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Purification and Properties of the Adenosine Diphosphate-Glucose and Uridine Diphosphate-Glucose Pyrophosphorylases of *Mycobacterium smegmatis*: Inhibition and Activation of the Adenosine Diphosphate-Glucose Pyrophosphorylase

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Crude extracts of *Mycobacterium smegmatis* catalyzed the synthesis of adenosine diphosphate-glucose (ADP-Glc), cytidine diphosphate-glucose, guanosine diphosphate-glucose (GDP-Glc), thymidine diphosphate-glucose (TDP-Glc), and uridine diphosphate-glucose (UDP-Glc). In these crude enzyme fractions, high concentrations of trehalose-P inhibited the ADP-Glc and GDP-Glc pyrophosphorylases but did not effect the UDP-Glc or TDP-Glc pyrophosphorylases. Both the ADP-Glc pyrophosphorylase and the UDP-Glc pyrophosphorylase were partially purified (about 140-fold and 60-fold, respectively), and their properties were compared. For the ADP-Glc pyrophosphorylase, the K_m for adenosine triphosphate was 6×10^{-4} M, whereas that for glucose-1-P was 8×10^{-4} M. The optimal concentration of Mg^{2+} was 1×10^{-3} M, and the *p*H optimum was 8.5. For the UDP-Glc pyrophosphorylase, the K_m for uridine triphosphate was 1×10^{-3} M and for glucose-1-P was 2×10^{-3} M. The optimal Mg^{2+} concentration was 1×10^{-3} M, and the *p*H optimum was about 8.0. The purified ADP-Glc pyrophosphorylase was inhibited by fructose-6-P, fructose-1,6-diphosphate, glucose-6-P, and phosphoenolpyruvate. On the other hand, trehalose, trehalose diphosphate, sodium pyruvate, and ribose-5-P did not effect the ADP-Glc pyrophosphorylase. None of these compounds, including trehalose-P, had any effect on the UDP-Glc pyrophosphorylase.

Previous studies (10, 11) from this laboratory have shown that the trehalose phosphate synthetase of *Mycobacterium smegmatis* catalyzes the synthesis of trehalose-P from glucose-6-P and any one of five glucose sugar nucleotides (adenosine diphosphate-glucose [ADP-Glc], cytidine diphosphate-glucose [CDP-Glc], guanosine diphosphate-glucose [GDP-Glc], thymidine diphosphate-glucose [TDP-Glc], or uridine diphosphate-glucose [UDP-Glc]). When the pyrimidine sugar nucleotides are used as glucosyl donors, the synthetase has an absolute requirement for a high-molecular-weight polyanion (ribonucleic acid, chondroitin sulfate, heparin) for activity. However, the enzyme is active with purine sugar nucleotides (ADP-Glc, GDP-Glc) in the absence of polyanion and is only stimulated two- to fourfold by polyanion.

Because of the unusual properties of the purified synthetase with regard to lack of specificity of sugar nucleotide donors, we decided to examine crude extracts of *M. smegmatis* for their ability to synthesize the various glucose sugar nucleotides. All five sugar nucleotide pyrophosphorylases (ADP-Glc, CDP-Glc, GDP-Glc, TDP-Glc, and UDP-Glc) were readily demonstrable in these extracts. Both the ADP-Glc and the GDP-Glc pyrophosphorylases were inhibited by high concentrations of trehalose-P (the product of the synthe-

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tase reaction), whereas TDP-Glc and UDP-Glc pyrophosphorylases were not inhibited. ADP-Glc pyrophosphorylase was purified about 140-fold, and its properties were studied. In addition to inhibition by trehalose-P, the enzyme is activated by fructose-6-P and phosphoenolpyruvate. For comparison, the UDP-Glc pyrophosphorylase was purified about 60-fold and separated from the ADP-Glc pyrophosphorylase. This enzyme was not inhibited by trehalose-P, nor was it affected by fructose-6-P or phosphoenolpyruvate.

MATERIALS AND METHODS

Materials. The following compounds were obtained from the indicated sources: nucleoside triphosphates, nucleoside diphosphates, nucleoside monophosphates, sugar nucleotides, and sugar phosphates from Sigma Chemical Co. or Calbiochem; inorganic pyrophosphatase from Worthington Biochemical Co.; and [^{32}P]sodium pyrophosphate and [*U*-^{14}*C*]-glucose-1-P from New England Nuclear Co. All other chemicals were obtained from reliable chemical sources. Trehalose-P was synthesized by the method of MacDonald and Wong (12). A sample of trehalose-P was kindly supplied by D. L. MacDonald.

Analytical methods. Protein was determined by the phenol method (21). Sugar nucleotides and nucleotides were measured by their ultraviolet absorption after isolation by paper chromatography. Glucose was determined with glucostat reagent (Worthington Co.). Radioactivity was measured by counting liquid samples in Bray's solution (5) in a liquid scintillation spectrometer. Radioactivity on paper was detected in a Packard strip scanner and was measured by cutting out radioactive areas of the paper and counting them in a liquid scintillation spectrometer.

Paper chromatography. Descending paper chromatography was done with Whatman 3 MM paper or SS 589 Blue Ribbon paper. The following solvents were used: (1) *n*-propanol-ethyl acetate-water (7:1:2); (2) ethanol-1 M ammonium acetate, *p*H 7.5 (7:3); and (3) isobutyric acid-concentrated ammonium hydroxide-water (57:4:39). Sugars were located with the silver nitrate dip (22), and nucleotides were located by their ultraviolet absorption.

