Biosynthesis of Bacterial Glycogen. IV. Activation and Inhibition of the Adenosine Diphosphate Glucose Pyrophosphorylase of *Escherichia coli* B*

Jack Preiss, Laura Shen, Elaine Greenberg, and Norman Gentner

ABSTRACT: An enzyme catalyzing the synthesis of adenosine diphosphate (ADP) glucose from adenosine triphosphate (ATP) and α-glucose 1-phosphate has been partially purified from *Escherichia coli* B. It was found that ADP glucose pyrophosphorylase activity could be stimulated by a number of glycolytic intermediates. Fructose 1,6-diphosphate was the most effective glycolytic intermediate. Fructose 1,6-diphosphate increased the V_{max} of pyrophosphorolysis 1.5-fold and the V_{max} of synthesis sevenfold. The apparent K_m for pyrophosphate and α-glucose 1-P were decreased in the presence of fructose 1,6-diphosphate 6- and 11-fold, respectively.

ATP and ADP-glucose gave sigmoid-shaped rate *vs.* concentration curves in the presence or absence of activator. The concentrations of ATP and ADP-glucose needed for 50% maximal velocity were decreased 12- and 8-fold, respectively in the presence of fructose 1,6-diphosphate. The enzyme was inhibited by phosphate, adenosine monophate (AMP), ADP, and sulfate. The relationship of these findings to the control of bacterial glycogen synthesis is discussed.

Recent reports indicate that the enzymatic synthesis of the α-1,4-glucosyl linkage of bacterial glycogen occurs *via* the transfer of glucose from adenosine diphosphate glucose (ADP[1]-glucose) to an α-1,4-glucan primer (Greenberg and Preiss, 1964; Shen *et al.*, 1964; Greenberg and Preiss, 1965; Preiss and Greenberg, 1965). The only other sugar nucleotide active as a glucosyl donor for the reaction was dADP-glucose. Thus the bacterial glycogen synthetases differed from the mammalian and yeast glycogen synthetases with respect to the glucosyl donor (Leloir and Goldemberg, 1960; Algranati and Cabib, 1962; Kornfeld and Brown, 1962; Villar-Palasi and Larner, 1958; Robbins *et al.*, 1959). Another difference between the bacterial and mammalian glycogen synthetases was that the mammalian glycogen synthetases were activated by glucose 6-P (Leloir *et al.*, 1959; Rosell-Perez and Larner, 1964). The bacterial glycogen synthetases, however, were not activated by glucose 6-P or any other glycolytic intermediate tested.

It was then shown by Shen and Preiss (1964) that ADP-glucose synthesis, catalyzed by the ADP-glucose pyrophosphorylase isolated from *Arthrobacter viscosus* extracts (Shen and Preiss, 1965), was activated by D-fructose 6-P, pyruvate, or D-ribose 5-P. The ADP-glucose pyrophosphorylase activity obtained from extracts of *Escherichia coli* B, however, was activated by fructose 1,6-diphosphate (FDP), phosphoenolpyruvate (PEP), and glyceraldehyde 3-P (Preiss *et al.*, 1965). The present paper reports on the purification of the ADP-glucose pyrophosphorylase from *E. coli* B, on the kinetic studies of the activation by the various metabolites as well as on the inhibition of the enzyme by P_i, 5′-AMP, and ADP.

Experimental Procedure

E. coli B[2] was grown in 15-l. cultures containing 1% glucose, 1.1% K_2HPO_4, 0.85% KH_2PO_4, and 0.6% Difco yeast extract at 37°. Growth was continued until the bacteria reached late log or stationary phase. The cultures were then centrifuged at 1–5° in a Sharples centrifuge and the bacterial paste was stored at −12°.

Sugar nucleotides were obtained from commercial sources or chemically synthesized according to a modification (Kochetkov *et al.*, 1962) of the procedure of Roseman *et al.* (1961). Xylulose 5-P was obtained from Dr. W. A. Wood of Michigan State University. Sedoheptulose 7-P was a gift from Dr. B. L. Horecker of the Albert Einstein Medical Center. Anhydro-D-glucitol 6-P and D-allose 6-P were given to us by Dr. R. K. Crane of Chicago University. D-Arabinitol 1,5-diphosphate, L-arabinitol 1,5-diphosphate, 1,5-pentane-

* From the Department of Biochemistry and Biophysics, University of California, Davis, California. *Received February 17, 1966.* This work was supported by a grant (AI-05520) from the U. S. Public Health Service.

[1] Abbreviations used: ADPG, adenosine diphosphate glucose, dADP, deoxy-ADP; ATP, GTP, CTP, UTP, ITP, and TTP, adenosine, guanosine, cytidine, uridine, inosine, and thymidine triphosphates; AMP, adenosine monophosphate; FDP, fructose 1,6-diphosphate; PEP, phosphoenolpyruvate; diP, diphosphate; P, phosphate; GSH, glutathione; TPN^+, triphosphopyridine nucleotide; DPN^+, diphosphopyridine nucleotide; DPNH, reduced DPN.

[2] We wish to thank Drs. A. Kornberg and P. Berg of the Stanford University School of Medicine for the generous supply of *E. coli* B cells in the initial phases of this work.

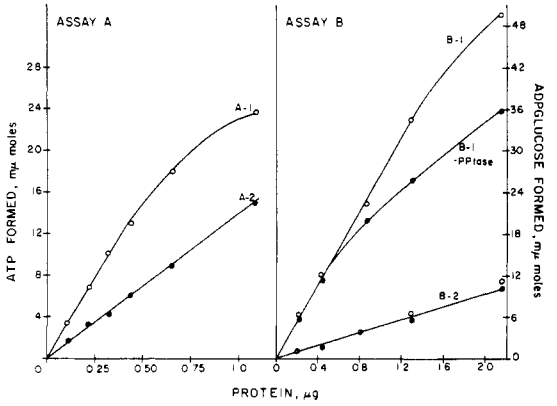

FIGURE 1: Effect of protein concentration (DEAE-Sephadex fraction) on the pyrophosphorolysis (assay A) and synthesis (assay B) of ADP-glucose. In assay B, ●—● represents incubation mixtures containing no yeast inorganic pyrophosphatase (PPtase). A1, 2, B1, and 2 represent the various incubation mixtures that are described in the test.

diol diP, D-glucitol 1,6-diP, and xylitol 1,5-diP were generously supplied to us by Dr. Robert Barker of the College of Medicine, State University of Iowa. 2-Keto-3-deoxy-D-gluconate and 6-phospho-2-keto-3-deoxy-D-gluconate were prepared as previously described (Cynkin and Ashwell, 1960). All other chemicals, radioactive or nonradioactive, were obtained commercially or as previously mentioned (Preiss and Wood, 1964).

The following solvent systems were used in descending paper chromatography on Whatman No. 1 filter paper: solvent A, 95% ethanol–1 M ammonium acetate, pH 7.5 (5:2); solvent B, 95% ethanol–1 M ammonium acetate, pH 3.8 (5:2); solvent C, isobutyric acid–1 M NH_3–0.1 M EDTA, pH 7.2 (10:6.0:0.16); solvent D, 600 g of ammonium sulfate in 1 l. of 0.1 M sodium phosphate, pH 6.8, and 20 ml of 1-propanol; solvent E, ethyl acetate–pyridine–water (3.6:1.0:1.15). Paper electrophoresis was done in 0.05 M citrate buffer, pH 3.9, in a GME electrophorator.

Total, acid-labile, and inorganic phosphate were determined by the method of Fiske and Subbarow (1925). Reducing sugars were assayed by the method of Nelson (1944) and detected on paper with silver nitrate (Anet and Reynolds, 1954). Protein concentration was determined by the method of Lowry *et al.* (1951).

Assay of ADP-Glucose Pyrophosphorylase. ASSAY A. Enzymatic activity was determined by following the synthesis of ATP-^{32}P from ADP-glucose and ^{32}P-P_i (Shen and Preiss, 1964). Two different incubation mixtures were used to measure pyrophosphorolysis. They are referred to as A1 and 2. The conditions of assay A1 are the conditions for the maximal rate of pyrophosphorolysis in the presence of the activator, fructose 1,6-diP. The conditions of assay A2 gave maximal rates of pyrophosphorolysis in the absence of the activator. The incubation mixtures of assay A1 contained 0.1 μmole of ADP-glucose, 0.5 μmole of ^{32}P-P_i (sp act. 5.0–25 × 10^5 cpm/μmole), 20 μmoles of Tris–Cl, pH 8.0, 2 μmoles of $MgCl_2$, 2.5 μmoles of KF, 0.3 μmole of fructose diP, 100 μg of bovine plasma albumin, and enzyme in a total volume of 0.25 ml. In incubation mixtures containing no fructose diphosphate (assay A2), the amounts of ADP-glucose and $MgCl_2$ were increased to 0.7 and 5 μmoles, respectively. The time of incubation was 10 min at 37°. The reaction was terminated by the addition of 3 ml of cold 5% trichloroacetic acid to the incubation mixture. The ATP-^{32}P product was isolated by adsorption to Norit A and measured as described previously (Shen and Preiss, 1964).

