

CHAPTER IV.

PURIFICATION AND PROPERTIES OF ADP-GLUCOSE :  
GLUCOHYDROLASE FROM MYCOBACTERIUM PHLEI.

## Introduction

Many gram negative bacteria and a few gram positive bacteria are known to possess enzymes on their surface which catalyse the hydrolysis of sugar nucleotides. These enzyme proteins are considered to be loosely attached to the cell surface (Ward and Glaser, 1968, 1969) between the cell wall and cell membrane. From yeast Glaser et al. (1967) have reported the purification and properties of UDP-glucose hydrolase, which liberates uridine, inorganic phosphate and glucose-1-phosphate from the UDP-sugar. UDP-sugar hydrolase has been known to participate in the regulation of UDP-sugar pool in E. coli (Glaser et al., 1967 and Ward and Glaser, 1968). The enzyme which can hydrolase ADP-glucose to 5'-AMP and alpha-D-glucose-1-phosphate has also been reported in E. coli (Glaser and Mauck, 1972). Bacillus subtilis W-23 is known to contain nucleotide diphosphate sugar hydrolase similar to E. coli enzyme, but with a much broader substrate specificity, when grown under conditions of phosphate limitation (Mauck and Glaser, 1970).

Sonnino et al. (1966, 1966 a) have reported the presence of GDP-glucose : glucosylhydrolase which hydrolases GDP-glucose into GDP and glucose. This is the only report on the existence of an enzyme involved in the direct release of sugar

from the sugar nucleotide (GDP-glucose) in microorganism.

Purification and properties of GDP-glucose : glucohydrolase have been reported (Cabib et al., 1972).

It has been mentioned in Chapter II that in the course of studies on the synthesis of glycogen from ADPG, ADP-glucose : glucohydrolase activity has been observed in the crude cell-free extracts of Mycobacterium smegmatis, M. phlei and M. tuberculosis H<sub>37</sub>Rv. In order to understand the exact role and regulation of this new enzyme, it is necessary to study the properties of the purified enzyme and hence the present work on purification has been undertaken.

## Experimental

### Purification of ADP-glucose : glucohydrolase.

Since the activity of this enzyme is higher in M. phlei than in other species of mycobacteria studied ADPglucose : glucohydrolase has been purified from M. phlei. M. phlei cells were grown as described in chapter III and used for the purification. All the steps of purification are carried out at 0-5°C unless otherwise mentioned.

### Preparation of cell-free extract.

For the preparation of cell-free extract, 30 per cent (w/v) suspension of cells was made in 0.05 M Tris-HCl buffer (pH 7.5) and subjected to sonication as described in the previous chapter. The sonicated extract was centrifuged at 20,000 x g for 20 minutes in a refrigerated centrifuge. The preliminary experiments showed that the enzyme is present in the supernatant fraction. Therefore, 20,000 x g supernatant of the sonicated extract was further spun at 105,000 x g for 1 hr in a Beckman ultracentrifuge Model L2 65B.

The supernatant obtained in the above step was used for the purification of the enzyme.

### Ammonium sulphate fractionation.

Preliminary experiments suggested that the enzyme was precipitated between 50-90 per cent saturation with ammonium sulphate.

To 110 ml of 105,000 x g supernatant fraction equal amount of cold saturated ammonium sulphate solution previously neutralised to pH 7.5 was added slowly in small volumes with continuous stirring using a magnetic stirrer. After 15 minutes the solution was centrifuged in the cold at 10,000 x g for 15 minutes. The precipitate obtained was discarded. To the supernatant 67 g of finely powdered ammonium sulphate was added in small amounts and the solution was continuously stirred while adding solid ammonium sulphate. After 15 minutes, the solution was centrifuged and the supernatant was discarded. The precipitate was dissolved in a minimum amount of 0.05 M Tris-HCl buffer (pH 7.5) and dialyzed overnight against the same buffer with several changes, till the last traces of ammonium sulphate were removed. The activity of the enzyme was determined in the protein fraction thus obtained. At this step it was found that the purification was only about 2 fold.

