Structural Studies and Chemistry of Bacterial Capsular Polysaccharides. Investigations of Phosphodiester-Linked Capsular Polysaccharides Isolated from *Haemophilus influenzae* Types a, b, c, and f: NMR Spectroscopic Identification and Chemical Modification of End Groups and the Nature of Base-Catalyzed Hydrolytic Depolymerization[1]

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Abstract: The capsular polysaccharide (Figure 1) from *Haemophilus influenzae* type b organisms was analyzed by a combination of NMR and chemical methods to determine its end-group composition. Phosphorus-containing end groups were present as D-ribosyl 2,3- and D-ribitol 4,5-cyclophosphates, as well as D-ribosyl C_3- and D-ribitol C_5-attached phosphate monoesters; reducing end groups were not found. A similar analysis of the *H. influenzae* type f capsular polysaccharide (Figure 5) showed cyclophosphate termini spanning the C_3 and C_4 positions of D-GalNAc residues and monophosphate end groups bonded to C_3 of D-GalNAc and C_1 of 3-(OAc)-D-GlcNAc residues. Capsular polysaccharide (Figure 8) derived from *H. influenzae* type c organisms contained phosphate monoester end groups at C_4 in the D-GlcNAc and 3-(OAc)-D-GlcNAc residues. In capsular polysaccharide (Figure 10) from *H. influenzae* type a organisms, the ^{31}P NMR-detectable monophosphate termini were tentatively assigned to D-ribitol C_5 or D-Glc C_4 or both of these positions. The ^{31}P NMR-derived molar ratio of phosphorus-containing end groups to repeating unit phosphodiester linkages was used to calculate number-average chain length. Comparisons of end-group composition and number-average chain length for capsular polysaccharide samples isolated from various strains of the four serotypes revealed appreciable differences. The base-catalyzed (pH 10) depolymerization of the capsular polysaccharides (cleavage of the phosphodiester linkages) was monitored by ^{31}P NMR spectroscopy, and pseudo-first-order kinetic analysis led to the serotype stability order a > c > f > b, with estimated relative rate ratios at 50 °C being 0.0052:0.0066:0.125:1, respectively. Types b and f capsular polysaccharide undergo depolymerization with formation of cyclophosphate and phosphate monoester end groups, while depolymerization of types c and a capsular polysaccharide was accompanied by phosphate monoester end-group formation without accumulation of detectable cyclophosphate termini. The type a polysaccharide gave evidence of phosphodiester linkage rearrangement. The phosphate monoester end groups in capsular polysaccharide types a and b were selectively derivatized (Scheme V) by a carbodiimide-mediated coupling with adipic acid dihydrazide, while similar nucleophilic trapping reactions with 4-amino-2,2,6,6-tetramethylpiperidinyl-1-oxy free radical and 7-amino-4-methylcoumarin afforded spin-labeled and fluorescence-labeled samples of the type a capsular polysaccharide; average yields were 50 ± 20%.

The presence of extracellular capsular polysaccharides on Gram-negative and Gram-positive bacteria is related to their ability to cause invasive disease in humans.[3,4] That the details of the structures of these polymers are important is evidenced by the observation that encapsulation is a necessary but not a sufficient condition for pathogenicity.[3,4] Thus, generically related, nonidentically encapsulated organisms can display markedly different abilities to cause disease; for example, of the six encapsulated *Haemophilus influenzae* serotypes, designated by the letters "a" through "f", only type b is associated with disease in humans.[5]

Immunity to encapsulated organisms is mediated by serum antibodies specific for the polysaccharide capsules.[3-5] Since the initial finding that capsule-derived polysaccharides could be immunogenic,[6] efforts have been made to actively immunize various populations againt a variety of pathogens by using purified polysaccharide preparations. The first unequivocal demonstration of the efficacy of a polysaccharide vaccine was provided by MacLeod and associates[7] in 1945; recently, capsular polysaccharide vaccines for the prevention of meningococcal and pneumococcal diseases have been licensed by the Food and Drug Administration. Unfortunately, success with polysaccharide vaccines has not been total. For example, polysaccharide vaccines are not, in general, highly effective in infants, the group for which they are often the most needed. This finding could reflect a preference, in infants, for T-cell-dependent vaccine antigens, although other factors affecting polysaccharide efficacy (e.g., molecular size) may be operative; in several instances, T-cell-dependent antigens have been formulated by conjugation of the polysaccharide with a protein carrier.[8]

The relationship between capsule structure and bacterial virulence, as well as the use of capsule-derived polysaccharides as immunogens, has prompted a number of structural studies of capsular polysaccharides, including those from *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *H. influenzae* and their commonly encountered, cross-reactive, nonpathogenic counterparts (usually *Escherichia coli*).[9,10] In contrast to the well-characterized

(1) Portions of this work have been presented at the International Symposium on Bacterial Vaccines, Sept 15-18, 1980, National Institutes of Health, Bethesda, MD.

(2) (a) Food and Drug Administration; (b) The Catholic University of America.

(3) Robbins, J. B. *Immunochemistry* **1978**, *15*, 839 and references therein.