ASSAY B. ADP-glucose formation was assayed as previously described (Shen and Preiss, 1964). As in assay A, two different incubation mixtures were used to measure ADP-glucose synthesis and they were designated as assay B1 and 2. The incubation mixtures of assay B1 contained 0.1 μmole of glucose-^{14}C 1-P (sp act. 8.0 × 10^5 cpm/μmole), 0.3 μmole of ATP, 1.0 μmole of $MgCl_2$, 10 μmoles of Tris–Cl buffer, pH 8.0, 100 μg of bovine plasma albumin, 0.3 μmole of fructose diP, 0.9 μg of crystalline yeast inorganic pyrophosphatase, and purified enzyme in a total volume of 0.20 ml. In incubation mixtures containing no fructose 1,6-diphosphate (assay B2) the amounts of ATP, glucose-^{14}C 1-P, and $MgCl_2$ present were 1.4, 0.2, and 5.0 μmoles, respectively. The incubation time was 10 min at 37°. The reaction was terminated by heating the incubation mixtures in a boiling water bath for 30 sec. *E. coli* alkaline phosphatase, 0.1 mg, was then added to the mixtures which were now incubated for 40 min at 37°. This treatment hydrolyzed glucose-^{14}C 1-P to glucose-^{14}C while leaving the formed sugar nucleotide intact. A portion of this phosphatase-treated mixture (0.1 ml) was adsorbed onto a disk (diameter 2.5 cm) of DEAE-cellulose paper. The disks were washed three times with 150 ml of deionized water to remove the glucose-^{14}C. The disks were dried and then counted by liquid scintillation technique as previously described (Shen and Preiss, 1964). Figure 1 shows that the rate of reaction in both assays A and B is linear with respect to enzyme concentration. In assay A1, *ca.* 1.5–2 times as much activity was observed as in assay A2. The rate of ADP-glucose synthesis was six to eight times greater in assay B1 than in assay B2. The effect of inorganic pyrophosphatase in assay B1 and 2 is also shown in Figure 1.

Results

Purification of ADP-Glucose Pyrophosphorylase. STEP 1. PREPARATION OF CRUDE EXTRACT. The *E. coli* B cells were disrupted by grinding in a Waring blendor. Thawed cells, 100 g, 80 ml of 0.05 M glycylglycine buffer, pH 7.0, containing 0.005 M GSH, and 300 g of chilled glass beads (Superbrite no. 100; average diameter 200 μ)[3] were stirred in a Waring blendor for 25 min. The tem-

[3] Obtained from Minnesota Mining and Manufacturing Co., St. Paul 6, Minn.

perature was kept <10° by stopping the grinding procedure at various times and cooling the blendor container in ice water. After completion of the grinding process, 300 ml of the glycylglycine–GSH buffer were added to the bacterial suspension. The suspension was stirred gently for 10 min and then the glass beads were allowed to settle. The supernatant fluid was poured off and the beads were extracted with another 200 ml of the above buffer. This wash was then combined with the supernatant fluid and centrifuged at 15,000*g* for 10 min. The supernatant fluid obtained from the centrifugation was used as the source of enzyme. All ensuing operations were carried out at 0–3°.

STEP 2. PROTAMINE SULFATE FRACTIONATION. A 1% protamine sulfate solution, 150 ml, was added slowly, with constant stirring, to 500 ml of the crude extract. After 10 min the suspension was centrifuged at 10,000*g* for 10 min; the precipitate contained the enzymatic activity. This precipitate was extracted twice with 300 ml of 0.3 M potassium phosphate buffer, pH 7.0, containing 0.01 M GSH. The eluates were combined and 305 g of ammonium sulfate were added to them. The resulting suspension was centrifuged at 15,000*g* for 10 min. The precipitate thus obtained was dissolved in 0.03 M phosphate, pH 7.2, containing 0.001 M GSH and then dialyzed overnight *vs.* 2 l. of the same buffer.

STEP 3. HEAT TREATMENT AND AMMONIUM SULFATE FRACTIONATION. The dialyzed enzyme (119 ml) was heated for 5 min in a beaker containing 4 l. of water at 70° and then quickly cooled. The denatured protein was removed by centrifugation and then washed with 30 ml of cold deionized water. The water wash and the supernatant solution obtained from the heat treatment were combined. *Ca.* 90–100% of the enzyme activity was still present after this treatment. A saturated ammonium sulfate solution, 141 ml, was then added to 141 ml of the combined enzyme solution and the resulting suspension was centrifuged at 20,000*g* for 10 min. The precipitate thus obtained was dissolved in 0.05 M Tris–Cl buffer, pH 7.2, containing 0.005 M GSH and dialyzed *vs.* 500 ml of the same buffer overnight.

TABLE I: Purification of ADPG Pyrophosphorylase.

Fraction	Vol. (ml)	Protein (mg/ml)	Sp Act. (units/mg)	Total Act.[a] (units)
(1) Crude extract	500	28.2	0.12	1700
(2) Protamine sulfate pptn	120	18.4	0.54	1200
(3) Heat treatment and $(NH_4)_2SO_4$	14	16.0	4.1	910
(4) DEAE-Sephadex	4	5.2	32	660

[a] Enzymatic activity (1 unit) is equal to 1 μmole of ATP formed in 10 min under conditions specified for assay A1.

STEP 4. DEAE-SEPHADEX CHROMATOGRAPHY. A 2 × 15 cm DEAE-Sephadex (A-50 medium particle size) column was equilibrated with a 0.015 M phosphate buffer, pH 7.5, containing 0.005 M GSH. Then 14 ml of the heat-treated fraction was adsorbed onto the column. The column was washed with 40 ml of the 0.015 M phosphate–0.005 M GSH buffer. The enzyme was then eluted with a linear gradient consisting of 1 l. of 0.015 M phosphate–0.005 M GSH buffer, pH 7.5, in the mixing flask and 1 l. of 0.2 M phosphate buffer, pH 7.0, containing 2.0 M NaCl and 0.005 M GSH in the reservoir flask. The eluates were collected in 30-ml fractions; the fractions containing enzyme were pooled (combined volume = 132 ml) and 68 g of ammonium sulfate were added. The resulting suspension was then centrifuged and the precipitate was dissolved in 0.05 M Tris, pH 7.2, containing 0.005 M GSH and dialyzed for 12 hr *vs.* the same buffer. Table I summarizes the purification of the pyrophosphorylase. The 260-fold purified enzyme contained no detectable amounts of phosphoglucomutase, aldolase, inorganic pyrophosphatase, phosphohexose isomerase, ATPase, nucleotide pyrophosphatase, adenylate kinase, or glucose 1-phosphatase activities.

Crude extracts of *E. coli* B cells grown in synthetic medium with glucose as the carbon source (Sigal *et al.*, 1964) contained the same amount of ADP-glucose pyrophosphorylase activity/mg of protein.

TABLE II: Requirements for Pyrophosphorolysis of ADP-Glucose.[a]

Conditions	ATP Formed (mμmoles) Assay A1	Assay A2
Complete	9.6	6.5
−ADP-glucose	<0.1	<0.1
$-MgCl_2$	<0.1	<0.1
−Bovine plasma albumin	7.4	4.0
−Fluoride	11.0	7.5
−Fluoride + 1 μg of inorganic pyrophosphatase	<0.1	<0.1
Reaction heat denatured at 0 time	0.28	0.20
$-P^{32}P_i$ + 1 μmole of $^{32}P_i$	0.27	0.20
−Fructose diP	0.90	...

[a] Conditions of the experiment were those of assay A1 and 2. The specific activities of the ^{32}P–P_i and $^{32}P_i$ were 7 × 10^5 cpm/μmole.

