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Rapid Turnover of Mannitol-1-Phosphate in *Escherichia coli*

H. ROSENBERG,* S. M. PEARCE, C. M. HARDY, AND P. A. JACOMB

Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. 2601, Australia

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The phosphate moiety of D-mannitol-1-phosphate in *Escherichia coli* is subject to rapid turnover and is in close equilibrium with P_i and the phosphorus of fructose-1,6-bisphosphate. These three compounds account for the bulk of ^{32}P label found in cells after several minutes of uptake of $^{32}P_i$ and mannitol-1-phosphate represents some 30% of this label. Mannitol-1-phosphate occurs in *E. coli* grown on a variety of carbon sources, in the absence of D-mannitol, and is synthesized de novo even in mutants lacking mannitol-1-phosphate dehydrogenase. The mannitol moiety of mannitol-1-phosphate was not affected during the total chase of the P moiety, which exchanged with a half-life of about 30 s. These findings suggest that the rapid equilibration of the phosphorus is a function of an enzyme, possibly a component of the phosphotransferase system, capable of forming a complex that allows the exchange of the phosphate without the equilibration of the mannitol moiety with free manitol.

D-Mannitol, the most ubiquitous natural hexitol, can serve as a carbon source for *Escherichia coli*, entering the cells through a specific phosphotransferase transport system (PTS) (27). The resultant mannitol-1-phosphate is oxidized by a specific dehydrogenase (29) in the presence of NAD to fructose-6-phosphate, which enters the general metabolic pathways. The PTS enzyme II and the dehydrogenase are controlled by the genes *mtlA* and *mtlD*, respectively. These genes, and a control gene (*mtlC*), form the *mtl* operon on the *E. coli* chromosome (12, 27).

There appears to be no evidence for any other metabolic role for mannitol-1-phosphate in *E. coli*, although it has been stated that its formation from fructose-6-phosphate may be important for the reoxidation of NADH (5). The enzymes necessary for the metabolism of the hexitols are induced by growth on these sugars, but there is a considerable difference between the kinetics of induction of the mannitol and glucitol (sorbitol) systems. Thus, although the induction of the mannitol system is immediate, and the level of its specific PTS enzyme II in uninduced cells ranges from 5 to 30% of the levels in induced cells, the glucitol system is induced after a lag of 25 to 90 min, and the uninduced activity of its enzyme II is 0.5 to 2.5% of induced levels (15). The possible explanation of this difference may be found in the report by Helle and Klungsøyr (5), who showed that *E. coli* grown on glucose contained mannitol-1-phosphate. Furthermore, both in whole cells and cell extracts, mannitol-1-phosphate incorporated considerable amounts of ^{32}P when $^{32}P_i$ was present. We confirmed this observation and found that, in short-term experiments, mannitol-1-phosphate was rapidly labeled and was in close dynamic equilibrium with P_i (25). Endogenously produced mannitol-1-phosphate serving as an inducer of the *mtl* operon could thus explain the presence of the specific enzymes for its metabolism in uninduced cells. The occurrence of mannitol-1-phosphate or of mannitol in cells grown in the absence of this sugar has also been reported in other microorganisms (3, 7). In this report, we deal with the production and turnover of mannitol-1-phosphate in wild strains of *E. coli* and in some mutants affected in the enzymes involved in mannitol metabolism.

* Corresponding author.

MATERIALS AND METHODS

Chemicals. Carrier-free $^{32}P_i$ and D-[1-^{14}C]mannitol were from the Radiochemical Centre, Amersham, United Kingdom. D-Glucitol was a product of Pfanstiehl Chemical Co., Wankegan, Ill. D-Mannitol, D-mannitol-1-phosphate, D-glucitol-6-phosphate, alkaline phosphatase (*E. coli* type III), and glucitol dehydrogenase were from Sigma Chemical Co., St. Louis, Mo., and fructose-6-phosphate was from Boehringer GMB, Mannheim, West Germany. All other chemicals were of the highest purity commercially available.

Bacterial strains. The strains used in this work are described in Table 1. Transductions with phage P1 were carried out by the method of Miller (17).