(4) Robbins, J. B.; Schneerson, R.; Egan, W.; Vann, W.; Liu, D. T. "The Molecular Basis of Microbial Pathogenicity"; Smith, H., Skehel, J. J., Turner, M. J., Eds.; Verlag Chemie: Weinheim, Germany, 1980; pp 115-132 and references therein.

(5) Robbins, J. B.; Schneerson, R.; Parke, J. C.; Liu, T.-Y.; Handzel, Z. T.; Orskov, I.; Orskov, F. "The Role of Immunological Factors in Infectious, Allergic, and Autoimmune Processes"; Beers, R. F., Bassett, E., Eds.; Raven Press: New York, 1976; pp 103-120 and references therein.

(6) Francis, T., Jr.; Tillett, W. S. *J. Exp. Med.* **1930**, *52*, 573.

(7) MacLeod, C. M.; Hodges, R. G.; Heidelberger, M.; Bernhardt, W. G. *J. Exp. Med.* **1945**, *82*, 445.

(8) Schneerson, R.; Robbins, J. B.; Hanson, L.; Kaisjer, B.; Sutton, A.; Vann, W.; Ahlstedt, S.; Egan, W.; Zon, G. *Semin. Infect. Dis.*, in press.

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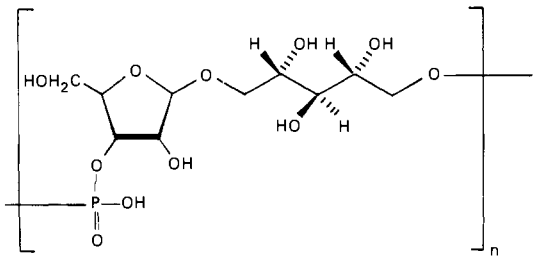


Figure 1. Repeating unit structure of *H. influenzae* type b capsular polysaccharide shown in its protonated form: →3)-β-D-Rib*f*-(1→1)-D-ribitol-5-(PO_4→.

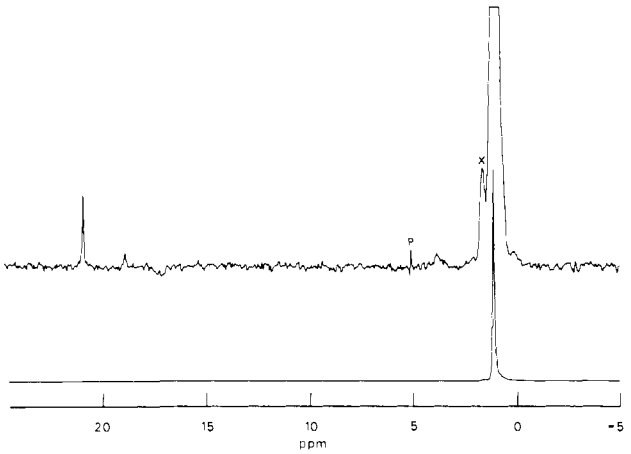


Figure 2. ^{1}H-decoupled 40.25-MHz ^{31}P NMR spectrum of *H. influenzae* type b capsular polysaccharide (strain 1482, 20 mg/mL); $\pi/2$ pulse, 30-s repetition time, no NOE, and 5048 accumulations; the middle trace was recorded at 50 times the amplitude of the lower trace; P refers to the pulse position and X refers to a spinning sideband. The upper trace was similarly obtained with a more concentrated sample (strain Eagen, 100 mg/mL); however, a signal at 3.95 ppm was not detected.

molecular structure of the repeating monomeric units in these polysaccharides, virtually nothing is known about their end-group composition. We have addressed this problem within the phosphodiester-containing *H. influenzae* capsular polysaccharides (types a, b, c, and f) and, following characterization, have used these end groups in selective chemical transformations. We have also studied the hydrolysis of phosphodiester linkages in these capsular polysaccharides to gain insight into the origin of the terminal groups and to aid in vaccine control and production. Our findings are reported herein.

Results and Discussion

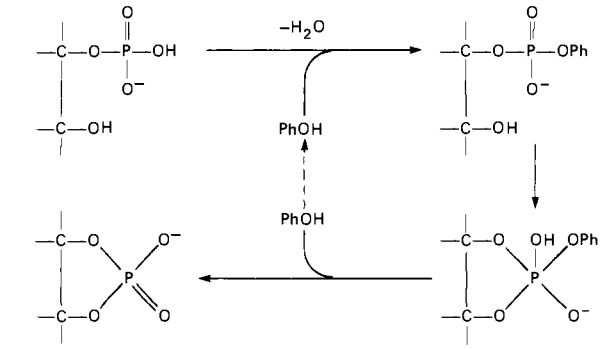
Type b. End-Group Studies. *H. influenzae* type b capsular polysaccharide has the repeating unit structure shown in Figure 1.[9] The ^{31}P NMR spectrum (Figure 2) of this material (strain 1482) featured an intense signal at 1.09 ppm for the repeating unit phosphodiester linkage as well as very low intensity signals at 3.95, 20.83, and 18.85 ppm. The large downfield chemical shifts of the latter pair of signals were suggestive of five-membered ring cyclophosphate end groups, which presumably spanned the C_3–C_2 positions of D-ribose and the C_5–C_4 positions of D-ribitol, on the basis of the connectivity of the phosphodiester linkage in the repeating unit. Isomers of cyclic adenosine monophosphate (cAMP) were used as ^{31}P NMR chemical shift model compounds, and the 20.25-ppm value measured for 2′,3′-cAMP was consistent with assignment of a D-ribosyl 2,3-cyclophosphate terminus to the 20.83-ppm signal exhibited by the polysaccharide. The ^{31}P chemical shift of 3′,5′-cAMP was −0.60 ppm, ruling out the presence of D-ribosyl 3,5-cyclophosphate end groups in the type b polysaccharide.

