ISOLATION OF A HIGH MOLECULAR WEIGHT POLYPHOSPHATE FROM *NEISSERIA GONORRHOEAE**

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The existence of a capsule on *Neisseria gonorrhoeae* remains controversial. A number of investigators have provided morphologic proof for its presence (1–4), yet its chemical identity is unknown. In the course of experiments to isolate the gonococcal capsular material we observed that this organism produces considerable amounts of polyphosphate. Polyphosphates are polymers of orthophosphate molecules that are linked together by phosphoric anhydride groups to form unbranched structures (5). Upon hydrolysis of each phosphate group, this type of linkage generates as much energy as the hydrolysis of the orthophosphate from ATP, indicating that polyphosphate is a high energy compound (6). The chain length can range from two to several thousand. Polyphosphate is stable in alkali and labile to acid. The latter property is a characteristic commonly used for its identification (7).

Polyphosphates have been found both in prokaryotes and eukaryotes (8). They usually occur in granules, the so-called volutin or metachromatic granules (i.e., they stain with basic dyes and shift the absorption maximum of these dyes to a shorter wavelength) (9). But they have been shown to be also present at or near the surface of some organisms (10, 11). Synthesis of polyphosphate by the organisms occurs only if phosphate is easily available. The synthesis pattern shows two distinct features. Polyphosphate is usually not present in large amounts in phases of active growth, but is accumulated in situations that are unfavorable to growth, as in the stationary growth phase or during nutritional imbalance, e.g., sulfur deficiency for *Klebsiella* and *Anacystis* (12, 13). Furthermore, polyphosphate formation increases enormously after a period of phosphate starvation. This phenomenon has been called "Überkompensation" (14).

In spite of the fact that the existence of polyphosphate in living organisms has been known since 1890 (15), and that it appears in a wide variety of organisms, its functions are not clearly defined. Two major hypotheses have been suggested, namely, phosphate storage or energy storage (7). To our knowledge, the occurrence of polyphosphate in the genus *Neisseria* has not been reported. In 1943, when Knaysi and Mudd (16) were looking for internal cell structures in bacteria they came to the conclusion that neither *N. gonorrhoeae* nor *N. meningitidis* contained polyphosphate. The closely related *Moraxella* contains poly-beta hydroxybutyrate granules as storage product (17).

We report the isolation and chemical characterization of polyphosphate, and its localization in *N. gonorrhoeae*. We also describe experiments that were designed to show whether polyphosphate synthesis in *N. gonorrhoeae* is dependent on growth phase,

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growth conditions, or phosphate concentration of the medium. These results are compared with those obtained with *Saccharomyces cerevisiae* and *Escherichia coli*, two organisms known to possess polyphosphate (8). Furthermore, we present the results of a survey of several *Neisseria* strains for polyphosphate production.

Materials and Methods

Bacterial Strains and Growth Conditions. The bacterial strains used in these experiments were *N. gonorrhoeae* R10, MS11, F62, 2686, and two strains isolated from patients with disseminated gonorrhea, 1896 and 1385; *N. meningitidis* B M986, M55, and a nonencapsulated variant of M986; *N. flava*, *N. sicca*, *N. lactamica*, *E. coli* B, and *Saccharomyces cerevisiae* (obtained from M. Ehrenberg, University of Würzburg, Federal Republic of Germany). *Neisseria* strains were grown on clear typing media (18) or chemically defined medium (19) as indicated; *E. coli* on Loria Bertani medium (10 g bacto-tryptone, 5 g yeast extract, 5 g NaCl/1,000 ml water), and *Saccharomyces cerevisiae* on YEPD medium as described (20). For the determination of limiting phosphate concentrations, *N. gonorrhoeae* was grown in Hepes-buffered chemically defined medium (30 mM Hepes, pH 7.2) and varying amounts of phosphate and thiamine pyrophosphate. The lowest concentration of phosphorus that allowed growth (although reduced) was 1×10^{-6} M phosphate and 5×10^{-6} M thiamine pyrophosphate.

