Phosphatase, Esterase, N-Acetylglucosaminidase, and Adenosine Triphosphatase of Group A Streptococci[1] (35639)

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(Introduced by T. N. Harris)

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Group A streptococci produce many extracellular antigens during growth in chemically defined media (1–3). Of these exoproducts, 6 have been identified as enzymes: deoxyribonuclease, ribonuclease, hyaluronidase, amylase, proteinase, and nicotinamide dinucleotidase. Stock *et al.* (4) described the presence in concentrated group A streptococcal culture supernates of an alkaline esterase which hydrolyzed β-naphthyl acetate (BNA). Lynn (5), first reported on the presence of alkaline and acid phosphatase activities in washed streptococci and streptococcal L-forms, but not in culture supernates. No further details on the properties of the esterase and phosphatase have been reported.

In the present investigation washed streptococci and streptococcal L-forms have been shown to have, in addition to acid phosphatase activity, an alkaline esterase and an *N*-acetylglucosaminidase (NAGAase) (6). Furthermore, cell-free extracts of streptococci and L-forms have been found to contain adenosine triphosphatase (ATPase), in addition to phosphatase, esterase, and NAGAase. This report also describes some preliminary experiments on the partial purification of these enzymes.

Materials and Methods. Streptococcal strains and L-forms. The following streptococcal strains and L-forms were obtained from the stock cultures of the Laboratory of Microbiology, NIAID, NIH.: group A, types 1, 2, 3, 4, 5, 6, 8, 10, 11, 12, 17–41, and 25, one strain each of groups C and G and one ADA L-form derived from group A, type 14 streptococci. Two group A-variant strains, K34 (type 1) and A 27 (type 27), were kindly supplied by Dr. R. Krause of the Rockefeller University, New York. The streptococci were cultivated in brain–heart infusion broth (Difco), the bacterial cells were harvested from different phases of growth, washed, and resuspended in saline solution (0.85% NaCl), and adjusted to 400 Klett units at 540 μm (Klett Summerson colorimeter, Model 800-3). Such streptococcal suspensions contained 5 mg of dry weight/ml and 0.1-ml amounts of cell suspension were employed in enzyme assays (see below). Strain ADA L-forms were cultivated in trypticase soy broth (BBL) containing 3% NACl. The L-forms were washed twice in saline and resuspended to 400 Klett units (540 μm) in 3% NaCl. A lyophilized preparation of L-forms (group A, type 19) was obtained from Dr. C. Panos, Albert Einstein Medical Center, Philadelphia, Pa.

Extracellular products. Concentrated streptococcal extracellular products (SEP) were obtained from group A, type 4 (SEP-A) and from group C (SEP-C) streptococci, all grown in a chemostat in a synthetic medium (1). These products were prepared and kindly supplied by Dr. T. N. Harris of The Children's Hospital of Philadelphia.

Cell-free extracts. a. Mechanical distintegration. 10-ml suspensions of type 4 streptococci were subjected to mechanical disinte-

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gration for 30 min at 4° in a Mickle disintegrator, using glass beads 0.003 in. in diameter (3M Co., St. Paul, Minn). The extent of disintegration was monitored spectrophotometrically. The cell debris was removed by centrifugation at 100,000*g* for 60 min. The supernatant fluid (SF) was filtered through a 0.22/μ Millipore filter and the proteins in the extract were precipitated by full saturation with ammonium sulfate and redissolved in distilled water. The extract was then freed of ammonium sulfate by passage through a column of Sephadex G-25 (20 × 1 cm) equilibrated with saline. The insoluble pellets (PT) derived by ultracentrifugation were resuspended in saline and recentrifuged at 100,000*g*. The supernatant fluid was discarded; and the pellet was resuspended to the original volume in saline. The SF contained approximately 3 mg of protein/ml by the procedure of Lowry *et al.* (7).

b. Phage-lysin extracts were prepared from 10-ml suspension of Type 4 streptococci following treatment with group C streptococcal phage-associated lysin (8). Supernatant fluids and pellets of such preparations were obtained as described above. The supernatant fluid contained approximately 5 mg of protein/ml.

c. L-forms. ADA and GL8 L-forms were lysed by repeated freezing and thawing five times. The suspensions were then centrifuged at 100,000*g* for 30 min, and both the supernatant fluids and insoluble pellets were assayed for enzymes (see below). The supernatant fluid contained approximately 3 mg of protein/ml.

