Metabolism of D-Mannose in *Aerobacter aerogenes*: Evidence for a Cyclic Pathway¹

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ABSTRACT

KAMEL, M. Y. (Michigan State University, East Lansing), AND R. L. ANDERSON. Metabolism of D-mannose in *Aerobacter aerogenes*: evidence for a cyclic pathway. J. Bacteriol. 92:1689-1697. 1966.—Evidence is presented which suggests a cyclic pathway for the constitutive utilization of D-mannose in extracts of *Aerobacter aerogenes* PRL-R3. D-Mannose is phosphorylated with D-glucose-6-phosphate to yield D-mannose-6-phosphate and D-glucose. D-Glucose-6-phosphate may be regenerated by isomerization of D-mannose-6-phosphate through D-fructose-6-phosphate, or by phosphorylation of D-glucose with adenosine-5'-triphosphate. The pathway involves the participation of four constitutive enzymes: D-glucose-6-phosphate isomerase, D-mannose-6-phosphate isomerase, a stereospecific D-glucokinase, and a phosphotransferase which phosphorylates D-mannose with D-glucose-6-phosphate, acetyl phosphate, or carbamyl phosphate. The absence of D-mannokinase (adenosine-5'-triphosphate:D-mannose phosphotransferase) activity in extracts of this organism suggests that the pathway may be of functional significance. Also, the pathway accounts for an apparent 2-epimerization of D-mannose to D-glucose that was observed in extracts.

Because of the widespread occurrence of hexokinase and D-mannose-6-phosphate isomerase in yeast, animal tissues, and bacteria, it is generally believed that D-mannose is normally metabolized by phosphorylation with adenosine triphosphate (ATP) to yield D-mannose-6-phosphate, followed by isomerization to D-fructose-6-phosphate. The constitutive hexokinase of *Aerobacter aerogenes* PRL-R3, however, has been purified over 1,000-fold and shown to be highly stereospecific for D-glucose (8). Attempts to demonstrate unequivocally the existence of a D-mannokinase (ATP:D-mannose phosphotransferase) in this organism have consistently yielded negative results in spite of the fact that D-mannose is metabolized constitutively. Rather, an apparent 2-epimerization of D-mannose to D-glucose was detected in extracts. In an effort to reconcile these observations, we have identified a sequence of reactions which comprise a cyclic pathway for the metabolism of D-mannose independent of the involvement of D-mannokinase (M. Y. Kamel and R. L. Anderson, Bacteriol. Proc., p. 82, 1965). In

addition to D-glucokinase, D-mannose-6-phosphate isomerase, and D-glucose-6-phosphate isomerase, the pathway involves the participation of an acyl phosphate:hexose phosphotransferase (9), which has now been purified and shown also to possess hexose phosphate:hexose phosphotransferase activity (M. Y. Kamel and R. L. Anderson, Federation Proc. 24:422, 1965). The present communication outlines the pathway and describes the experiments which led to its proposal.

MATERIALS AND METHODS

Growth of organism. *A. aerogenes* PRL-R3 was grown aerobically at 30 C for 18 hr and harvested by centrifugation. Unless otherwise specified, the medium consisted of 1.35% Na₂HPO₄·7H₂O, 0.15% KH₂PO₄, 0.3% (NH₄)₂SO₄, 0.02% MgSO₄·7H₂O, 0.0005% FeSO₄·7H₂O, and 0.5% D-glucose (autoclaved separately). The peptone-beef extract medium used in one experiment consisted of 0.5% Difco peptone and 0.3% Difco beef extract, pH 7.0.

Preparation of cell extracts. Cell extracts were prepared by treatment of cell suspensions for 5 to 10 min in a Raytheon model DF-101, 250-w, 10-kc sonic oscillator circulated with ice water. The supernatant fluid, after removal of the cellular debris by centrifugation at 31,000 × g, was the crude extract.