Requirements for Pyrophosphorolysis of ADP-Glucose. Table II shows that pyrophosphorolysis of ADP-glucose was dependent on the simultaneous presence of magnesium ion, inorganic pyrophosphate, ADP-glucose, and enzyme. Fluoride, which inhibits inorganic

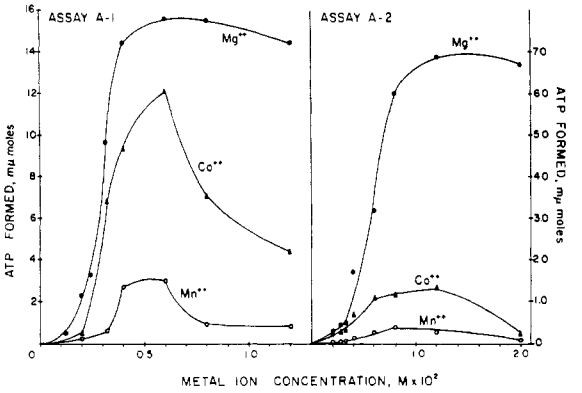

FIGURE 2: Effect of various metal ion concentrations on the rate of pyrophosphorolysis of ADP-glucose. The incubation mixtures are those of assays A1 and 2.

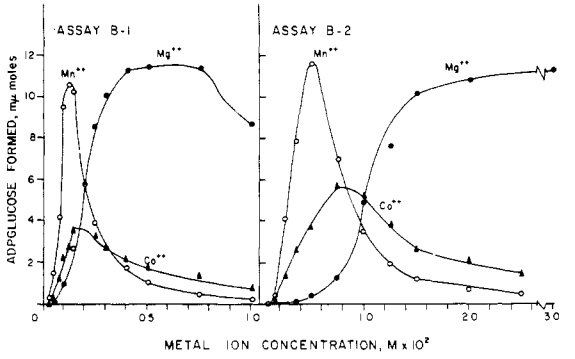

FIGURE 3: Effect of various metal ion concentrations on the rate of synthesis of ADP-glucose. The incubation mixtures are those of assays B1 and 2. Five times as much enzyme was used in assay B2 as in assay B1.

pyrophosphatase in the crude extract, was not required in the assay of the purified enzyme. Addition of 0.9 μg of crystalline yeast inorganic pyrophosphatase to the reaction mixture before addition of pyrophosphorylase inhibited the reaction over 95%. Substitution of $^{32}P_i$ for $P^{32}P_i$ resulted in no formation of Norit-adsorbable radioactivity. The requirements for pyrophosphorolysis were the same for the conditions of assay A1 or 2. However, fructose diP gave a 10–15-fold stimulation of the pyrophosphorolytic rate in reaction mixtures containing 4×10^{-4} M ADP-glucose and 8×10^{-3} M $MgCl_2$. Bovine plasma albumin usually stimulated the rate of pyrophosphorolysis 30–40%. When ADP-glucose was replaced by GDP-glucose, UDP-glucose, TDP-glucose, IDP-glucose, or CDP-glucose, little or no formation of Norit-adsorbable radioactivity was observed. The crude extract, however, did contain UDP-glucose and TDP-glucose pyrophosphorylase activities. The purified enzyme was able to catalyze the pyrophosphorolysis of dADP-glucose. Pyrophosphorolysis of dADP-glucose proceeded at a rate of 5% of that of ADP-glucose under conditions of assay A1 or 2. The radioactive product dATP was identified and distinguished from ATP by paper chromatography in solvents B–D.

Requirements for Synthesis of Sugar Nucleotides. Synthesis of ADP-glucose was dependent on the presence of Mg^{2+} ion, ATP, glucose 1-P, and enzyme (Table III). Bovine plasma albumin stimulated the reaction rate 33–50%. Omission of fructose diP from incubation mixtures of assay B1 resulted in a 30-fold decrease in the rate of ADP-glucose synthesis. Sugar nucleotide synthesis was observed when UTP, GTP, ITP, CTP, and dATP were used in place of ATP in assay B2. The rates of sugar nucleotide synthesis from the various nucleoside triphosphates under the conditions of assay B2 were *ca.* 1.5–4% the rate of synthesis of ADP-glucose. In the presence of activator (assay B1) the rates of synthesis of sugar nucleotides from GTP, CTP, UTP, and dATP were only 0.07–0.34% the rate of synthesis of ADP-glucose from ATP.

TABLE III: Requirements for Synthesis of ADP-Glucose.[a]

Conditions	ADP-Glucose Formed (μmoles/mg/hr) Assay B1	Assay B2
Complete	192	37.8
$MgCl_2$ omitted	2.0	2.0
Enzyme omitted	<0.1	<0.1
ATP omitted	<0.1	<0.1
−Fructose diP omitted	6.0	...
−Bovine plasma albumin omitted	150	27.6
ATP replaced by		
GTP	0.19	1.03
CTP	0.65	1.52
UTP	0.14	1.38
dATP	0.41	1.00
ITP	0.10	0.60
TTP	<0.10	<0.10

[a] Conditions of the experiment were those of assays B1 and 2, except where noted.

Specificity of Divalent Metal Requirement. As mentioned previously Mg^{2+} was required for the synthesis or pyrophosphorolysis of ADP-glucose. Co^{2+} and Mn^{2+} were found to be able to replace Mg^{2+} in these reactions. In pyrophosphorolysis Co^{2+} and Mn^{2+} were not as effective as Mg^{2+} either under conditions of assay A1 or A2 (Figure 2). However, the maximal rates of ADP-glucose synthesis in the presence of Mg^{2+} or Mn^{2+} were about the same and greater than the maximal rates obtained when Co^{2+} was the divalent cation (Figure 3). Manganese was more effective than Mg^{2+} at lower concentrations. Figure 3 also shows that the concentration range of Mn^{2+} for maximal ADP-glucose synthesis was very narrow. The concentration range of Mg^{2+} for maximal ADP-glucose synthesis was rela-

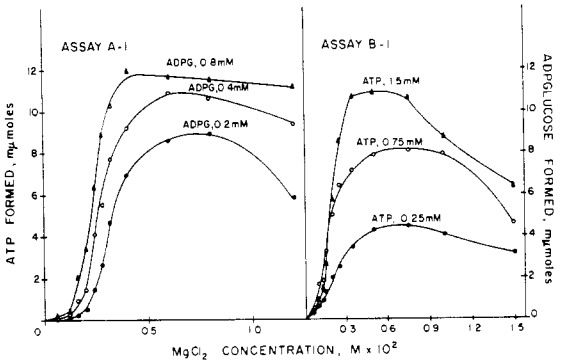

FIGURE 4: Effect of ADP-glucose and ATP on the $MgCl_2$ concentration *vs.* rate curve. The incubation mixtures of assay A1 and assay B1 are described in the text. The concentrations of ADP-glucose (ADPG) and ATP are indicated in the graph.

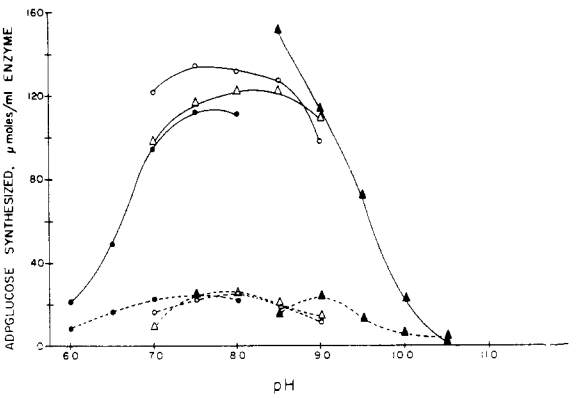

FIGURE 5: Effect of pH on ADP-glucose synthesis. The reaction mixtures of assays B1 (solid lines) and B2 (dotted lines) are described in the text. ●—● and -●- - -●- represent reaction mixtures containing histidine buffer. Δ—Δ and -Δ- - -Δ- are for reaction mixtures containing Tris–Cl buffer. ○—○ and -○- - -○- represent reaction mixtures containing glycylglycine buffer. ▲—▲ and -▲- - -▲- represent reaction mixtures containing glycine–NaOH buffer.

tively broad. Because Mg^{2+} did catalyze maximal rates of both pyrophosphorolysis and synthesis and did have a relatively broad concentration range for maximal activity it was used as the divalent cation for further kinetic studies. It should be noted that the rate *vs.* divalent cation concentration curve is sigmoid shaped suggesting that there was more than one binding site on the enzyme for the divalent cations. The sigmoid nature of the curve or the concentration of Mg^{2+} necessary for optimal activity did not appear to be affected by the concentration of ATP or ADP-glucose present in the reaction mixtures (Figure 4).

pH Optimum. Figure 5 shows that the pH optimum for ADP-glucose synthesis was rather broad, being between pH 7 and 9. This was independent of the buffer used in the reaction mixtures as well as the assay conditions. Optimal activation of the enzymatic activity by fructose diP also occurred in the range of 7–9. The pH optimum of pyrophosphorolysis was essentially the same as that of synthesis.

