Periplasmic Nucleoside Diphosphate Sugar Hydrolase from *Bacillus subtilis**

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ABSTRACT: A nucleoside diphosphate sugar hydrolase is produced by *Bacillus subtilis* W-23 under conditions of phosphate limitation. When cells are converted into spheroplasts, the enzyme is specifically released into the medium. Enzyme activity can also be demonstrated with no crypticity with whole cell preparations. Both these observations suggest that the enzyme is located near the cell surface. The enzyme catalyzes the hydrolysis of nucleoside diphosphate sugars to the corresponding nucleoside, inorganic phosphate, and sugar phosphate. The enzyme shows 5′-nucleotidase activity and evidence is presented that both activities are catalyzed by the same protein.

The nucleoside diphosphate sugar hydrolase is relatively unspecific, it has an approximate molecular weight of 137,000, a pH optimum of 8, and very high apparent affinity for its substrates (K_m of uridine diphosphate D-glucose 1.8×10^{-6} M). It shows no requirement for the addition of divalent cations.

In the course of examining teichoic acid synthesis in *Bacillus subtilis* W-23 grown under conditions of phosphate limitation, we noted the presence of an enzyme which hydrolyzed CDP-ribitol to cytidine, inorganic phosphate, and ribitol phosphate. Further examination showed that this reaction is catalyzed by a relatively nonspecific enzyme which hydrolyzes a variety of nucleoside diphosphate sugars. Two criteria suggest that this enzyme is localized near the cell surface. (1) It is selectively released from *B. subtilis* W-23 during spheroplast formation. (2) The enzymatic activity can be demonstrated in whole cells with very little crypticity. These are essentially the criteria which have been used to classify certain enzymes in *Escherichia coli* and other gram-negative organisms as "periplasmic" (Heppel, 1967).

To the best of our knowledge, enzymes with these characteristics have not been described in gram-positive organisms. Indeed, it has been suggested (Cashel and Freese, 1964) that enzymes which are periplastic in gram-negative organisms, are secreted into the medium in gram-positive organisms. These observations were based on data obtained with alkaline phosphatase. Although a variety of hydrolytic enzymes are secreted by *B. subtilis*, in some strains the alkaline phosphatase is membrane bound, rather than released into the medium (Takeda and Tsugita, 1966).[1]

We report in this communication the partial purification and some of the properties of the nucleoside diphosphate sugar hydrolase from *B. subtilis* W-23.

Materials and Methods

Phosphate-limited cultures of *B. subtilis* W-23 were grown in a medium of the following composition in grams per liter: NH_4Cl, 1.5; KCl, 0.45; Tris base, 12.1; $MgSO_4 \cdot 7H_2O$, 0.05; $FeSO_4 \cdot 7H_2O$, 0.005; $MnCl_2 \cdot 4H_2O$, 0.005; and K_2HPO_4, 0.035; and adjusted to pH 7.6. Glucose was autoclaved separately and added to a final concentration of 6 g/l. Cells were grown in a rotary shaker at 37° in 2-l. flasks with 600 ml of medium. Cell mass was estimated from optical density at 650 mμ. An absorbancy of 3.7 at 650 mμ equals 1 mg/ml.

The following chemicals and enzymes were obtained from the sources indicated. CDP-D-glucose, GDP-D-glucose, ADP-D-glucose, GDP-D-mannose, TDP-glucose, and α-D-glucose 1,6-diphosphate from Calbiochem. UDP-D-glucose, DPN, TPN, *E. coli* alkaline phosphatase, phosphoglucomutase, ADP, ATP, lactic dehydrogenase, pyruvic kinase, pyruvate, and phosphoenolpyruvate from Sigma Chemical Co.

Ribitol dehydrogenase were prepared by the method of Fromm (1962). CDP-ribitol and CDP-glycerol were prepared as described previously (Glaser, 1964). UDP-D-glucose-^{14}C and UMP-^{14}C were obtained from New England Nuclear. Uridine-^{14}C-labeled UDP-D-glucose was prepared as described previously (Glaser *et al.*, 1967).

Assays for lactic dehydrogenase, pyruvic kinase, phosphofructokinase, glucose 6-phosphate dehydrogenase, and phosphoglucose isomerase were carried out by standard techniques (Bergmeyer, 1965). When enzymes were assayed in crude cell extracts, assays were carried out at three different concentrations of enzyme to ensure proportionally to enzyme concentration. Inorganic pyrophosphatase was assayed by the method of Tono and Kornberg (1967). Inorganic phosphate was measured by the method of Fiske and Subbarow (1925). Nucleoside diphosphate sugar hydrolase activity was measured by one of the following three assays. (A) The reaction mixture contained 37 μmoles of Tris-Cl (pH 8.0), 7 μmoles of $MgCl_2$, 0.7 μmole of EDTA, 0.5 μmole of UDP-D-glucose, 0.5 μmole of TPN, 1 μmole of α-D-glucose-1-6-di-P, 0.2 unit of phosphoglucomutase, 0.2 unit of glucose 6-phosphate de-

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[1] We have confirmed these observations with *B. subtilis* W-23 and *B. subtilis* ATCC. 6051 (Marburg Strain).