Growth media and buffers. The mineral salt medium contained 60 mM K_2HPO_4, 40 mM NaH_2PO_4, 15 mM $(NH_4)_2SO_4$, and 1 mM $MgSO_4$. The pH was adjusted to 6.5 with HCl. The medium was supplemented with 20 mM glucose, mannitol or *K*-DL-lactate and 3 μM thiamine. Arginine (1 mM) and 10 μM 2,3-dihydroxybenzoate were added where required. Sterile solutions of the growth supplements were added to the sterilized mineral salts base. The phosphate-free buffer used for phosphate deprivation and in the assay of phosphate uptake and the conditions for growth, washing, and starving of cells were described elsewhere (25).

Uptake assay. The procedure (22) and apparatus (21) for the uptake assay were as described previously.

Extraction of cells and enzyme purification. All steps were carried out at 1 to 3°C. Cells grown as described above were collected by centrifugation and washed once in a buffer (pH 7.0) containing 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 20 μM phenylmethylsulfonyl fluoride. The washed pellets were suspended in the same buffer at a ratio of 1 g of cells per ml of buffer, and crystalline DNase and RNase (1 mg of each per 100 ml) were added. The suspension was disrupted in a Ribi cell fractionator at a pressure of 20,000 lb/in^2 under N_2, and the debris was removed by centrifugation for 15 min at 12,000 × *g*. The supernatant fluid was centrifuged for 2 h at 100,000 × *g*, and the clear cell-free extract was collected. Mannitol-1-phosphate dehydrogenase was partially purified by the method of Klungsøyr from such extracts of mannitol-grown *E. coli* strain AN710; the purifi-

TABLE 1. Strains of *E. coli* K12

Strain	Relevant genetic loci	Reference or source
AN710	*phoT101 argH entA xyl*	23
236	*mtlA xyl*$^+$	27
239	*mtlD mtlC mtlA*$^+$ xyl$^+$	27
HR27	*phoT101 argH entA mtlD mtlC mtlA*$^+$ *xyl*$^+$ P1^r	239 × AN710, Xyl$^+$ selected, *mtlD* and *mtlA*^{c} tested[a]
HR28	*phoT101 argH entA mtlA xyl*$^+$	236 × AN710, Xyl$^+$ selected, Mtl$^-$ tested
HR33	As HR27, but P1^{sb}	

[a] *mtlC* strains were constitutive for the uptake of mannitol, which was tested by the radioautographic method of Kornberg and Smith (11) with nutrient agar-lactate plates containing 100 μM [1-^{14}C]mannitol of a specific radioactivity 0.13 mCi/mol.

[b] Strain HR33 was constructed similarly to HR27 and tested for correct phenotype, after HR27 was found to be resistant to P1 and thus unsuitable for transduction. Strain HR33 was used routinely after that, but some of the early work was done with strain HR27.

cation was taken to the passage through QAE-Sephadex A15 (10). Mannitol-1-phosphate dehydrogenase was assayed by the method of Wolff and Kaplan (29).

Chromatography. Hexitols were chromatographed on silica gel 60 plates (Merck, Darmstadt, West Germany) that had been dipped in 0.1 M H_3BO_3 in 90% (vol/vol) aqueous ethanol and dried at 110°C. The solvent used was benzene-acetic acid-methanol (2:1:20, vol/vol/vol). Hexitols were visualized by spraying the dried plates with the periodato-cuprate reagent of Bonner (1).

Determination of metabolic intermediates. Triose phosphate and fructose-1,6-bisphosphate analyses were carried out in the laboratory of M. J. Weidemann (Department of Biochemistry, Faculty of Science, Australian National University) by a fluorimetric adaptation of the method of Bücher and Hohorst (2).

Phosphate analysis. P_i was determined by the method of Harris and Popat (4). Ester phosphate was determined by the same method after hydrolysis by autoclaving 45 min at 121°C in 0.32 M sodium persulfate.

Assay of ^{32}P esterification. The original method for the assay of ^{32}P esterification Ohnishi (18) was modified as described previously (25).

