6-diphosphates on Dowex-1-formate columns.) In developing such a system, the use of dilute mineral acids was avoided because of the acid lability of these esters, and borate was not used because of possible interference with colorimetric analyses. It was found that separation of the anomers could be achieved on Dowex-1 columns, by elution with ammonium chloride solution.

Columns of Dowex-1-X8-chloride, 200 to 400 mesh, 14 × 0.9 cm. were used for samples of 1 to 10 mg. Samples were adsorbed on the column

at about pH 7.5, and eluted with 0.04 M ammonium chloride at a flow rate of 1 ml./min. Hexose was determined in 0.5 or 1.0 ml. aliquots of the fractions by the cysteine-sulfuric acid method.

The separation of α- and β-galactose 1-phosphates by this procedure is shown in Fig. 2. Each peak was identified by comparing its elution volume with those obtained for each anomer chromatographed separately under identical conditions. The small peak (X) which was eluted just before α-Gal-1-P is due to material of unknown composition which was found in both synthetic compounds, and in the enzymatic product. This peak contained sugar, total phosphate, and acid-labile phosphate in approximately equimolar proportion, and was not further identified. Gal-6-P, ATP, or ADP was not eluted from a column of this size with 500 ml. of 0.04 M ammonium chloride; a concentration of at least 0.1 M was required for elution of Gal-6-P, and even higher salt concentration for the nucleotides.

Analysis of the peak fractions of the two galactose 1-phosphates gave molar ratios of acid-labile phosphorus to sugar of 1.2 for α, and 1.1 for β.

ISOLATION OF THE REACTION PRODUCT OF β-GALACTOSE

Incubations of galactokinase with β-galactose were carried out with a large excess of enzyme, and with the sugar added in small portions at intervals. It was thought that these conditions would favor a rapid phosphorylation of β-galactose, if such a reaction could

occur, before the sugar mutarotated to the reactive α-anomer. The sugar 1-phosphates do not mutarotate after their formation.

Each enzyme preparation was assayed just before use in an incubation. In a typical experiment, 0.01 ml. aliquots of galactokinase were used in the spectrophotometric assay, with 0.3 mg. of mutarotation galactose (0.1 ml. of 3 mg./ml. solution) or 0.3 mg. of solid β-galactose. It was found that the galactose solution reacted at a rate of 0.056 μmole/min., whereas 0.012 μmole of the β-anomer reacted during the first minute, and an additional 0.029 μmole during the second minute.

For isolation of the product, the incubation mixture was similar to that used by Anderson et al. (19): 4.0 ml. of 0.16 M potassium phosphate buffer at pH 7.0 (572 μmoles), 23.2 mg. (114 μmoles) of magnesium chloride, 41.6 mg. (68.6 μmoles) of ATP dipotassium salt, and 2.0 ml. of galactokinase. A total of 7.2 mg. (40 μmoles) of solid β-galactose was divided into 20 to 30 approximately equal portions. A portion was added to the incubation mixture, at room temperature, every 90 seconds. (Rough extrapolation from the spectrophotometric assay indicated that this amount of galactose should react in less than 90 seconds.) After the additions were complete, the mixture was heated in a boiling water bath for 1 minute, and centrifuged to remove the protein. The pH was adjusted to 8.3 with KOH, and 0.1 ml. of saturated barium hydroxide per ml. supernatant solution was added. After centrifugation to remove water-insoluble barium salts, four volumes of cold absolute ethanol were added, and the mixture was

allowed to stand overnight at 5°. An excess of potassium sulfate solution was added to the precipitate, and the barium sulfate removed. The supernatant solution of potassium Gal-1-P was used for ion exchange chromatography as described above.

When this solution was chromatographed, the elution pattern of Fig. 3 was obtained. In the column eluate, 34 μmoles of phosphorylated product were recovered from 40 μmoles of β-galactose incubated with the enzyme. The large peak is in the position corresponding to α-Gal-1-P. Further identification of this peak was obtained by co-chromatography with each of the authentic galactose 1-phosphates. The three fractions from the peak shown in Fig. 3 were combined, and frozen in three equal portions. One portion was mixed with an approximately equal amount of α-Gal-1-P and chromatographed, giving an elution pattern with a single large α peak (Fig. 4A). Another portion was chromatographed with β-Gal-1-P, and showed distinct peaks in both the α and β regions (Fig. 4B). Thus the product of the enzyme reaction appeared to be only α-Gal-1-P.