Characterization of Products. The products formed from glucose-^{14}C 1-P plus either ATP, CTP, GTP, UTP, dATP, or ITP (assay B2) were isolated by chromatography in solvent B. The radioactive spots were eluted with water and rechromatographed in solvents B–D. In each instance the radioactive sugar nucleotide cochromatographed with its respective standard. Thus the sugar nucleotides formed from ATP, CTP, GTP, UTP, dATP, or ITP were ADP-glucose, CDP-glucose, GDP-glucose, UDP-glucose, dADP-glucose, and IDP-glucose. The ADP-glucose product was isolated from large scale incubation mixtures (100-fold, assay B1) by Dowex-1 × 8 (Cl) column chromatography. The ADP-glucose fractions from the column were pooled and then desalted by adsorption on charcoal followed by elution with an aqueous solution of 50% ethanol–0.015 M NH_3. Table IV shows the chemical and spectral analysis of this product. The ratio of nucleotide to total phosphate to acid-labile phosphate was that expected for a nucleotide diphosphate sugar. The specific activity of the sugar nucleotide was equal to that of the substrate, glucose 1-P. Furthermore this product cochromatographed with an authentic sample of ADP-glucose in solvents A–D. Only one ultraviolet-absorbing spot was seen with each product in each of the four solvent systems and the radioactivity was associated with this spot.

TABLE IV: Chemical Analysis of Sugar Nucleotide Product.

Anal	Concn (μmoles/ml)	Spectral Constants (pH 2.0)
Adenosine	4.0	λ_{max} 257 mμ
Acid-labile phosphate	4.2	$A_{280}:A_{260}$ 0.21
Total phosphate	8.3	$A_{250}:A_{260}$ 0.84
Glucose-^{14}C (sp act.)	3.87	
Reducing sugar before acid hydrolysis	0.0	$\lambda_{max}^{pH7.0}$ 260 mμ
Reducing sugar after acid hydrolysis	3.85	$A_{280}:A_{260}$ 0.15
Glucose after acid hydrolysis[a]	3.67	$A_{250}:A_{260}$ 0.79
Glucose 1-P after phosphodiesterase treatment	3.95	

[a] Determined with hexokinase, ATP, TPN, and glucose 6-P dehydrogenase.

Hydrolysis of the sugar nucleotide product by snake venom phosphodiesterase, a procedure which had been used previously in the characterization of sugar nucleo-

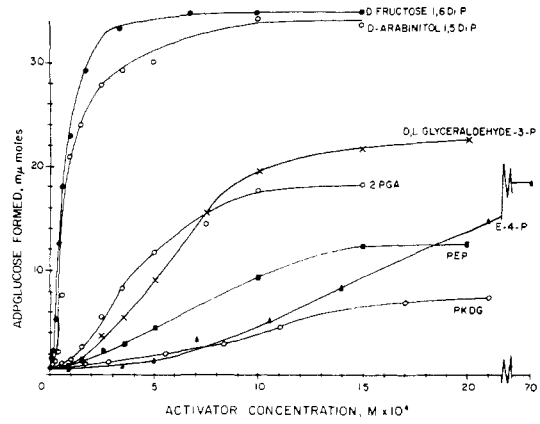

FIGURE 6: Effect of the concentrations of various activators on the synthesis of ADP-glucose. The incubation mixtures are those of assay B1.

tides (Preiss and Wood, 1964; Ginsburg *et al.*, 1962a,b), resulted in the formation of 5′-AMP and glucose-^{14}C 1-P. AMP was identified by paper chromatography and paper electrophoresis and glucose 1-P was identified by its reaction with phosphoglucomutase, TPN, and glucose 6-P dehydrogenase.

Hydrolysis of the ADP-glucose product in 0.01 N HCl at 100° for 10 min gave rise to a reducing sugar which cochromatographed with glucose in solvent E. All of the radioactivity of the product corresponded to the glucose spot, which was detected by the $AgNO_3$ test. The amount of glucose released by acid hydrolysis was *ca.* 1 μmole/μmole of sugar nucleotide (Table IV). With ADP-glucose acid hydrolysis caused the formation of 0.89 μmole of ADP/μmole of ADP-glucose, as determined by the method of Kornberg and Pricer (1950). ADP was also identified by chromatography in solvents A–D. A trace amount of 5′-adenylate was also observed.

Stoichiometry of Reaction. Table V shows that pyrophosphorolysis of ADP-glucose leads to concomitant formation of equal amounts of glucose 1-P and ATP. The synthesis of 1 μmole of ADP-glucose led to the disappearance of 1 μmole of glucose 1-P and 1 μmole of ATP.

Activation of the Enzyme. SPECIFICITY OF ACTIVATION. Our preliminary report (Preiss *et al.*, 1965) indicated that a number of glycolytic intermediates were capable of activating ADP-glucose pyrophosphorolysis or synthesis. Figures 6 and 7 show that fructose 1,6-diphosphate was the most effective activator of all glycolytic intermediates tested. Activation under conditions of assay B1 were *ca.* 30–40-fold. Sedoheptulose 1,7-diP and D-arabinitol 1,5-diP were just as effective as fructose 1,6-diP in the activation of ADP-glucose synthesis. Compounds not as effective as the above three but which activated to an appreciable extent were glucose 1,6-diP, DL-glyceraldehyde 3-P, 2-phosphoglycerate, erythrose 4-P, phosphoenolpyruvate, and phosphoketodeoxygluconate (Figures 6 and 7). Compounds which were tested at concentrations of 0.05 and 1.5 mM, that gave no stimulation of ADP-glucose synthesis or pyrophosphorolysis were 2-deoxy-D-glucose 6-P, D-fructose 1-P, D-fructose 6-P, D-glucosamine 6-P, D-glucose 6-P, β-glycerol-P, D-galactose 6-P, D-ribose 5-P, D-2-deoxyribose 5-P, D-sedoheptulose 7-P, dihydroxyacetone P, glycoaldehyde P, 2,3-diphosphoglycerate, 3′,5′-cyclic adenylate, glyceric acid, lactic acid, *o*-serine P, pyruvate, fructose, glucose, fumarate, succinate, malate, α-ketoglutarate, and $NaHCO_3$. Compounds that gave stimulations of ADP-glucose synthesis between 1.1- and 2-fold at a concentration of 1.5 mM were 3-phosphoglycerate, acetyl P, and acetyl CoA. Both phosphohydroxypyruvate and ribulose 1,5-diP at a concentration of 1.5 mM gave stimulations of ADP-glucose synthesis of *ca.* fourfold. The UDP-glucose and TDP-glucose pyrophosphorylase activities found in the crude extracts were not stimulated by fructose diP.

A common property of all these activators is that their velocity *vs.* concentration curves are sigmoid

TABLE V: Stoichiometry of ADP-Glucose Pyrophosphorylase.[a]

Compound	0 Time	10 min	Δ10 min	30 min	Δ30 min
			μmoles		
Pyrophosphorolysis of ADP-glucose					
Glucose 1-P	0.00	0.87	0.87		
ATP	0.00	0.85	0.85		
Synthesis of ADP-glucose					
Glucose 1-P	0.57	0.27	−0.30	0.01	−0.56
ATP	1.13	0.82	−0.31	0.55	−0.58
ADP-glucose	0.0	0.28	+0.28	0.55	+0.55

[a] The pyrophosphorolysis incubation mixture contained: Tris–Cl, pH 8.3, 100 μmoles; $MgCl_2$, 10 μmoles; PP_i, 5 μmoles; ADP-glucose, 1.05 μmoles; fructose diP, 3.0 μmoles; bovine plasma albumin, 1.0 mg; and DEAE-Sephadex enzyme in a volume of 2.5 ml. Portions, 0.5 ml, of the reaction mixture were taken at the time indicated for the assay of glucose 1-P (determined with phosphoglucomutase, TPN, and glucose 6-P dehydrogenase) and ATP (determined with glucose, hexokinase, TPN, and glucose 6-P dehydrogenase). The reaction mixture used to measure the synthesis of ADP-glucose-^{14}C contained: Tris, pH 8.3, 60 μmoles; $MgCl_2$, 6.0 μmoles; glucose-^{14}C 1-P, 570 mμmoles; ATP, 1.13 μmoles; crystalline yeast inorganic pyrophosphatase, 1.8 μg; bovine plasma albumin, 600 μg; fructose 1,6-diP, 1.8 μmoles; and DEAE-Sephadex enzyme in a volume of 1.2 ml. Samples, 0.01 ml, were taken at 0 time and 10 and 30 min for the determination of glucose 1-P, ATP, and ADP-glucose (assay B).