Reagents. Intestinal alkaline phosphatase and glu-

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cose oxidase (Glucostat) were obtained from the Worthington Biochemical Corp., Freehold, N.J. Glucose-6-phosphate dehydrogenase of suitable purity for enzyme-coupled assays was obtained from several commercial sources. D-Mannose-6-phosphate isomerase (about 50-fold purified) and D-glucose-6-phosphate isomerase (about 100-fold purified) were obtained from *A. aerogenes* PRL-R3 by fractionation of extracts with protamine sulfate, ammonium sulfate, alumina C₇ gel, and Sephadex G-100. D-Glucokinase was the preparation described by Kamel, Allison, and Anderson (8), and purified acyl phosphate:hexose phosphotransferase was the preparation reported by Kamel and Anderson (Federation Proc. 24:422, 1965). D-Mannose (chemically pure) was twice recrystallized (7) to remove interfering amounts of D-glucose. All other chemicals were used as obtained from commercial sources.

Analytical procedures. Unless otherwise stated, all reactions were thermostated at 25 C, and were terminated by heating in a boiling-water bath for 2 to 5 min, rapidly cooled, and centrifuged to remove turbidity. Spectrophotometric measurements of reduced pyridine nucleotides were made at 340 m μ with a Gilford absorbance-recording spectrophotometer, by use of microcuvettes with a 1-cm light path. Manometric measurements were made by use of conventional techniques (19). Descending paper chromatography of sugars was done by use of Whatman no. 1 paper (washed with 1 N HCl and water) with 80% phenol or butanol-pyridine-water (6:4:3) as the solvent. The sugars on the chromatograms were visualized with silver nitrate (18) or with *N,N*-dimethyl-*p*-phenylenediamine monohydrochloride (10). Protein was determined spectrophotometrically, with the aid of a nomograph (courtesy of Calbiochem), based on the data of Warburg and Christian (20). D-Glucose was measured with glucose oxidase or by measuring nicotinamide adenine dinucleotide phosphate (NADP) reduction in the presence of excess purified stereospecific D-glucokinase (8), glucose-6-phosphate dehydrogenase, and ATP.

Enzyme assays. D-Glucokinase and acyl phosphate:hexose phosphotransferase were assayed as previously described (8, 9). D-Glucose-6-phosphate isomerase was assayed spectrophotometrically by measuring NADP reduction in the presence of D-fructose-6-phosphate and glucose-6-phosphate dehydrogenase. D-Mannose-6-phosphate isomerase was assayed spectrophotometrically by measuring NADP reduction in the presence of D-mannose-6-phosphate, D-glucose-6-phosphate isomerase, and glucose-6-phosphate dehydrogenase. Units of enzyme refer to the micromoles of substrate reacted per hour at 25 C, pH 7.5, and with saturating levels of substrate.

Radioactivity experiment. The reaction mixture for the experiment depicted in Fig. 5 contained 200 μ moles (2 μ c) of D-mannose-1-C¹⁴ and crude cell extract (432 mg of protein, adjusted to pH 7.5) in a volume of 15 ml. Samples (7-ml) were removed at zero-time and after incubation at 25 C for 3 hr, heated in a boiling-water bath for 5 min, cooled, and centrifuged. Absolute ethyl alcohol (28 ml) was added to each supernatant solution, and, after cooling to 0 C, the precipitates were removed by centrifugation and discarded. The

samples were then evaporated to dryness under reduced pressure and redissolved in 3 ml of water. They were then treated three times with Dowex 50W-X8 (H⁺), after which the pH was 3, followed by three treatments with Amberlite CG-4B, after which the pH was 6.4. The volumes were then reduced to about 0.1 ml under reduced pressure, and samples [1.0 μ liter (2,500 count/min) of the 3-hr sample and 1.3 μ liters (2,800 count/min) of the zero-time sample] were chromatographed on paper with 80% phenol as the solvent. Radioactivity scans were made with a Nuclear-Chicago model 1036 4-pi Actigraph II scanner, Nuclear-Chicago model 1620CS analytical count ratemeter, and Sargent model SRL recorder. The following were used in scans: a 0.25-inch (0.6 cm) slit width, a scan speed of 0.2 inch (0.5 cm) per min, and a 20-sec time constant.