MUTAROTATION OF β-GALACTOSE

The appearance of α-Gal-1-P as the only product of phosphorylation of β-galactose indicated that the sugar was rapidly mutarotating to the α-anomer, followed by phosphorylation. A rapid mutarotation might be catalyzed by the components of the reaction mixture, or by mutarotase activity (20) in the enzyme preparation.

These possibilities were examined by following the mutarotation of β-galactose in the presence of buffer alone, buffer with two levels of enzyme (without ATP), or the incubation mixture (without enzyme) as used in the isolation experiments described above.

In each case 80 mg. of dry, pulverized β-galactose was dissolved as rapidly as possible in the solution to be used, made up to 2.0 ml., and transferred to a 1-dm. polarimeter tube. Zero time was taken as the time of addition of sugar to the solvent, and readings were taken at short intervals for 30 minutes at room temperature. The enzyme used had been fractionated as described above. Each reading was corrected by the value of the optical rotation observed with a corresponding blank solution (without galactose) in the same polarimeter tube. The validity of the correction is shown by the fact that the corrected rotations of the four solutions were within 0.04° after 30 minutes.

It is evident from Fig. 5 that the incubation mixture (ATP, $MgCl_2$, 0.16 M phosphate buffer) catalyzed a much more rapid mutarotation (curve A) than that observed in 0.05 M buffer alone (curve C). The presence of a low concentration of enzyme (0.27 mg. protein/ml.) gave a curve which is indistinguishable from that observed in the presence of the same concentration of buffer alone (curve C). A higher concentration of enzyme (0.68 mg./ml.) slightly increased the rate of mutarotation (curve B).

DISCUSSION

The difficulty of studying the enzymatic reactions of individual mutarotation forms of a sugar is illustrated by the small number of such studies which have appeared. Gottschalk (21) took advantage of the low mutarotation rate of fructose at 0° to show that β-D-fructofuranose was the only form fermented by yeast. He also found that both anomers (α and β) of mannopyranose and of glucopyranose were fermented (22). The findings with fructose were later confirmed by Slein et al. (23), for crystalline hexokinase, again using a low temperature to slow mutarotation. In their studies on glucose oxidase, Keilin and Hartree showed that this enzyme oxidized only the β anomer (24, 25). Although α-glucose reacted to some extent, that reaction was accounted for by the calculated rate of mutarotation under the conditions used.

In the present studies with yeast galactokinase, a high ratio of enzyme to substrate has been used in an attempt to obtain rapid phosphorylation before the substrate is altered by mutarotation. When the substrate was β-galactose, the product isolated was exclusively α-Gal-1-P, suggesting that mutarotation occurred prior to phosphorylation. It was not possible to compare the rate of phosphorylation and mutarotation directly, by polarimetry of the incubation mixture, because of the high sugar concentrations required for those measurements. Calculation of the amount of mutarotation to be expected in the short time intervals used here is not feasible because galactose exhibits complex mutarotation. Although rate equations can be derived

empirically for such a sugar (26), they are of doubtful significance in the calculation of the amount of α-galactose formed by mutarotation in 90 seconds under the conditions of the experiment. Isbell and Pigman (27, 28) pointed out that the mutarotation equilibrium mixture of galactose contains the anomers of furanose, pyranose, and heptanose rings, plus free aldehyde. More recently, gas chromatography of trimethylsilyl (29, 30) or methyl (31) derivatives of galactose has confirmed the occurrence of these forms in appreciable amount. Such derivatives have, of necessity, been made in organic solvents, and under fairly rigorous conditions, so that we do not yet know the quantitative composition of an aqueous mutarotation equilibrium solution.

The demonstration in these studies of the absence of any significant increase in the mutarotation rate of β-galactose in the presence of galactokinase would indicate that there is no mutarotase activity associated with this enzyme preparation. The slight increase in rate at the higher enzyme concentration is probably due to the ionic strength of the protein itself. These results also underscore the efficiency of salts and organic compounds as catalysts of mutarotation.