Assay of the sugar nucleotide pyrophosphorylases. Two different assays were used to detect enzymatic activity. One of these utilized ^{32}P-pyrophosphate in the direction of phosphorolysis (assay A), whereas the other utilized ^{14}C-glucose-1-P in the direction of synthesis (assay B) (16, 20).

Assay A. The complete reaction contained the following components (in micromoles) in a final volume of 0.3 ml: sugar nucleotide, 1.0; $MgCl_2$, 2; NaF, 0.1; tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 8.0), 10; sodium pyrophosphate, 0.5 (6×10^5 counts per min per μmole); and an appropriate amount of enzyme. Incubations were at 37 C for 10 min, and the reaction was terminated by the addition of 3 ml of cold 5% trichloroacetic acid. The precipitated protein was removed by centrifugation, and 10 μmoles of unlabeled pyrophosphate were added to the supernatant fluid. The nucleotides were then absorbed by the addition of 0.2 ml of acid-washed charcoal (150 mg/ml). After 30 min at room temperature, the suspension was centrifuged and the supernatant fluid was discarded. The charcoal was washed twice with 3 ml of cold trichloroacetic acid and once with 3 ml of cold water. The washed charcoal was suspended in 2 ml of 50% ethanol containing 0.1% NH_3, and 0.5-ml samples were pipetted into planchets and counted in a gas-flow counter.

Assay B. Pyrophosphorylase activity was also assayed with ^{14}C-glucose-1-P in the direction of sugar nucleotide synthesis (9). Complete reactions contained the following components (in micromoles) in a final volume of 0.25 ml: nucleoside triphosphate, 1; $MgCl_2$, 2; Tris buffer (*p*H 8.0), 10; ^{14}C-glucose-1-P, 1 (100,000 counts/min); inorganic pyrophosphatase, 1 μg; and an appropriate amount of enzyme. After 10 min at 37 C, the reaction was terminated by the addition of 3 ml of 5% cold trichloroacetic acid. Protein was removed by centrifugation, and 0.2 ml of acid-washed charcoal (150 mg/ml) was added to the supernatant fluid. After several minutes, the suspension was centrifuged and the supernatant fluid was discarded. The charcoal was washed two times with cold 0.02 N HCl and then was suspended in 1 ml of 0.02 N HCl and heated at 100 C for 15 min. The charcoal was removed by centrifugation, and a 0.4-ml fraction of the supernatant fluid was placed in a scintillation vial with 0.4 ml of 1 N NH_3 and 10 ml of Bray's solution and was counted in a liquid scintillation counter.

RESULTS

Demonstration of glucose nucleotide pyrophosphorylases. As shown in Table 1, pyrophosphorylase activity could be demonstrated for each of the five glucose sugar nucleotides (ADP-Glc, CDP-Glc, GDP-Glc, TDP-Glc, UDP-Glc) that are glucosyl donors for the synthesis of trehalose-P. ADP-Glc pyrophosphorylase activity was highest, whereas UDP-

TABLE 1. *Sugar nucleotide pyrophosphorylase activity in the ammonium sulfate fraction (0–70%) of M. smegmatis*[a]

Sugar nucleotide added	Nucleotide triphosphate formed (nmoles)	
	3.5 mg of protein added	7.0 mg of protein added
ADPG	13.4	24.6
CDPG	0.2	0.8
GDPG	4.0	8.3
TDPG	4.1	7.4
UDPG	3.9	7.2

[a] Reaction mixtures were as described in the text by using assay A.

Glc, GDP-Glc, and TDP-Glc pyrophosphorylases showed somewhat lower activity. CDP-Glc pyrophosphorylase activity, while detectable, was quite low in these extracts. In each case, the ^{14}C-product, formed in the forward reaction from nucleoside triphosphate and ^{14}C-glucose-1-P, was characterized as the expected glucose nucleotide by the following criteria. (i) Both the radioactivity and the ultraviolet-absorbing material had the same mobility as the expected glucose sugar nucleotide (based on the nucleoside triphosphate used) in solvents 2 and 3. (ii) In each case, the isolated and purified sugar nucleotide had the expected ultraviolet absorption spectrum. (iii) In each case, the ratio of glucose to nucleoside was approximately 1:1. (iv) In each case, the ^{14}C-sugar released by mild acid hydrolysis (0.02 N HCl, 100 C, 10 min) was identified as glucose by paper chromatography in solvent 1.

Inhibition of trehalose-P. Early in these studies it was found that trehalose-P inhibited the purine sugar nucleotide pyrophosphorylases (ADP-Glc and GDP-Glc) but had no effect on the pyrimidine sugar nucleotide pyrophosphorylases. These results are shown in Fig. 1. This inhibition could be demonstrated either in the crude extract or in a 0 to 70% $(NH_4)_2SO_4$ fraction. As shown in the figure, 50% inhibition of these two enzymes occurred at a trehalose-P concentration of about 0.01 M. UDP-Glc pyrophosphorylase was not inhibited even at very high concentrations of trehalose-P. As shown in Table 2, the inhibition was specific for trehalose-P. Other sugar phosphates and intermediates, such as fructose-6-P, trehalose diphosphate, trehalose, glucose-6-P, phosphoenolpyruvate, etc., did not inhibit these enzymes. In fact, some of these compounds have been found to be activators of the ADP-Glc pyrophosphorylase (*see* Figure 6).