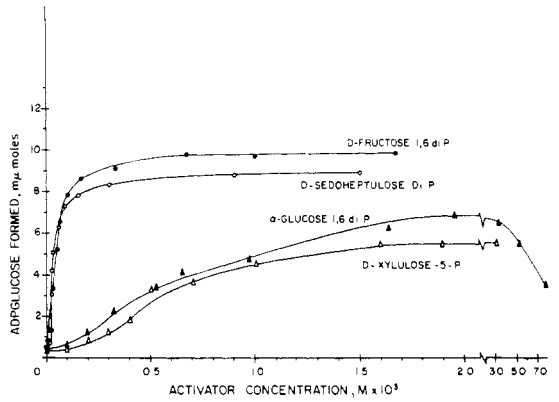

FIGURE 7: Effect of the concentrations of various activators on the synthesis of ADP-glucose. The incubation mixtures are those of assay B1.

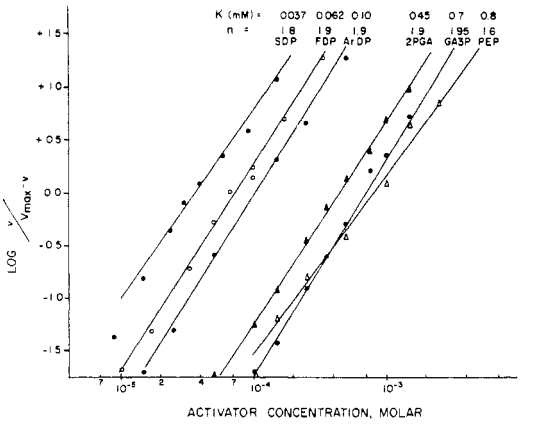

FIGURE 8: The plot of some of the data of Figures 6 and 7 as log $v/V_{max} - v$ *vs.* log activator concentration. The values of V_{max} were estimated from reciprocal plots of rate *vs.* activator concentration. If the rate became independent of further increase in activator concentration, it was assumed that this maximal rate was V_{max}. v is the increase in velocity due to addition of activator, *i.e.*, the velocity obtained upon addition of a certain amount of activator to the reaction mixture minus the velocity of reaction mixtures containing no activator. The following abbreviations are used: SDP, sedopheptulose 1,7-diP; FDP, fructose 1,6-diP; ArDP, arabinitol diP; 2PGA, 2-phosphoglycerate; GA3P, glyceraldehyde 3-P; PEP, phosphoenol pyruvate.

shaped suggesting that more than one molecule of activator is bound to the enzyme and that these activator sites are interacting; *i.e.*, the binding of the first activator molecule facilitates the binding of additional activator molecules. The expression

$$\log \frac{v}{V_{max} - v} = n \log (\text{substrate or activator}) - \log K$$

has been used for the treatment of kinetic data whose reaction rate *vs.* concentration curves were sigmoid shaped. v is the reaction velocity of enzyme; V_{max} is the velocity of reaction at saturating concentration of activator. K is known as the product of the n dissociation constants of the n binding sites. n is known as the interaction coefficient or apparent order of reaction and is dependent on the total number of actual binding sites as well as their strength of interaction. If the interactions are strong, then n will approximate the actual number of binding sites. If the interactions are weak, then n will be less than the number of binding sites. If there is no interaction, $n = 1$. Changeux (1963) introduced this expression as the Hill equation. Atkinson *et al.* (1965) and Taketa and Pogell (1965) have derived similar expressions using Michaelis–Menten assumptions. A plot of log $v/V_{max} - v$ *vs.* log of activator concentration would then give a straight line with a slope equal to n. K is the value of S where

$$\log \frac{v}{V_{max} - v} = 0$$

Figure 8 shows the plot of Log $v/V_{max} - v$ *vs.* Log [activator concentration] for some of the data of Figure 6 and 7. It also lists the n values for a number of activators as well as their respective K values. The data suggests that there are at least two binding sites for the activators.

Hartman and Barker (1965) have shown that certain isosteric analogs of fructose 1,6-diP, competitively inhibited rabbit muscle aldolase. These compounds were also able to activate the *E. coli* ADP-glucose pyrophosphorylase. Figure 6 shows the activation caused by D-arabinitol 1,5-diP. Other analogs isosteric with fructose 1,6-diP and activating the *E. coli* ADP-glucose pyrophosphorylase as effectively as D-arabinitol 1,5-diP were L-arabinitol 1,5-diP, 1,5-pentanediol diP, D-glucitol 1,6-diP, and xylitol 1,5-diP.

The activator sites appear to be nonspecific since compounds dissimilar to fructose 1,6-diP, such as 2-P-glycerate and phosphoenolpyruvate, are also capable of serving as activators. It was of interest to know if activators like 2-phosphoglycerate were bound to the same sites as fructose 1,6-diP. Figure 9 shows that 2-phosphoglycerate enhanced the activation caused by unsaturating concentrations of fructose diphosphate. However, 2-phosphoglycerate inhibited slightly the activation caused by higher concentrations of fructose 1,6-diP. These results suggest that 2-phosphoglycerate binds to the same sites as does fructose diP. At low concentrations 2-phosphoglycerate would facilitate the binding of fructose 1,6-diP to these sites. At higher concentrations of fructose diP, 2-phosphoglycerate would act as a competitor for the activator sites since it is inferior to fructose diP as an activator. In addition, 2-phosphoglycerate affects n, the interaction coefficient of fructose diphosphate. Similar results were obtained with 3-phosphoglyceraldehyde and phosphoenolpyruvate. Thus, it is believed that the various activators of

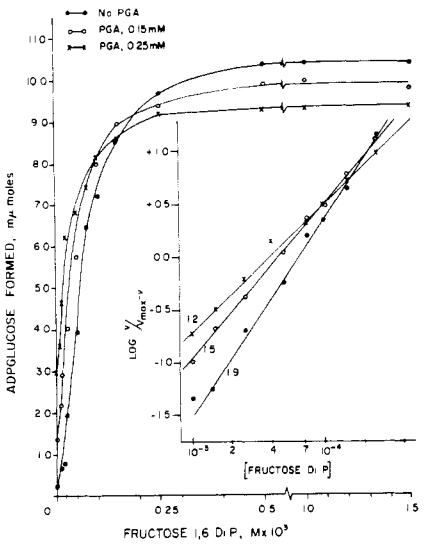

FIGURE 9: The effect of 2-phosphoglycerate on the activation of ADP-glucose synthesis by fructose diphosphate. The incubation mixtures are those of assay B1. The amounts of fructose diphosphate and 2-phosphoglycerate are indicated in the figure.

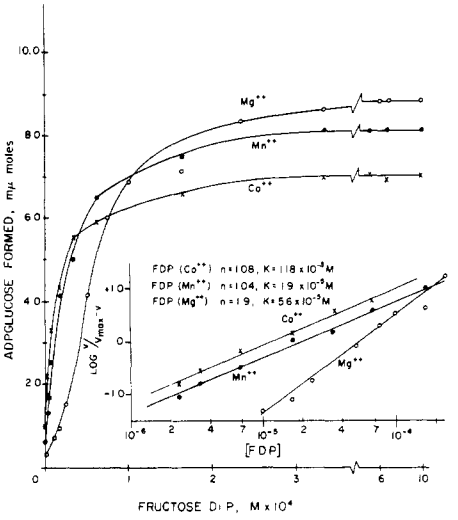

FIGURE 10: The effect of metal ions on the activation of ADP-glucose synthesis by fructose 1,6-diP. The incubation mixtures are essentially those of assay B1. The concentrations of $MnCl_2$, $CoCl_2$, and $MgCl_2$ were 1.25, 1.5, and 5.0 mM, respectively. The reaction mixtures which had Co^{2+} as the cation contained three times more enzyme than the reaction mixtures containing Mn^{2+} and Mg^{2+}.