### DEAE Sephadex A 50 chromatography.

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DEAE <sup>S</sup> sephadex A-50 chromatography was tried for further purification of the enzyme. The DEAE <sup>S</sup> sephadex ion exchanger

was suspended in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.05 M KCl, and washed several times with the same buffer <sup>until</sup> till the pH of the washings was the same as that of the buffer used. A suspension was made and packed into in a column (45 x 2.2 cms) and equilibrated with the same buffer overnight. Enzyme protein from step 2 was loaded onto the DEAE-sephadex column. The enzyme protein was eluted using a linear concentration gradient of potassium chloride from 0.05 M to 1.0 M, in 0.05 M Tris-HCl buffer (pH 7.5) by adjusting the flow rate at 16 ml/hr. Eight ml fractions were collected and protein and enzyme activity were determined in each fraction. The enzyme activity appeared as a single peak as shown in Fig. 1. The fractions containing the enzyme activity were pooled. For concentrating the enzyme the combined fraction was precipitated by the addition of ammonium sulphate (90 per cent saturation). The precipitate thus obtained was dissolved in a minimum volume of 0.05 M Tris-HCl (pH 7.5) and dialyzed overnight with several changes of the <sup>same</sup> buffer. There was a considerable increase in the specific activity of the enzyme and the purification was found to be 37-fold over the crude enzyme.

#### Sephadex gel filtration.

Further purification was attempted by sephadex G-200 gel filtration. Swelling of the sephadex G-200 gel was

FIGURE 1.

DEAE SEPHADEX A-50 CHROMATOGRAPHY OF ADP-GLUCOSE :  
GLUCOHYDROLASE.

The elution was carried out using 300 ml of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.05 M KCl, in a linear gradient of KCl from 0.05 M to 1.0 M. The flow rate was 16 ml per hour and 8 ml fractions were collected.

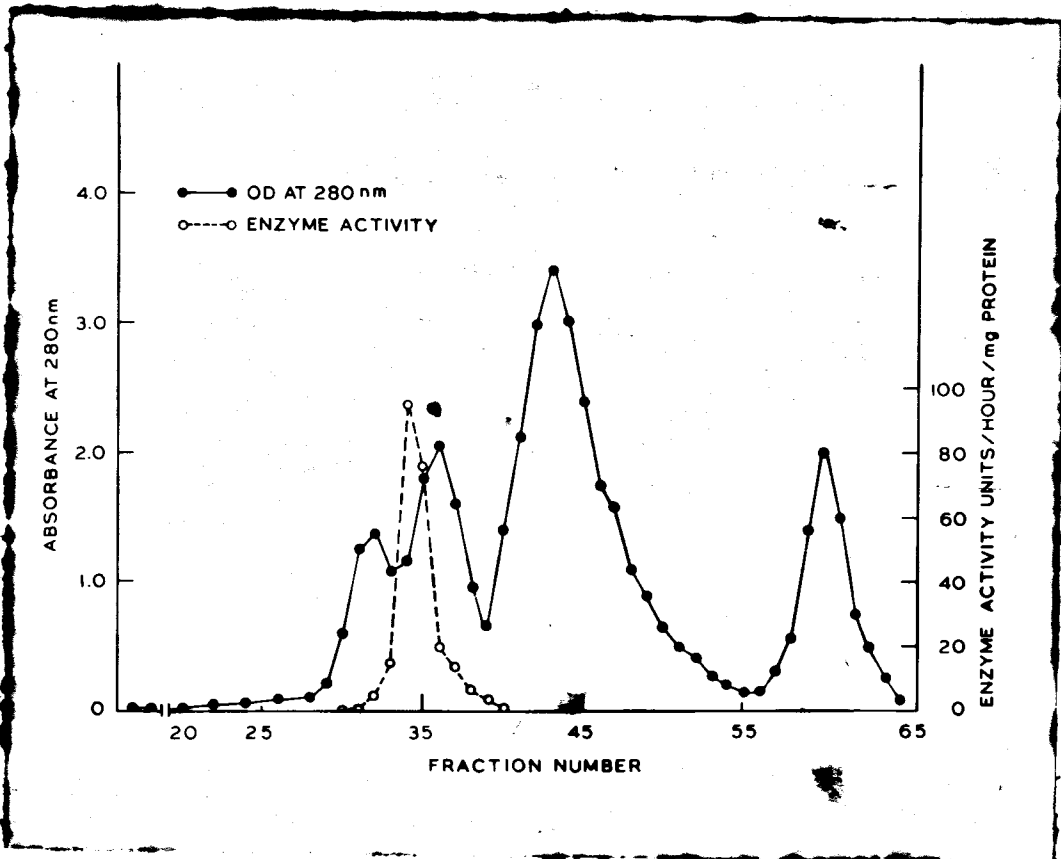


FIGURE 2.

SEPHADEX G-200 GEL FILTRATION.

The elution was carried out using 0.05 M Tris-HCl buffer (pH 7.5). The flow rate was adjusted at 6.0 ml per hour and 3.0 ml fractions were collected.