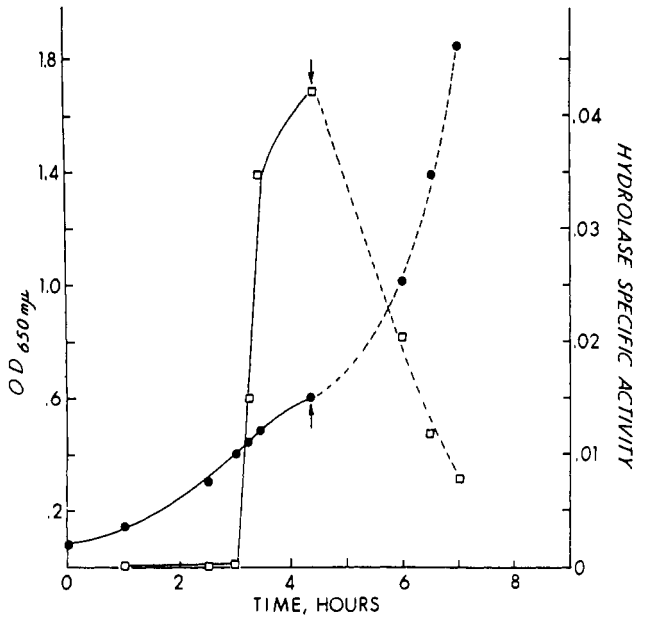

FIGURE 1: Induction of nucleoside diphosphate sugar hydrolase. *B. subtilis* W-23 was grown in phosphate-limited medium. At the times indicated the cells from 10 ml of the culture were collected by centrifugation, washed twice with 0.10 M Tris-Cl (pH 8.0)–0.01 M $MgCl_2$–0.001 M EDTA (pH 8.0), and resuspended in 0.5 ml of the same buffer at 37° containing 0.2 μmole of CDP-ribitol. After incubation at 37° for 15 min, the cells were removed by centrifugation at 15,000*g* for 5 min. To the supernatant fluid was added 50 μg of *E. coli* alkaline phosphatase and the reaction mixture was incubated for 15 min at 37° and then heated for 1 min at 100° to inactivate the phosphatase. The solution was then assayed for ribitol with ribitol dehydrogenase. At the arrow sterile phosphate was added to the culture to give a final concentration of 0.01 M. (●) Optical density of culture at 650 mμ. (□) Nucleoside diphosphate sugar hydrolase as μmoles of nucleotide hydrolyzed per mg of cell dry weight per 15 min.

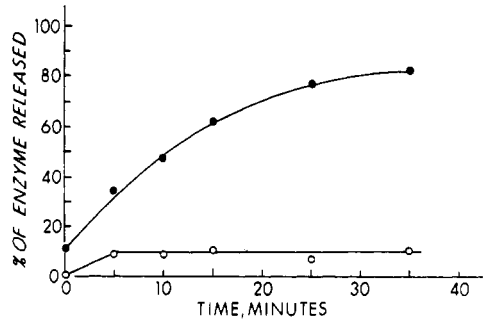

FIGURE 2: Release of nucleoside diphosphate sugar hydrolase during spheroplast formation. *B. subtilis* W-23 in phosphate-limited medium was harvested by centrifugation at an optical density of 0.520 at 650 mμ and washed twice with 0.10 M Tris-Cl (pH 8.0)–0.01 M $MgCl_2$–0.001 M EDTA, and finally suspended in 0.1 M Hepes (pH 7.0)–0.01 M $MgCl_2$–10 mM $CaCl_2$–20% w/v sucrose at 25° at a density of 17 mg/dry wt per ml; 0.2 mg of lysozyme was added per ml, and at the times indicated 2-ml aliquots were pipetted into 20 ml of the same Hepes–sucrose buffer at 0° and centrifuged (15,000-*g*) at 0° for 5 min. The supernatant was removed and the residue was gently suspended in 5 ml of Hepes–sucrose and centrifuged again. The pellet was suspended in 5 ml of 0.10 M Tris-Cl–0.01 M $MgCl_2$–0.001 M EDTA (pH 8.0) containing 0.2 mg/ml of lysozyme and incubated at 37° for 15 min and the residual cells were broken in a 10-kc Ratheon sonic oscillator for 1 min. Cell debris was removed by centrifugation at 105,000*g* for 2 hr. Enzyme activities were assayed in the supernatant fluid from the spheroplast formation and in the soluble cell extract. The total enzyme obtained by adding the enzyme measured in the two fractions was essentially the same at all time periods. (●) Nucleoside diphosphate sugar hydrolase. (○) Glucose 6-phosphate dehydrogenase. The nucleoside diphosphate sugar hydrolase in the 105,000*g* pellet was negligible.

hydrogenase, and enzyme in a final volume of 1 ml. The rate of TPNH formation was followed at 340 mμ in a Gilford spectrophotometer. (B) The reaction mixture contained 10 μmoles of Tris-Cl (pH 8.0), 2 μmoles of $MgCl_2$, 0.2 μmole of EDTA, and 1 mμmole of UDP-D-glucose-^{14}C (1×10^4 cpm/mμmole) in a final volume of 0.4 ml. The reaction was stopped by the addition of 0.5 ml of 3 N formic acid. α-D-Glucose-1-P (1 μmole) was added as carrier and the mixture was placed on a 0.5 × 4 cm column of Dowex 1 (formate . α-D-Glucose-1-P was eluted with 10 ml of 4 N formic aci , evaporated to dryness, dissolved in water, and counted in a Packard liquid scintillation counter using Bray's solution (Bray, 1960). Unincubated controls were always included. The recovery of carrier α-D-glucose-1-P was about 90% in all samples. (C) The reaction mixtures contained 10 μmoles of Tris-Cl (pH 8.0), 2 μmoles of $MgCl_2$, 0.2 μmole of EDTA, 0.2 μmole of CDP-ribitol, 50 μg of *E. coli* alkaline phosphatase, and enzyme. The reaction was terminated by heating at 100° for 2 min, and the ribitol formed was measured with ribitol dehydrogenase (Glaser, 1964).