Purification of ADP-Glc pyrophosphorylase: (i) preparation of crude extract. *M. smegmatis* was grown in Trypticase soy broth on a rotary shaker at 37 C for 2 to 4 days. Cells were harvested by centrifugation, washed with ice-cold distilled water, and stored as a paste at −20 C. One hundred grams of cell paste was suspended in 500 ml of 0.01 M Tris buffer, *p*H 8.0, containing 0.001 M mercaptoethanol and subjected to sonic oscillation (3 min followed by 5 min of cooling, three times). The extract was then centrifuged at 20,000 × *g* for 15 min, and the supernatant liquid was removed and saved. All steps were done at 0 to 5 C unless otherwise specified.

(ii) Ammonium sulfate fractionation. To 450 ml of supernatant liquid (11 mg of protein/ml), an equal volume of an ice-cold, saturated ammonium sulfate solution, previously neutralized with ammonium hydroxide, was added slowly with stirring. The precipitate was removed by centrifugation, dissolved in a minimal volume of distilled water (75 ml), and dialyzed against 0.01 M Tris buffer, *p*H 8.0, containing 0.001 M mercaptoethanol. The supernatant fluid from this centrifugation was saved since it contained almost all of the UDP-Glc pyrophosphorylase. The UDP-Glc pyrophosphorylase was precipitated by the addition of solid ammonium sulfate (13.6 g/liter) to 70% saturation.

(iii) DEAE-cellulose chromatography. Diethylaminoethyl (DEAE)-cellulose was washed with 1 N NaOH and then with 1 N HCl before being suspended in 1 M KCl. A 50-ml amount of the ammonium sulfate fraction (10 mg of protein/ml) was placed on a DEAE column (4 by 40 cm) previously equilibrated with 0.01 M Tris buffer, *p*H 8.0, containing 0.001 M mercaptoethanol. The column was then washed with buffer followed by 500 ml of the following KCl solutions in buffer: 0.1, 0.2, 0.3, and 0.4 M. The ADP-Glc pyrophosphorylase was eluted at 0.4 M KCl. An equal

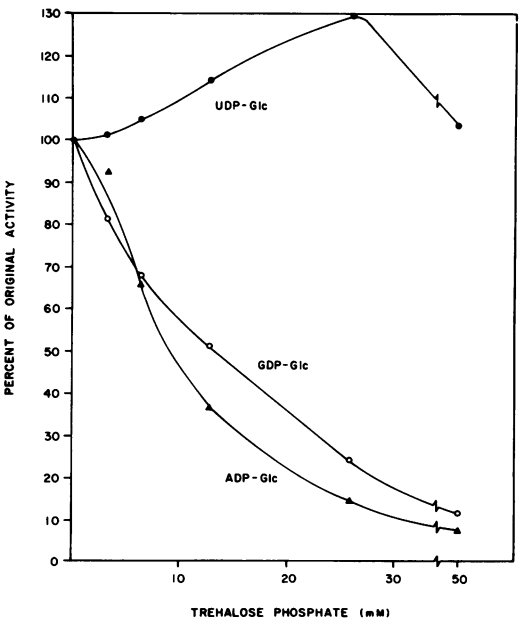

FIG. 1. *Effect of trehalose-P concentration on pyrophosphorylase activity. Incubation mixtures were as described in the test (assay B) with either ATP, GTP, or UTP. Trehalose-P was added as indicated. One milligram of the 0.70% ammonium sulfate fraction was used. In the absence of inhibitor, 3.7 nmoles of ADP-Glc, 1.5 nmoles GDP-Glc, and 1.0 nmole UDP-Glc were synthesized in this experiment (100% of original activity).*

TABLE 2. *Effect of various metabolites on sugar nucleotide pyrophosphorylase activity*

Metabolite (μmoles)	Percentage of original activity with			
	ADPG	GDPG	TDPG	UDPG
Trehalose-P				
10	61	42	109	122
20	31	21	119	105
30	19	12	124	92
Trehalose-diP				
10	96	102	119	85
20	101	82	103	106
30	85	57	126	102
Fructose-6-P				
1	145	115	115	110
3	158	100	102	104
6	160	109	112	102
Phosphoenol-pyruvate				
1	122	108	114	105
3	125	95	108	91
6	153	99	105	93
Trehalose				
1	101	98	114	94
3	99	114	88	89
6	97	105	74	91
Glucose-6-P				
1	146	109	110	94
3	165	118	110	101
6	224	96	108	111

volume of neutralized saturated $(NH_4)_2SO_4$ was added slowly with stirring to precipitate the enzyme. The precipitate was collected by centrifugation, dissolved in distilled water (15 ml), and dialyzed against Tris buffer.