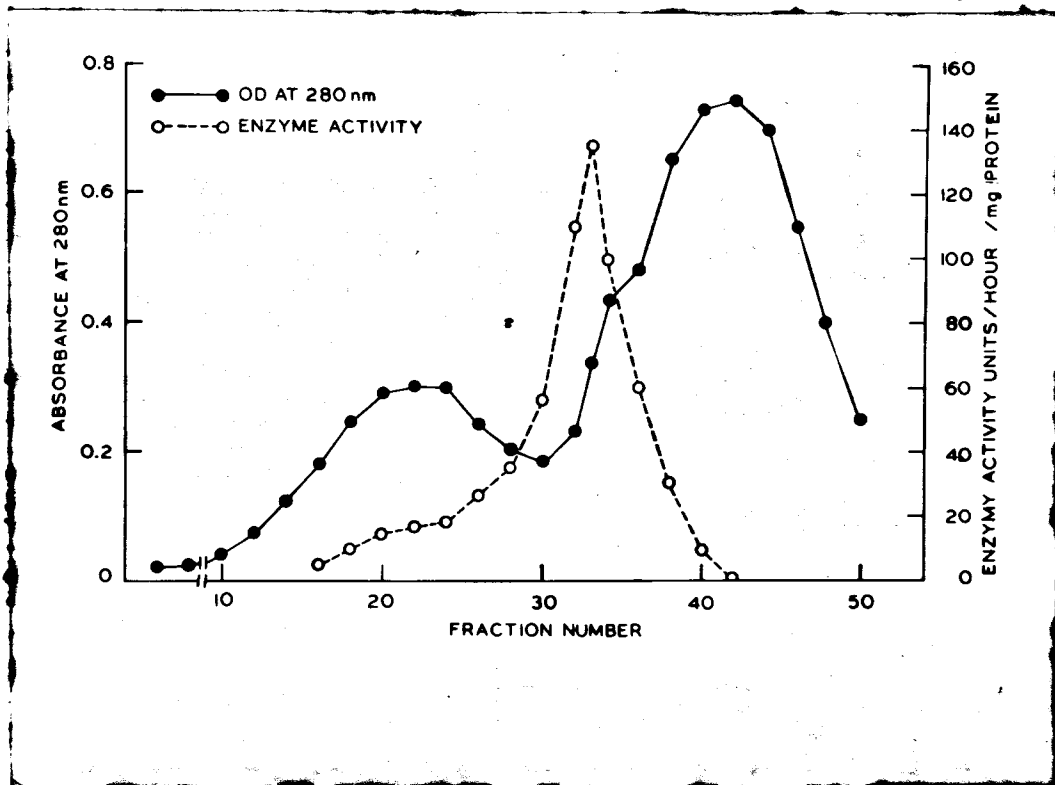


Table 1.

PURIFICATION OF ADP-GLUCOSE : GLUCOHYDROLASE FROM M. PHLEI.

The enzyme activity was measured by assay A described in chapter II.

Steps	Volume (ml)	Total protein (mg)	Specific activity units/hour/mg protein	Total activity	Fold purification	Percentage recovery
Crude extract	130	1630.0	0.9	1467	-	100
50-90% ammonium sulphate fraction	31	491.0	2.0	982	2	67
DEAE-sephadex A-50 fraction	12	4.8	37.0	178	41	12
Sephadex G-200 fraction	16	1.8	67.0	121	74	8

carried out in 0.05 M Tris-HCl buffer (pH 7.5) as described in chapter III. The gel thus prepared was packed into a column (43 x 1.5 cms) and equilibrated overnight with the same buffer. Void volume was determined as described in the previous chapter by adjusting the flow rate at 6 ml/hour.

The fraction obtained from the DEAE-sephadex column was then layered on the sephadex G-200 column and eluted with 0.05 M Tris-HCl buffer (pH 7.5). The bed height <sup>and</sup> on the flow rate <sup>were</sup> ~~was~~ same as used for determining the void volume. 3.0 ml fractions were collected and protein concentration and enzyme activity determined in each fraction. It was observed that the enzyme fraction was not eluted in the void volume indicating that the enzyme protein is of low molecular weight, i.e. below 200,000. The fractions containing the enzyme activity were pooled (together) and used for studying the properties. The purification obtained was about 74 fold over the crude enzyme. Summary of the purification procedure used is given in Table 1.

The estimation of activity of ADP-glucose glucohydrolase was carried out according to the assay A given in chapter II unless otherwise mentioned. Protein was determined according to the method of Warburg and Christian (1941).

Results and Discussion

Properties of ADP-glucose : glucohydrolase.

Identification of the products of reaction.

With the partially purified enzyme preparation, the products of the reaction were identified as ADP and glucose by using two different methods of assay. In assay A described in chapter II, the activity was determined by converting ADP formed from ADPG into ATP in presence of a specific enzyme, pyruvate kinase and phosphoenol pyruvate. This establishes that one of the products formed is ADP.

