NUCLEOTIDE DIPHOSPHATE HEXOSE PYROPHOSPHATASES [1]

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In the course of examining several nucleotide diphosphate sugar pyrophosphorylases in extracts of E. coli and Salmonella, we noted the presence of several nucleotide diphosphate sugar pyrophosphatases. These enzymes are of interest because they are inactive in freshly prepared sonic extracts. Activity was elicited by heating the extracts at 58° for a few minutes which destroyed an inhibitor. Nucleotide diphosphate sugar pyrophosphatases may serve to prevent accumulation of nucleotide diphosphate sugars (Glaser, 1965). In this communication we present some observations on the specificity of a few of these enzyme, their interaction with the inhibitor, and the location of the enzyme(s) and inhibitor(s) in the cell.

Analytical Methods - Bacteria were grown in Difco Antibiotic medium 3 at 37° in a rotary shaker. Sonic extracts were centrifuged at 100,000 x g for 1 hour before assay. The activity of nucleotide diphosphate hexose pyrophosphatases was measured as follows: The reaction mixture contained 20 μmoles Tris-Cl pH 7.5, 4 μmoles of $MgCl_2$,

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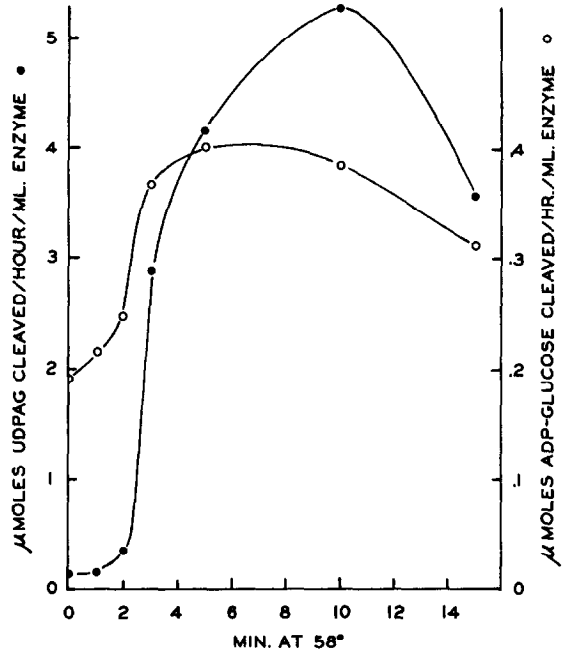

Fig. 1. A sonic extract of E. coli ATCC 12793 was heated at 58° C for the time intervals indicated, and the activity of the nucleotide diphosphate hexose pyrophosphatases assayed. The data also show that in aged sonic extracts (compare Table I) the ADP-glucose pyrophosphatase is no longer completely inhibited. The activation of the UDP-glucose pyrophosphatase was the same as that shown for the UDP-N-acetylglucosamine pyrophosphatase.

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1 μmole of nucleotide diphosphate sugar, 50 μg of E. coli alkaline phosphatase and enzyme in a final volume of 0.5 ml. After incubation at 37° the reaction was stopped by heating 1 min at 100°, and the free sugar liberated was measured. Glucose was measured with hexokinase and glucose-6-phosphate dehydrogenase and amino sugars by the method of Levvy and McAllan (1959). In extracts of Salmonella weslaco the enzyme activity could be determined by measuring the formation of α-D-glucose-1-P in a coupled assay with phosphoglucomutase and glucose-6-P dehydrogenase (Melo and Glaser, 1965). The cleavage of UDP-D-galactose was measured by the release of radioactive galactose from the corresponding labelled nucleotide. The disappearance of UDP-glucose when the nucleotide was

incubated with whole cells was measured with UDP-D-glucose dehydrogenase. Nucleotides were chromatographed in ethanol/1 M NH_4 acetate pH 7.8 (7.5/3) (Paladini and Leloir, 1952).

Results - As shown in Table I, extracts of E. coli ATCC 12793 or E. coli O-17 [a], show no nucleotide diphosphate hexose pyrophosphatase activity unless heated at 58° for a few minutes. Addition of unheated sonic extract to the heat activated enzyme resulted in inhibition which suggested the presence of excess inhibitor. Fig. 1 illustrates the effect of time of heating on enzyme activity. The sonic extract used in these experiments was an aged preparation in which a part of the ADP-glucose pyrophosphatase activity had become active. The presence of at least two inhibitors in the sonic extract, specific for the ADP-glucose pyrophosphatase and the UDP-N-acetylglucosamine pyrophosphatase respectively is suggested by this experiment.