Since the galactokinase preparation catalyzes phosphorylation rather than mutarotation, the high concentration of enzyme used in these experiments should favor phosphorylation of β-galactose if it is a substrate. The failure to find β-Gal-1-P as a product strongly indicates that β-galactose is not a substrate, but mutarotates to α-galactose, which is then phosphorylated. This sequence

of steps explains the "lag period" seen in the spectrophotometric assay, if we assume that mutarotation is more rapid than phosphorylation during the early part of the reaction, so that substrate can accumulate.

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FIGURE LEGENDS

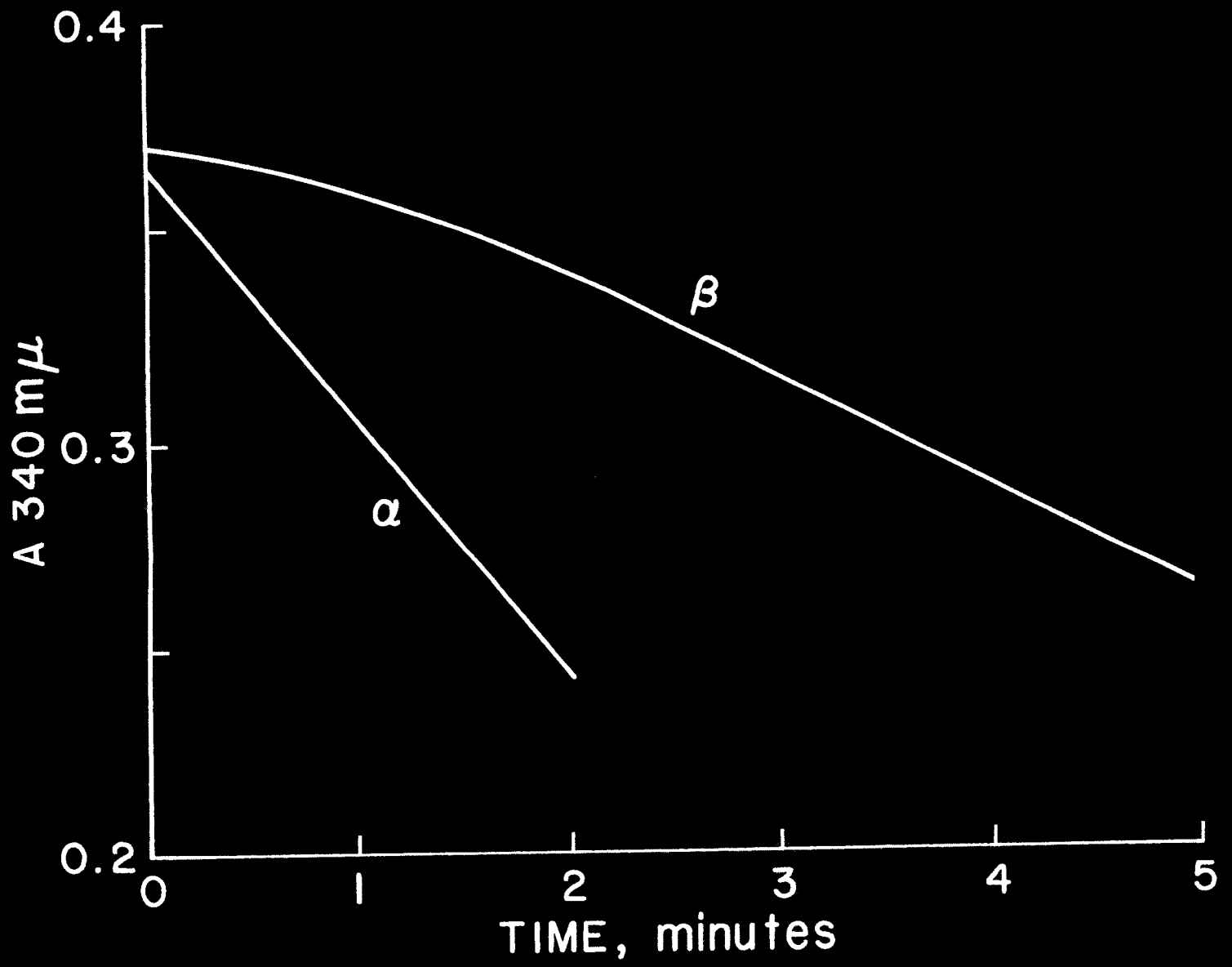
Fig. 1. Reaction of galactose anomers with galactokinase. Spectrophotometric assay (see text). Enzyme for each assay was 0.03 mg. of protein. Galactose anomers added as solids, 0.13 mg. of α or 0.14 mg. of β.