5′-Nucleotidase activity was assayed by measuring the formation of uridine from UMP-^{14}C as described previously (Glaser *et al.*, 1967).

Phosphomonesterase activity was assayed as follows. The reaction mixture contained 25 μmoles of Tris-Cl (pH 8.0), 2.5 μmoles of $MgCl_2$, 0.25 μmole of EDTA, 0.4 μmole of *p*-nitrophenyl phosphate, and enzyme in a final volume of 0.4 ml. After incubation at 37°, the reaction was stopped by the addition of 0.6 ml of 1 M Na_2CO_3, and the liberated *p*-nitrophenol was measured at 420 mμ. Phosphodiesterase activity was measured with the same assay using bis(*p*-nitrophenyl phosphate) as a substrate.

Nucleotides were chromatographed by descending paper chromatography on Whatman No. 3MM paper with ethanol–1 M ammonium acetate (pH 7.8) as the solvent (Paladini and Leloir, 1952).

Results

If *B. subtilis* W-23 is grown in medium with limiting phosphate, intact cells acquire the ability to hydrolyze CDP-ribitol (as well as other nucleoside diphosphate sugars) at the end of the logarithmic phase of growth. This is illustrated in Figure 1, where it is seen that the addition of phosphate stops further enzyme synthesis and the enyzme is then diluted out by growth. Under these growth conditions less than 10% of the total enzyme activity can be detected in the growth medium, whether it is assayed directly or assayed after concentration 100-fold by pressure filtration. The ability of the cells to hydrolyze nucleoside diphosphate sugars does not simply represent a change in cell permeability since cell-free extracts obtained by sonic oscillation are unable to degrade CDP-ribitol before enzyme activity can be detected in whole cells.

TABLE I: Release of Enzymes from *B. subtilis* During Spheroplast Formation.[a]

	Enzyme	Act. in Spheroplast Supernatant Fluid	Act. in Spheroplast (μmoles/min ml)	% Released
Expt 1	Glucose 6-phosphate dehydrogenase	0.017	0.29	6
	Phosphoglucose isomerase	0.23	1.44	14
	Phosphofructokinase	0.041	0.44	9
	Nucleoside diphosphate sugar hydrolase	0.018	0.004	82
Expt 2	Glucose 6-phosphate dehydrogenase	0.04	0.28	12.5
	Inorganic pyrophosphatase	2.75	14.4	13
	Nucleoside diphosphate sugar hydrolase	0.0076	0.0025	75

[a] Cells were incubated with lysozyme as indicated in the legend to Figure 1 for 30 min. Enzyme assays were carried out as described in Materials and Methods. Nucleoside diphosphate sugar hydrolase was measured by the hydrolysis of CDP-ribitol (assay C). The spheroplasts were ruptured in a volume of buffer, equivalent to the corresponding spheroplast supernatant fluid so that activities per unit volume are directly comparable.

Figure 2 shows that when cells containing nucleoside diphosphate sugar hydrolase are converted into spheroplasts with lysozyme in 20% sucrose, the enzyme activity is almost entirely released into the supernatant fluid, while glucose 6-phosphate dehydrogenase is retained in the spheroplast pellet. Table I shows that under these conditions phosphoglucose, isomerase, phosphofructokinase, and inorganic pyrophosphatase are all retained in the spheroplast. These observations strongly suggest that the nucleoside diphosphate sugar hydrolase is located at the cell surface and is preferentially released during spheroplast formation.[2]

In Table II the crypticity of the enzyme in whole cells is studied by measuring the activity of the enzyme in whole cells *vs.* cell-free extracts under three different sets of conditions: (I) Cells harvested and suspended in Tris buffer; (II) cells harvested and suspended in Hepes[3] containing 20% sucrose; and (III) measurement of the enzyme activity in cells directly in the growth medium without harvesting. Essentially the same results are obtained under all three conditions in that more activity can be detected in intact cells than in cell-free extracts. We have no explanation at present for the higher activity of whole cells as compared with cell-free extracts. This difference in activity is also observed if the enzyme is released from the cells by lysozyme treatment rather than by sonic oscillation, suggesting that this difference in activity is not due to enzyme denaturation during cell breakage. These experiments suggest that the activity of the enzyme is higher when attached to the cell surface than when free in solution. However, it is very difficult to rule out the possibility that whole cells contain an additional enzyme which is destroyed by our extraction methods.

Because cells that have been harvested may show altered permeability, expt III in Table II is particularly relevant, since in this experiment the rate of CDP-ribitol hydrolysis was measured in the culture without prior harvesting of the cells, and under these conditions the cells still retained the ability to hydrolyze CDP-ribitol. The difference in activity observed in different experiments in Table II appears to reflect small variations in phosphate concentrations in the medium so that the cells are not induced to the same extent, although they are harvested at the same optical density.