TABLE I

Activity of Nucleotide Diphosphate Hexose Pyrophosphatase in Sonic Extracts

	Pyrophosphatase Activity μmoles cleaved/hour/mg/ protein		
Enzyme Source and Treatment	UDP-glucose	UDP-N-acetylglucosamine	ADP-glucose
E. coli ATCC 12793			
none	< 0.01	< 0.01	< 0.01
Heated at 58° for 5 min.	0.142	0.183	0.11
E. coli O-17			
none	< 0.01	< 0.01	< 0.01
Heated at 58° for 5 min.	0.02	0.05	0.12
Salmonella weslaco ATCC 12010			
none	0.64	-	-
Heated at 55° for 5 min.	1.38	-	-

[a] E. coli O-17 was kindly provided by Dr. Otto Luderitz.

The UDP-glucose pyrophosphatase activity in extracts of Salmonella weslaco is only partially inhibited, Table I. Addition of the E. coli inhibitor to the enzyme from S. weslaco has no effect.

The inhibitor(s) present in extracts of E. coli react with the enzyme in a stoichiometric manner, and appropriate controls show that the apparent inhibition is not due to destruction of either the substrate or the product of the reaction by the inhibitor. The inhibitor(s) are resistant to digestion with RNase or DNase, can be precipitated with ammonium sulfate, and chromatographs on Sephadex G-100 as a defined peak (Fig. 2).

When spheroplasts are prepared from E. coli or S. weslaco, 70 to 100% of the nucleotide diphosphate hexose pyrophosphatases are released into the medium. They can be released also by the osmotic shock treatment (Neu and Heppel, 1965) in about 70% yield. The enzymes released by this treatment are fully active, and the inhibitor is retained in the spheroplasts.

The nucleotide diphosphate sugar pyrophosphatases, share many properties in common with a variety of hydrolases, whose location can be operationally defined as between the cell wall and the cell membrane (Malamy and Horecker, 1964; Neu and Heppel, 1964, 1965). Together with the 5'-nucleotidase and the α-aldose-1-phosphate phosphatase described previously (Neu and Heppel, 1965), provide a mechanism for the complete breakdown of nucleotide diphosphate sugars to the corresponding monosaccharide and nucleoside, both of which can penetrate the cell membrane.

As predicted by the location of these enzymes in the cell, intact cells of E. coli and S. weslaco will catalyze the breakdown of UDP-glucose. In E. coli this can be determined by the disappearance of UDP-glucose and in S. weslaco which lacks α-aldose-1-P phosphatase, also by the appearance of a stoichiometric quantity of α-glucose-1-P.

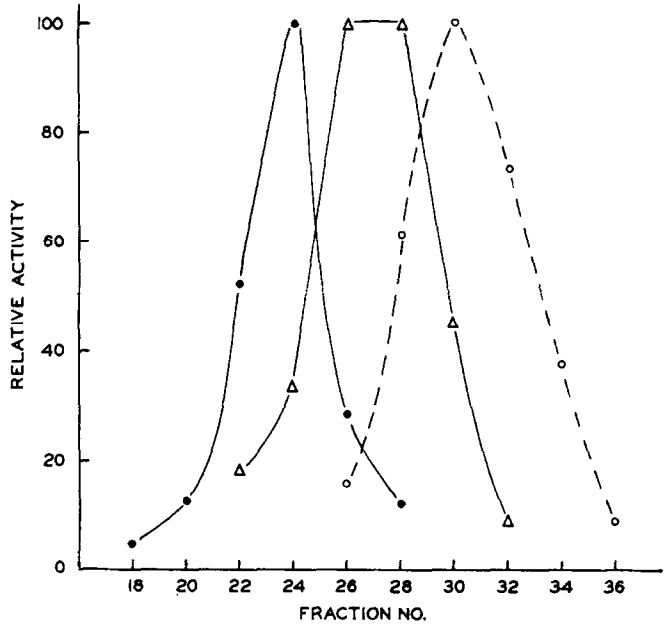

Fig. 2. Chromatography of UDP-N-acetylglucosamine pyrophosphatase on Sephadex G-100. The enzyme was chromatographed on a 2.5 x 50 cm column of Sephadex G-100 in 0.05 M Tris-Cl - 0.01 M $MgCl_2$ - 0.001 M EDTA, pH 8.0, and 3 ml fractions were collected. The dotted line shows the elution of the active enzyme (Expt. 1). The solid lines show the elution position in a separate experiment, on the same column of (O) inhibited enzyme assayed after heating the fractions at 58° for 6 min, and of (Δ) inhibitor, assayed by addition of the fraction to active enzyme (Expt. 2). Activities are expressed in relation to the most active fraction as 100%. In experiment 1, 100% enzyme activity = 1.7 μmoles of UDP-N-acetylglucosamine cleaved/hour/ml of enzyme. In experiment 2, 100% enzyme activity = 5.2 μmoles/hour/ml of enzyme, and 100% inhibitor is a quantity of inhibitor per ml that will inhibit enzyme catalyzing the breakdown of 6.0 μmoles of UDP-N-acetylglucosamine/hour. In both experiments the UDP-glucose pyrophosphatase activity was superimposable with the UDP-N-acetylglucosamine pyrophosphatase activity, and the ADP-glucose pyrophosphatase activity was maximal in fraction 32.