Radioactivity assays. Radioactivity was determined in a Packard 300 counter. For ^{32}P alone, samples were in water or in 50% aqueous ethanol, and the Cerenkov radiation was counted with channel settings 2 to 25 keV. Samples containing ^{14}C alone were made to 1.5 ml in water and mixed with 10 ml of a scintillation cocktail containing 0.5% (wt/vol) 2,5-diphenyloxazole in xylol–Triton X-100 (2:1, vol/vol). The settings for channels I and II were 0 to 156 keV and 4 to 156 keV, respectively. Mixed samples containing ^{14}C and ^{32}P were counted in the same cocktail with channels I and II set at 5 to 160 keV and 200 to 1,200 keV, respectively. The instrument was programmed to yield results as disintegrations per minute and was calibrated with a set of quenched standards for ^{14}C supplied by Packard Instruments Co. and a set of quenched standards for ^{32}P prepared from $^{32}P_i$ converted to the tri-*n*-octylamine salt. In the dual counting program, the final printout showed no spillover of either nuclide into the count of the other when single nuclides were counted. All counts were corrected for chemiluminescence.

Elemental analysis was carried out by the Australian National University Analytical Services.

RESULTS

We reported previously (25) that the Pit transport system for phosphate in *E. coli* catalyzed a rapid exchange reaction between intra- and extracellular P_i, and that cells that accumulated $^{32}P_i$ lost most of the label when chased with unlabeled P_i. Since at the start of chase some 60% of the $^{32}P_i$ in the cells was already in ester form, and all of the ^{32}P chased out as $^{32}P_i$, it followed that the bulk of the organically bound $^{32}P_i$ had also been chased by P_i and must be in rapid equilibrium with it. As shown below, this ^{32}P was confined to two esters: mannitol-1-phosphate and fructose-1,6-bisphosphate. The compounds were identified in extracts of cells of the strain AN710 that had been allowed to take up $^{32}P_i$ for 5 min. Chromatography of the acid-soluble material from the cells revealed three major peaks of labeled compounds (Fig. 1). A check of the middle fraction from each peak for esterified ^{32}P (see above) showed peaks A and C to be ester phosphate and peak B to be P_i. The contents of each peak were pooled, and $KHCO_3$ was removed by treatment