Analytical Methods. Carbon, hydrogen, and nitrogen analyses were performed by the microanalytical service of The Rockefeller University. Phosphorus was determined by the method of Chen et al. (21) with slight modifications. Samples containing 1–10 μg of phosphorus were dried at 100–120°C, 0.1 ml of a mixture containing equal parts of concentrated sulfuric acid and 70% perchloric acid was added, and the samples were boiled for 90 s. After cooling, 4 ml of water were added and 4 ml of reagent (H_2O/6 N H_2SO_4/2.5% ammonium molybdate/10% ascorbic acid) (2:1:1:1). After 2 h at 37°C the color developed was read at 820 nm. To determine acid-labile phosphate, HCl was added to a 1 M concentration, the samples were hydrolyzed in a boiling waterbath for 15 min and processed as described above. Total nucleic acid content was determined by absorption at 260 nm, DNA content by the diphenylamine assay (22), and protein by the Coomassie blue reaction (23). Sugars were identified by gas liquid chromatography as their alditol acetate derivatives (24).

Identification of Polyphosphate. Polyphosphate was identified by two criteria. (*a*) Hydrolysis: the amount of orthophosphate after hydrolysis in 1 M HCl at 100°C for 15 min is identical to the amount obtained after complete hydrolysis. (*b*) Metachromatic reaction: the experiment was performed as described by Griffin et al. (25).

Isolation and Determination of Polyphosphate. Bacteria were grown in liquid medium or on agar plates, harvested, and washed with 10 mM Tris-HCl, pH 7.4. The Tris-HCl wash was dialyzed in the cold against water and lyophilized. Material obtained from the Tris-HCl supernatant was further purified by column chromatography on DEAE-Sepharose. The column was run in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and eluted with a linear salt gradient, 0–1 M NaCl, in the Tris-EDTA buffer. Polyphosphate-containing fractions were dialyzed against water and lyophilized. For the isolation of polyphosphate from washed cells, two different methods were used depending on the experiment. (*a*) Sodium hypochlorite method (26): The cell pellet was resuspended in a 5.4% sodium hypochlorite solution (7.5 ml/g wet weight of cells), stirred for 45 min at room temperature, and centrifuged at 16,000 *g* for 30 min. The pellet was washed twice with ice-cold 1.5 M NaCl containing 1 mM EDTA and extracted twice with water in the cold. Polyphosphate was precipitated by adding NaCl to 0.1 M and 1 vol of ethanol, and purified further by ion-exchange chromatography as described above. (*b*) Perchloric acid extraction (12): Washed cell pellets were extracted twice with cold perchloric acid (0.5 M) for 5 min, once with ethanol at room temperature, once with an ethanol-ether mixture (3:1) at 96°C for 1 min, and twice with hot 0.5 M perchloric acid at 70°C for 5 min. The latter fraction contained the polyphosphate, which could be precipitated by adding $BaCl_2$ to a final concentration of 25%. The pellet was dissolved in 1 M HCl and the phosphate content determined after hydrolysis by boiling for 15 min.

Enzyme Assays. Glucose-6-phosphate dehydrogenase was assayed by the method of Noltmann et al. (27) and aldolase according to Pinto (28).

Results

Isolation of Polyphosphate from N. gonorrhoeae. Several methods exist for the isolation of polyphosphate. Extraction by cold followed by hot acids (e.g., perchloric acid) is effective. It is based on the fact that high molecular weight polyphosphate is insoluble in cold acid. The disadvantage of this method is that polyphosphate is an acid-labile compound and is partially depolymerized during the acid treatment (7). In the method used by Harold (26) for isolation of polyphosphate from *Klebsiella*, the organisms are rapidly lysed with sodium hypochlorite and centrifuged, and polyphosphate is extracted from the insoluble pellet with water. The latter is the gentler method, enabling the isolation of intact high molecular weight polyphosphate, and was used for the isolation of polyphosphate from *N. gonorrhoeae* after initial studies showed that yields of both methods were comparable.