Purification of Nucleoside Diphosphate Sugar Hydrolase. The following procedure was used to obtain a partially purified nucleoside diphosphate sugar hydrolase. *B. subtilis* W-23 was grown in phosphate-limited medium to an optical density of 0.65 at 650 mμ. The cells from 4.8 l. of medium were harvested by centrifugation and washed twice with 25 ml of 0.1 M Tris-Cl (pH 8.0)–0.01 M $MgCl_2$–0.001 M EDTA and then suspended in 100 ml of 0.1 M Hepes (pH 7.0)–0.01 M $MgCl_2$–0.01 mM $CaCl_2$–20% w/v sucrose at 25°, containing 0.2 mg/ml of lysozyme and incubated at 25° until most of the cells had been converted into spheroplasts as judged by phase microscopy (usually 25–30 min).[4] The spheroplasts were removed by centrifugation at 15,000*g* for 15 min and small particles by centrifugation at 105,000*g* for 2 hr. The supernatant fluid was dialyzed against two 8-l. changes of 0.1 M Tris-Cl (pH 8.0)–0.01 M $MgCl_2$–0.001 M EDTA for 24 hr and concentrated by pressure filtration using an Amicon XM-50 membrane.

The enzyme was placed on a 2.5 × 30 cm column of DEAE-cellulose equilibrated with 0.1 M Tris-Cl (pH 8.0)–0.01 M $MgCl_2$–0.001 M EDTA and eluted with a linear gradient. The mixing flask contained 500 ml of this buffer and the reservoir 500 ml of 0.5 M KCl in the same buffer. The enzyme was eluted between 140 and 170 ml. The peak

[2] It is important that spheroplast formation be carried out at 25°. When the same experiment is repeated at 37°, the results are rather variable in that considerable leakage of glycolytic enzymes is observed in some experiments. We use the word spheroplast, rather than protoplast, because no measurements have been carried out to show that the osmotically fragile forms obtained under our conditions are totally devoid of cell wall components.

[3] The following nonstandard abbreviations are used: Hepes, *N*-2-hydroxylethyl-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholine)ethansulfonic acid.

[4] All subsequent steps in the purification were carried out at 3°.

TABLE II: CDP-ribitol Hydrolysis by Intact Cells of *B. subtilis* W-23.[a]

Expt	Enzyme Source	NDP[b] Sugar Hydrolase Act. (mμmoles/ mg dry wt min)	Ratio Whole Cells:Cell Extract
I	Whole cells	2.74	2.2
	Cell supernatant	0	
	Cell extract	1.27	
II	Whole cells	0.61	
	Cell supernatant	0	2.6
	Cell extract	0.23	
III	Whole cells	6.7	
	Culture medium	0	2.6
	Cell extract	2.6	

[a] *B. subtilis* W-23 was grown in phosphate-limited medium. In expt I cells were harvested from 1.2 l. of medium at $OD_{650m\mu}$ 0.52, washed twice by centrifugation with 0.1 M Tris-Cl (pH 8.0)–0.01 M $MgCl_2$–0.001 M EDTA, and suspended in 10 ml of the same buffer and aliquots were assayed directly for their ability to cleave CDP-ribitol. A second aliquot of cells was ruptured by sonic oscillation and the cell-free extract was assayed for its ability to hydrolyze CDP-ribitol. As a control for enzyme leakage during the assay, whole cells were incubated without CDP-ribitol under assay conditions. The cells were removed by centrifugation, and the supernatant fluid was assayed for its ability to hydrolyze CDP-ribitol. These samples are referred to as cell supernatant. In expt II the same procedure was used with a different cell culture, but 0.1 M Hepes (pH 7.0)–0.01 M $MgCl_2$–10 μM $CaCl_2$–20% w/v sucrose was used instead of Tris buffer to suspend the cells. In expt III the hydrolysis of CDP-ribitol-^{14}C by whole cells in the culture medium was measured as follows. To 3-ml aliquots of culture at $OD_{650m\mu}$ 0.55 was added 30 mμmoles of CDP-ribitol-^{14}C (5.4×10^5 cpm/μmole) and incubated at 37° in a rotatory shaker for 5- and 10-min periods. The cells were then removed by filtration through a 0.45 μ Millipore filter. To the filtrate was added carrier CDP-ribitol (1 μmole) and ribitol-5-P (0.86 μmole) and the ribitol-5-P was hydrolyzed to ribitol by incubating with 0.1 mg of *E. coli* alkaline phosphatase for 10 min at 37°. After incubation with alkaline phosphatase, the samples were deionized by passage through a 1×7 cm column of Amberlite MB-3. The column was washed with three volumes of H_2O, and the pooled elutes were concentrated to dryness and dissolved in 1 ml of H_2O. Aliquots were assayed for ribitol and counted in a liquid scintillation counter. As a control an aliquot of the culture was filtered through a 0.45 μ Millipore filter and the filtrate was incubated with CDP-ribitol-^{14}C, as described for the whole culture. In order to obtain the activity of cell-free extract, cells from the remainder of the culture in expt III were collected by centrifugation, disrupted in a sonic oscillator, and assayed for their ability to break down CDP-ribitol by assay C. [b] NDP = nucleoside diphosphate.