Fig. 2. Separation of α- and β-galactose 1-phosphates. 7.4 μmoles of each compound (potassium salt) on Dowex-1-chloride, 14 × 0.9 cm. Eluted with 0.04 M NH_4Cl; 5-ml. fractions. Sugar was determined in 1 ml. aliquots by the cysteine-H_2SO_4 method.

Fig. 3. Chromatography of the reaction product of galactokinase with β-galactose. The product of the enzymatic reaction with 40 μmoles of β-galactose, chromatographed as in Fig. 2. Fraction size: 10 ml. Sugar was determined in 0.5-ml. aliquots.

Fig. 4. Co-chromatography of the enzyme reaction product with synthetic Gal-1-P anomers. Equal portions of the product from the peak tubes of Fig. 3 were mixed with: (A) α-Gal-1-P or (B) β-Gal-1-P, and each mixture chromatographed as in Figs. 2 and 3. Sugar was determined in 1-ml. aliquots.

Fig. 5. Mutarotation of β-galactose. Concn.: 4%; 1-dm. tube; Hg line, 546.1 mμ. β-galactose dissolved at zero time in: (A) 0.16 M K phosphate buffer, pH 6.8, 0.02 M $MgCl_2$, 0.01 M ATP; (B) 0.05 M ammonium phosphate buffer, pH 7.0, containing 0.68 mg. of galactokinase; (C) 0.05 M ammonium phosphate buffer alone, or with 0.27 mg. of galactokinase.



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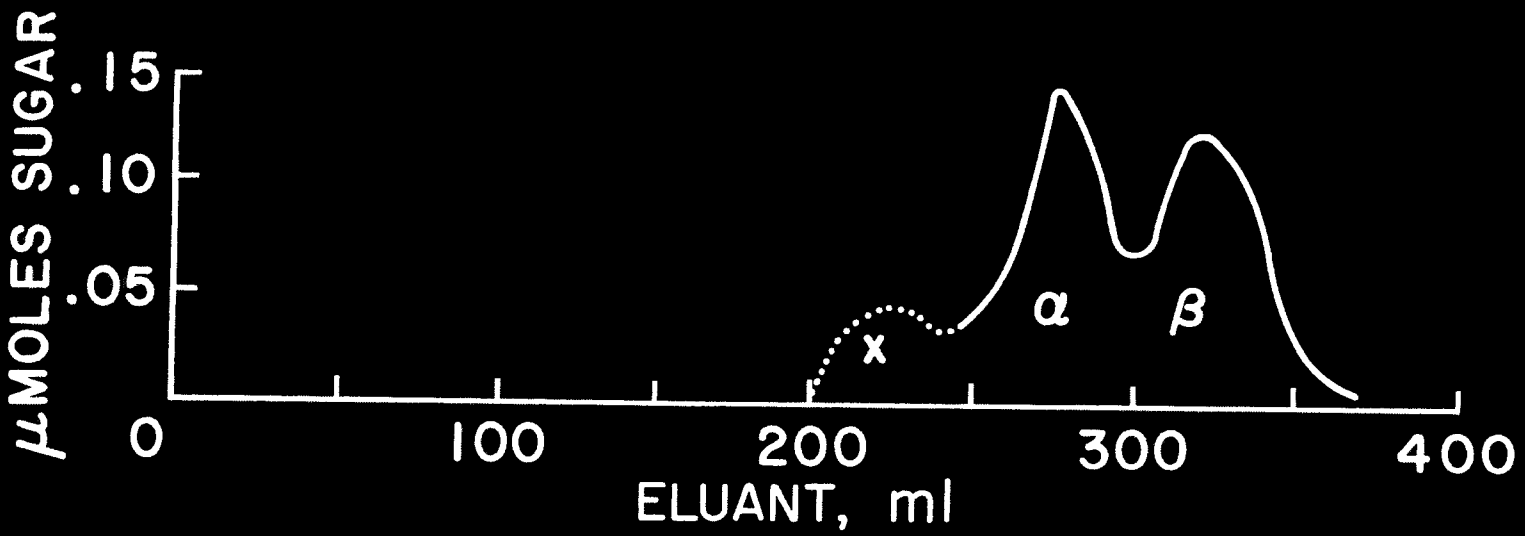