For the identification of glucose as the other product of reaction, assay B described in chapter II was used. In this procedure ADP-(glucose-<sup>14</sup>C) was used as the substrate. <sup>14</sup>C-glucose formed was identified after separation by paper chromatography.

Effect of pH.

Two different buffers were employed for the determination of optimum pH for the enzyme activity. The results shown in Fig. 3 reveal that optimum pH for the activity of this enzyme is around 10.0. It may be pointed out here that there are enzymes in mycobacteria exhibiting high alkaline pH optimum.

FIGURE 3.

EFFECT OF pH ON THE ACTIVITY OF ADP-GLUCOSE :  
GLUCOHYDROLASE.

The assay mixture in a final volume of 0.5 ml contained, 10  $\mu$  moles of Tris-HCl buffer (●—●) or glycine-NaOH buffer (○—○), 0.75  $\mu$  mole of ADP-glucose, 2.0  $\mu$  moles of  $MgCl_2$ , 1.0  $\mu$  mole of phosphoenol pyruvate; 10  $\mu$ g of pyruvate kinase and 8  $\mu$ g of enzyme protein. Other experimental condition were as described assay A, given in the section on 'Materials and Methods' in Chapter II.

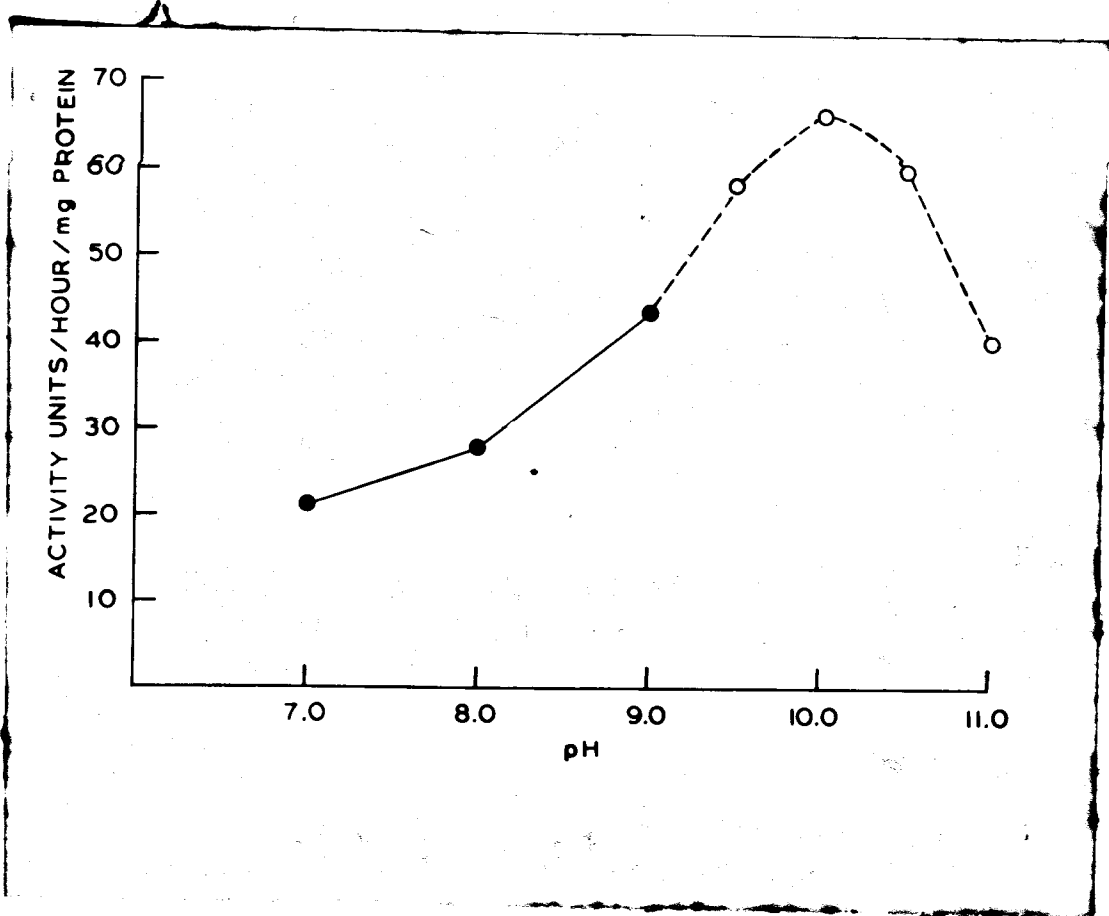


FIGURE 4.