N. gonorrhoeae was grown on solid chemically defined medium, harvested after 18–20 h of growth, washed with Tris-HCl buffer (10 mM, pH 7.4), pelleted, and digested with NaOCl solution. The material obtained was essentially free of protein but contained a substantial amount of nucleic acid as determined by absorbance at 260 nm, as well as a phosphate-rich compound that could be hydrolyzed by boiling in dilute acid (Table I). To separate the different components, the material was subjected to DEAE-Sepharose chromatography. The fractions were assayed for their nucleic acid content (absorbance at 260 nm) and phosphorus content (Fig. 1). Material with high phosphorus content and little absorption at 260 nm eluted before a nucleic acid peak. The phosphorus-rich fractions were pooled and dialyzed, and the material precipitated with a 1/10 vol of 1 M NaCl and 1 vol of alcohol. Elemental analysis showed a phosphorus content of >20% and almost no carbon or nitrogen (Table II). Boiling in 1 M HCl for 15 min released all the phosphate present when compared with a total hydrolysis, indicating that the material was polyphosphate (Table III). When assayed with toluidine blue, the material caused a shift in the absorbance

TABLE I

Characterization of Polyphosphate from N. gonorrhoeae at Different Stages of Purification

Purification stage	Protein*	Nucleic acid‡	Total phosphorus§	Acid-labile phosphorus‖
			μg/100 μg	
Water extract of the insoluble pellet after NaOCl digestion of cells	1.0	10.0	14.0	12.8
Polyphosphate from NaOCl-digested cells after DEAE-Sepharose chromatography	ND¶	ND	22.0	22.0
Tris-released material	3.0	3.0	6.8	4.0
Tris-released polyphosphate after DEAE-Sepharose chromatography	ND	ND	24.0	24.0

* Protein content was determined by the Coomassie blue assay.
‡ Nucleic acid content was estimated from the absorbance at 260 nm.
§ Phosphorus content after complete hydrolysis.
‖ Phosphorus released by hydrolysis in 1 M HCl.
¶ Not detected.

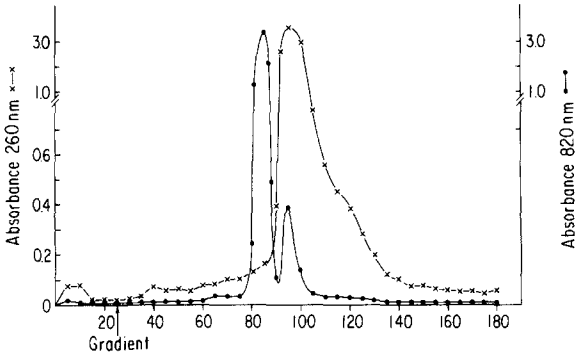

FIG. 1. Separation of the components of NaOCl-extracted *N. gonorrhoeae* R10 on a DEAE-Sepharose column. Chromatography was performed in 10 mM Tris-HCl pH 8.0, 1 mM EDTA, with a linear NaCl gradient from 0 to 1 M. The fractions were monitored for absorbance at 260 nm, and for phosphorus content.

TABLE II

Elemental Analysis of Polyphosphate Obtained From N. gonorrhoeae Cells by NaOCl Digestion and from the Tris-released Material

Polyphosphate preparation	Percent N	Percent C	Percent H	Percent Ash	Percent H_2O	Percent P
Tris-released polyphosphate	2.30	8.06	1.87	72	3.9	23.99
Polyphosphate obtained by NaOCl digestion	0.17	1.2	1.44	83	13.2	22.06

TABLE III

Differential Hydrolysis of Isolated Polyphosphate

Polyphosphate	Phosphorus per 100 μg material after:		
	Complete hydrolysis	Partial hydrolysis (boiling 1 M HCl)	No hydrolysis
		μg	
Isolated			
(*A*)	27.2	26.0	4.0*
(*B*)	22.1	23.0	4.0
Synthetic	27.8	27.0	4.9

Polyphosphate was isolated from either (*A*) NaOCl-digested cells or (*B*) the Tris-wash of intact cells.

* This degree of hydrolysis occurs during the 2-h incubation period at 37°C in the presence of the molybdate reagent.

maximum characteristic for polyphosphate (Fig. 2).

Localization of Polyphosphate in N. gonorrhoeae. Experiments designed to isolate putative capsular material from *N. gonorrhoeae* led to the initial finding of the existence of polyphosphate in the gonococcus. In these experiments *N. gonorrhoeae* cells grown on solid chemically defined medium for 18–20 h at 37°C were scraped off the plates, gently washed with Tris-HCl buffer (10 mM, pH 7.4), pelleted, and the supernatant dialyzed, lyophilized, and analyzed. Usually a yield of 10–20 mg material/g wet