RESULTS AND DISCUSSION

Whole-cell fermentation. Cells of *A. aerogenes* PRL-R3 grown on either the D-glucose-mineral medium or on a peptone-beef extract medium fermented D-glucose and D-mannose at equivalent rates (Fig. 1), indicating that the metabolism of these two hexoses is constitutive in this organism.

Absence of D-mannokinase. Several different assay procedures were used in attempts to detect the possible phosphorylation of D-mannose with ATP. These included measurement of the disappearance of reducing sugar (4) after removal of the phosphate esters with barium (17); measurement of acid production manometrically (16), spectrophotometrically (3), and by titration with NaOH to maintain a constant pH; and the meas-

FIG. 1. Fermentation of D-glucose, D-mannose, and D-fructose by cells of *Aerobacter aerogenes* PRL-R3 grown on D-glucose-mineral salts and on peptone-beef extract. Each Warburg vessel contained, in a volume of 0.5 ml: 10 μ moles of NaHCO₃, 10 μ moles of hexose, and washed cells (1.86 mg, dry weight). The gas phase was 5% CO₂ in nitrogen, and the temperature was 30 C. An endogenous rate of about 6 μ liters of CO₂ per hr per mg (dry weight) has been subtracted from the rates shown.

urement of adenosine diphosphate (ADP) formation in a pyruvate kinase-lactate dehydrogenase-linked assay (2). Although the results obtained with the different methods varied, an apparent activity could always be detected in crude extracts by manometric and titrimetric assays. Attempts to purify the apparent D-mannokinase by various fractionation procedures, however, invariably led to a loss of activity. Typical data are shown in Fig. 2. The titrimetric assay for kinases showed activity on both D-glucose and D-mannose in the crude extract, but only on D-glucose in the ammonium sulfate fraction. Other fractions were also devoid of D-mannokinase activity. The use of ethylenediaminetetraacetate, reduced glutathione, or mercaptoethanol during fractionation and assay had no effect on preserving apparent D-mannokinase activity. D-Mannokinase activity was also not detected in particulate fractions of broken-cell suspensions. Assays with cytidine triphosphate (CTP), guanosine triphosphate (GTP), inosine triphosphate (ITP), and uridine triphosphate (UTP) in place of ATP were also negative. Cell extracts prepared by procedures other than sonic vibration, such as with a French pressure cell, also contained no detectable D-mannokinase activity after fractionation.

Although it is possible that a very labile or otherwise peculiar D-mannokinase does exist in *A. aerogenes* PRL-R3, the apparent activity that was detected in crude extracts can also be explained on a basis other than the direct phosphorylation of D-mannose with ATP. In the titrimetric assay shown in Fig. 3, D-mannose was preincubated with crude extract before the addition of ATP. With the addition of ATP to the

FIG. 2. Titrimetric assay for D-glucokinase and D-mannokinase in a crude extract and an ammonium sulfate fraction. Each reaction mixture contained, in a volume of 1.64 ml: 40 μ moles of ATP, 80 μ moles of $MgCl_2$, 80 μ moles of the indicated hexose, and extract (25 mg of protein for the crude extract and 20 mg of protein for the ammonium sulfate fraction). The pH was maintained between 7.2 and 7.4 with the periodic addition of NaOH.

FIG. 3. Titrimetric assay for D-mannokinase-type activity in a crude extract. Each reaction mixture contained, in a volume of 1.44 ml: 10 μ moles of ATP, 20 μ moles of $MgCl_2$, 200 μ moles of D-mannose, and extract (18 mg of protein). The endogenous control was minus D-mannose. The reaction mixtures were preincubated with D-mannose for the times indicated before the addition of ATP, and adjusted to pH 7.4 with NaOH before the clock was started for zero-time. The pH was then maintained between 7.2 and 7.4 with the periodic addition of NaOH. Controls in which the extract was preincubated without D-mannose before the addition of ATP gave the same rates as the indicated endogenous rate.

preincubated mixture, a rapid initial rate was observed, followed by a slow rate approximating that which was obtained without preincubation. This suggested that D-mannose was not phosphorylated directly, but was first converted to another compound which was phosphorylated. Since an ATP:hexose phosphotransferase was known for D-glucose, but could not be demonstrated for D-fructose, a likely candidate for the unknown compound was considered to be D-glucose.