(iv) Calcium phosphate gel adsorption and elution. To the DEAE-cellulose fraction (15 ml, about 2 mg of protein/ml) an equal volume of calcium phosphate gel (17 mg/ml) was added. After standing for 5 min in ice, the suspension was centrifuged, and the supernatant fluid was discarded. The gel was washed with water, and the enzyme was eluted with 0.01 M phosphate buffer, *p*H 7.0. In each case, the gel was homogenized in 10 ml of phosphate buffer and allowed to stand at room temperature for 5 min. The gel was isolated by centrifugation, and the supernatant fluid was removed and saved. The gel was again suspended in phosphate buffer and allowed to stand for 5 min, and the supernatant liquid was removed by centrifugation and saved. This phosphate buffer treatment was repeated four times. The combined supernatant fluids were treated with an equal volume of saturated $(NH_4)_2SO_4$ to precipitate the enzyme which was then dissolved in 5 ml of distilled water and dialyzed against buffer.

(v) Hydroxyapatite column chromatography. Enzyme (3 ml) from the calcium phosphate gel treatment (about 1.5 mg of protein/ml) was placed on a column (1.5 by 10 cm) of hydroxyapatite mixed with cellulose (equal parts of hydroxyapatite and cellulose). The column was washed with water and eluted with a 0 to 0.15 M gradient of phosphate buffer, *p*H 7.0. The enzyme was eluted at about 0.01 to 0.02 M phosphate buffer. The enzyme was then precipitated by the addition of an equal volume of saturated $(NH_4)_2SO_4$. However, in some cases, the amount of protein was too small to precipitate. Therefore, the enzyme solution was concentrated on an Amicon filtration cell with a 50,000 molecular weight filter, that is, a filter which retains proteins with a molecular weight of 50,000 or above.

By the use of these purification procedures, the ADP-Glc pyrophosphorylase was purified about 140-fold, with a recovery of about 10%. These data are shown in Table 3. At the calcium phosphate gel purification stage, the enzyme could be stored in the frozen state for up to 2 weeks with no apparent loss of activity. The enzyme was also stable for at least several days at 0 C. This fraction was used in most of these studies. However, the hydroxyapatite fraction also appeared to be stable for at least a week when kept in the frozen state.

Purification of the UDP-Glc pyrophosphorylase: (i) ammonium sulfate fractionation. The crude extract which was prepared for the purification of the ADP-Glc pyrophosphorylase also contained UDP-Glc pyrophosphorylase activity. These two enzymes were separated by ammonium sulfate fractionation. Whereas the ADP-Glc pyrophosphorylase was

TABLE 3. *Purification of ADP-Glc and UDP-Glc pyrophosphorylases*

Fraction	ADP-Glc		UDP-Glc	
	Specific activity[a]	Total units[a]	Specific activity	Total units
Crude	0.0083	41.1	0.00085	4.2
$(NH_4)_2SO_4$ 0–50%	0.029	22.1		
50–70%	0.001		0.0014	2.4
DEAE-cellulose 0.4 M	0.12	12		
0.3 M			0.017	2.0
Calcium phosphate gel plus $(NH_4)_2SO_4$	0.59	6.5	0.05	0.8
Hydroxyapatite	1.1	4.0		

[a] One unit is that amount of enzyme that catalyzes the synthesis of 1 μmole of sugar nucleotide under the conditions described in assay B. Specific activity = units/mg of protein.

precipitated at 0 to 50% $(NH_4)_2SO_4$ saturation, the UDP-Glc pyrophosphorylase was precipitated at 50 to 70% $(NH_4)_2SO_4$ saturation. The protein was isolated by centrifugation, dissolved in 50 ml of distilled water, and dialyzed against 0.01 M Tris buffer, *p*H 8.0, containing 0.001 M mercaptoethanol.

(ii) DEAE-cellulose column chromatography. Fifty milliliters of the above enzyme solution (about 15 mg of protein/ml) was placed on a column (4 by 40 cm) of DEAE-cellulose. The column was washed with 500 ml of Tris buffer and then with 500 ml of each of the following KCl solutions in buffer: 0.1, 0.2, and 0.3 M. UDP-Glc pyrophosphorylase activity was eluted at 0.3 M KCl. The 0.3 M KCl fraction was brought to 50% saturation by the addition of an equal volume of saturated, neutralized $(NH_4)_2SO_4$, and any protein precipitate was removed by centrifugation and discarded. The supernatant liquid was then adjusted to 70% saturation by the addition of solid $(NH_4)_2SO_4$ (13.7 g/100 ml). The precipitate was collected by centrifugation, dissolved in distilled water (30 ml), and dialyzed against 0.01 M Tris buffer, *p*H 8.0, containing 0.001 M mercaptoethanol.

(iii) Calcium phosphate gel treatment and ammonium sulfate fractionation. To 30 ml of DEAE fraction (about 1.2 mg of protein/ml) 5 ml of calcium phosphate gel (17 mg/ml) was added. The mixture was allowed to stand for 5 min in ice, and the calcium phosphate gel was removed by centrifugation and discarded. The supernatant liquid was then subjected to $(NH_4)_2SO_4$ fractionation, first by the addition of an equal volume of saturated $(NH_4)_2SO_4$ to 50% saturation and then by the addition of solid $(NH_4)_2SO_4$ to 60%. The protein precipitating between 50 and 60% $(NH_4)_2SO_4$ saturation was isolated by centrifugation, dissolved in distilled water (5 ml), and dialyzed against buffer. At this stage, the UDP-Glc pyrophosphorylase was purified about 60-fold with a recovery of about 20% (Table 3). When kept in the frozen state, the enzyme slowly lost activity over a period of several weeks.