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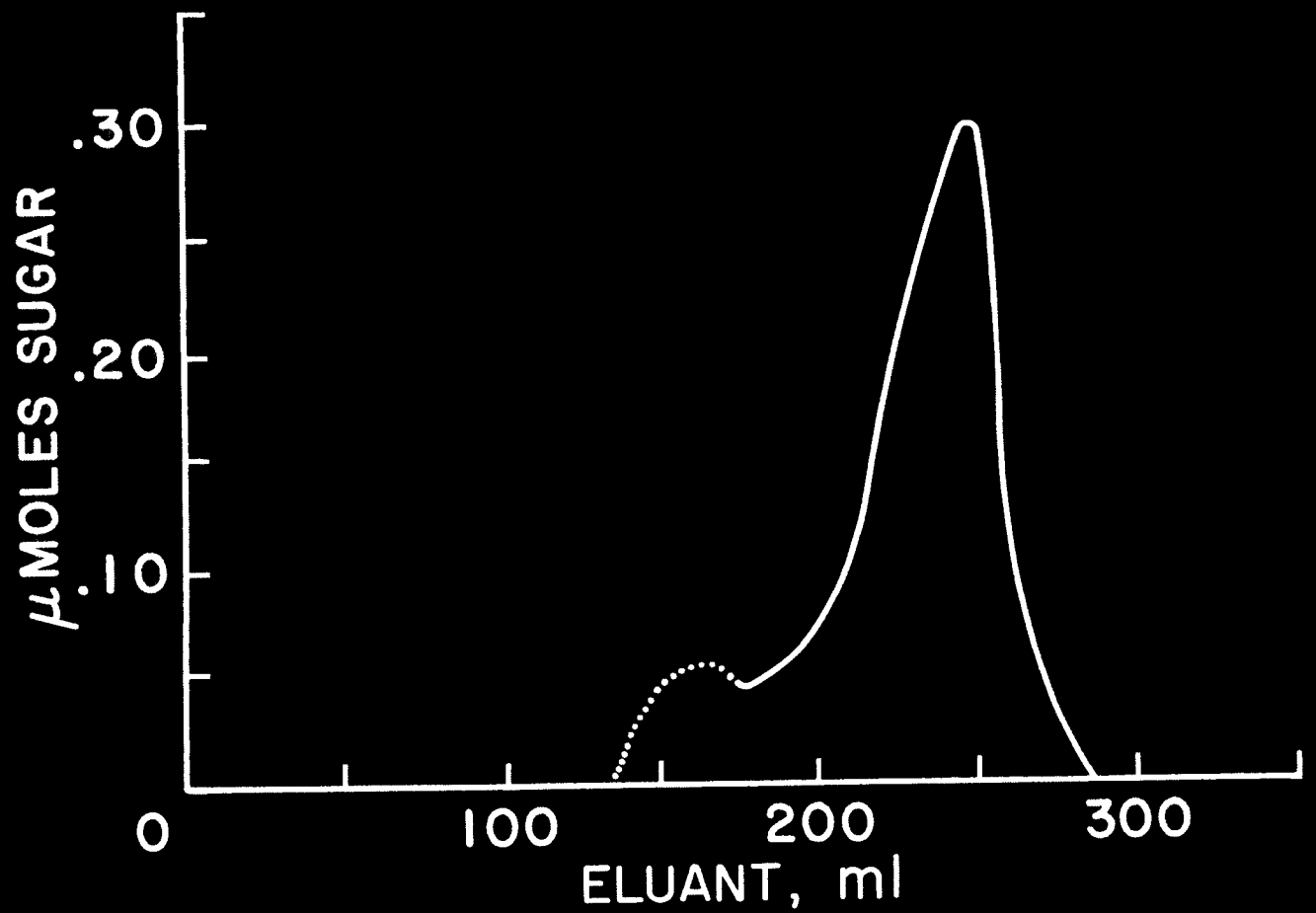