Apparent 2-epimerization of D-mannose to D-glucose. The experiments described above suggested that D-mannose was converted to D-glucose, which then served as the phosphoryl acceptor for ATP by the action of D-glucokinase. To test this possibility, crude extracts of *A. aerogenes* PRL-R3 were incubated with D-mannose at 25 C and at pH 7.5, and the accumulation of D-glucose was measured with glucose oxidase and with the stereospecific D-glucokinase. D-Glucose was found to be formed at rates up to about 0.3 μ mole per hr per mg of protein. The rate of D-glucose formation was half maximal at a D-mannose concentration of about 20 mM (Fig. 4).

The identification of the product as D-glucose by the use of glucose oxidase and D-glucokinase

as specific reagents was corroborated by paper chromatography. With either 80% phenol or butanol-pyridine-water (6:4:3) as the solvent, a spot which migrated at a rate corresponding to D-glucose was detected with AgNO_3 and with *N,N*-dimethyl-*p*-phenylenediamine monohydrochloride. The size of the glucose spot increased with increasing times of incubation of D-mannose with the crude extract. An endogenous formation of D-glucose was slight, and was not detected at the shorter incubation times. To show that the D-glucose was derived from added D-mannose rather than from an endogenous compound by glycosyl exchange reactions, C^{14} -D-mannose was used. Figure 5 shows that, at zero-time, essentially all of the radioactivity on the chromatogram migrated with D-mannose, whereas, after 3 hr of incubation, the spots corresponding to both D-glucose and D-mannose were radioactive.

The observed conversion of D-mannose to D-glucose could not have involved aldose-ketose isomerization with D-fructose as a free intermediate. We have detected the isomerization of D-mannose to D-fructose in extracts of this organism only after growth on D-lyxose (1); the isomerization of D-glucose to D-fructose, as determined by the Roe procedure (15), has never been detected in extracts of this organism. Thus, an apparent 2-epimerization of D-mannose to D-glucose was indicated.

Inactivation of the D-mannose to D-glucose conversion by charcoal. Treatment of a crude extract with 10 and 20% charcoal (Darco G-60) caused a loss of the ability to convert D-mannose

to D-glucose 53 and 100%, respectively, suggesting the involvement of a cofactor. This observation was consistent with the participation of an epimerase, certain of which are known to require charcoal-adsorbable cofactors. For example, *N*-acyl-D-glucosamine 2-epimerase requires ATP (6), and UDP-galactose 4-epimerase requires nicotinamide adenine dinucleotide (NAD) (13). However, attempts to reactivate the charcoal-inactivated extract with ATP, ITP, UTP, GTP, CTP, thymidine triphosphate, or NAD were unsuccessful. Also, attempts to elute from the charcoal a cofactor which would reactivate the extract were unsuccessful. The possibility existed that a protein was adsorbed by the charcoal, although analysis of the charcoal-treated extract for enzymes such as D-glucokinase, D-glucose-6-phosphate isomerase, and D-mannose-6-phosphate isomerase indicated that they were not adsorbed. The possibility also existed that the suspected 2-epimerase acted on nucleoside diphosphate derivatives of D-mannose and D-glucose rather than on the free sugars, and that the necessary sugar nucleotides were adsorbed on the charcoal. This reaction would require another enzyme, a transferase which would catalyze a glycosyl exchange of D-mannose with a nucleoside diphosphate-D-glucose to yield a nucleoside diphosphate-D-mannose and D-glucose. Experiments which are described below, however, indicate that the conversion of D-mannose to D-glucose can be explained on a basis other than one involving two hypothetical enzymes.

Inhibition of the D-mannose to D-glucose conversion by alkaline phosphatase. When a crude extract was supplemented with alkaline phosphatase and incubated with D-mannose, no D-glucose was formed (Table 1). This suggested the participation of phosphomonoesters in the D-mannose to D-glucose conversion.