Properties of the purified ADP-Glc and UDP-Glc pyrophosphorylases: effect of time and protein concentration. The synthesis of ADP-Glc and UDP-Glc by assay B with the purified enzymes was linear with time over a 20-min incubation period and with protein concentration over a sixfold range of concentration (15–100 μg for ADP-Glc; 80–400 for UDP-Glc). When 20 μg of ADP-Glc pyrophosphorylase was used, 22 nmoles of ADP-Glc was formed in 10 min (2% of substrate converted), whereas with 400 μg of UDP-Glc pyrophosphorylase 22 nmoles of UDP-Glc was formed (2.2% of substrate converted). This assay was used in most of these studies. Assay A was also linear with time and protein concentration but in this case for only about 10 min and over a three- or fourfold range of protein concentration.

Requirements for enzymatic activity. Table 4 shows the requirements for activity of the ADP-Glc and UDP-Glc pyrophosphorylases. In assay A, essentially no activity was observed with either enzyme when sugar nucleotide or Mg^{2+} was omitted or when $^{32}P_i$ was added at the end of the incubation or when boiled enzyme was used. NaF appeared to be inhibitory, since when it was omitted activity was nearly doubled in both cases. In assay B, essentially no activity was observed when nucleoside triphosphate or Mg^{2+} was omitted or when boiled enzyme was used. With the ADP-Glc pyrophosphorylase, guanosine triphosphate (GTP) was somewhat effective in replacing adenosine triphosphate (ATP), but little activity was observed with uridine triphosphate (UTP). With the UDP-Glc pyrophosphorylase, thymidine triphosphate (TTP) was somewhat effective in place of UTP, and some activity was also observed with ATP. Whether these

TABLE 4. *Requirements for the purified ADP-Glc and UDP-Glc pyrophosphorylases*

Conditions	Product formed (nmoles) from pyrophosphorylase	
	ADP-Glc	UDP-Glc
Assay A[a]		
Complete	12.1	5.2
Minus ADPG or UDPG	1.1	0.1
Minus $MgCl_2$	1.0	0.7
Minus NaF	21.0	9.0
$^{32}P_i$ added at end	0.8	0.5
Boiled enzyme	1.1	0.7
Assay B[a]		
Complete	29	14.5
Minus ATP or UTP	1.1	1.5
Minus $MgCl_2$	0.9	1.3
Boiled enzyme	0.7	1.1
Minus ATP or UTP, plus GTP	6.5	1.3
Minus ATP, plus UTP	1.4	
Minus UTP, plus ATP		3.6
Minus UTP, plus TTP		6.4

[a] Assay conditions were as described in the text. Calcium phosphate gel fraction (26 μg) was used for the ADP-Glc pyrophosphorylase and 400 μg of the purified UDP-Glc pyrophosphorylase.

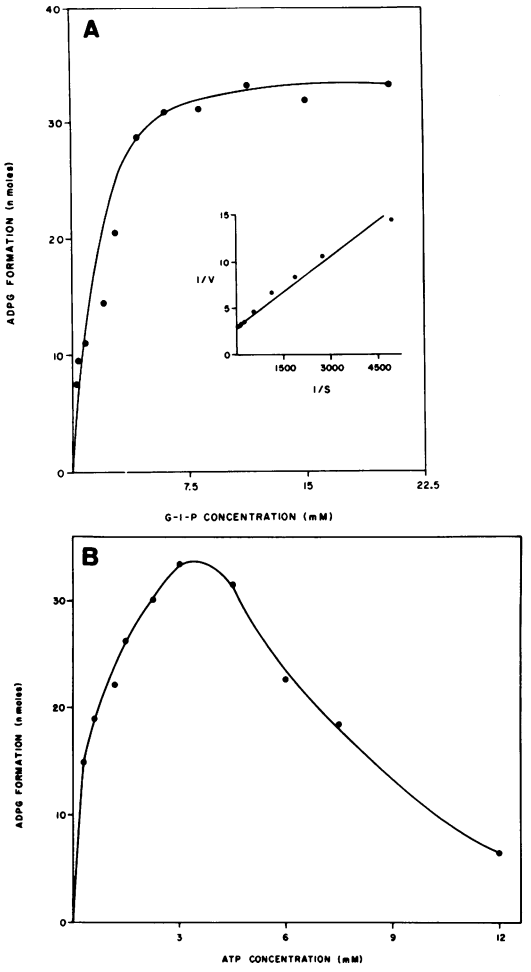

FIG. 2. *Effect of substrate concentration on the synthesis of ADP-Glc. Reaction mixtures were as described in the text under assay B with 26 μg of purified ADP-Glc pyrophosphorylase. In A, glucose-1-P concentration was varied, whereas in B, ATP concentration was varied. An incorporation of 1,000 counts/min is equivalent to synthesis of 10 nmoles in assay B.*

activities with other nucleotides represent contaminating pyrophosphorylases or nonspecificity is not known at the present time.

Effect of substrate concentration. The effect of substrate concentration on the synthesis of ADP-Glc is shown in Fig. 2A and B. From reciprocal plots, the K_m for ATP was estimated to be 6×10^{-4} M and that for glucose-1-P to be 8×10^{-4} M. High concentration of ATP was found to be inhibitory. This inhibition was observed even when the Mg^{2+} concentration was increased two- to fourfold. Figure 3A and B show the effect of substrate concentration on the synthesis of UDP-Glc. From reciprocal plots, the K_m for UTP was estimated to be 1×10^{-3} M and for glucose-1-P to be 2×10^{-3} M.