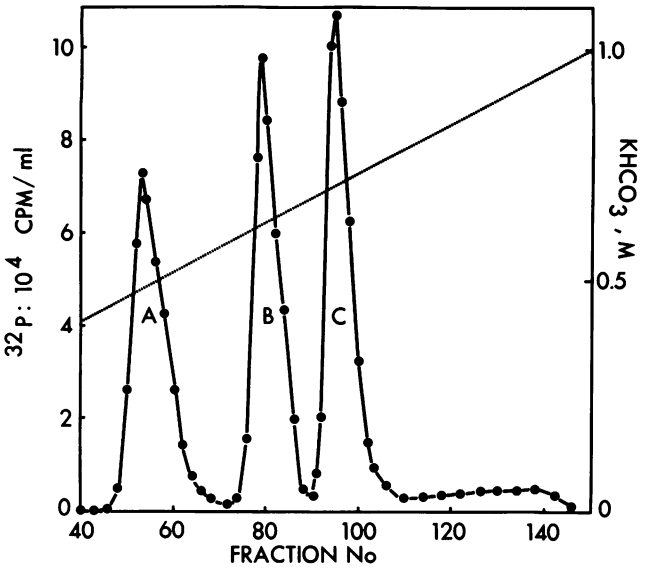

FIG. 1. Chromatography of acid-soluble P esters from *E. coli* AN710. Lactate-grown cells (see the text) were harvested at an absorbancy at 660 nm of 1.2, washed three times in phosphate-free medium, and suspended at an absorbancy at 660 nm of 1.0 in the same medium supplemented with 20 mM glucose. They were stored at 3°C until required and processed in batches of 320 ml as follows. Flasks were shaken at 400 oscillations per min at 37°C for 5 min, at which time P_i was added to 50 μM. The suspensions were shaken for 5 min with the P_i, at which stage *N*-ethylmaleimide was added to 2 mM, and shaking was continued for 1 min. The suspensions were rapidly centrifuged, and the pellets were extracted at 0°C with 160 ml of 0.75 N $HClO_4$. After centrifugation the pellets were extracted with 60 ml of 0.25 N $HClO_4$. The combined extract was brought to pH 7.2 with bicarbonate-free 10 N KOH and, after standing on ice for 30 min, was filtered. The extracts from 20 batches (about 6.3 liters of cell suspension) were pooled, the clear neutral filtrate was evaporated under reduced pressure from 250 ml to about 15 ml and cooled on ice, and a further crop of $KClO_4$ crystals was filtered off. The filtrate was applied to a column (550 by 35 mm) of Dowex 1 × 8 ion-exchange resin (mesh 100-200, bicarbonate form) as described by Martonosi (16). The column was eluted with 1.5 l of $KHCO_3$ in a linear gradient from 0.2 to 1.0 M. Fractions (10 ml) were collected, and 50 μl from each was counted. No radioactivity appeared before fraction 40. The dotted line represents the calculated $KHCO_3$ gradient.

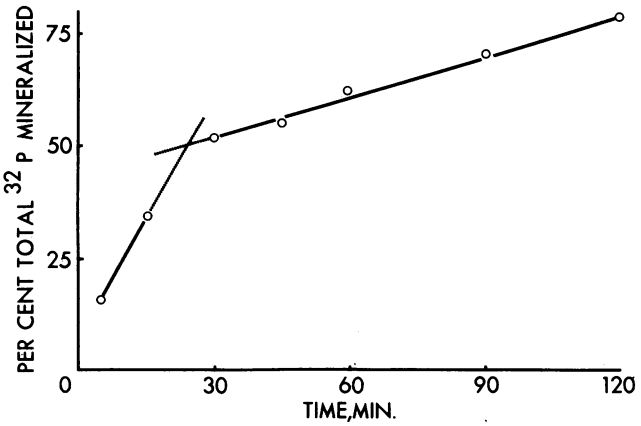

FIG. 2. Hydrolysis of compound C in 1 N $HClO_4$. A sample (600 μl) of the stock solution of the Na salt (see the text) was mixed with 600 μl of 2 N $HClO_4$. The mixture was heated on a boiling water bath, and 100-μl samples were withdrawn into ice-cooled tubes each containing 400 μl of 0.25 M sodium acetate. After all samples were withdrawn they were processed to determine the release of $^{32}P_i$ from ester P (see the text).

with $HClO_4$ at 0°C. Fraction B (P_i) was retained for determination of specific radioactivity (see below), and fractions A and C were each rechromatographed as described above, but with shallower gradients of $KHCO_3$ within the appropriate concentration range. Each fraction emerged as a symmetrical peak and with small, well separated peaks of P_i.

Identification of fraction A as mannitol-1-phosphate. The material was precipitated as the Ba^{2+} salt at pH 8.5 in 80% ethanol. The dried solid was not pure and analyzed close to $C_6H_{14}PO_9Ba$. It was treated with Dowex 50-H^+ ion-exchange resin to remove Ba^{2+} and was neutralized with NaOH and precipitated with ethanol. The Na salt analyzed as $C_6H_{14}P_{0.95}Na_{1.47}O_{9.1}$. The high H content precluded a hexose, but was compatible with hexitol. The material was incubated at 30°C in 5.0 ml of 0.2 M $(NH_4)_2CO_3$–10 mM $MgCl_2$ containing 2 mg of *E. coli* alkaline phosphatase per ml. The loss of esterified ^{32}P and appearance of $^{32}P_i$ was monitored as described above, and the reaction was stopped after 3 h with 1 ml of 4 M $HClO_4$. By this time 63% of the ^{32}P had been mineralized. The reaction mixture was cooled and centrifuged, and the supernatant was brought to pH 7.0 with KOH and cooled to 0°C. After filtration to remove $KClO_4$ the material was passed through a small bed of Dowex-1 (HCO_3^-), and the effluent from the column was treated with Dowex 50 (H^+). The deionized material was taken to a small volume under reduced pressure and was treated with excess ethanol. Crystals appeared on standing and were collected and dried. Mass spectroscopy of the product after silylation indicated a molecular weight of 614 (as expected from a fully silylated hexitol) without any indication of steric structure. The hexitol behaved as mannitol in chromatography, showing no trace of glucitol (data not shown). In a mixed melting point determination, authentic mannitol melted at 167 to 168°C, the isolated material melted at 167.5 to 168.5°C, and the mixture melted at 166 to 168°C.