ATP dependence of the D-mannose to D-glucose conversion. Molecular sieving of a crude extract by passage through Sephadex G-25 abolished its ability to convert D-mannose to D-glucose. The activity could be restored, however, by the addition of ATP to the reaction mixture (Fig. 6). The rate was maximal at an ATP concentration of about 0.5 mM. At higher ATP concentrations, the D-glucose formed was phosphorylated to D-glucose-6-phosphate. Preliminary experiments on the stoichiometry of the reaction indicated that more than 2 μ moles of D-glucose were formed per μ mole of ATP, suggesting that the ATP was acting catalytically. Thus, the reaction had a superficial resemblance to the ATP-dependent 2-epimerization of *N*-acyl-D-glucosamine described by Ghosh and Roseman (6). However, experiments which are described below indicate

FIG. 4. Double reciprocal plot of the rate of D-glucose formation as a function of D-mannose concentration. Each reaction mixture contained, in a volume of 1.0 ml: 100 μ moles of glycylglycine buffer (pH 7.5), crude extract (12 mg of protein), and varying concentrations of D-mannose.

FIG. 5. Formation of C^{14} -D-glucose from C^{14} -D-mannose in a crude extract. The details are described in Materials and Methods.

that the D-mannose to D-glucose conversion observed in extracts of *A. aerogenes* PRL-R3 was mediated by the concerted action of several enzymes rather than a single ATP-dependent enzyme.

Evidence that D-glucose was not derived from D-glucose phosphate by the hydrolytic action of a phosphatase. In view of the ATP dependence and alkaline phosphatase sensitivity of the D-mannose to D-glucose conversion, the possibility existed that D-mannose was somehow phosphorylated to D-mannose-6-phosphate and converted to D-glucose-6-phosphate and D-glucose-1-phosphate by isomerase- and mutase-catalyzed reactions. The D-glucose might then arise from the hydrolysis of D-glucose-6-phosphate or D-glucose-1-phosphate. This pathway has been implicated in the conversion of D-mannose to D-glucose in mammalian

tissues (11). Evidence which militates against this possibility in the present study is illustrated in Fig. 7; a crude extract formed D-glucose from D-mannose but not from the phosphate esters of D-mannose, D-glucose, or D-fructose.

Measurement of the conversion of D-mannose to D-glucose-6-phosphate in a continuous spectrophotometric assay; discovery of acyl phosphate: hexose phosphotransferase. Because the crude extracts contained an active D-glucokinase with a high affinity for D-glucose ($K_m = 8 \times 10^{-5}$ M) (8), the ATP-dependent conversion of D-mannose to D-glucose could conveniently be measured spectrophotometrically in a continuous assay consisting of D-mannose, ATP, NADP, glucose-6-phosphate dehydrogenase, and crude or Sephadex G-25-treated extract. Subsequently, it was found that acetyl phosphate could replace ATP in this

TABLE 1. *Effect of added alkaline phosphatase on the formation of D-glucose from D-mannose by a crude cell extract^a*

Incubation time. <i>min</i>	D-Glucose formed			
	Minus phosphatase		Plus phosphatase	
	Plus D-mannose	Minus D-mannose	Plus D-mannose	Minus D-mannose
0	0 ^b	0	7	7
20	56	0	17	20
40	86	0	23	20
60	126	0	23	23

^a The complete reaction mixture contained, in a volume of 1.0 ml: 60 μ moles of glycylglycine buffer (pH 7.5), 50 μ moles of D-mannose, 12 μ moles of MgCl₂, crude cell extract (11.4 mg of protein), and 2 mg of alkaline phosphatase. Controls were minus phosphatase or D-mannose as indicated.

^b Results expressed as millimicromoles of D-glucose formed per milligram of protein.

FIG. 6. Inactivation of the ability of a crude extract to convert D-mannose to D-glucose by chromatography on Sephadex G-25 and reactivation with ATP. Crude extract (10 ml) was passed through a column (20 by 3.5 cm) of Sephadex G-25 at 4 C. The activity of the resulting protein fraction was then tested in reaction mixtures consisting of 60 μ moles of glycylglycine buffer (pH 7.5), 12 μ moles of MgCl₂, 50 μ moles of D-mannose, extract (2.4 mg of protein), and ATP in amounts varying from 0 to 5 μ moles, in a volume of 1.0 ml. G-6-P, D-glucose-6-phosphate.

assay. This led to the discovery of a phosphotransferase which phosphorylated D-glucose (9) and D-mannose (Kamel and Anderson, *Federation Proc.* 24:422, 1965) with acetyl phosphate to

yield the respective 6-phosphates. This enzyme, which was found to be inactivated by, or adsorbed by, charcoal would account for the acetyl phosphate-dependent conversion of D-mannose to D-glucose-6-phosphate when coupled with D-mannose-6-phosphate isomerase and D-glucose-6-phosphate isomerase. It would not, however, account for the ATP-dependent conversion of D-mannose to D-glucose.