TABLE VI: The Concentration of Various Activators in Reaction Mixtures Synthesizing ADP-Glucose.[a]

Activator	Incubation Time (min)	ADP-Glucose Formed (μmoles)	Amount of Activator (μmoles)
Fructose 1,6-diphosphate	0	0.00	0.26
	15	0.17	0.27
	30	0.30	0.26
Phosphoenolpyruvate	0	0.00	1.28
	10	0.10	1.28
	30	0.26	1.28
DL-3-phosphoglyceraldehyde	0	0.00	1.17
	10	0.103	1.17
	30	0.285	1.15

[a] The reaction mixtures contained 80 μmoles of Tris-Cl buffer pH 8.0, 0.80 μmole of glucose-^{14}C 1-P, 1.6 μmoles of ATP, 8.0 μmoles of $MgCl_2$, 0.80 mg of bovine plasma albumin, 1.6 μg of inorganic pyrophosphatase, activator in the amount indicated in the table, and enzyme in a volume of 1.6 ml. Fructose diphosphate was analyzed by coupling it to DPNH oxidation with aldolase, triose phosphate isomerase, and dihydroxyacetone phosphate dehydrogenase. Phosphoenolpyruvate was measured with DPNH, ADP, pyruvokinase, and lactate dehydrogenase. DL-3-phosphoglyceraldehyde was measured with DPN, arsenate, and triose phosphate dehydrogenase. The value obtained from this measurement was multiplied by two to give the total amount of the D plus L forms. ADP-glucose was measured as described in assay B.

the enzyme bind to similar sites. The addition of saturating concentrations of either sedoheptulose diphosphate or arabinitol 1,5-diP to reaction mixtures containing saturating amounts of fructose diP did not cause any further activation of ADP-glucose synthesis. These results would be consistent with the concept that these activators are bound to the enzyme at common sites.

EFFECT OF METALS ON ACTIVATION OF ADP-GLUCOSE SYNTHESIS BY FRUCTOSE DIPHOSPHATE. Figure 10 shows the effect of fructose diphosphate concentrations on ADP-glucose synthesis in the presence of the metal ions Mn^{2+}, Co^{2+}, and Mg^{2+}. There appears to be little or no interaction between the fructose diphosphate sites when Mn^{2+} or Co^{2+} are used as the metallic cofactors. In the presence of Mg^{2+}, however, there appears to be interaction between the fructose diphosphate sites. The K value of the Hill equation (or concentration of activator where 50% of maximal activation occurs) for fructose diphosphate, however, is lower when Mn^{2+} or Co^{2+} are used as the divalent metals than when Mg^{2+} is the divalent metal.

ACTIVATOR CONCENTRATION DURING ADP-GLUCOSE SYNTHESIS. Table VI shows that the concentrations of the activators fructose diP, phosphoglyceraldehyde, and phosphoenolpyruvate remained constant during the formation of ADP-glucose. Thus the activators are not consumed during the reaction and they appear to be directly stimulating ADP-glucose pyrophosphorylase activity.

EFFECT OF FRUCTOSE 1,6-DIP ON K_m AND V_{max}. Figures 11–14 show the effect of substrate concentrations on the kinetics of synthesis and pyrophosphorolysis. Both

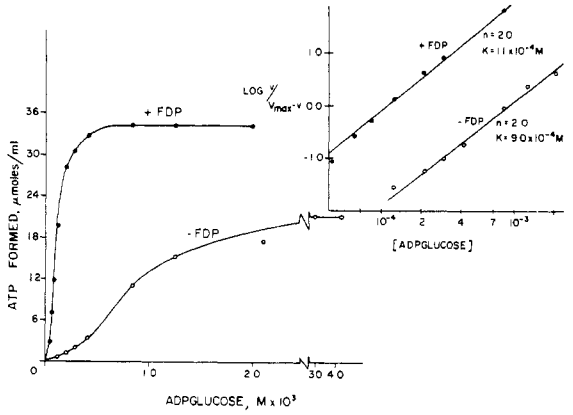

FIGURE 11: Effect of ADP-glucose concentrations on enzyme activity. The reaction mixtures of the experiment were those of assay A1 (+fructose diphosphate, FDP) and of assay A2 (−fructose diphosphate).

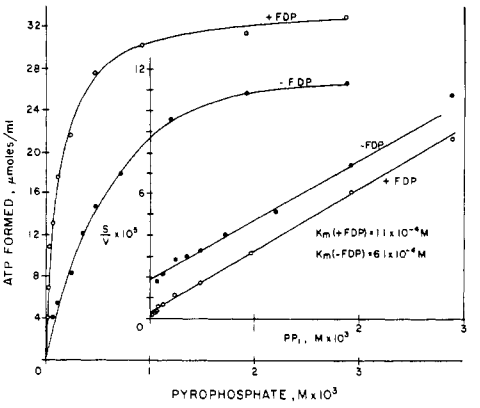

FIGURE 12: Effect of pyrophosphate concentration on enzyme activity. The reaction mixtures of the experiment were those of assay A1 (+ fructose diphosphate) and of assay A2 (− fructose diphosphate).

glucose 1-P and pyrophosphate follow first-order (Michaelis–Menten) kinetics. However, the ATP and ADP- glucose concentration *vs.* rate curves were sigmoidal, indicating higher than first-order kinetics. The data from these figures are summarized in Table VII. The V_{max} of pyrophosphorolysis and of synthesis

TABLE VII: Kinetic Constants of *E. coli* B ADP-Glucose Pyrophosphorylase.

Substrate	Fructose diP	K_m (mM)	K (mM)	V_{max} (μmole mg^{-1} min^{-1})	n
ADP-glucose	−		0.90	1.87	2.15
	+		0.11	2.8	2.0
PP_i	−	0.61		2.3	1.01
	+	0.11		2.8	1.06
ATP	−		3.3	0.32	1.7
	+		0.26	2.1	1.7
Glucose 1-P	−	0.33		0.30	0.94
	+	0.029		2.1	0.96
Mg^{2+} Pyrophosphorolysis	−		6.0		3.4–3.9
	+		3.0		3.7–3.9
Mg^{2+} Synthesis	−		11.5		3.1–3.9
	+		2.0		3.7–4.4

of ADP-glucose were increased *ca.* 1.5- and 7-fold, respectively, by fructose diP. The apparent K_m of glucose 1-P was decreased 11-fold and the apparent K_m of PP_i was decreased sixfold in the presence of fructose diP. The K values of ATP and ADP-glucose were decreased 13- and 8-fold, respectively, in the presence of

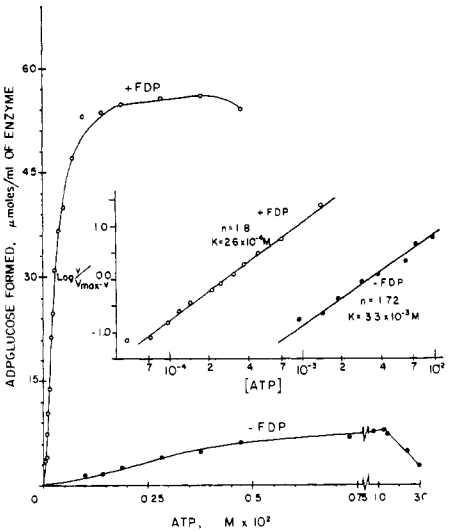

FIGURE 13: Effect of ATP concentration on enzyme activity. The reaction mixtures of the experiment were those of assay B1 (+ fructose diphosphate) and of assay B2 (− fructose diphosphate).

fructose diP. The K values for Mg^{2+} were also decreased by the presence of fructose 1,6-diP. It is interesting to note that n, the apparent order of reaction for the substrates, was not altered by the presence of the activator, fructose diP.

THE ACTIVATION OF ADP-GLUCOSE PYROPHOSPHORYLASE FROM OTHER BACTERIAL EXTRACTS. It had already been reported that the ADP-glucose pyrophosphorylase of *A. viscosus* NRRL B1973 was activated by fructose 6-P, pyruvate, or ribose 5-P. Table VIII shows that the ADP-glucose pyrophosphorylases present in other bacterial extracts were also activated by glycolytic intermediates. The enzyme from *Aerobacter aerogenes* was activated by the same intermediates that affect the *E. coli* enzyme, namely, fructose diP,

TABLE VIII: The Activation of ADP-Glucose Synthesis in Enzyme Preparations Isolated from Various Bacteria.[a]

Enzyme Source	ADP-Glucose Formed (mμmoles)						
	None	FDP	PEP	3PG[b] Aldehyde	F 6-P	Pyr	R 5-P
A. aerogenes	0.82	11.9	11.5	10.7	0.90	0.81	0.80
A. viscosus	4.0	3.0	1.6	2.0	32.5	21	12.5
A. tumefaciens	6.0	5.4	1.3	...	35.7	32.7	31.1
R. rubrum	0.69	0.67	0.59	...	0.65	3.52	0.66