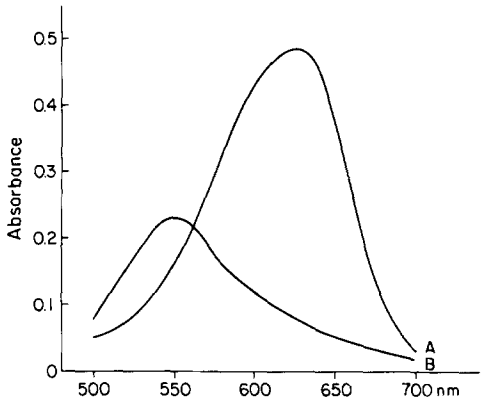

FIG. 2. Absorption spectrum of toluidine blue in the presence and absence of NaOCl-extracted polyphosphate from *N. gonorrhoeae* R10. (*A*) toluidine blue alone; (*B*) toluidine blue in the presence of 50 μg/ml NaOCl-extracted polyphosphate.

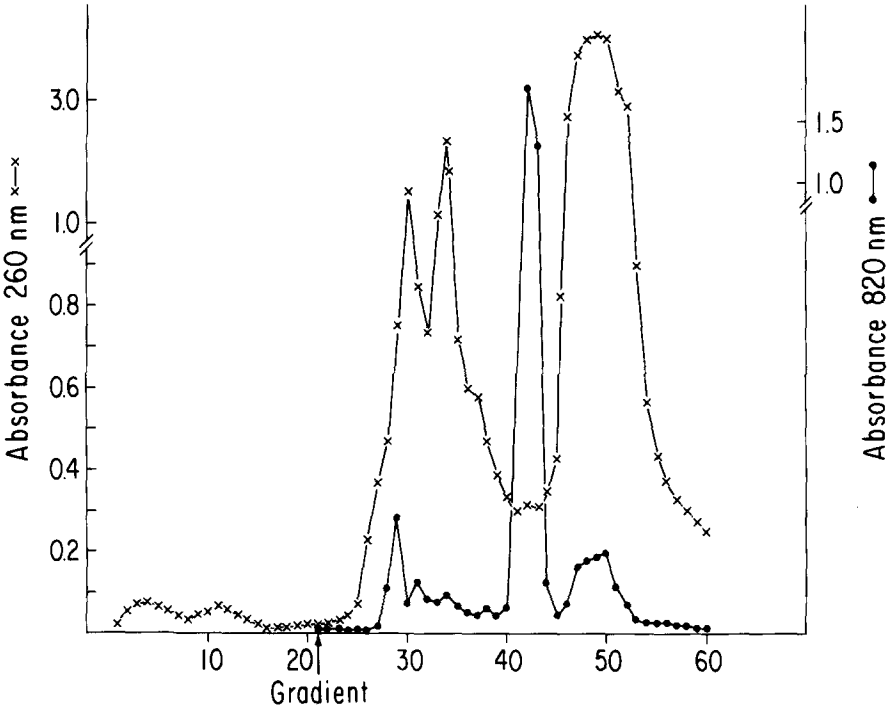

FIG. 3. Separation of the components of the Tris-released material from *N. gonorrhoeae* R10 on a DEAE-Sepharose column. Chromatography was performed in 10 mM Tris-HCl pH 8.0, 1 mM EDTA, with a linear gradient of NaCl from 0 to 1.0 M. The eluate was monitored for absorbance at 260 nm, and for phosphorus content.

weight of cells was obtained. The different components comprising the material were separated on a DEAE-Sepharose column, and the fractions were tested for phosphorus, and for absorption at 260 nm (Fig. 3). An unknown compound with high phosphorus content and no absorbance at 260 nm eluted at about 0.4 M NaCl. It was analyzed as described above and proved to be a polyphosphate (Tables II and III). In addition, when the compound was added to toluidine blue, a typical metachromatic shift in

absorbance was observed (data not shown).

Since *N. gonorrhoeae* is known to be a highly autolytic organism (29) and polyphosphate usually occurs in cytoplasmic granules, the question arose whether the polyphosphate isolated by gently washing the cells was of cytoplasmic origin. To answer this question, "internal" polyphosphate from intact cells was isolated by perchloric acid extraction or sodium hypochlorite treatment and that amount compared with the amount of "external" polyphosphate. To be able to estimate the degree of autolysis a similar comparison was made for the activities of cytoplasmic enzyme markers (glucose-6-PO_4-dehydrogenase and aldolase) in the Tris-HCl wash and in the cells after lysis by sonication. The results of these experiments (Table IV) indicated that in *N. gonorrhoeae*, at least half the polyphosphate is located externally. In contrast, the activity of the cytoplasmic enzymes outside the cell is between 1 and 2% of the total activity. Furthermore, a comparison of particle and viable counts showed that the buffer treatment of the cells did not impair their viability.