Fractionation of the enzymes involved in the acetyl phosphate-dependent and ATP-dependent conversions of D-mannose to D-glucose-6-phosphate. Chromatography of a crude extract on Sephadex G-75 and analysis of the fractions for the ability to convert D-mannose to D-glucose-6-phosphate in the continuous spectrophotometric assay revealed a loss of most of the activity. The activity could be restored, however, by the recombination of certain fractions. A typical experiment is shown in Fig. 8. Fraction 14, which contained almost no activity, activated fractions 6 through 9. Fraction 6, which contained no activity alone, greatly stimulated the activity in fractions 10 through 14. Essentially identical results were obtained when ATP replaced acetyl phosphate in the reaction mixture. Fractions 6 through 9 were subsequently found to contain acyl phosphate:hexose phosphotransferase, whereas fractions 10 through 14 were found to contain D-glucokinase, D-glucose-6-phosphate isomerase, and D-mannose-6-phosphate isomerase.

Hexose - 6 - phosphate:hexose - 6 - phosphotransferase. To explain the ATP-dependent conversion of D-mannose to D-glucose, it was neces-

FIG. 7. Measurement of the D-glucose formed from D-mannose and from hexose phosphates in a crude extract. The reaction mixtures contained, in a volume of 7 ml: 100 μ moles of hexose phosphate or 500 μ moles of D-mannose, and crude extract (252 mg of protein), adjusted to pH 7.5. M-6-P, D-mannose-6-phosphate; G-1-P, D-glucose-1-phosphate; F-6-P, D-fructose-6-phosphate; G-6-P, D-glucose-6-phosphate.

FIG. 8. Chromatography of a crude extract on Sephadex G-75 and assay of the fractions (singly and in combination) for the ability to catalyze an acetyl phosphate-dependent conversion of D-mannose to D-glucose-6-phosphate. A crude extract was passed through a column (30 by 2.5 cm) of Sephadex G-75 at 4°C and collected in 4.5-ml fractions. Samples (0.03 ml) of the fractions were then assayed by measuring the rate of absorbance increase at 340 mμ in reaction mixtures consisting of 10 μmoles of glycylglycine buffer (pH 7.5), 5 μmoles of D-mannose, 0.5 μmole of acetyl phosphate, 1 μmole of MgCl₂, 0.2 μmole of NADP, and excess glucose-6-phosphate dehydrogenase in a volume of 0.15 ml. Where combinations of fractions are indicated, 0.03 ml of each fraction was used.

sary to postulate a reaction wherein the phosphoryl group of D-glucose-6-phosphate, which could be formed from a D-glucokinase-catalyzed phosphorylation of D-glucose, could be transferred to the 6-position of D-mannose. This reaction was first demonstrated by measuring the reaction in reverse; i.e., the D-glucose-dependent conversion of D-mannose-6-phosphate to D-glucose-6-phosphate was measured by coupling the reaction to glucose 6-phosphate dehydrogenase. The complete reaction mixture contained the following, in a volume of 0.15 ml: 10 μmoles of glycylglycine buffer (pH 7.5), 0.1 μmole of NADP, 1 μmole of MgCl₂, 1 μmole of D-glucose, 1 μmole of D-mannose-6-phosphate, excess glucose-6-phosphate dehydrogenase, and a Sephadex G-75 fraction (200 μg of protein) of a crude extract. D-Glucose-6-phosphate was formed at a rate of 196 μmoles per hr per mg of protein in the complete reaction mixture, but none was formed if one of the components was omitted, indicating the presence of a hexose-6-phosphate:hexose-6-phosphotransferase. It should be emphasized that this reaction was first detected in a Sephadex G-75 fraction; it is difficult to detect in unfractionated extracts, because the conversion of