Gel filtration and ion-exhange chromatography. Cell-free extracts of the streptococci were subjected to gel filtration on Sephadex G-200 columns (70 × 2.0 cm) and to ion-exchange chromatography on DEAE; Ecteola, and Sulfoethyl-Cellulose, (Bio-Rad Laboratories, Richmond, Calif.) using stepwise salt elution techniques (9). Enzyme activities were assayed on the various fractions eluted from the columns.

Substrates. The following substrates were employed: *p*-nitrophenyl-phosphate (PNPP), Sigma Chemical Co., St. Louis, Mo.; *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (PNP-NAGA) and *o*- and *p*-nitrophenyl-β-D-galactopyranoside (PNP-GAL), Calbiochem, Los Angeles, Calif.; *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide (PNP-NAGAL), Cyclo, Los Angeles, Calif., *p*-nitrophenyl and β-naphthyl derivatives of acetic, propionic, butyric, lauric, myristic, palmitic, and stearic acids, (Nutritional Biochemical Co., Cleveland, Ohio.) PNPP, PNP-NAGA, PNP-NAGAL, PNP-GAL, and the PNP derivatives of acetic, propionic, and butyric acids were dissolved in water, while the other PNP derivatives were dissolved in ethylene glycol monomethyl ether (Methyl Cellosolve, Fisher, Fairlawn, N.J.). β-naphthyl derivatives were dissolved in acetone and jetted into distilled water. Adenosine-5-triphosphate (ATP), adenosine-5-diphosphate (ADP), adenosine-5-phosphate (AMP), uridine triphosphate (UTP), cytidine triphosphate (CTP), inosine triphosphate (ITP), and guanosine triphosphate (GTP) were obtained from Sigma Chemical Co., St. Louis, Mo. Alpha- and β-glycerophosphate, *o*-phosphorylcholine, glycerophosphocholine, spermidine phosphate, creatine phosphate and *o*-phosphoryl ethanolamine were obtained from Nutritional Biochemical Co., Cleveland, Ohio.

Buffer solutions. Solutions of 0.1 *M* acetate buffer (pH 4.5–6.5), 0.1 *M* citrate buffer (pH 5.5), 0.1 *M* phosphate buffer (pH 7.0–7.8). 0.1 *M* Tris (hydroxymethylaminomethane (pH 7.0–9.0) and 0.1 *M* glycine buffer (pH 4.0–12.0) were employed.

Assays of enzyme activities. These were performed on 0.1-ml aliquots either of washed streptococcal suspensions or L-forms, or of cell-free extracts.

a. Phosphatase. A reaction mixture of 0.1 ml of enzyme preparation, 0.6 ml of 0.1 *M* acetate buffer (at pH 5.5), 0.1 ml of 0.1 *M* $Mg\ Cl_2$, and 0.2 ml of 0.01 *M* PNPP was incubated at 37° for 30 min. The reaction was stopped and the color was developed by the addition of 1 ml of 0.2 *N* NaOH. PNP release was determined colorimetrically at 420 μm (Klett). One unit of phosphatase is defined as the amount of enzyme which releases 1 μmole/min of PNP.

b. Esterase. Esterase activity was determined by the method of Hardin *et al.* (10),

using BNA as the substrate. One unit of esterase is defined as the amount of enzyme which releases 1 μmole of β-naphthol/min.

c. NAGAase and NAGALase. Assays were performed in 0.1 *M* acetate buffer (pH 5.5), utilizing 0.25 ml of 0.01 *M* PNP-NAGA or PNP-NAGAL. The release of PNP was determined as described in the assay of phosphatase. One unit of enzyme is defined as that amount of enzyme which releases 1 μmole/min of PNP.