High Molecular Weight Nature of the Neisserial Polyphosphate. Polyphosphates of different chain lengths have been isolated from living material (7). The size of the neisserial polyphosphate was determined on a Sepharose 4B column. NaOCl-extracted polyphosphate migrates as a single, relatively sharp peak (Figure 4*A*). The Tris-released material migrates in an identical manner (data not shown). Synthetic polyphosphate with an average chain length of 200 phosphate residues elutes in a heterogenous pattern with a major peak at a similar position as the neisserial polyphosphate, suggesting that the molecular weight of the neisserial polyphosphate is ~20,000 (Fig. 4*B*). These results suggest that the isolated neisserial polyphosphates represent a rather homogeneous species and that the external and internal polyphosphate are of identical size.

Special Features of Polyphosphate Synthesis in N. gonorrhoeae. In several species, polyphosphate synthesis is induced if the organisms are exposed to conditions where phosphate is available in limited amounts only (30). We investigated whether this phenomenon applied to *N. gonorrhoeae* by growing the organisms to early log phase in chemically defined medium with normal phosphate concentration (33 mM), washing them twice with saline, and then allowing them to grow for 3 h in the same medium, containing 1×10^{-6} M phosphate and 5×10^{-6} M thiamine pyrophosphate (during which time the optical density increased only slightly). After this period the cells were transferred to the normal medium. Samples were taken before starvation, at 0, 30,

TABLE IV

Localization of Polyphosphate in N. gonorrhoeae

Substance assayed	Tris-extracted material	Intact cells after Tris-wash
Polyphosphate (*mg*)	1.0	0.52*
Glucose-6 PO_4-dehydrogenase (*U*)‡	2,079	244,300
Aldolase (*mU*)§	102	4,270

* Polyphosphate obtained by NaOCl extraction.
‡ Units are as defined by Noltmann et al. (27).
§ Units are as defined by Pinto et al. (28).

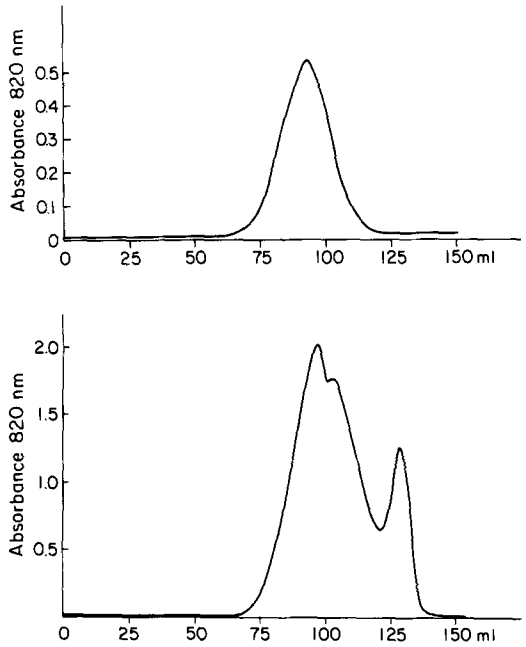

FIG. 4. (*A*) Gel filtration of 0.5 mg of NaOCl-extracted polyphosphate from *N. gonorrhoeae* R10 on a Sepharose 4B column (1.5 × 85 cm) equilibrated with 0.2 M ammonium acetate buffer, pH 7.0. Chromatography was carried out at a flow rate of 7.5 ml/h and the fractions monitored for acid-labile phosphate. The void volume and the total volume were 46.2 and 142.9 ml. (*B*) Gel filtration of 1.2 mg of synthetic polyphosphate with an average chain length of 200 phosphate residues. The conditions of the chromatography were as described for (*A*).