EFFECT OF PROTEIN CONCENTRATION.

The reaction mixture in a final volume of 0.5 ml contained 10  $\mu$  moles of glycine NaOH buffer (pH 10.0); 0.75  $\mu$  mole of ADP-glucose; 2.0  $\mu$  moles of  $MgCl_2$ ; 1.0  $\mu$  mole of PEP; 10  $\mu$ g of pyruvate kinase and varying amounts of enzyme protein. Other experimental conditions were as described in Chapter II for the enzyme assay.

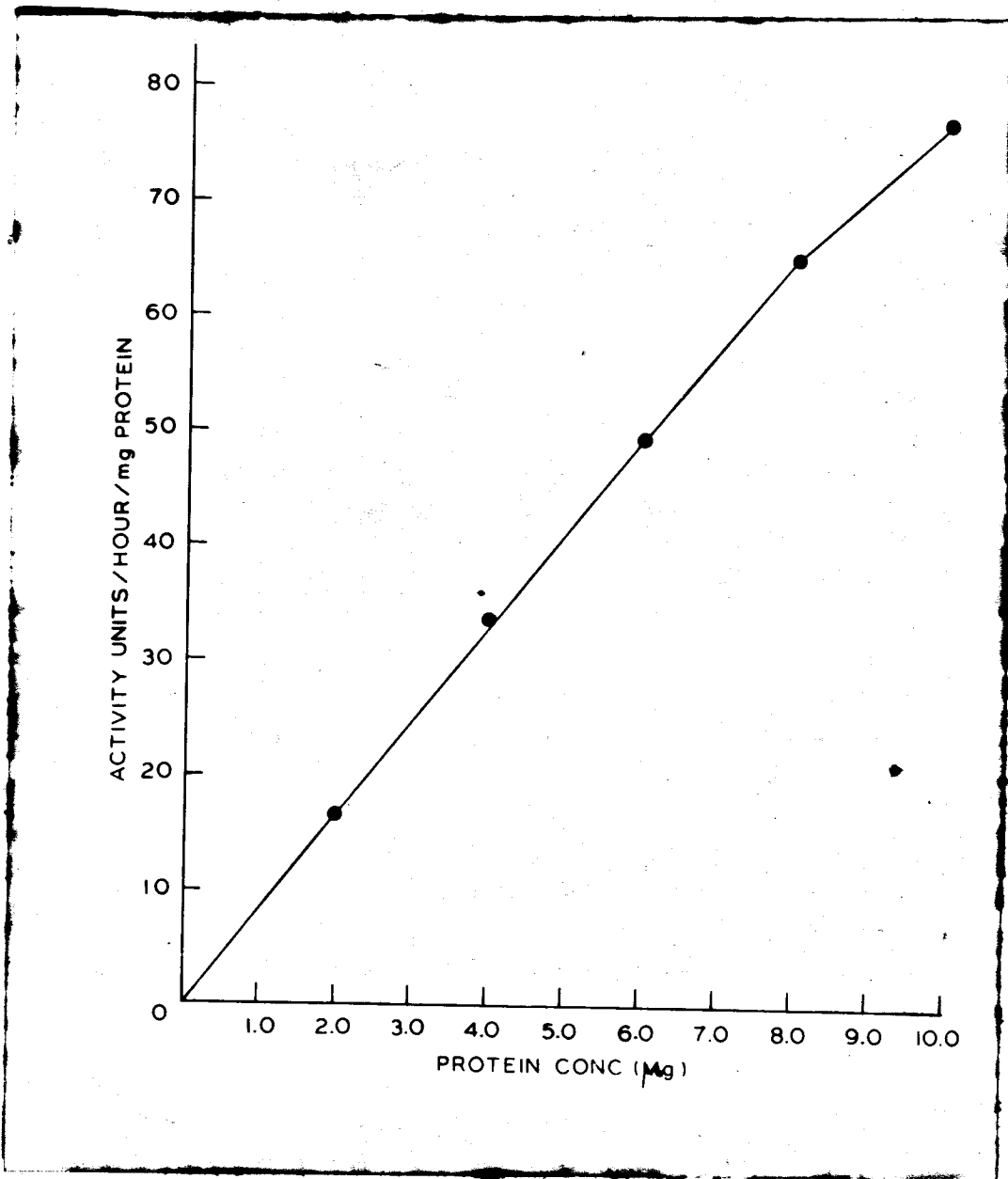


Table 2.**SUBSTRATE SPECIFICITY OF ADPGLUCOSE : GLUCOHYDROLASE.**

Reaction mixture consisted of 10  $\mu$  moles glycine-NaOH buffer (pH 10.0); 2.5  $\mu$  moles  $MgCl_2$ ; 1.0  $\mu$  mole phosphoenol pyruvate; 10  $\mu$ g pyruvate kinase; enzyme protein 8  $\mu$ g and 0.75  $\mu$  mole of sugar nucleotide. Other experimental conditions were as described in Chapter II.