The isolated mannitol failed to reduce NAD in the standard assay (14) in the presence of sheep liver glucitol dehydrogenase, which was active with authentic glucitol. The (unhydrolyzed) isolated mannitol-1-phosphate actively reduced NAD in the presence of partially purified mannitol-1-phosphate dehydrogenase (29) from strain AN710.

Identification of fraction C as fructose-1,6-bisphosphate. Fraction C (Fig. 1) was chromatographed on Dowex-1–HCO_3 with an expanded gradient of $KHCO_3$ from 0.1 to 0.6 M. Emergence of the material was monitored as ^{32}P label. The main peak emerged in the gradient between 0.47 and 0.54 M $KHCO_3$ and was symmetrical throughout, except for minor trailing. It was completely separated from a small P_i peak at about 0.4 M $KHCO_3$. The contents of the tubes (with the exception of the trailing end) were pooled, and the material was processed essentially as described for the mannitol-1-phosphate peak above. A water-insoluble barium salt prepared from the material showed, on analysis, to contain C, P, and Ba in the ratio of 3.0:1.1:1.02, compatible with either triose phosphate or hexose diphosphate. Acid hydrolysis of this material produced a biphasic plot, and the extrapolated lines intersected at 50% hydrolysis, indicating the presence of two nonequivalent P groups hydrolyzing with half-lives of 14 and 100 min, respectively (Fig. 2). The Ba salt was converted to the Na salt with Dowex 50-sodium, a stock solution was prepared, and its total phosphate was assayed as 1.8 μmol of P per ml. A sample of this solution was then assayed enzymically (2) and was found to contain 0.91 μmol of fructose-1,6-bisphosphate and 0.026 μmol of triose phosphate per ml. Fraction C is therefore largely fructose-1,6-bisphosphate.

Other fractions. A small, irregular, and trailing peak normally emerged after peak C in the first fractionation and was greatly reduced when the extracts were treated with charcoal before chromatography. This fraction was presumed to contain nucleotides as well as some other minor components that were not further investigated.

Determination of specific radioactivities. Material for the analysis of specific radioactivities was taken either from purified fractions or from the midpeak tube of each fraction from the first fractionation such as shown in Fig. 1. In all cases the percent esterified ^{32}P was determined, and only samples showing 98 to 100% esterification were used (samples from the P_i peak were invariably better than 99% P_i). P_i estimations were done directly on the P_i fraction and after hydrolysis (see above) on all organophosphate fractions. A comparison of several experiments is shown in Table 2. Clearly both fructose-1,6-bisphosphate and mannitol-1-phosphate exhibit a turnover rate high enough to effect about 90% equilibration with P_i in about 2 min. This is in good agreement with the observed half-time decay of radioactivity in these fractions (26 s) during phosphate chase (25).

Mannitol-1-phosphate in mutants affected in mannitol metabolism. The higher turnover of mannitol-1-phosphate observed in short-term P_i uptake and chase indicated that a cyclic set of reactions must occur, during which phosphate is

TABLE 2. Specific radioactivities of ^{32}P in the three major labeled fractions[a]

Uptake time (s)	Specific radioactivity (cpm/nmol of P)			Relative specific radioactivity (P_i = 1.0)	
	Pi	M-1-P	FDP	M-1-P	FDP
20	386	292	315	0.76	0.82
120	853		795		0.93
150	908	797	875	0.88	0.96
300	822	773	895	0.94	1.09

[a] Phosphate estimations were done directly on the P_i fraction and after hydrolysis (see the text) on the organic phosphates. M-1-P, D-Mannitol-1-phosphate; FDP, fructose-1,6-bisphosphate.