TABLE V

Inducibility of Polyphosphate Synthesis in N. gonorrhoeae R10

Phosphate concentration in medium	Experimental conditions	Yield of polyphosphate (percent of total PO_4)
M		%
33×10^{-3}	Growth in chemically defined medium to mid-log phase. Centrifuge and wash cells twice in saline.	23
1×10^{-6}*	Resuspend in chemically defined medium; grow for 3 h. Centrifuge and wash.	26
33×10^{-3}	Resuspend in chemically defined medium;	
	grow for 30 min	27
	60 min	27
	12 h	29

* The chemically defined medium also contains 5×10^{-6} M thiamine pyrophosphate.

and 60 min, and at 12 h after the starvation period; polyphosphate was isolated by the perchloric acid method and related to the total phosphate content of the cells (Table V). Three conclusions can be drawn from this experiment: (*a*) There seems to be no induction of polyphosphate synthesis after the starvation period. The amount of polyphosphate per total phosphate content is comparable to the result obtained

from the uninduced cells. (*b*) Polyphosphate is still present after a 3-h starvation period. The total phosphate content of the cells is lowered, but polyphosphate still represents >20% of the total phosphate. (*c*) Polyphosphate is present in constant amounts during all growth phases in *N. gonorrhoeae.*

The experiment described above showed that polyphosphate is present after a starvation period, but it allows no conclusion as to whether it is newly synthesized when the phosphate concentration is kept at a minimum. To answer this question, *N. gonorrhoeae* was grown on defined agar medium with 1.1×10^{-5} M added phosphorus and 500 μCi ^{32}P as phosphoric acid. The cells were harvested at 18 h and washed in Tris-HCl buffer, and the wash was applied to a G-50 column. We could separate a high molecular weight phosphorus-containing material from unincorporated ^{32}P. This material was extracted twice with phenol and precipitated with ethanol. To determine the acid lability of the compound, a method described by Kornberg et al. (31) was used that is based on the ability of polyphosphate to precipitate with bovine serum albumin. Hydrolysis by boiling in 1 M HCl for 15 min released all ^{32}P as judged by the fact that the radioactivity was no longer precipitable with bovine serum albumin. By the criteria of having a high molecular weight and being completely acid labile, it was identified as newly synthesized polyphosphate.

Comparison of the Polyphosphate Content of N. gonorrhoeae with Other Organisms. The polyphosphate and the total phosphate content were determined on *E. coli* and *Saccharomyces cerevisiae* harvested in late logarithmic growth phase. The polyphosphate was isolated after NaOCl digestion of the cells. In yeast, 9% of the total phosphate of the cell is present in the form of high molecular weight polyphosphate. In the *E. coli* strains tested, polyphosphate represented <2% of the total phosphate (Table VI). We were unable to isolate polyphosphate from yeast and *E. coli* by washing with Tris-HCl buffer. In *N. gonorrhoeae*, the Tris-HCl-extractable polyphosphate, which is at least half of the polyphosphate content, accounts for 5% or more of the total cell phosphorus.

Occurrence of Polyphosphate in Other Neisseria Strains. All the experiments reported above were done with the *N. gonorrhoeae* strain R10. In subsequent experiments we confirmed the presence of polyphosphate in all gonococcal strains examined (MS11, F62, 2686). Two strains (1819 and 1385) isolated from patients with disseminated gonorrhea were also investigated. The meningococcal strains as well as the nonpathogenic *Neisseria flava*, *N. sicca*, and *N. lactamica* contained polyphosphate (Table VII).

Discussion

The occurrence of polyphosphate has been reported for various bacteria including several pathogenic organisms like *Corynebacterium diphtheriae* and *Mycobacterium tubercu-*

TABLE VI

Comparison of the Polyphosphate Content in N. gonorrhoeae, E. coli, and Saccharomyces cerevisiae

Strain	Percent polyphosphate of total phosphate
E. coli K12	0.44
N. gonorrhoeae R10	9.83
S. cerevisiae (no induction)	9.0

TABLE VII
Presence of Polyphosphate in Different Neisseriae

Strain	Presence of polyphosphate	Percent polyphosphate per total phosphate
N. gonorrhoeae R10	+	>10
N. gonorrhoeae 102776	+	ND*
N. gonorrhoeae MS11	+	ND
N. gonorrhoeae 2686	+	ND
DGI strains 1896	+	25
DGI strains 1385	+	13
N. meningitidis B NCV	+	>10
N. meningitidis B M55	+	>10
N. meningitidis B M986	+	26
N. sicca	+	16
N. flava	+	15
N. lactamica	+	20