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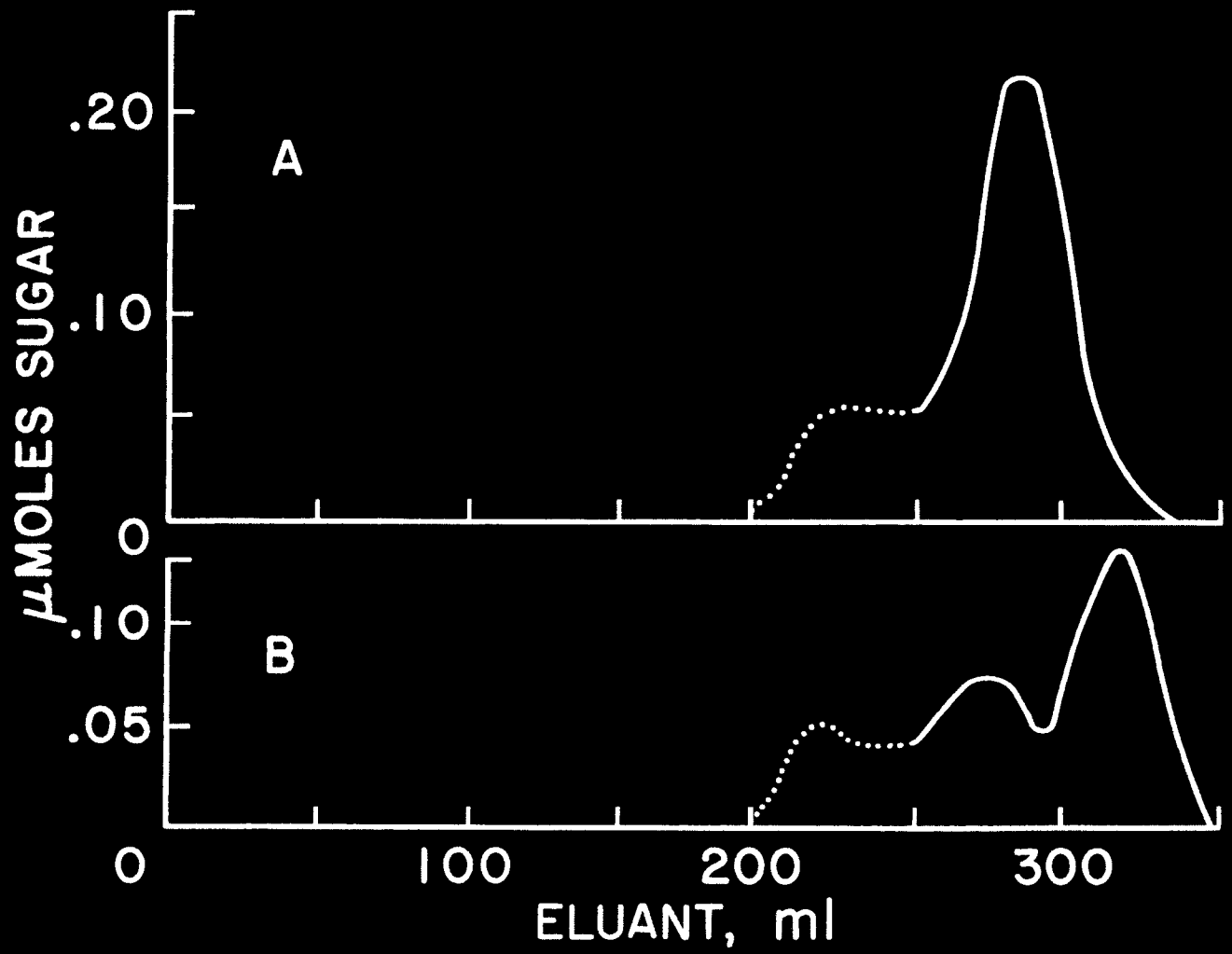