d. ATPase. Assays were performed in 0.1 *M* acetate buffer (pH 5.5), utilizing 0.1 ml of 0.5 *M* ATP and 0.1 ml of 0.1 *M* $MgCl_2$. Following incubation for 45 min at 37° the amount of inorganic phosphate (P_i) released was determined by the method of Fiske and Subbarow (11). ATPase activity was given in micrograms of P_i released/min.

pH optima. The pH optima of the various enzymes were determined on cell-free extracts of streptococci following ultracentrifugation and gel filtration on Sephadex G-200 as well as on solutions of SEP-A. Aliquots of enzyme solutions (0.25 ml) were incubated with the various substrates in 0.5 ml of the appropriate 0.1 *M* buffers; and assays were performed as described above.

Inhibitors and activators. The following compounds were tested: 0.001, 0.01, and 0.1 *M* $ZnSO_4 \cdot H_2O$, $CuSO_4 \cdot 5H_2O$, $FeCl_3 \cdot 6H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, $HgCl_2$, NaF, and iodoacetic acid (IAA), ethylene diaminotetraacetic acid (EDTA) and NaN_3 NaCl was used at various concentrations from 0.02 to 2 *M*. *N*-Acetylglucosamine, glucose, galactose, galactosamine, and rhamnose were used at 0.05–0.5 *M*. C-Polysaccharide and mucopeptide were used at 5 mg/ml.

Results. The presence of enzymes in washed streptococcal suspensions. Phosphatase activity was found in all streptococcal strains tested, ranging between 5 and 10 units/ml of streptococcal suspension (5 mg of dry wt). NAGAse was found in all group A streptococci and in the group C strain tested (1.0–3.0 units/ml of streptococci). Esterase activity was present in all group A, group C, and group G strains (1.0–5.0 units). ATPase activity was not detected in any of the washed suspensions, appearing only after the cells had been broken. All 4 enzymes were found in washed suspensions of ADA L-forms (phosphatase, 1 unit; NAGAase, 2.5 units; esterase, 2.0 units; and ATPase, 0.5 units/ml of suspension), and in crude membranes isolated from GL-8 forms (phosphatase, 2.0 units; NAGAase, 0.4 units/mg of dry wt; and traces of esterase). ATPase was not assayed in the membranes. Also, highly purified L-form preparations (AED) were found to contain 2.0 units of phosphatase and 0.4 units of NAGAase/mg of dry weight, and traces of esterase. The activity of the various enzymes in streptococcal suspensions varied with their phase of growth. Phosphatase activity increased slightly over the growth period, reaching maximal values at the end of the logarithmic phase of growth. NAGAase activity was constant throughout the growth curve. Esterase activity was highest at the early logarithmic phase of growth and declined markedly thereafter. Streptococci frozen at —25° for long periods of time retained their enzymatic activity. All 4 enzymes were also found in SEP-A, with 23 units of phosphatase, 11 units of esterase, 0.5 units of NAGAase, and 0.6 units of ATPase/mg of protein.

In experiments designed to isolate the various enzymes from the bacterial cells, it was found that the extracts obtained from 1 ml of streptococcal suspension by mechanical means (Mickle disintegrator) or by group C phage-associated lysin, showed ATPase activity in addition to the other 3 enzymes. The distribution of enzymatic activity in supernatants and pellets of disrupted streptococci and L-forms is shown in Table I. More than 90% of the NAGAase and ATPase activity was released by both Mickle disintegration and phase-associated lysin; while phosphatase and esterase were equally distributed between supernatant fluids and pellets. Usually there was 90–100% recovery of the total enzymatic activity. Slightly different distributions were obtained from ADA L-forms (Table I). Attempts to release more enzymes from pellets of both streptococci and L-forms by use of detergents failed (Tween 40, Triton X-100, and Triton X-305, at 1 mg/

TABLE I. Distribution of Enzymes in Fractions of Streptococci and L-forms.