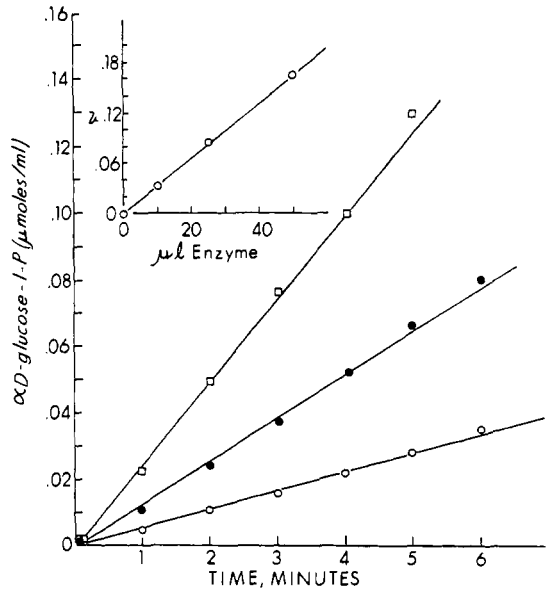

FIGURE 3: Proportionality of assay to time and enzyme concentration. Assay A was used. (○) 10 μl of enzyme; (●) 25 μl of enzyme; (□) 50 μl of enzyme. The insert shows a replot of these data to show proportionality to enzyme concentration. In insert ν = μmoles/ml of glucose-1-P formed in 5 min.

tubes were concentrated by pressure filtration as described above to a volume of 6 ml; 1-ml aliquots were further purified by chromatography on a 1×60 cm column of Sephadex G-200, equilibrated with 0.1 M Tris-Cl (pH 8.0)–0.01 M $MgCl_2$–0.001 M EDTA–0.2 M KCl. Fractions of 1 ml were collected.

The purified enzyme could be kept frozen in the buffer used in the gel filtration step for at least 1 month with only minor loss in activity. The purification is summarized in Table III.

Properties of the Nucleoside Diphosphate Sugar Hydrolase. The proportionality of assay A to time and enzyme concentration is shown in Figure 3. The pH optimum is 8.0 as shown in Figure 4.

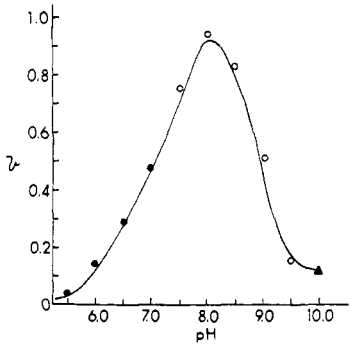

FIGURE 4: Effect of pH on enzyme activity. The reaction mixture contained 0.1 M buffer as indicated, 0.5 μmole of UDP-D-glucose, and 0.024 unit of enzyme and were incubated at 25° for 30 min. The reaction was stopped by heating at 100° for 1 min. α-D-Glucose-1-P formed was assayed with phosphoglucomutase and D-glucose 6-P-dehydrogenase. Buffers used were: (●) MES, (○) Tris-Cl, (▲) glycine, and ν = μmoles of UDPG hydrolyzed per min per mg of protein.

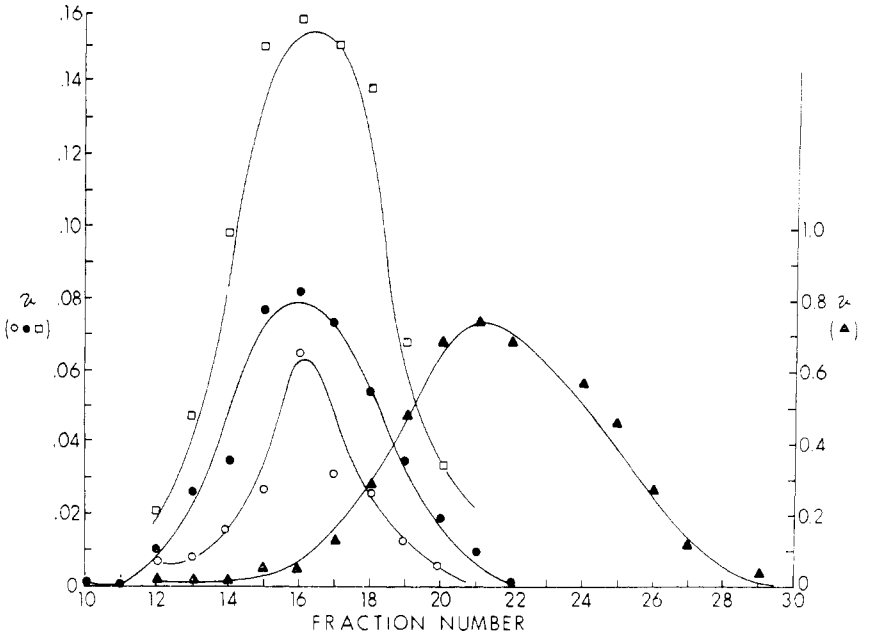

FIGURE 5: Fractionation of nucleoside diphosphate sugar hydrolase on Sephadex G-200. The experiment was carried out as described under enzyme purification. (●) UDP-D-glucose hydrolysis, (○) nitrophenolphosphate hydrolysis, (□) bis(*p*-nitrophenyl phosphate) hydrolysis, (▲) inorganic pyrophosphatase, and ν = μmoles of substrate hydrolyzed per min per ml of enzyme.

TABLE III: Purification of Nucleoside Diphosphate Sugar Hydrolase.[a]

Fraction	Vol (ml)	Act. (units)	Sp Act. (units/mg of Protein)	5′-Nucleotidase:Nucleoside Diphosphate Sugar Hydrolase
Spheroplast Supernatant	152	6.1	0.31	
DEAE-cellulose Eluate	6	3.3	1.2	1.3
Sephadex G-200[b]	3	0.22	2.3	1.2

[a] For details see text. [b] Only 1 ml of the DEAE-cellulose eluate was chromatographed on Sephadex G-200.