Properties of the enzymes - By chromatography on DEAE cellulose [4] the E. coli nucleotide diphosphate sugar pyrophosphatases could be separated into 2 fractions. One fraction catalyzing the cleavage of uridine nucleotides, and the other of adenine nucleotides. This is in agreement with

[4] The enzymes were chromatographed on a 2.5 x 12 cm column of DEAE cellulose and eluted with linear gradient, the mixing vessel contained 200 ml of 0.05 M Tris-Cl pH 8.0, 0.01 M $MgCl_2$, 0.001 M EDTA and the reservoir 200 ml of the same buffer + 0.5 M KCl. 4 ml fractions were collected. The UDP-glucose and UDP-N-acetylglucosamine pyrophosphatases were eluted at fraction 37-47 and the ADP-glucose pyrophosphatase in fractions 70-80.

the observation that the inhibitors for the ADP-glucose pyrophosphatase and the UDP-N-acetylglucosamine pyrophosphatase are distinct. The specificity of these enzymes, as well as of the enzyme(s) from Salmonella weslaco are shown in Table II. A variety of fractionation procedures have failed to separate the UDP-D-glucose pyrophosphatase from the UDP-N-acetyl-D-glucosamine pyrophosphatase. It is possible that activities may be catalyzed by the same protein.

The Michaelis constants for all the pyrophosphatases are approximately 8×10^{-5} M, they all have an absolute requirement for Mg^{++}, and a pH optimum between pH 7 and pH 8.

In crude enzyme preparation the products of the reaction are the corresponding nucleosides due to the presence of 5'-nucleotidase (Neu and Heppel, 1965), and free hexose due to the presence of phosphatase that cleaves α-aldose-1-phosphates (Neu and Heppel, 1965). Salmonella weslaco lacks this phosphatase and the products of UDP-glucose cleavage are uridine and α-D-glucose-1-P. With the purified ADP-glucose pyrophosphatase the products are AMP and α-D-glucose-1-P. The most purified E. coli UDP-glucose pyrophosphatase still contains 5'-nucleotidase activity and the products are α-D-glucose-1-P, uridine and P_i.

The nucleotide diphosphate sugar pyrophosphatases described in this communication are clearly distinct from the nucleotide diphosphate phosphorylases, (Carminatti and Cabib, 1965; Danker, Goncalves and Recondo, 1964), since they do not require inorganic phosphate and attempts to demonstrate the formation of UDP or ADP by carrying out the reaction in the presence of excess pyruvic kinase and lactic dehydrogenase have been unsuccessful.

It seems unlikely that the function of the nucleotide diphosphate sugar pyrophosphatases is to hydrolyze exogenously supplied nucleotide diphosphate sugars. It appears much more likely that they function to hydrolyze excess nucleotides, preventing nucleotide accumulation in the

TABLE II

Nucleotide Specificity of Nucleotide Diphosphate Hexose Pyrophosphatase

	Salmonella weslaco[1] UDP-hexose pyrophosphatase	*E. coli*[2] UDP-hexose pyrophosphatase	*E. coli*[3] ADP-glucose pyrophosphatase
	Relative Activity		
UDP-glucose	100	84	< 5
UDP-galactose	100	-	< 5
UDP-N-acetylglucosamine	4	100	< 5
UDP-N-acetylgalactosamine	-	100	< 5
ADP-glucose	8	< 5	100
CDP-glucose	10	< 5	< 5
GDP-glucose	6	< 5	< 5
dTDP-glucose	3	< 5	< 5
DPN	-	0	0

1/ The enzyme was purified by ammonium sulfate fractionation and had a specific activity of 10 μmoles/hour/mg of protein.

2/ Purified by chromatography on DEAE cellulose. The activity with UDP-N-acetylglucosamine was 2 μmoles/hour/mg protein.

3/ Purified by chromatography on DEAE cellulose. The activity with ADP-glucose was 40 μmoles/hour/mg.

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cell. This mechanism is an alternative to feedback inhibition, for those nucleotides, whose hydrolysis products are readily metabolizable as has been discussed previously (Glaser, 1965). For the enzymes described in this communication, a mechanism must also be provided for bringing together the enzymes and the substrate across the cell membrane. It is possible to distinguish this type of mechanism from feedback inhibition since it predicts a rapid turnover of the intracellular nucleotide diphosphate hexose pool, even in the absence of polymer synthesis. Attempts to measure this turnover are currently in progress.

The possible function of the intracellular inhibitor remains obscure. Preliminary attempts to obtain changes in the level of the enzyme(s) or inhibitor(s) by changes in the growth conditions have been unsuccessful. It should be mentioned that specific protein inhibitors for hydrolytic enzymes have been described previously, both for a bacterial DPN pyrophosphatase (Kern and Natale, 1958) and a bacterial DPNase (Swartz, Kaplan and Lamborg, 1958).

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