Requirement for Mg^{2+}. Figure 4A and B show the effect of $MgCl_2$ concentration on the activity of the ADP-Glc and UDP-Glc pyrophosphorylases. The optimal concentration of Mg^{2+} using assay B was 3×10^{-3} M for ADP-Glc formation and 2×10^{-3} M for UDP-Glc formation. No activity was detected in either case in the absence of $MgCl_2$.

Effect of pH. Figure 5A and B show the effect of *p*H on the ADP-Glc and UDP-Glc pyrophosphorylases. The *p*H optimum for ADP-Glc synthesis was found to be 8.5 in Tris buffer, whereas that for UDP-Glc formation was 8.0 in Tris buffer. The low activity observed in the presence of citrate buffer (particularly with the ADP-Glc pyrophosphorylase)

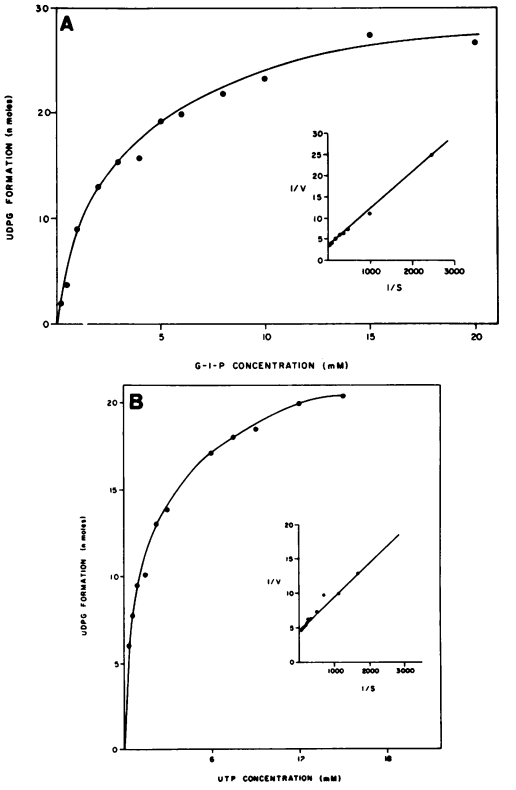

FIG. 3. *Effect of substrate concentration on the synthesis of UPD-Glc. Assay conditions were as described in the text under assay B except that in A glucose-1-P concentration was varied, whereas in B, ATP concentration was altered. Purified UDP-Glc pyrophosphorylase was used (400 μg).*

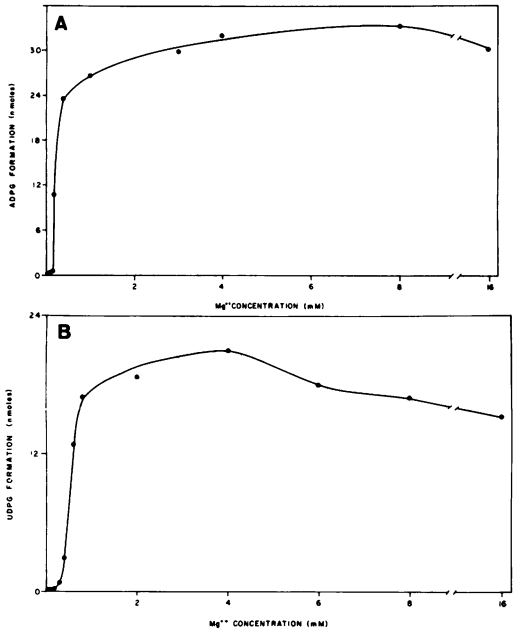

FIG. 4. *Effect of Mg^{2+} concentration on the synthesis of ADP-Glc and UDP-glc. Reaction mixtures were as described in assay B except that the Mg^{2+} concentration was varied as indicated. In A, 26 μg of ADP-Glc pyrophosphorylase was used, whereas in B, 400 μg of UDP-Glc pyrophosphorylase was used.*

may be due to chelation of Mg^{2+}, but this has not been investigated further.

Effect of inhibitors and activators. Figure 6A and B demonstrate the effect of increasing concentration of trehalose-P on the synthesis of ADP-Glc and UDP-Glc. Trehalose-P inhibited the purified ADP-Glc pyrophosphorylase. A number of other sugar phosphates and intermediates were also tested to determine their effect on these enzymes. Ribose-5-P, sodium pyruvate, 3-phosphoglyceraldehyde, and trehalose had no effect on either enzyme at the various concentrations tested. However, others such as fructose-6-P, fructose-1,6-diphosphate, and phosphoenolpyruvate were found to activate the ADP-Glc pyrophosphorylase but had no effect on the UDP-Glc pyrophosphorylase. Thus, at a concentration of 1 mM, fructose-6-P caused a 50% increase in ADP-Glc formation. Figure 7A and B show the effect on fructose-6-P, phosphoenolpyruvate, and trehalose-P on the synthesis of ADP-Glc as a function of ATP concentration. Both fructose-6-P and phosphoenolpyruvate at 3 mM concentration stimulated ADP-Glc synthesis regardless of the ATP concentration, whereas trehalose-P at 10 mM concentration inhibited at all ATP concentrations.