Source	Treatment	Fraction	Total Activity (%)[a]			
			Phosphatase	Esterase	NAGAase	ATPase
Streptococci	Phage lysin[b]	Supernate	47	49	93	98
		Pellet	53	51	7	2
ADA L-forms	Osmotic lysin	Supernate	62	65	71	53
		Pellet	38	35	29	47

[a] Total amounts of enzyme per milliliter of streptococcal suspension: Phosphatase, 8 units; esterase, 3 units; NAGAase, 1 unit. No ATPase activity was detected in unbroken cells. Total amounts of enzyme per milliliter of L-form suspension: Phosphatase, 1 unit; esterase, 2 units; NAGAase, 2.0 units; ATPase 0.5 unit.

[b] Results were similar for Mickle disintegration except for a 2–3-fold increase in the total yield of esterase.

ml). In other experiments it was found that these detergents caused an increase of 25% in the activity of phosphatase, esterase, and NAGAase.

Separation of the enzymes by gel filtration. Cell-free extracts derived from a type 4 streptococcus by mechanical disintegration and ultracentrifugation were subjected to gel filtration on Sephadex G-200. Figure 1 shows that the phosphatase was eluted from the column with the void volume associated with the high molecular weight fraction. This fraction also contained a cell-sensitizing factor (12) and traces of streptolysin O. NAGAase emerged from the column in a sharp peak immediately following phosphatase, but complete separation of the 2 enzymes could not be achieved. On the other hand, ATPase activity was concentrated in a fraction of lower molecular size which was completely devoid of phosphatase and NAGAase activity. Under the same conditions esterase activity deteriorated markedly, and the small amounts eluted from the column were associated with

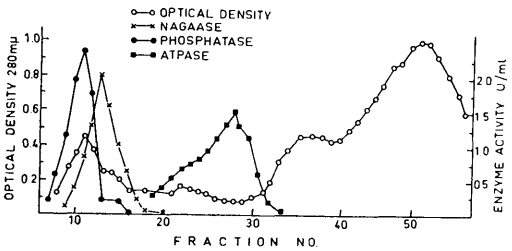

FIG. 1. Distribution of phosphatase, NAGAase, and ATPase in cell-free extracts of type 4 streptococci; gel-filtration on Sephadex G-200.

both high and low molecular weight fractions. Treatment of the extracts with DNAse and RNAse prior to gel filtration did not improve separation.

Ion-exchange chromatography of cell-free extracts. Supernatant fluids derived from streptococci by mechanical disintegration were subjected to chromatography on a variety of anion and cation exchangers (9). Phosphatase, esterase, and NAGAse activities were strongly bound to DEAE-Sephadex columns equilibrated with 0.05 *M* phosphate at pH 7.4. (ATPase was not assayed in any of the following experiments.) Only 5–10% of the activity of the various enzymes could be eluted from the columns by stepwise elution up to 1 *M* NaCl, and no separation among the various enzymes could be achieved. Yields were not improved by using less strongly charged exchangers such as ECTEOLA-cellulose or aminoethyl-cellulose, or by pretreatment of the extracts with nucleases.

Properties of the enzymes. Since preparations of the enzymes separated from each other were not available, studies on some of the properties of the enzymes were performed on fractions of cell-free extracts eluted from Sephadex G-200 columns (Fig. 1).

Activators. Both phosphatase and ATPase activities were enhanced by Mg^{2+} at 0.01 *M* (Table II). Phosphatase activity was increased 2- to 3-fold, while ATPase was enhanced at least 10-fold. Zn^{2+} (0.01 *M*) could replace Mg^{2+} as an activator of ATPase. None of the metal compounds tested were

TABLE II. The Effect of Various Agents on Enzyme Activity.