D-mannose-6-phosphate to D-glucose-6-phosphate is not then D-glucose-dependent owing to the action of D-mannose-6-phosphate isomerase and D-glucose-6-phosphate isomerase. We have subsequently purified this enzyme and have obtained evidence that it is the same as that previously reported to phosphorylate D-glucose with carbamyl phosphate and acetyl phosphate (Kamel and Anderson, *Federation Proc.* 24:422, 1965). The details of its purification and properties will be published elsewhere.

The dependence of the conversion of D-mannose to D-glucose-6-phosphate on either acetyl phosphate or ATP and on two Sephadex G-75 fractions may now be rationalized. With acetyl phosphate, D-mannose was phosphorylated to D-mannose-6-phosphate by the acyl phosphate:hexose phosphotransferase, and converted to D-glucose-6-phosphate by the action of D-mannose-6-phosphate isomerase and D-glucose-6-phosphate isomerase. With ATP, the D-glucose which contaminated even the recrystallized D-mannose at a level of 0.08% was phosphorylated by the action of D-glucokinase. The D-glucose-6-phosphate formed then served as a phosphoryl donor for D-mannose by the action of the hexose phosphate:hexose phosphotransferase, yielding D-mannose-6-phosphate and free D-glucose which could be phosphorylated again with ATP. The D-mannose-6-phosphate was then converted to D-glucose-6-phosphate by isomerization reactions.

Reconstitution of the reactions involved in the apparent 2-epimerization of D-mannose to D-glucose. The apparent 2-epimerization of D-mannose to D-glucose that was observed in crude extracts may be explained by the reactions shown in Fig. 9, involving hexose phosphate:hexose phosphotransferase, D-glucose-6-phosphate isomerase, and D-mannose-6-phosphate isomerase. This sequence was reconstituted by purified enzymes by incubating 200 μmoles of D-mannose, 0.2 μmole of D-glucose-6-phosphate, 200 μmoles of glycylglycine buffer (pH 7.5), 0.18 unit of phosphotransferase, 0.09 unit of D-mannose-6-phosphate isomerase, and 0.09 unit of D-glucose-6-phosphate isomerase in a volume of 1.8 ml. D-Glucose (1.5 μmoles) was formed in 2 hr in the complete reaction mixture, but none (< 0.02

FIG. 9. Proposed reaction sequence for the conversion of D-mannose to D-glucose in crude extracts. The D-glucose is derived from D-glucose-6-phosphate by the action of a hexose phosphate:hexose phosphotransferase.

μmole) was formed if any of the reaction components was omitted. Consistent with this explanation for the D-mannose to D-glucose conversion are the observations that (i) the K_m of purified hexose phosphate:hexose phosphotransferase for D-mannose is about the same as the concentration which effects the half-maximal rate of D-glucose formation ($2 \times 10^{-2} \text{ M}$, see Fig. 4) in crude extracts, and (ii) the specific activity of the hexose phosphate:hexose phosphotransferase in the crude extract (about $0.2 \mu\text{mole}$ of hexose phosphorylated per hr per mg of protein) is about the same as the rate of D-glucose formation by the crude extract, as described earlier in this communication. The specific activities of D-glucokinase, D-mannose-6-phosphate isomerase, and D-glucose-6-phosphate isomerase in crude extracts were always higher than that of the phosphotransferase and were, therefore, nonlimiting.

Cyclic pathway for the metabolism of D-mannose.