Sugar nucleotide added	Specific activity units/hr/mg protein
ADP-glucose	67.16
GDP-glucose	8.90
CDP-glucose	0
UDP-glucose	0
ADP-mannose	0
ADP-ribose	0

Table 3.

**EFFECT OF SULPHYDRYL REAGENTS ON ADP-GLUCOSE : GLUCOHYDROLASE  
ACTIVITY.**

The reaction mixture consisted of glycine-NaOH buffer (pH 10.0) 10  $\mu$  moles; ADP-(Glucose- $^{14}\text{C}$ ), 0.75  $\mu$  mole; enzyme protein, 8  $\mu\text{g}$  and sulphhydryl reagents (5  $\mu$  moles) in a final volume of 0.5 ml.

Sulphydryl reagent added	$^{14}\text{C}$ - glucose formed cpm x $10^{-3}$ /mg protein
None	299
10 mM DTT	220
10 mM GSH (reduced)	217
10 mM cysteine	129
10 mM beta-mercaptoethanol	621

The fatty acid synthetase of M. 607 (Parvin Khan and Venkita-subramanian, 1964), asparaginase in M. tuberculosis H<sub>37</sub>Rv and M. tuberculosis H<sub>37</sub>Ra (Jayaraman et al., 1968) and ADPG pyrophosphorylase of M. smegmatis (Lapp and Elbein, 1972) are <sup>some</sup> few examples of enzymes with high alkaline pH.

Substrate specificity.

Several sugar nucleotides were examined as substrates for the activity of the enzyme. From the results presented in Table 2, it is evident that ADPG is the substrate for the enzyme. With GDPG as a substrate the activity was insignificant as compared to that with ADPG, whereas other sugar nucleotides employed in the studies were unable to serve as substrates. These findings clearly show that ADPG is the natural substrate for the activity of this enzyme.

Effect of sulphhydryl reagents.

The effect of certain sulphhydryl reagents were examined on the activity of the enzyme. The results (Table 3) indicate that DTT, reduced GSH and cysteine did not stimulate the enzyme activity. Cysteine showed some inhibition. On the other hand, beta-mercaptoethanol stimulated the activity of the enzyme to a considerable extent.

Table 4.  
EFFECT OF METAL IONS.

The activity was determined according to assay B described in Materials and methods. The assay mixture in a final volume of 0.5 ml consisted of 10  $\mu$  moles glycine-NaOH buffer (pH 10.0); 0.75  $\mu$  mole of ADP-(Glucose-<sup>14</sup>C); enzyme protein 8  $\mu$ g and metal ions in concentrations as shown in the table below.

Metal ion added	Concentration	<sup>14</sup> C-glucose formed cpm x 10 <sup>-3</sup> /mg protein
None	-	325
MgCl <sub>2</sub>	1 mM	374
MgCl <sub>2</sub>	5 mM	1080
MnCl <sub>2</sub>	5 mM	1069
CaCl <sub>2</sub>	5 mM	759
CoCl <sub>2</sub>	5 mM	63
BaCl <sub>2</sub>	5 mM	55

Table 5.

EFFECT OF NUCLEOTIDES ON THE ACTIVITY OF ADP-GLUCOSE : GLUCO-  
HYDROLASE.

The following assay procedure was used for the determination of the activity. The reaction mixture in a final volume of 0.5 consisted of 10  $\mu$  moles of glycine-NaOH buffer (pH 10.0), 0.75  $\mu$  moles of ADP-(glucose- $^{14}\text{C}$ ); 8  $\mu\text{g}$  of enzyme protein and nucleotides in concentrations mentioned below.  $^{14}\text{C}$ -Glucose formed was estimated as described in Table 3.