Compound tested	Conc (M)	Inhibition or activation[a] (%)			
		ATPase	Phosphatase	Esterase	NAGAase
Hg^{2+}	0.01	68	15	90	84
Ca^{2+}	0.01	+25[b]	18	0	0
Co^{2+}	0.01	+37	30	0	26
Cu^{2+}	0.01	0	36	90	87
Fe^{3+}	0.01	0	52	0	23
Zn^{2+}	0.01	+1000	50	90	65
Mg^{2+}	0.01	+1000	+250	0	0
EDTA	0.001	50	17	25	0
NaF	0.01	0	100	0	0
Iodoacetate	0.01	0	25	0	0
NaN_3	0.001	50	0	85	0
NaCl	0.4	50	ND[c]	ND	ND

[a] ATPase, phosphatase, and NAGAase activities were obtained from fractions of cell-free extracts eluted from Sephadex G-200 (Fig. 1). The source of esterase was the unfractionated cell-free extract.

[b] Activation.

[c] ND = not done.

found to enhance the activity of esterase or NAGAase.

Inhibitors. The strongest inhibitor of phosphatase was NaF (0.01 M), while Hg^{2+}, Cu^{2+}, Zn^{2+}, and NaN_3 showed the strongest inhibition of esterase (Table II). NAGAase was inhibited maximally by Hg^{2+}, Cu^{2+}, and Zn^{2+}, while ATPase activity was markedly affected by Hg^{2+}. ATPase was also inhibited by NaCl, with 50% inhibition occurring at approximately 0.4 M. NAGAase activity was strongly inhibited by NAGA, glucosamine, and glucose, 50% inhibition being attained with 0.02, 0.033, and 0.045 M, respectively (Fig. 2). No inhibition occurred with NAGAL, galactosamine, galactose, rhamnose, or streptococcal mucopeptide.

Substrate specificity. Appropriate fractions eluted from Sephadex G-200 columns were assayed for their activity against a variety of fatty acid esters and nucleotides. The fractions which split PNPP did not split α- or β-glycerophosphate, creatine phosphate, or ATP. The same peak was found to hydrolyze PNP-acetate and PNP-propionate (74 and 29%, respectively, of the value for PNPP), but neither the longer chain fatty acid derivatives nor PNP-sulfate were split.

Fractions containing ATPase activity also split CTP, ITP, and GTP at 90, 40, and 40% of the ATP values, respectively. Preincubation of the enzyme with AMP (0.01 M) for 10 min at 37° produced at 50% inhibition of ATPase activity.

Fractions containing NAGAase activity did not split PNP-NAGAL, PNP-glucopyranoside, or PNP-galactopyranoside, but did hydrolyze PNP-glucuronide, indicating that this fraction also contained glucuronidase activity. The latter substrate was not split by whole cells, and no requirement for Mg^{2+} was found. Fractions containing NAGAase activity were concentrated 10–

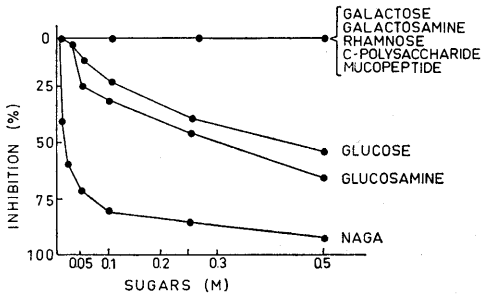

FIG. 2. Inhibition of NAGAase by sugars and cell-wall components of group A streptococci. Sugars tested at molarities as shown. The C-polysaccharide and mucopeptide were tested at 5 mg/ml.

20-fold by ammonium sulfate precipitation and desalted by gel filtration on Sephadex G-25. Prolonged incubation of this preparation with purified C-polysaccharide or with streptococcal cell walls (both 10 mg/ml) did not result in the release of free NAGA (13).

Esterase activity was assayed on unfractionated Mickle supernatants, since most of the activity was lost on the columns. In addition to BNA, activity was present against β-naphthylbutyrate only (15% release of β-naphthol compared to NA). None of the longer chain fatty acid derivatives were split.