A variety of other compounds were tested as activators or inhibitors of these enzymes including adenosine monophosphate (AMP) and ADP. Although some inhibition of the ADP-Glc pyrophosphorylase was occasionally observed, this inhibition was not consistent and required fairly high concentrations of these nucleotides. Furthermore the inhibition varied from one enzyme preparation to another and even from experiment to experiment. No explanation for these observations is apparent at this time.

DISCUSSION

The finding that all five glucose sugar nu-

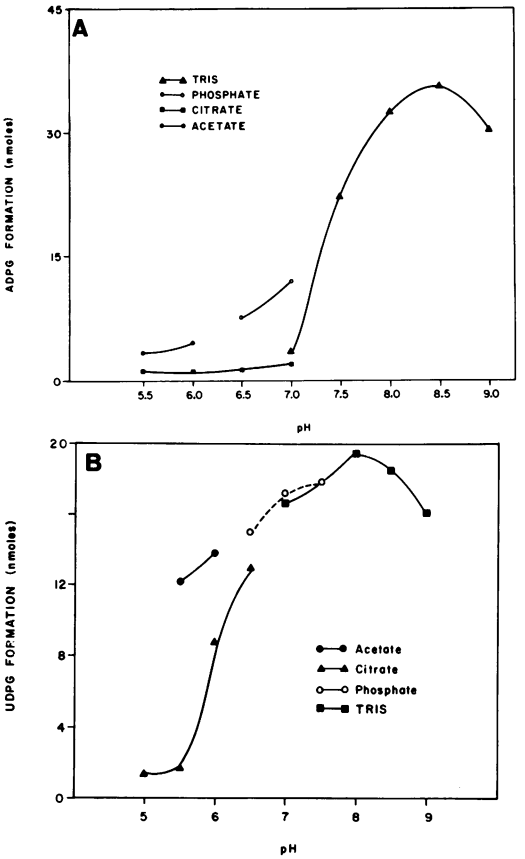

FIG. 5. *Effect of pH on ADP-Glc and UDP-Glc pyrophosphorylases. Assay mixtures were as described in the text except that pH was varied as indicated. ADP-Glc pyrophosphorylase (26 μg) was used in A, and 400 μg of UDP-glc pyrophosphorylase was used in B. All buffers were used at a final concentration of 0.04 M.*

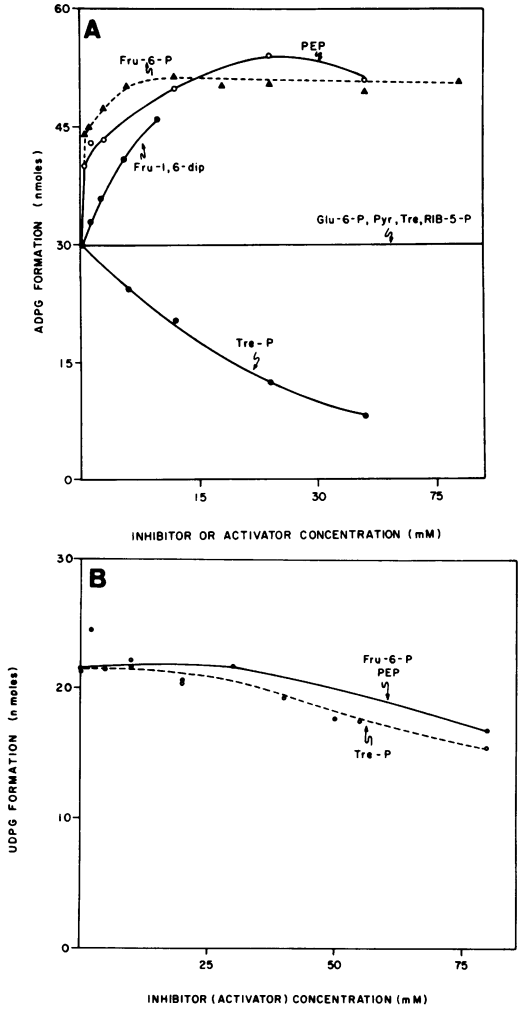

FIG. 6. *Effect of various activators and inhibitors on ADP-Glc and UDP-Glc pyrophosphorylases. Incubation mixtures were as described in the text except that various compounds were added as indicated. ADP-Glc pyrophosphorylase (26 μg) was used in A, and 400 μg of UDP-Glc pyrophosphorylase was used in B.*

cleotide pyrophosphorylases (ADP-Glc, CDP-Glc, GDP-Glc, TDP-Glc, and UDP-Glc) are present in *M. smegmatis* lends credence to the possibility that all five glucose nucleotides are glucosyl donors for the synthesis of trehalose-P. All five of these sugar nucleotides have been shown to be substrates for the purified trehalose-P synthetase of *M. smegmatis* (11, 10). The synthetase has been found to require a high-molecular-weight polyanion to be active with the pyrimidine sugar nucleotides. However, when the purine sugar nucleotides are used as substrate for trehalose-P synthesis, the enzyme no longer requires a polyanion for activation. In this case, the enzyme is only stimulated about two- to fourfold by the addition of polyanion.