Figure 10 summarizes the reactions which we propose to be involved, although not necessarily exclusively, in the constitutive utilization of D-mannose by *A. aerogenes* PRL-R3. The essential features of this scheme are that D-glucose can be phosphorylated with ATP, but D-mannose cannot be. Instead, D-mannose is phosphorylated with D-glucose-6-phosphate to yield D-mannose-6-phosphate and D-glucose. D-Glucose-6-phosphate may be regenerated by isomerization of D-mannose-6-phosphate through D-fructose-6-phosphate, or by phosphorylation of D-glucose with ATP. Hexose phosphates that are depleted by further metabolism may be replenished by phosphorylation of D-mannose with acetyl phosphate or carbamyl phosphate. The apparent 2-epimerization of D-mannose to D-glucose that was observed in crude extracts may be explained by a cyclic process involving D-mannose-6-phosphate isomerase, D-glucose-6-phosphate isomerase, and acyl phosphate:hexose phosphotransferase (hexose phosphate:hexose phosphotransferase). Ob-

servations in support of the pathway depicted in Fig. 10 are as follows. D-Mannose is utilized constitutively; the constitutive hexokinase is stereospecific for D-glucose, and D-mannokinase activity cannot be detected. Crude extracts convert D-mannose to D-glucose as determined with glucose oxidase, a stereospecific D-glucokinase, and paper chromatography by use of C^{14} -D-mannose. D-Fructose is not a free intermediate in the conversion of D-mannose to D-glucose. The D-mannose to D-glucose conversion is inactivated by alkaline phosphatase, charcoal, and chromatography on Sephadex G-25, and the Sephadex G-25 inactivated extract can be reactivated with ATP but the charcoal-inactivated extract cannot be. The accumulated D-glucose is not derived from D-glucose-1-phosphate or D-glucose-6-phosphate by the action of a phosphatase. Acetyl phosphate can replace ATP in the conversion of D-mannose to D-glucose-6-phosphate, and an enzyme which phosphorylates D-mannose with D-glucose-6-phosphate or acetyl phosphate has been identified. D-Mannose-6-phosphate isomerase, D-glucose-6-phosphate isomerase, D-glucokinase, and acyl phosphate:hexose phosphotransferase are constitutive enzymes; acyl phosphate:hexose phosphotransferase is adsorbed by or inactivated by charcoal. The ATP-dependent and acetyl phosphate-dependent conversions of D-mannose to D-glucose-6-phosphate are inactivated by chromatography on Sephadex G-75, but can be restored by combining two of the fractions, one of which contains acyl phosphate:hexose phosphotransferase and the other which contains D-mannose-6-phosphate isomerase, D-glucose-6-phosphate isomerase, and D-glucokinase. The conversion of D-mannose to D-glucose can be reconstituted by D-mannose-6-phosphate isomerase, D-glucose-6-phosphate isomerase, acyl phosphate:hexose phosphotransferase, and a catalytic amount of D-glucose-6-phosphate. The K_m value of the phosphotransferase for D-mannose is the same as the D-mannose concentration which effects a half-maximal rate of conversion of D-mannose to D-glucose in crude extracts, and the specific activity of acyl phosphate:hexose phosphotransferase in crude extracts is the same as the specific activity at which D-mannose is converted to D-glucose in crude extracts. Of interest is the fact that, even though D-glucokinase is stereospecific for D-glucose, it can presumably function in D-mannose metabolism by supplying a suitable phosphoryl donor (D-glucose-6-phosphate).

Whether the observed rate of D-mannose metabolism in extracts (an average of about $0.2 \mu\text{mole}$ per hr per mg of protein at pH 7.5 and

FIG. 10. Summary of reactions proposed for the metabolism of D-mannose in extracts of *Aerobacter aerogenes* PRL-R3.

25 C) is adequate to account for the rate at which cells of *A. aerogenes* PRL-R3 metabolize D-mannose is difficult to ascertain because the degree of inactivation during extraction is not known, nor is the effect of disruption of cellular organization. Furthermore, the enzymes involved have widely divergent pH optima [e.g., pH 9 for acyl phosphate:hexose phosphotransferase (Kamel and Anderson, Federation Proc. 24:422, 1965) and less than pH 6 for D-mannose-6-phosphate isomerase], and so the conditions used were not necessarily optimal. At higher pH values and higher temperatures, rates have been obtained which were several-fold greater and, therefore, approached the specific activities of certain other enzymes involved in monosaccharide metabolism in this organism (8, 14). However, the data presented do not exclude the possibility that other phosphotransferases which use different phosphoryl donors (5, 12) may also be involved in hexose metabolism in this organism.

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