Differentiation between ATPase and phosphatase. Tosteson *et al.* (14) have shown that membranes of red blood cells contain both acid ATPase and acid phosphatase activities. A differentiation between the two enzymes was made by the selective inhibition of ATPase by PNP while phosphatase activity was inhibited at ATP. Similar experiments were performed on streptococcal supernatants derived by mechanical disintegration and ultracentrifugation. Preincubation with PNP (0.015 *M*) caused a 50% inhibition of ATPase activity while no inhibition was obtained with PNPP (0.02 *M*). Phosphatase activity, on the other hand, was inhibited 50% by ATP (0.001 *M*) or by ADP and AMP (0.1 *M*) but not by CTP, GTP, UTP, or ITP.

Sodium azide (0.075 *M*) caused a 50% inhibition of ATPase activity, while phosphatase activity was unaffected. At 0.5 *M* NaN_3, a 30% inhibition of phosphatase was found, compared with an 80% inhibition of ATPase.

pH optima. The optimum for phosphatase and NAGAase was pH 5.5, in either acetate or citrate buffers. The optimum for esterase was pH 9 in either Tris or phosphate buffers. (Esterase activity was, however, routinely measured in phosphate at pH 7.5, because of the instability of the substrate BNA at higher pH.) Maximal ATPase activity occurred at pH 5–6 in either glycine or acetate buffers. Tris buffer was found to be inhibitory to ATPase.

Heat inactivation. The various enzymes differed in their resistance to heat: 50% of the activities of phosphatase and esterase were lost after 30 min at 39°, 50% of ATPase activity was lost at 46°, while NAGAase withstood heating at 55° and declined to 50% at 60°.

Discussion. The present data give the localization and some of the properties of a phosphatase, esterase, NAGAase, and ATPase of Group A streptococci and their L-forms. Phosphatase, esterase, and NAGAase activities were present in washed streptococcal cells and L-forms, supernatant fluids from steady-state cultures of streptococci, and cell-free extracts of streptococci and L-forms. These 3 enzymes appear to be present uniformly in almost all Group A streptococcal strains. ATPase however, was found only in cell free extracts, and in supernates of streptococcal cultures (SEP-A).

The acid phosphatase described in this report is probably the same as that described by Lynn (5). We have confirmed his observations, and have also found this enzyme in cell-free extracts and culture supernatants, in addition to washed streptococci and L-forms. However we failed to find an alkaline phosphatase in streptococci, as described by Lynn (5). The fact that only 50% of the total activity of this enzyme can be released by disrupting the organisms, and that it is present in highly purified L-form membranes, would indicate that, at least in part, this enzyme may be considered to be membrane-bound. This is consistent with data available on phosphatases of other organisms (15, 16). From its behavior in gel filtration, it can be concluded that the enzyme is associated with high molecular weight material and may be bound to large molecular fragments of cell wall or membrane not sedimented at 100,000*g*. The enzyme is similar to other acid phosphatases in its substrate specificity, Mg^{2+} requirement, and inhibition patterns. It is also present in the most purified toxic fraction of SEP-A, and its role in the pathogenicity of this fraction is at present under study (Ginsburg and Gallis, unpublished data). The role of this enzyme in the metabolism of streptococci is unknown.

This report corroborates the finding of Stock *et al.* (4) of an alkaline esterase in

concentrated culture supernatants of Group A streptococci. In addition, we found the enzyme to be present in washed streptococci, cell-free extracts, and in intact L-forms. However, only trace amounts were found in purified (washed) L-form membranes. The distribution in supernates and pellets of distrupted organisms was similar to that of the acid phosphatase. The inactivation of the enzyme during fractionation precluded study of more purified preparations. The role of this enzyme in the metabolism of streptococci is also unknown.