TABLE 3. Phosphate uptake and exchange in *E. coli* mutants affected in mannitol metabolism[a]

Strain	Relevant genotype	P_i taken up in 2 min (nmol/mg [dry wt])	% ^{32}P in ester form at start of chase (at 2 min)	% ^{32}P chased in next 2 min
AN710	*phoT*	73	71.7	71
HR27	*phoT mtlD mtlC mtlA*$^+$	75.5	73.0	67
HR28	*phoT mtlA*	83	74.0	70

[a] Cells were grown on lactate and treated as for P_i uptake. They were allowed to take up 50 μM $^{32}P_i$ in the presence of 10 mM lactate for 2 min then chased with 2 mM unlabeled P_i for a further 2 min. The assay of ester-bound ^{32}P is described in the text. The data given are means of two experiments.

cleaved from and rejoined to the hexitol. One such possibility could involve the exit of free mannitol and its reentry through the specific mannitol PTS carrier as mannitol-1-phosphate. Another may involve a reversible reaction or exchange catalyzed by the mannitol-1-phosphate dehydrogenase. To check this possibility, we examined the chase of P_i and ester P in three isogenic strains, the parent AN710 and strains HR27 and HR28, carrying the *mtlD mtlC* and *mtlA* mutations, respectively. Phosphate uptake and chase patterns in these strains were very similar to those found in the wild strain (Table 3). Some 70% of the total ^{32}P was chased from the cells, although at the start of chase less than 30% of the total ^{32}P was P_i. It follows that, in all three strains, about the same proportion of the bound ^{32}P was chased out, even though the mutants lacked either the PTS enzyme II^{mtl} (strain HR28) or the mannitol-1-phosphate dehydrogenase (strain HR27).

Identification of mannitol-1-phosphate in strains HR27 and HR28. The cells were grown, prepared, labeled, and extracted as described in the legend to Fig. 1. The extracts were chromatographed also in the same manner. The elution profiles (not shown) were the same as in the parent strain, AN710 (Fig. 1). The respective peaks corresponding to peak A (Fig. 1) were collected from the eluates of strains HR27 and HR28, and the solutions were processed as described above. They behaved identically to mannitol-1-phosphate in the rate of release of $^{32}P_i$ when heated at 100°C in either 1.0 N HCl or 0.1 N NaOH (data not shown). The material from strain HR27 was hydrolyzed with *E. coli* alkaline phosphatase, and the non-ionic moiety was isolated as described above for strain AN710. It cochromatographed precisely with authentic mannitol TLC. Since the presence of mannitol-1-phosphate in strain HR27 was unexpected, the isolated material from it was tested as a substrate for the partially purified mannitol-1-phosphate dehydrogenase. It behaved like authentic mannitol-1-phosphate (data not shown).

The finding of normal exchange in the *mtlA* mutant eliminated the cycling of mannitol-1-phosphate through the PTS system as a mechanism of chase. We then considered the possibility of continuous mannitol-1-phosphate synthesis and breakdown. Direct phosphorylation (by ATP) of mannitol does not occur in *E. coli* (12). The only known source of mannitol-1-phosphate is fructose-6-phosphate, through the action of the dehydrogenase. The failure of the *mtlD* mutation to abolish the exchange and the actual presence of mannitol-1-phosphate in lactate-grown (or glucose-grown) *mtlD* mutants suggested the existence of an alternative biosynthetic pathway. The enzymatic activities of crude extracts of the *mtlD* strains 239 and HR27 were tested for such activities. We confirmed (Table 4) the findings of Solomon and Lin (27) that mannitol-1-phosphate dehydrogenase activity could not be detected in *mtlD* mutants. However, the reverse activity, namely, the reduction of fructose-6-phosphate by NADH, was found in crude cell-free extracts of these strains (Table 4). Although this observation explained the presence of mannitol-1-phosphate in these mutants, it cannot shed any light on the rapid chase.