TABLE IV: Stoichiometry of Nucleoside Diphosphate Sugar Hydrolase.[a]

Substrate	Sugar 1-Phosphate Formed (μmole)	P_i Formed (μmole)
ADP-glucose	0.39	0.41
GDP-glucose	0.38	0.41
GDP-mannose		0.42
dTDP-D-glucose	0.11	0.12
CDP-D-glucose	0.22	0.28

[a] The reaction mixtures contained 15 μmoles of Tris-Cl (pH 8.0), 3 μmoles of $MgCl_2$, 0.3 μmole of EDTA, 0.5 μmole of the indicated nucleotide, and 0.014 unit of enzymes except in the case of dTDP-glucose where 0.028 unit of enzyme was used, and were incubated at 37° for 40 min. The reaction was stopped by heating at 100° for 1 min. α-D-Glucose-1-P and P_i were determined as described in Materials and Methods.

As shown in Table IV the enzyme hydrolyzed essentially all of the nucleoside diphosphate sugars tested. In each case where it was examined, the stoichiometry was one molecule of inorganic phosphate formed per molecule of sugar phosphate released, suggesting that the product in each case was the nucleoside, P_i, and sugar phosphate. The fact that the nucleoside was the product of the reaction was confirmed for each of the substrates in Table IV by paper chromatography of the reaction products using ethanol–1 M ammonium acetate (pH 7.8) as the solvent. As shown in Table III the enzyme shows considerable 5′-nucleotidase activity. Evidence will be presented below that these two activities are catalyzed by the same enzyme.

In addition to nucleoside diphosphate sugar, the purified enzyme catalyzes the hydrolysis of DPN, but not ATP or ADP and shows no RNase or DNase activity. As shown in Figure 5, the purified enzyme contained both phosphomonoesterase and phosphodiesterase activity. Both of these activities could be inhibited by CDP-ribitol, a substrate of the enzyme (see below).[5]

[5] The inorganic pyrophosphatase activity seen in Figure 5, represents the small fraction of the intracellular inorganic pyrophosphatase that is released into the medium during spheroplast formation (Table I), presumably due to damage to a small number of cells.

TABLE V: Hydrolysis of Uridine-^{14}C-Labeled UDP-D-glucose in the Presence of UMP.[a]

Expt	Time	Uridine		UMP		α-D-Glucose-1-P
		μmoles	Sp Act.	μmoles	Sp Act.	(μmoles)
I	0	0	0	1.58	606	0.011
	30	0.92	6400	0.8	1350	0.258
II	0	0	0	1.85	695	0
	70	1.39	7500	0.71	1580	0.385

[a] The reaction mixtures contained 60 μmoles of Tris-Cl (pH 8.0), 6 μmoles of $MgCl_2$, 0.6 μmole of EDTA, 2 μmoles of UDP-D-glucose uridine-^{14}C (28,000 cpm/μmole), 1.7 μmoles of UMP, and 0.01 unit of enzyme in expt I and 0.005 unit of enzyme in expt II. At the times indicated an aliquot was removed and the reaction was stopped by heating at 100° for 1 min. The samples were then assayed for glucose-1-P with phosphoglucomutase and glucose-6-P-dehydrogenase and a 0.15-ml aliquot was placed on a 0.5 × 2.0 cm Dowex 1-X8 (formate) column. The column was washed with 12 ml of 0.01 N formic acid to elute uridine and with 12 ml of 4 N formic acid to elute UMP (Glaser *et al.*, 1967). The samples were evaporated to dryness and dissolved in H_2O. The nucleoside concentration was determined from the absorbancy at 262 mμ using an extinction coefficient of 10^4. Radioactivity was determined in a liquid scintillation counter.

The enzyme showed no metal requirement, although it was somewhat more stable in buffers containing Mg^{2+}. Addition of EDTA during the assay of the enzyme or dialysis against large volumes of Tris, resulted in no loss of enzyme activity.

In Figure 6 is demonstrated the inactivation of the enzyme at 58°. As can be seen the ability to hydrolyze UDP-D-glucose, CDP-ribitol, and UMP decrease together, consistent with the idea that these reactions are catalyzed by the same enzyme.

If the 5′-nucleotidase and the nucleoside diphosphate sugar hydrolase activities are catalyzed by the same protein, then the reaction can be described in a simplified way as follows (where E = enzyme)

$$E + \text{UDP-Glu} \rightleftharpoons \text{E-UDP-Glu} \longrightarrow \text{E-UMP} + \text{glucose-1-P}$$
$$\text{E-UMP} \rightleftharpoons \text{E} + \text{UMP}$$
$$\text{E-UMP} \longrightarrow \text{E} + \text{uridine} + P_i$$

If the presumed enzyme UMP complex equilibrates slowly with free UMP relative to rate of the reaction, then when the enzyme is allowed to act on a mixture of uridine-^{14}C-labeled UDP-glucose and unlabeled UMP, the uridine formed should have a higher specific activity than the UMP as we have previously shown to be the case with a uridine diphosphate sugar hydrolase from *E. coli* (Glaser *et al.*, 1967). The results of such experiments with the nucleoside diphosphate sugar hydrolase from *B. subtilis* W-23 are shown in Table V which shows that the uridine from UDP-glucose did not equilibrate with the UMP pool, in accord with the proposed mechanism.

The K_m values for UDP-glucose and other substrates are surprisingly low. From the data in Figure 7, the K_m of UDP-D-glucose can be calculated as 1.8×10^{-6} M. Both CDP-glycerol and CDP-ribitol are competitive inhibitors of the hydrolysis of UDP-glucose, the K_i for CDP-ribitol is 4×10^{-7} and the K_i for CDP-glycerol is 1.6×10^{-6} M.