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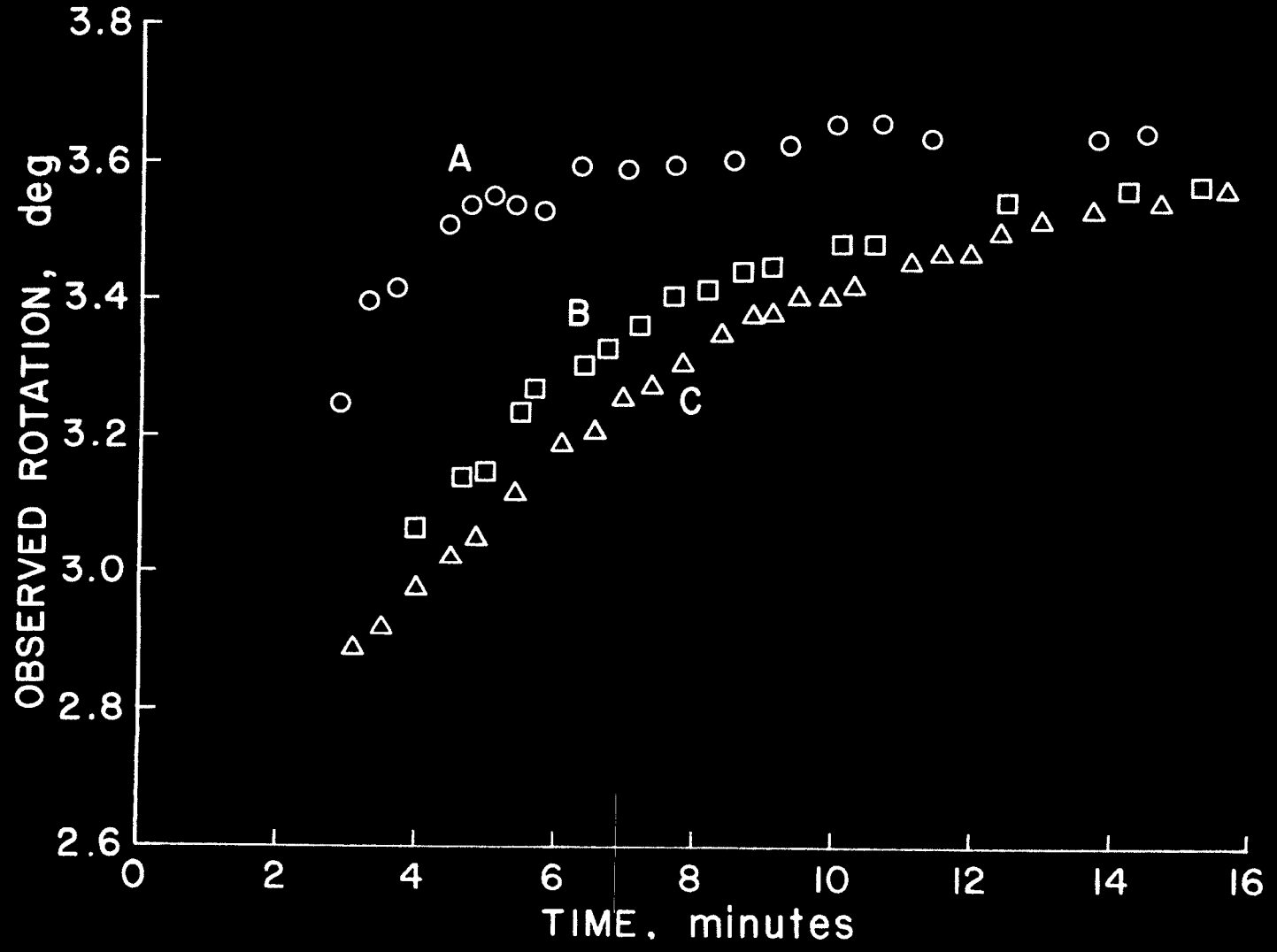

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