Nucleotides added	Concentration	$^{14}\text{C}$ -glucose formed cpm x $10^{-3}$ /mg protein
None	-	357
5'-AMP	1 mM	748
5'-AMP	5 mM	415
ADP	1 mM	517
ADP	5 mM	211
ATP	1 mM	532
ATP	5 mM	143

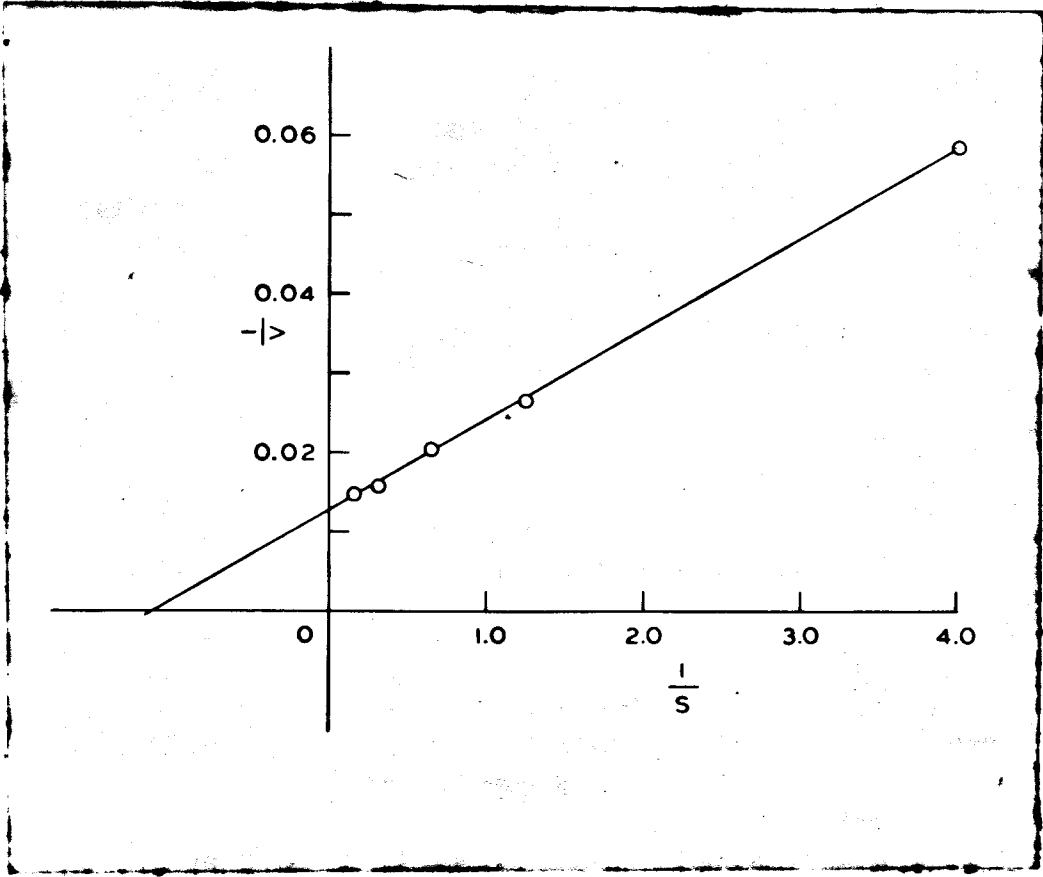
0.2  $\mu\text{Ci}$  of ADP-(Glucose- $^{14}\text{C}$ ) was used.

FIGURE 5.

LINEWEAVER-BURK PLOT OF THE RELATIONSHIP BETWEEN ADP-GLUCOSE : GLUCOHYDROLASE ACTIVITY AND ADP-GLUCOSE CONCENTRATION.

The assay was carried out according to the procedure (assay B) described in chapter II for the enzyme.

The reaction mixture in a final volume of 0.5 ml contained 10  $\mu$  moles of glycine-NaOH buffer (pH 10.0), 0.2  $\mu$ Ci of ADP-(Glucose-<sup>14</sup>C), 8  $\mu$ g of enzyme protein and varying amounts of cold ADP-glucose. S is expressed as  $\mu$  mole of ADP-glucose per ml and V as <sup>14</sup>C-glucose formed ( $\text{cpm} \times 10^{-4}$ ) / mg protein).



### Effect of metal ion.

The results presented in Table 4 show that considerable stimulation in the enzyme activity was observed by  $Mg^{++}$ ,  $Mn^{++}$  and  $Ca^{++}$  at a concentration of 5 mM in the assay mixture. The stimulation by  $Mg^{++}$  and  $Mn^{++}$  are almost the same but with  $Ca^{++}$  it is less than the other metal ions.  $Co^{++}$  and  $Ba^{++}$  significantly inhibited the enzyme activity.

### Effect of substrate concentration.

The effect of substrate concentration on the activity of the enzyme was studied. The  $K_m$  for ADP-glucose, from Lineweaver-Burk plot was found to be  $0.89 \times 10^{-3}M$  (Fig. 5). After studying the cofactor requirements and the optimal conditions, for assaying the enzyme activity the effect of some compounds which are likely to modify the enzyme activity was studied.