In Table VI are listed a variety of nucleotides which have been tested as inhibitors of UDP-D-glucose hydrolysis at single nucleotide concentrations. As can be seen, the enzyme shows very little nucleotide specificity and all these nucleotides must have an affinity of the same order of magnitude as UDP-D-glucose since they showed significant inhibition when added to a reaction mixture in a concentration equivalent to that of the UDP-D-glucose used as a substrate. Inorganic

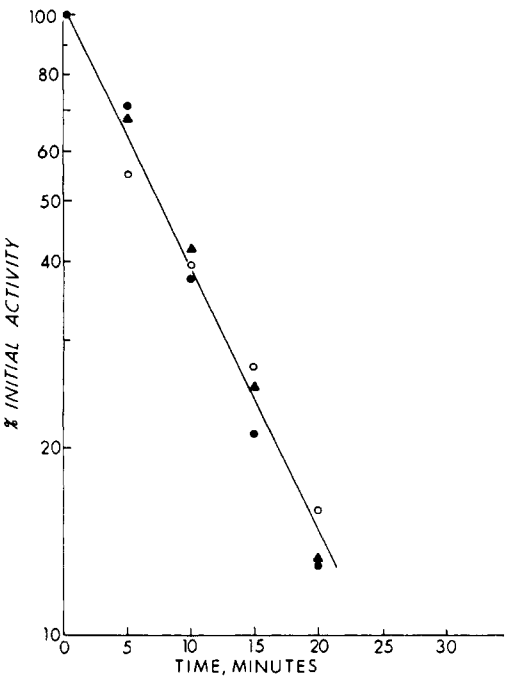

FIGURE 6: Heat inactivation of nucleoside diphosphate sugar hydrolase. Purified enzyme (0.022 unit) was heated at 58° in 0.3 ml of 0.10 M Tris-Cl (pH 8.0)–0.01 M $MgCl_2$–0.001 M EDTA for the times indicated, cooled, and assayed for enzyme activity at 25°. The activities before heating were UDP-D-glucose hydrolysis, 0.022 μmole/min; CDP-ribitol hydrolysis, 0.0078 μmole/min; 5′-nucleotidase, 0.0354 μmole/min. (●) UDP-D-glucose hydrolysis, (○) CDP-ribitol hydrolysis, and (▲) 5′-nucleotidase.

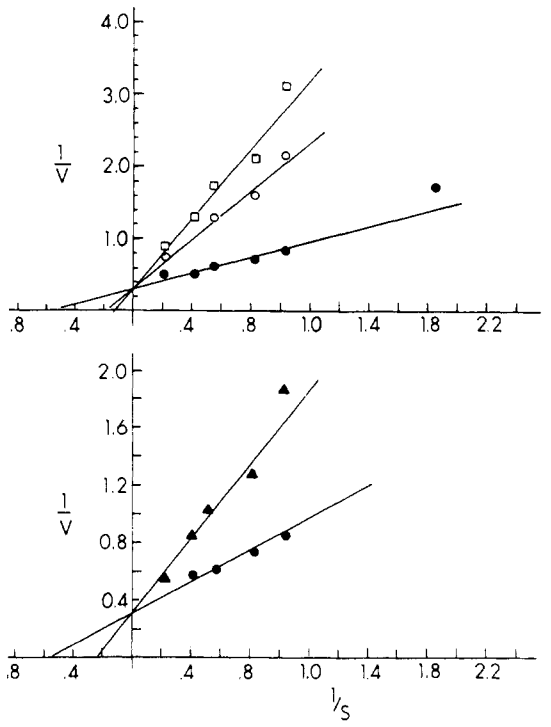

FIGURE 7: Effect of substrate concentration of enzyme velocity. Assay B was used with varying concentration of UDP-glucose as indicated. (●) No inhibitor, (○) 1.8×10^{-6} M CDP-ribitol, (□) 3.5×10^{-6} M CDP-ribitol, and (▲) 2.2×10^{-6} M CDP-glycerol. S = concentration on UDP-D-glucose, M $\times 10^{-6}$; v = velocity in cpm $\times 10^{-3}$.

TABLE VI: Inhibition of Nucleoside Diphosphate Sugar Hydrolase by Various Substrates.[a]

Substrate	Inhibitor	% Inhibn
UDP-D-glucose	None	0
	DPN^+	73
	AMP	69
	CMP	60
	GMP	74
	UMP	50
	ADP-glucose	58
	CDP-glucose	48
	GDP-glucose	72
	GDP-mannose	67
	CDP-glycerol	52
p-Nitrophenyl phosphate	None	0
	CDP-ribitol	92
Bis(*p*-nitrophenyl phosphate)	None	0
	CDP-ribitol	54

[a] The hydrolysis of UDP-D-glucose-^{14}C was measured with assay B. Inhibitors were added at equimolar concentration to that of the UDP-glucose. The hydrolyses of *p*-nitrophenyl phosphate and bis(*p*-nitrophenyl phosphate) were measured as described under Materials and Methods. When indicated, 0.4 μmole of CDP-ribitol was added to the reaction mixture.

phosphate inhibited the enzyme 50% at a concentration of 3×10^{-3} M. This inhibition appeared to be independent of the substrate level.