[a] The reaction mixture used to assay ADP-glucose synthesis with the *A. aerogenes* enzyme was the same as described for assay B1 in the text. The reaction mixture used to assay the *A. viscosus* and *A. tumefaciens* enzymes contained 10 μmoles of Tris–Cl, pH 8.3, 2.0 μmoles of $MgCl_2$, 0.1 μmole of glucose-^{14}C 1-P, 0.2 μmole of ATP, 0.9 μg of inorganic pyrophosphatase, and enzyme in a final volume of 0.2 ml. The incubation mixture used to assay the *R. rubrum* enzyme contained 20 μmoles of Tris–Cl pH 8.5, 2.0 μmoles of $MgCl_2$, 0.1 μmole of ATP, 0.1 μmole of glucose-^{14}C 1-P, 50 μg of bovine plasma albumin, 0.9 μg of inorganic pyrophosphatase, and enzyme in a final volume of 0.2 ml. All reaction mixtures were incubated at 37° for 10 min. The *A. aerogenes* was purified in the same manner as the *E. coli* enzyme except that step IV was omitted. The enzyme was purified 12-fold by this procedure and 12 μg of the purified fraction was added to the reaction mixtures in the above experiment. The purification of the *A. viscosus* enzyme has been described (Shen and Preiss, 1965); 0.1 μg of the purified enzyme was used. The preparation of the 100,000*g* supernatant fluid from *A. tumefaciens* has been described previously (Greenberg and Preiss, 1964). The amount of protein added to each reaction mixture was 75 μg. The *R. rubrum* enzyme was purified 70-fold (C. Furlong and J. Preiss, unpublished data) and 0.5 μg of protein was added to each reaction mixture. [b] 3PG, 3-phosphoglyceraldehyde; F, fructose; Pyr, pyruvate; R, ribose.

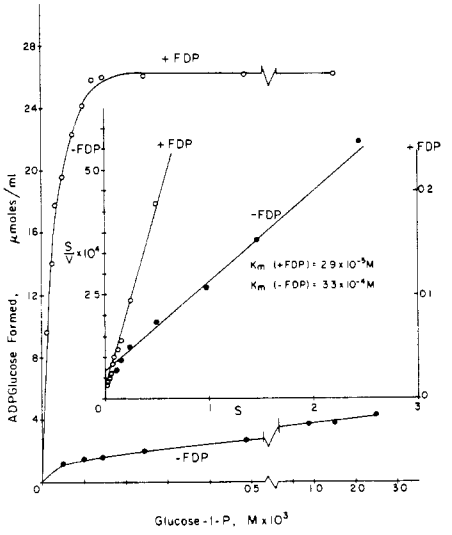

FIGURE 14: Effect of glucose 1-P concentration on enzyme activity. The reaction mixtures of the experiment were those of assay B1 (+ fructose diphosphate) and of assay B2 (− fructose diphosphate). *S*, equals molarity of glucose-^{14}C 1-P × 10^3.

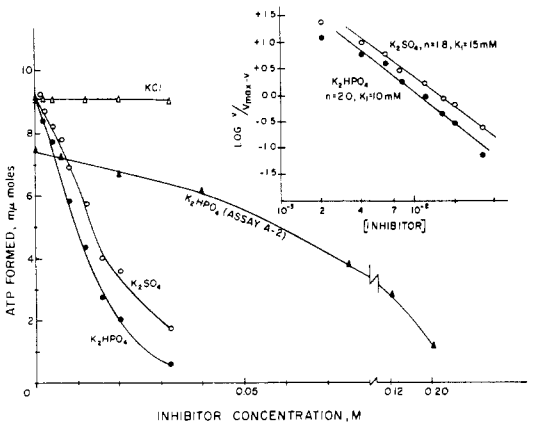

FIGURE 15: Inhibition of ADP-glucose pyrophosphorolysis by anions. The reaction mixtures of the experiment were those of assay A1 and 2.

phosphoenolpyruvate, and glyceraldehyde 3-P. The *Agrobacterium tumefaciens* enzyme was activated by fructose 6-P, pyruvate, and ribose 5-P and, therefore, similar to the *A. viscosus* enzyme. The ADP-glucose pyrophosphorylase from *Rhodospirillum rubrum*, however, was found to be activated only by pyruvate and no other glycolytic metabolite.

Inhibition of ADP-Glucose Pyrophosphorylase. INHIBITION BY PHOSPHATE AND SULFATE. Figure 15 shows that inorganic phosphate inhibited the pyrophosphorolysis of ADP-glucose. KCl at the same concentration or at the same ionic strength did not inhibit pyrophosphorylase activity. Sulfate, however, appeared to be as effective as phosphate as an inhibitor. The phosphate inhibition curve appeared to be sigmoid shaped. Plotting the data according to the Hill equation (Taketa

and Pogell, 1965)

$$\log \frac{V_{max} - v}{v} = n \log (\text{inhibitor}) - \log K_i$$

gave interaction coefficients of 2.0 for phosphate and 1.8 for sulfate. The K_i for sulfate and phosphate were 15 and 10 mM, respectively. In the absence of the activator, fructose diP (assay A2), the enzyme was relatively insensitive to phosphate inhibition.

INHIBITION BY ADENYLATE AND ADENOSINE DIP. ADP-glucose pyrophosphorylase activity was also inhibited by 5′-AMP and ADP (Figure 16). AMP was the more effective inhibitor; its K_i was 8.4×10^{-5} M. The K_i for ADP was 1.1×10^{-3} M. The inhibitor curves for AMP and ADP were both sigmoid shaped. In the absence of the activator, fructose diP (assay B2), the enzyme was relatively insensitive to AMP inhibition. Under the conditions of assay B1, other 5′-mononucleotides at a concentration of 10^{-3} M gave the following per cent of inhibition; IMP, 20%; GMP, 50%; UMP, 10%; CMP 50%. At 2.5×10^{-4} M, these 5′-mononucleotides inhibited ADP-glucose synthesis <15%. AMP at 2.5×10^{-4} M inhibited ADP-glucose synthesis >90%.

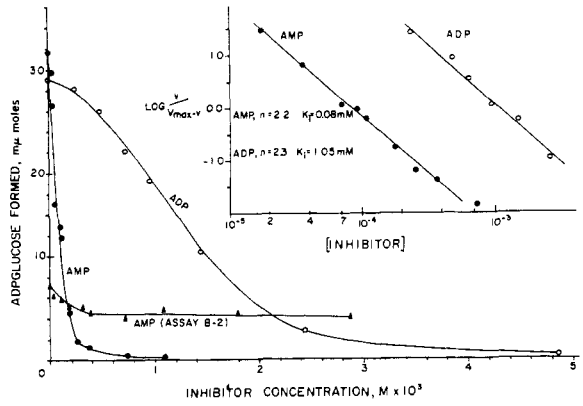

FIGURE 16: Inhibition of ADP-glucose synthesis by 5′-adenylate and adenosine diphosphate. The reaction mixtures of the experiment were those of assay B1 and 2 except that inorganic pyrophosphatase was omitted.

Discussion

The above data indicate that the synthesis of ADP-glucose in *E. coli* occurs by condensation of glucose 1-P and ATP with the concomitant formation of inorganic pyrophosphate. The reaction is catalyzed by a specific enzyme which is distinct from the UDP-glucose and TDP-glucose pyrophosphorylases present in crude extracts of *E. coli* (Bernstein and Robbins, 1965). The most purified fraction of ADP-glucose pyrophosphorylase, however, was able to catalyze to a small extent the synthesis of UDP-glucose, GDP-glucose, IDP-glucose, CDP-glucose, and dADP-glucose. Whether the syntheses of these sugar nucleotides are catalyzed specifically by ADP-glucose pyrophosphorylase is not known. A divalent metal is required for both the synthesis and pyrophosphorolysis of ADP-glucose. Mn^{2+}, Mg^{2+}, or Co^{2+} can fulfill this requirement for the *E. coli* enzyme. At low concentrations Mn^{2+} is more effective than Mg^{2+} in catalyzing ADP-glucose synthesis. Manganese, however, is also a potent inhibitor of ADP-glucose synthesis at higher concentrations and is not as effective as Mg^{2+} or Co^{2+} in the pyrophosphorolysis of ADP-glucose. The differences in the relative activities of these metals in pyrophosphorolysis and synthesis may be a reflection in the binding affinities of the nucleotides, ATP and ADP-glucose for these metals.