Mannitol is not displaced from mannitol-1-phosphate during chase. To test the possibility that the ^{31}P chase reflected a rapid de novo synthesis of mannitol-1-phosphate, we used cells in which the mannitol-1-phosphate carried the dual ^{14}C–^{32}P label. Cells of strain HR33 (*mtlD*) were allowed to take up $^{32}P_i$ and [1-^{14}C]mannitol, and the ^{32}P was chased with $^{31}P_i$. The cells were then processed, and the extracts were chromatographed as described above (Fig. 1). The radioactivities of both ^{32}P and ^{14}C were assayed in all fractions by dual-label counting. The results clearly showed that, although ^{32}P was nearly completely chased from the mannitol-

TABLE 4. Mannitol-1-phosphate dehydrogenase—forward and reverse reaction—in extracts of wild-type and *mtlD* strains[a]

Strain	Relevant genotype	Growth substrate	M-1-P (nmol/min per mg of protein)	
			Forward reaction NAD reduced by M-1-P	Reverse reaction[b] NADH oxidized by F-6-P
AN710	Parent	Mannitol	462 ± 13	217 ± 22
		Glucose	134 ± 1.4	44.5 ± 0.5
		Lactate	70 ± 8.1	49 ± 12
HR27	*mtlD*	Lactate	0.24 ± 0.01	30 ± 4.2
239	*mtlD*	Lactate	0.17 ± 0.01	11.3 ± 0.3

[a] The results represent means (with standard errors) of two to four determinations done on one extract. The crude cell-free extracts were prepared as described in the text. M-1-P, D-Mannitol-1-phosphate; F-6-P, fructose-6-phosphate.

[b] The reaction was always started with fructose-6-phosphate, and all rates were corrected for endogenous oxidation of NADH by the crude extracts.

1-phosphate, the ^{14}C label remained (Fig. 3). The result excludes de novo synthesis of mannitol-1-phosphate as a possible explanation of the observed rapid turnover.

DISCUSSION

The observed rapid chase of the phosphorus moiety of mannitol-1-phosphate in *E. coli* may occur either through the cyclic processes involving the removal and replacement of the phosphate moiety or by the catabolism of the mannitol-1-phosphate molecule and its synthesis de novo. After hydrolysis by a phosphatase, mannitol may exit into the periplasmic space and could be recaptured there by the PTS enzyme II^{mtl}, reentering the cell as mannitol-1-phosphate. Its phosphate moiety will be in equilibrium with that of phosphoenolpyruvate and therefore subject to rapid chase. This scheme would require mannitol to be capable of leaving the cell and the PTS system to be operative. The possibility of mannitol exit was demonstrated by Solomon et al. (28), who found that the exit depended on the specific mannitol enzyme II. We showed (Table 3) that the chase of the mannitol-1-phosphate occurred readily in *mtlA* strains lacking the PTS enzyme II^{mtl}. Thus a cycle involving recapture through the PTS system cannot be responsible for the observed chase results. Lengeler (13) stressed that free mannitol was not found in cells after it entered through the PTS, nor was there any indication of systems capable of transporting free hexitols directly in *E. coli*. Removal of the D-mannitol by oxidation to fructose by NAD is unknown in *E. coli*, although these reactions form part of a NADH-NADP transhydrogenase cycle in fungi (7, 8).