Although the enzyme will utilize a variety of nucleotides as substrate, the data in Table VII show that the maximal velocity of various nucleoside diphosphate glucose and a variety of 5′-nucleoside monophosphates is a function of the purine or pyrimidine base. This observation implies that the catalytic efficiency of the enzyme is in a subtle way determined by the structure of the nucleoside base bound to it in what can be formally described as induced fit (Koshland and Neet, 1967). It should be noted that the structure

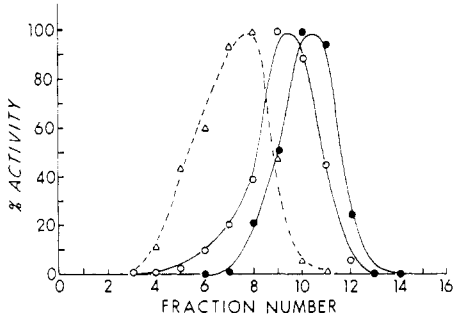

FIGURE 8: Sucrose density centrifugation of nucleoside diphosphate sugar hydrolase. Nucleoside diphosphate sugar hydrolase, together with marker skeletal muscle, pyruvic kinase, and lactic dehydrogenase was centrifuged in a 4–20% sucrose gradient in 0.1 M Tris-Cl (pH 8.0)–0.01 M $MgCl_2$–0.001 M EDTA for 12 hr at 38,000 rpm at 3° in a Spinco SW 39 rotor. Fractions were collected as described by Martin and Ames (1961). The activities are expressed as per cent of the activity of the most active fraction. The 100% values are (●) nucleoside diphosphate sugar hydrolase 0.021 μmole/min per ml; (○) lactic dehydrogenase 16.5 μmoles/min per ml; (△) pyruvic kinase 4.3 μmoles/min per ml.

TABLE VII: Velocity of Nucleoside Diphosphate Sugar Hydrolase with Various Substrates.[a]

Nucleotide	Velocity Rel to UDP-D-glucose Hydrolysis
UDP-D-glucose	1
CDP-D-glucose	0.40
ADP-D-glucose	0.35
GDP-D-glucose	0.05
dTDP-D-glucose	0.018
UMP	1.22
CMP	0.78
AMP	2.10
GMP	1.35

[a] Assay A was used to measure the hydrolyses of nucleoside diphosphate sugars, using enzyme eluted from Sephadex G-200. The hydrolysis of 5′-nucleotides was measured by the release of inorganic phosphate. All values are average of two or three separate assays.

of the substrate has a much more pronounced effect when the enzyme catalyzes the hydrolysis of nucleoside diphosphate sugars as compared with when it catalyzes the hydrolysis of 5′-nucleotides.

We have obtained a rough estimate of the molecular weight of the nucleoside diphosphate sugar hydrolase by sucrose density gradient centrifugation (Figure 8). Because of the possibility that the enzyme when released from the cells is initially attached to a membrane fragment, we have also examined crude enzyme preparations with essentially the same result. Using a molecular weight of 150,000 for lactic dehydrogenase and 237,000 for pyruvic kinase, the data in Figure 8, calculated by the method of Martin and Ames (1961), yield a molecular weight of 137,000 for the nucleoside diphosphate sugar hydrolase.

Discussion

The nucleoside diphosphate sugar hydrolase from *B. subtilis* W-23 bears a striking resemblance to the UDP sugar hydrolase from *E. coli* (Glaser *et al.*, 1967; Neu and Heppel, 1965; Neu, 1967). Both of these enzymes appear to be periplasmic and both show 5′-nucleotidase activity. The *B. subtilis* enzyme, unlike the *E. coli* enzyme, shows very little nucleotide specificity although it is clear that different nucleotide substrates have slightly different apparent Michaelis constants and different maximal velocities.

Of the substrates we have examined the enzyme shows the highest apparent affinity for CDP-ribitol. Whether this is any indication of its physiological substrate remains to be determined.

We use the word periplasmic to indicate that the enzyme is specifically released during spheroplast formation and is available to external substrate in intact cells, but we specifically would like to avoid the implication that the enzyme is not attached to the cell surface. Indeed, there is evidence for several periplasmic proteins in *E. coli* for specific attachment sites for these enzymes on the cell surface (Anraku, 1968; Ward and Glaser, 1968, 1969), and that these enzymes are not simply suspended in a periplasmic space.

It has been suggested the periplasmic enzymes in gram-positive organism are exoenzymes in gram position bacteria (Cashel and Freese, 1964). Although a number of hydrolytic enzymes are in fact secreted into the medium by various strains of *B. subtilis*. The nucleoside diphosphate sugar hydrolase has a considerably larger molecular weight than any of these proteins or than most periplasmic enzymes of *E. coli* for which the molecular weights have been determined, and it may be impossible for a protein of this size to cross the cell wall.

The release of the nucleoside diphosphate sugar hydrolase during spheroplast formation is clearly not primarily determined by molecular size. Thus, the inorganic pyrophosphatase (mol wt 58,000) (Tono and Kornberg, 1967) is not released during spheroplast formation.

It has previously been reported by Taniguchi and Tsugita (1966) that *B. subtilis* grown under conditions of phosphate limitation produces two enzymes capable of hydrolyzing bis(*p*-nitrophenyl phosphate). One is excreted into the medium and the other is a cellular protein.

It is possible that the cellular phosphodiesterase reported by these authors is the nucleoside diphosphate sugar hydrolase that we have described. The properties of the two enzymes are similar although these authors did not examine the hydrolysis of nucleotides by their phosphodiesterase, nor did they examine whether this enzyme was released during spheroplast formation.

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