* Not determined.

losis (8). In this paper, we show the presence of polyphosphate in *N. gonorrhoeae* and several other *Neisseriae.* Thus far we have been unable to find a *Neisseria* strain lacking this compound. The presence and synthesis of polyphosphate in organisms that have been extensively investigated usually show very distinct features (7), which we have tried to identify in *N. gonorrhoeae* as well. Polyphosphate is generally localized in cytoplasmic granules. We were able to isolate polyphosphate from intact cells after digestion. In addition to this internal polyphosphate, *N. gonorrhoeae* possesses a polyphosphate fraction that is easily obtainable from the organisms by gentle washings. We could show that this polyphosphate is not due to autolysis but seems to be located outside the cytoplasmic membrane. A localization of polyphosphate beyond the cytoplasmic membrane has been shown for several microorganisms including *Mycobacterium smegmatis* (by nuclear magnetic resonance spectroscopy) (11) and *Saccharomyces mellis* (32).

Although polyphosphate is frequently a compound of living cells, its function is still unclear. Its usual synthesis pattern (overproduction after phosphate starvation) has led to the assumption that it may serve as a phosphate storage pool. This function is thought to apply to organisms living in environments where the phosphate concentration is low (33). When phosphate is available, it is taken up and stored in the form of polyphosphate. However, in the gonococcus the response of polyphosphate synthesis to phosphate starvation is unlike that reported for other organisms. The polyphosphate can be isolated from gonococci grown in phosphate-rich medium (10^{-3} M), but is also produced in low-phosphate medium (10^{-5} M). From these results, it seems unlikely that polyphosphate serves as a phosphate storage product in *N. gonorrhoeae.*

The second major hypothesis for polyphosphate function is its possible role as an energy storage pool. This role is suggested by the fact that several organisms have a polyphosphate kinase, which can transfer a phosphate group from polyphosphate to ADP (34). Thus far, we have not been able to identify a comparable activity in *N. gonorrhoeae* using the methodology developed for the isolation of the *E. coli* enzyme (31). Several other possible roles for polyphosphate exist. Its localization beyond the

cytoplasmic membrane has evoked the suggestion that it may play a role in the uptake of certain substances, e.g., glucose (32). The chemical properties of polyphosphate suggest additional possible functions. By chelating metal ions such as Ca^{++}, Fe^{++}, and Mn^{++}, it could influence processes in which these ions play a role (e.g., complement fixation). There is also a report (35) on the possible involvement of polyphosphate in the pathogenicity of an organism. It was shown that *Corynebacteria*, when freshly isolated or grown on serum, contained a higher amount of polyphosphate than after several passages in the laboratory.

Since the polyphosphate was found while attempting to isolate a capsular material from the gonococcus, we would like to comment on its possible role as a capsule. The existence of the capsule remains controversial, with morphologic evidence for (1–4) and against (36). James and Swanson (2) reported that the capsule appeared to be very fragile and easily removed by shearing. This observation is in keeping with our finding that the polyphosphate is readily extracted with neutral buffer. Polysaccharide capsules most often are polyanions and are believed to have an antiphagocytic function. Polyphosphate certainly fulfills the first requirement and might even be capable of the antiphagocytic function by complexing divalent cations in the immediate environment (37). However, the stability of polyphosphate in various compartments in the human host remains to be established. Polyphosphatases have been reported to occur in human serum and intestinal mucosa (33). Cervical mucus has levels of alkaline and acid phosphatases that vary with the estrus cycle (38), and their effect on polyphosphate is not known.

Summary

Neisseria gonorrhoeae, as well as other *Neisseriae*, produce polyphosphate. This polyphosphate exists in two forms. Approximately half of it is loosely associated with the cells and can be recovered by washing in neutral buffers under conditions in which no significant lysis of the cells is observed. The other half is either intracellular or tightly associated, because it requires digestion of the cells with perchloric acid or sodium hypochlorite. Polyphosphate obtained by both methods was purified by column chromatography and chemically characterized. In contrast to other organisms, gonococci do not respond with increased polyphosphate synthesis when shifted from phosphate starvation to a phosphate-rich medium. In addition, gonococcal polyphosphate does not serve as a depletable phosphate source during phosphate starvation. All strains of *Neisseriae* examined produce substantial amounts of polyphosphate.

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