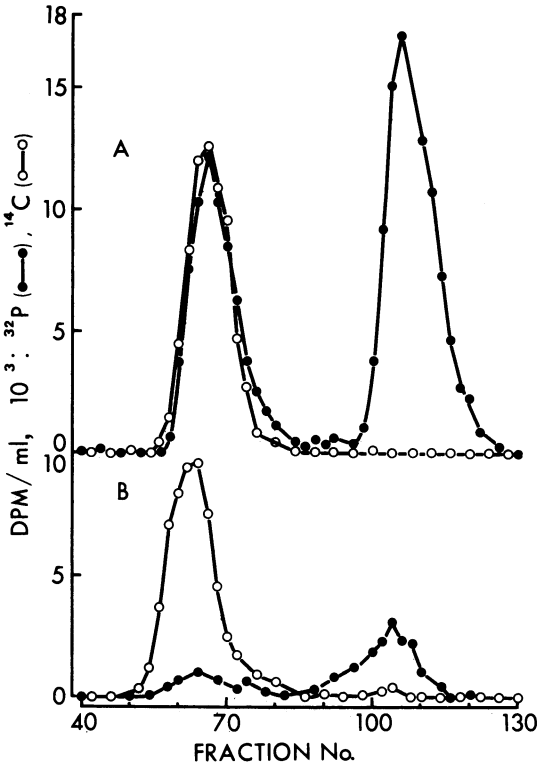

FIG. 3. Phosphate chase in cells labeled with [1-^{14}C]mannitol and $^{32}P_i$. Lactate-grown cells of strain HR33 (*mtlD*, *mtlA*^{c}) were prepared as described in the legend to Fig. 1, but lactate was used as the energy source. The cells were then given 50 μM $^{32}P_i$ (30 μCi) at zero time and 0.29 μM [1-^{14}C]mannitol (5 μCi) at 2 min. After an additional 5 min of shaking, one half of the suspension (control) was treated with 1 mM *N*-ethylmaleimide and placed on ice, and the other half (chase) was given $^{31}P_i$ to 40 mM and allowed to shake for a further 5 min, when it was also treated with *N*-ethylmaleimide and cooled. The cells were centrifuged, extracted, and chromatographed as described in the legend to Fig. 1, and the fractions were sampled for dual counting of ^{14}C (○) and ^{32}P (●). (A) control suspension; (B) chased suspension. The first and second peaks in each case represent mannitol-1-phosphate and P_i, respectively.

Noncyclic reactions compatible with the observed chase of ^{32}P from mannitol-1-phosphate must again involve the disposal of the compound as well as its de novo synthesis. This is contradicted by our finding (Fig. 3) that the mannitol moiety of the mannitol-1-phosphate is not affected by the phosphate chase.

We suggest that these results are compatible with the existence of an exchange reaction that allows the phosphate moiety of mannitol-1-phosphate to equilibrate either with P_i or with a phosphorylated intermediate in close equilibrium with P_i, without the liberation of mannitol. A similar exchange of the mannitol moiety of mannitol-1-phosphate through the PTS enzyme II system has been demonstrated, as has that for other sugars (26). The involvement of a phosphorylated enzyme II in such an exchange (with fructose) has been suggested (19) for *Bacillus subtilis* and (with glucose) for *Streptococcus faecalis* (6), although kinetic studies of the PTS-mannose of *Salmonella typhimurium* exclude such an intermediate (20). For a corresponding phosphate exchange one might envisage an interaction with another component of the PTS or with some other protein, involving an enzyme-mannitol complex, which would permit the exchange.

In the course of these experiments, we found mannitol-1-phosphate in the *mtlD* mutant strains in amounts comparable to those in wild-type cells. This finding raised two questions. Why, and by what route, is mannitol-1-phosphate synthesized in the absence of the dehydrogenase? Since mannitol-1-phosphate cannot be oxidized to fructose-6-phosphate, and its exit from the cells is minimal, how is it disposed of? If allowed to accumulate, it reaches toxic levels (27).

The first question was answered by the finding that crude extracts of *mtlD* strains reduced fructose-6-phosphate to mannitol-1-phosphate in the presence of NADH, but virtually no activity in the reverse direction was detected (Table 4). As for function of this ester during growth on a variety of carbon sources, Helle and Klungsøyr (5) concluded that the reduction of fructose-6-phosphate to mannitol-1-phosphate was important for the reoxidation of NADH. This may be so under anaerobic conditions or under inadequate aeration in cell suspensions as dense as 20% (wt/vol) (5). Since mannitol-1-phosphate is found in lactate-grown aerobic cells, and since such cells have considerable aerobic NADH oxidase

activity, some other explanation may be necessary. We believe that this explanation may well be found in the answer to the second question—the fate of this compound. In the accompanying paper (24) we show that, in strains blocked in mannitol-1-phosphate dehydrogenase, the bulk of carbon from the mannitol moiety ends up in ribose and deoxyribose of the nucleic acids. While this route provides a steady drain of mannitol-1-phosphate (and may well explain the reason why mannitol-1-phosphate is produced in *E. coli*) it is not rapid enough to account for the observed turnover rates during P_i chase, with half-times of less than 30 s.

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