NAGAases have recently been described in a variety of microorganisms (17, 18). Woollen *et al.* (17) sought this enzyme in a variety of washed organisms, including Streptococcus pyrogenes, but found no trace of the enzyme in the latter. However, we have found activity in all strains of Group A streptococci tested. In contrast to the phosphatase and esterase, the NAGAase is readily solubilized during disruption of the organisms. This enzyme could be partially separated from the phosphatase by gel filtration (Fig. 1). Fractions containing NAGAase activity did not hydrolyze PNP-NAGAL, differing from the finding of Woollen *et al.* (17), who showed that NAGAase of other bacteria was usually associated with NAGALase activity. The NAGAase described here has many properties in common with the NAGAase of *D. pneumoniae* (18): pH optimum of 5.5, inhibition by Hg^{2+}, and lack of divalent cation requirement for activation.

The presence of this enzyme in Group A streptococci is especially interesting in relation to the possible role of such enzyme in the synthesis and/or degradation of the cell wall. Unlike the NAGAase of mammalian macrophages (19) we could not demonstrate any release of NAGA from highly purified C-polysaccharide or group A cell walls. It is also of interest that group A-variant streptococci, whose cell walls contain only small amounts of NAGA, were found to have large amounts of the enzyme. This is interesting in light of the finding (20) that A-variant organisms grown at 22° regain their reactivity with group A antisera, indicating that the synthesis of NAGA into cell walls may be temperature dependent. Therefore, it would be of interest to study the role of NAGAase in the synthesis and degradation of cell wall in A-variant strains.

ATPase activity of group A streptococci was completely solubilized by treatment with phage-associated lysin or mechanical disruption (in the absence of Mg^{2+} or Ca^{2+}), and could not be sedimented at 100,000*g*. This differs from the findings (21, 22) that ATPase activity of Group H streptococci would be sedimented at 105,000*g* (in the absence of divalent cations, but in 0.5 *M* sucrose), or that ATPase of group D streptococci were released from membranes with repeated washings (8–10×) in the absence of Mg^{2+} or Ca^{2+}. As shown above, release of the enzyme from Group A L-forms was more difficult, *i.e.*, 50% release vs 98% release in the parent strain. This difference may be related to the extraction procedure, as purified L membranes (repeated washings) contained no measurable ATPase activity, yet did retain phosphatase activity. This ATPase was fully activated by Mg^{2+} or Zn^{2+}, and slightly by Ca^{2+} (25%). This dependence upon divalent cations is the rule for ATPases. Inhibition patterns were not unusual. The specificity of this enzyme was demonstrated by its greater activity against ATP than against other nucleotide phosphates, and its partial inhibition by AMP and ADP. The separation pattern by gel filtration indicates that this enzyme is either somewhat smaller in size than the phosphatase and NAGAase or that it is not associated with wall or membrane fragments. Further studies on the purification and localization of the enzyme in the streptococcal cells may shed more light on their role in the biology of the streptococcus.

Summary. Washed suspensions of group A, C, and G streptococci and group A L-forms possess phosphatase, esterase, and *N*-acetylglucosaminidase, respectively. Cell-free extracts, obtained from streptococci and L-forms by mechanical disintegration or by treatment of group A streptococci with phage-associated lysin, possess, in addition to these enzymes, an adenosine triphosphatase (ATPase). Over 90% of the total

ATPase and NAGAase and 50% of phosphatase and esterase activities were solubilized by cell breakage, indicating that the latter 2 enzymes are membrane-bound.

A partial separation between the phosphatase, NAGAase, and ATPase was achieved by gel filtration of cell-free extracts on Sephadex G-200. Phosphatase was eluted with high molecular weight material excluded from the column. NAGAase and ATPase were associated with much lower molecular weight fractions, while esterase activity was present in both high and low molecular weight fractions. Studies on substrate specificity showed that fractions which split PNP-phosphate also split PNP-acetate and PNP-propionate fractions which split ATPase also split CTP, ITP, and GTP but to a lesser extent. Fractions which were active against β-napthylacetate also split β-naphthyl butyrate (15% efficiency); no activity against longer fatty acid esters of PNP or β-naphthyl derivatives was present.

The activation and inhibition of the enzyme activities by di- and trivalent cations and by sugars is also reported.

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