### Effect of nucleotides.

The effect of certain nucleotides were examined on the activity of the enzyme (Table 5). The results indicate that 5'-AMP, at low concentrations (1 mM) stimulated the activity of the enzyme whereas high concentrations of ADP and ATP inhibited the enzyme activity. Sommino et al. (1966) have

Table 6.

EFFECT OF SOME METABOLITES ON THE ACTIVITY OF ADPG :  
GLUCOHYDROLASE.

The assay system in a final volume of 0.5 ml consisted of 10  $\mu$  moles of glycine-NaOH buffer (pH 10.0), 0.75  $\mu$  moles of ADP-(Glucose- $^{14}\text{C}$ ) (0.2  $\mu\text{Ci}$ ), 8  $\mu\text{g}$  of enzyme protein and 1.0  $\mu$  mole of metabolite as mentioned in the table.

Metabolite added	$^{14}\text{C}$ -glucose formed cpm x $10^{-3}$ /mg protein
None	330
Glucose-1-P	326
Fructose-1-P	324
Fructose-1,6-diphosphate	335
Pyruvate	320
Succinate	326
Fumarate	299

observed inhibition of GDP-glucose : glucosyltransferase by GDP and ATP, and these authors have attributed the GDP inhibition as competitive. Hence, decreased activity observed in presence of ADP may be due to competitive inhibition. The inhibition of ADPG glucosyltransferase by high concentrations of ATP provides an explanation for the accumulation of glycogen in M. phlei and other mycobacteria grown in nitrogen deficient media. In normal cells role of ADPG glucosyltransferase may be to hydrolyse ADPG and thus prevent its accumulation. However, in nitrogen deficiency it is known that there is excessive accumulation of ATP which is shown in the present studies to inhibit ADPG glucosyltransferase. This results in accumulation of ADPG which is likely to be utilized for glycogen synthesis. Thus intracellular levels of ATP may regulate the activity of this enzyme.

#### Effect of certain metabolites.

In order to find out whether any of the intermediates of glycolytic pathway and the tricarboxylic acid cycle modify the activity of ADPG : glucosyltransferase, the effect of some of these metabolites has been studied. It is evident from the results presented in Table 6, that these metabolites do not have any effect on the activity of the enzyme.

Table 7.

EFFECT OF SULPHYDRYL INHIBITORS ON THE ACTIVITY OF ADP-GLUCOSE :  
GLUCOHYDROLASE.

The assay system contained 10  $\mu$  moles of glycine-NaOH buffer (pH 10.0); 0.75  $\mu$  mole of ADP-(Glucose- $^{14}\text{C}$ ) (0.2  $\mu\text{Ci}$ ); 8  $\mu\text{g}$  of enzyme protein and 1.0  $\mu$  mole of inhibitor in a final volume of 0.5 ml. After inactivation  $^{14}\text{C}$ -glucose formed was separated and counted.

Inhibitors	$^{14}\text{C}$ -glucose formed cpm x $10^{-3}$ /mg protein
None	315
PCMB	280
N-ethyl maleimide	195
Iodoacetate	30

Table 8.**EFFECT OF ANIONS ON THE ACTIVITY OF ADP-GLUCOSE : GLUCOHYDROLASE.**

The reaction mixture in a final volume of 0.5 ml contained the following: Glycine-NaOH buffer (pH 10.0), 10  $\mu$  moles; ADP-(Glucose-<sup>14</sup>C) (0.2  $\mu$ Ci), 0.75  $\mu$  mole; enzyme protein 8  $\mu$ g and anions at a concentration of 2.0 mM.

Anion added	<sup>14</sup> C-glucose formed cpm x 10 <sup>-3</sup> /mg protein
None	335
Phosphate	210
Sulphate	305
Borate	310
Nitrate	325
EDTA	1067

### Effect of sulphydryl inhibitors.

The studies on the effect of several sulphydryl inhibitors on the activity of the enzyme show (Table 7) that iodoacetate is a strong inhibitor of the enzyme thus confirming the requirement of -SH groups for the activity. The inhibition caused by N-ethyl maleimide <sup>than that with</sup> ~~is more as compared to~~ PCMB. ✓

### Effect of some anions.

Effect of some anions has been studied (Table 8) on the activity of the enzyme. The results indicate that phosphate at concentration employed in the present studies showed some inhibition, whereas other anions failed to produce any effect on the enzyme activity. EDTA caused nearly 3 fold increase in activity. ✓

Thus the above studies with the partially purified ADPG glucosylase show that its optimum pH is around 10 and is stimulated by divalent metal ions (like  $Mg^{++}$  and  $Mn^{++}$ ) and beta-mercaptoethanol. Its activity appears to be regulated by intracellular levels of ATP.