These observations on the trehalose-P synthetase suggest that the purine nucleotides are distinguished from pyrimidine nucleotides in this system. This is further implied from the findings described in this paper with the sugar nucleotide pyrophosphorylases. In crude enzyme preparations of *M. smegmatis*, both the ADP-Glc and the GDP-Glc pyrophosphorylases were inhibited by high concentrations of trehalose-P, whereas the UDP-Glc and TDP-Glc pyrophosphorylases were not inhibited by trehalose-P, even at very high concentrations. Sugar nucleotide pyrophosphorylases are frequently subject to end product inhibition, particularly in prokaryotic cells. Thus, Melo and Glaser (15) found that deoxyTDP-L-rham-

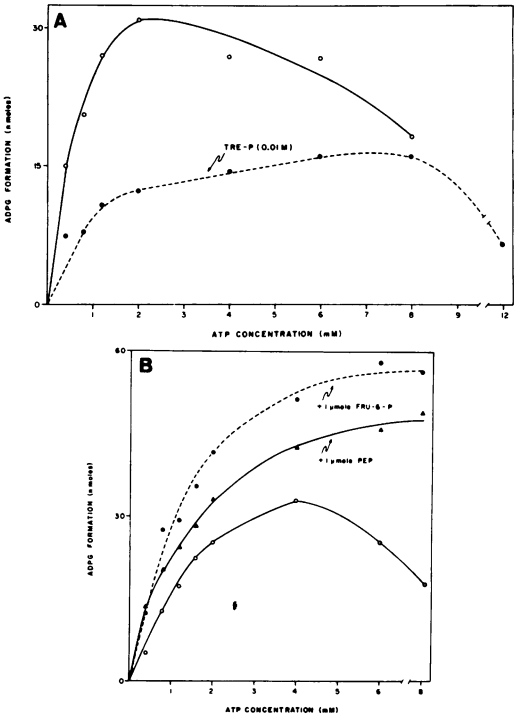

FIG. 7. *Effect of various activators and inhibitors on the synthesis of ADP-Glc. Reaction mixtures were as described in the text. A, Trehalose-P (10 μmoles/ml) was added at various ATP concentrations, and 26 μg of ADP-Glc pyrophosphorylase (DEAE fraction) was used. B, Fructose-6-P and phosphoenolpyruvate were added (1 μmole/ml) at various ATP concentrations, and 26 μg of ADP-Glc pyrophosphorylase (calcium phosphate fraction) was used.*

nose inhibits deoxyTDG-Glc pyrophosphorylase, the first enzyme of its biosynthetic pathway. Similar results were shown by Bernstein and Robbins (4) who also found that TDP-rhamnose and TDP-glucose competitively inhibited the UDP-Glc pyrophosphorylase of *Escherichia coli* B. Mayer and Ginsburg (14) showed that CDP-abequose inhibited the CDP-Glc pyrophosphorylase of *Salmonella paratyphi* A. Kornfield and Ginsburg (9) have found that GDP-mannose inhibits its own synthesis. In the mycobacterial system, the pyrophosphorylases are inhibited by trehalose-P which can be considered to be an end product of the pathway. Although the concentration of trehalose-P required to inhibit these enzymes is fairly high, this may not be surprising since trehalose is thought to function as a reserve carbohydrate in these organisms. Thus, if the concentration of trehalose-P in the cells builds up, this can "turn off" the synthesis of ADP-Glc and GDP-Glc. Accumulation of trehalose-P could occur in vivo by inhibition of the trehalose-P phosphatase. This enzyme was purified from *M. smegmatis* and was found to be inhibited by citrate (13). Although this inhibition was thought to be due to chelation and removal of Mg^{2+}, which is required by the phosphatase, it could have a physiological function as a control mechanism. It should be pointed out that the amount of trehalose-P (and trehalose) in these organisms under various physiological conditions has not yet been determined. It is of interest to note that Winder and Brennan (25) and Winder and Rooney (26) have found that when growing cells of *M. tuberculosis* BCG were exposed to isoniazid, there was an accumulation of both trehalose and an insoluble glucan. It might be of interest to examine the levels of trehalose-P and the activity of the enzymes involved in its synthesis in isoniazid-inhibited cells.

It should also be noted that mycobacteria contain glycogen as well as trehalose (2, 6, 7). This polysaccharide accumulates when growth is limited by nitrogen or sulfur (3). Although virtually nothing is known about the synthesis of this polysaccharide, it seems likely that either ADP-Glc or UDP-Glc is the glucosyl donor. Preiss (17) has found that ADP-Glc is the glucosyl donor for α-1,4-glucans in a number of different bacteria. Depending on the pathway of metabolism of the organisms in question, various intermediates such as fructose-6-P, fructose 1,6-di-P, phosphoenolpyruvate, 3-phosphoglyceraldehyde, etc., may activate the ADP-Glc pyrophosphorylase. On the other hand in some organisms AMP and ADP have been shown to be inhibitors of the ADP-Glc pyrophosphorylase (8, 17–19). Shen and Atkinson (19) have found that this inhibition is related to the energy charge of the adenylate pool.

In reference to the UDP-Glc pyrophosphorylase, none of the compounds tested as activators or inhibitors had any effect on enzymatic activity. However, one control on activity of this enzyme may be the rather low affinity for the substrates UTP and glucose-1-P. Several investigators (3, 23, 24, 16) have found that UDP-Glc inhibits the synthesis of UDP-Glc from UTP and glucose-1-P. This inhibition has not been examined in this system.

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