Of special interest is the ability of various glycolytic intermediates and their analogues to activate the *E. coli* ADP-glucose pyrophosphorylase. The activation appears to be independent with respect to what metal ion is used to carry out the reaction. There also appears to be a lack of specificity for the activator since diverse compounds like fructose diphosphate and 2-phosphoglycerate are active. The tetracyclohexylammonium salt of fructose diphosphate is just as active as the sodium salt. It is not known whether all activators are bound to the same site. However, the data of Figure 9 suggest that 2-phosphoglycerate and fructose diphosphate share common binding sites.

A number of compounds that were found to activate the *E. coli* enzyme can be classified into one group. Fructose diphosphate, sedoheptulose diphosphate, 3-phosphoglyceraldehyde, and erythrose 4-phosphate are all known to be substrates of aldolase. L- and D-arabinitol 1,5-diphosphate, 1,5-pentanediol diphosphate, D-glucitol 1,6-diphosphate, and xylitol 1,5-diphosphate were shown by Hartman and Barker (1965) to be competitive inhibitors of rabbit muscle aldolase. All these compounds are effective activators of the *E. coli* ADP-glucose pyrophosphorylase and it is tempting to speculate that there are certain similarities between the substrate site of rabbit muscle aldolase and the activator sites of the *E. coli* ADP-glucose pyrophosphorylase. Inconsistent with this view is the fact that dihydroxyacetone phosphate, a substrate of aldolase, does not activate ADP-glucose synthesis.

The activator concentration *vs.* rate curves are sigmoid shaped when Mg^{2+} is the metal ion used, indicating that the activator sites are interacting. However, when Co^{2+} and Mn^{2+} are used in place of Mg^{2+}, the activator concentration *vs.* rate curve becomes hyperbolic, suggesting that the activator sites are not interacting. Effective activation of ADP-glucose synthesis by fructose diphosphate still occurs under these conditions. It should be noted that the kinetic data for the *E. coli* enzyme appears to follow the Hill equation over the initial velocity range investigated for the substrates, ATP and ADP-glucose and the various activators. The data show that the activator, fructose diphosphate, does not change n, the interaction coefficient of the various substrates. It is not known what the relationship is between n and the actual number of binding

sites of the various activators and substrates of the *E. coli* enzyme. Since sigmoid-shaped rate *vs.* concentration curves are obtained with ATP and ADP-glucose, indicating that more than one molecule of these substrates is being bound to the enzyme, one may consider ATP or ADP-glucose as activators as well as substrates of the *E. coli* enzyme.

At the present time there is insufficient data to postulate any working model for the *E. coli* ADP-glucose pyrophosphorylase. In recent years a number of models have been postulated to explain the effect of inhibitors and activators which presumably affect enzymatic activity by binding at sites on the enzyme other than the substrate sites (Monod *et al.*, 1965; Atkinson *et al.*, 1965; Koshland *et al.*, 1966). At present however, no attempt will be made to use the data presented in this paper to correlate the *E. coli* ADP-glucose pyrophosphorylase with any of these models for "allosteric" or regulatory proteins. Nevertheless it should be pointed out that in the model proposed by Monod *et al.* (1965) it is postulated that n, the interaction coefficient of the substrates, should change in the presence of the effector molecule (activator or inhibitor). This appears not to be the case for the *E. coli* ADP-glucose pyrophosphorylase even though the activator, fructose diphosphate, decreases the substrate concentrations for half-maximal velocity *ca.* 10-fold.

The most important implications of these data pertain to the physiological function of this enzyme in the *E. coli* cell. Since ADP-glucose appears to be the sole glycosyl precursor for glycogen synthesis in bacteria, the effect of various metabolites like P_i, ATP, AMP, ADP, FDP, and any other glycolytic intermediates may be of utmost physiological significance. Under certain conditions (low pH, nitrogen or phosphate deficiency) glycogen may accumulate >20% of the bacterial cell dry weight (Zevenhuizen, 1964; Sigal *et al.*, 1964). Thus under conditions where cell growth is limited, the accumulation of glycogen may occur. It is thought that glycogen accumulation is a result of excess ATP production occurring in situations where the cell does not require (or requires to a lesser extent) formation of new macromolecules such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein. Where there is an excess of carbon source, it is presumed that the rate of formation of ATP is greater than its rate of utilization under these conditions. Glycolytic intermediates such as fructose diphosphate may also accumulate in this situation. These conditions would be optimal for ADP-glucose synthesis from ATP and glucose 1-P since the ADP-glucose pyrophosphorylase would be activated by ATP as well as by glycolytic intermediates.

Since the rate of formation of ADP-glucose appears to be the rate-limiting step in glycogen synthesis in bacteria (Greenberg and Preiss, 1964), an increase in the rate of ADP-glucose formation would thus lead to an increase in the amount of glycogen formed. In periods where ATP is being utilized faster than its synthesis, the concentrations of ADP, AMP, and P_i would increase relative to the ATP concentration. The higher concentrations of ADP, AMP, and P_i would inhibit the pyrophosphorylase and, therefore, ADP-glucose and glycogen synthesis. Since the enzyme is more sensitive to adenylate, it is thought to be the more important inhibitor. Glycogen synthesis may then be thought of being regulated in part by the ratio of the cellular levels of ATP/AMP.

The role of fructose diphosphate and other glycolytic intermediates in activating ADP-glucose synthesis is somewhat unclear. It may be visualized that under conditions of ATP excess and a carbon-source excess, the glycolytic intermediates would accumulate and act as precursors of glucose 1-P as well as activators of the pyrophosphorylase and thereby increase the rate of ADP-glucose synthesis. However, preliminary evidence indicates that fructose diphosphate may render the ADP-glucose pyrophosphorylase more sensitive (Figure 16) to AMP and phosphate inhibition. Since the concentrations of the various components in the reaction mixtures containing fructose diphosphate are different when compared to those containing no fructose diphosphate, additional studies would be required to determine the interactions between the inhibitors, fructose diphosphate, and the various substrates. It would also be of interest to examine the various concentrations of glycolytic intermediates, adenine nucleotides, and P_i in the cell under conditions where glycogen is accumulating as compared to situations where little or no glycogen accumulation is occurring. Data of this nature at present is not available and would be required to substantiate the above concepts.

Thus it appears that the regulation of glycogen synthesis at the enzyme level in bacteria is different than that found in mammalian systems and in yeast (Leloir *et al.*, 1959; Kornfeld and Brown, 1962; Robbins *et al.*, 1959; Villar-Palasi and Larner, 1958; Algranati and Cabib, 1962). Whereas activation of mammalian and yeast glycogen synthesis occurs at the transglucosylase level, it seems that bacterial glycogen synthesis is regulated at the level of ADP-glucose synthesis (Shen and Preiss, 1964; Preiss *et al.*, 1965). It may be pointed out that UDP-glucose in the mammalian cell has many functions. It is utilized for synthesis of galactose and glucuronic acid which in turn are transformed into other cellular substituents. It would be advantageous to the cell then to regulate glycogen synthesis not at the level of UDP-glucose synthesis but at the glycogen synthetase level. With respect to bacteria, where ADP-glucose is the glycosyl donor for α-1,4-glucan synthesis and perhaps has no other physiological function, it would then be advantageous for the bacterial cell to regulate synthesis of the α-1,4-glucan at the level of ADP-glucose synthesis. Table VII indicates that the activation of the bacterial ADP-glucose pyrophosphorylases is found in a number of bacterial species. It is not apparent why the activators for the various bacterial ADP-glucose pyrophosphorylases are different. It is possible they reflect different patterns of carbohydrate metabolism in these organisms.

The *E. coli* ADP-glucose pyrophosphorylase belongs to the group of enzymes that are effected by adenine

nucleotides. The enzyme phosphofructokinase (Mansour, 1963; Passonneau and Lowry, 1962; Ramaiah *et al.*, 1963; Atkinson and Walton, 1965) is inhibited by ATP and this inhibition is overcome by 5′-AMP (or ADP). The DPN-specific isocitrate dehydrogenase requires AMP or ADP for activity (Hathaway and Atkinson, 1963; Chen and Plaut, 1963; Sanwal *et al.*, 1964; Sanwal and Stachow, 1965). Phosphofructokinase and isocitrate dehydrogenase are both involved in the process of carbohydrate utilization to form ATP. Fructose diphosphate diphosphatase, an enzyme important in glucogenesis is, however, inhibited by AMP (Taketa and Pogell, 1965; Krebs *et al.*, 1964; Salas *et al.*, 1964). The finding that ADP-glucose pyrophosphorylase, an enzyme involved in the formation of glycogen, an energy reserve polysaccharide, is inhibited by AMP or ADP and stimulated by ATP is in accord with the effect of the adenine nucleotides on the previously mentioned enzymes.

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