Table I. ^{31}P NMR-Derived[a] End-Group Composition for Samples of Capsular Polysaccharide from *H. influenzae* Type b (Strain 1482)

sample[b]	end group	composition,[c] %	ratio
before EDAC	D-ribosyl 2,30cyclophosphate	0.33	3.3/1
	D-ribitol 4,5-cyclophosphate	0.10	
	phosphate monoester	0.22	
		0.65	
after EDAC	D-ribosyl-2,3-cyclophosphate	0.60	3.5/1
	D-ribitol-4,5-cyclophosphate	0.17	
	phosphate monoester	*d*	
		0.77	

[a] See Experimental Section for spectral acquisition parameters. [b] See text. [c] Relative molar percentages; calculated from integrated signal intensities relative to the total value for all end groups and the repeating unit phosphodiester linkage. [d] Not detached.

Scheme I



Intramolecular cyclization of the phosphate monoester group in D-ribitol 5-phosphate by reaction with a carbodiimide was investigated as a convenient synthetic route to D-ribitol 4,5-cyclophosphate, which would then serve as a chemical shift model compound for the ribitol segment of the polysaccharide. An aqueous solution of D-ribitol 5-phosphate was reacted at pH 3–5 with a 2-fold molar excess of 1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide hydrochloride (EDAC). ^{31}P NMR monitoring of the mixture showed a gradual disappearance of the starting material signal at 3.12 ppm with formation of a product signal at 19.06 ppm; the reaction was complete after 2 h at 25 °C, and there was no evidence for intermolecular pyrophosphate formation (no signals at ca. −10 ppm). ^{13}C NMR data for D-ribitol 5-phosphate and the isolated product were consistent with identification of the latter material as D-ribitol 4,5-cyclophosphate.[11] The close chemical shift correspondence for this model compound (19.06 ppm) and the polysaccharide (18.85 ppm) was preliminary evidence for D-ribitol 4,5-cyclophosphate termini in the bacterial polymer.

(9) Egan, W. "Magnetic Resonance in Biology"; Cohen, J. S., Ed.; Wiley: New York, 1980; Vol. 1, Chapter 5 and references therein.
(10) Larm, O.; Lindberg, B. *Adv. Carbohydr. Chem. Biochem.* **1976**, *33*, 295.

(11) The ^{13}C NMR spectrum for D-ribitol 5-phosphate was as follows (tentative resonance assignments in parentheses): 64.86 ppm (C_1); 68.71 ppm, J_{CP} = 4.9 Hz (C_5); 73.51 ppm, J_{CP} = 7.3 Hz (C_4); 74.61 and 74.19 ppm (C_3 and C_2); for D-ribitol 4,5-cyclophosphate: 65.11 ppm (C_1); 74.83 ppm (C_2); 73.95 ppm, J_{CP} = 7.3 Hz (C_3); 78.31 ppm (C_4); 68.61 ppm (C_5). With D-ribitol 4,5-cyclophosphate, the absence of observable spin couplings of phosphorus to C_4 and C_5 and the presence of a relatively large spin coupling of phosphorus to C_3 are similar to the coupling patterns found in 2′,3′-cyclic nucleotides wherein small (<∼2 Hz) couplings are observed between phosphorus and $C_{2'}$ or $C_{3'}$ and a larger coupling is seen for phosphorus and $C_{4'}$ (Lapper, R. D.; Smith, I. C. P. *J. Am. Chem. Soc.* **1973**, *95*, 2880). Unambiguous identification of the C_1 signal for D-ribitol 5-phosphate and D-ribitol 4,5-cyclophosphate follows from the synthesis of their C_1-*d* analogues, starting with $NaBD_4$ reduction of D-ribose 5-phosphate. For each of the deuterium-labeled compounds, the C_1-*d* resonance at ∼65 ppm appeared as the expected triplet. The possibility of a degenerate rearrangement of the cyclophosphate moiety between C_4–C_5 and C_1–C_2 positions was excluded by this labeling experiment.

Table II. ^{31}P NMR-Derived[a] Structural Data for Samples of Capsular Polysaccharide from *H. influenzae* Type b

strain	end-group composition,[b] % D-ribosyl 2,3-cyclophosphate	D-ribitol 4,5-cyclophosphate	phosphate monoester	cyclophosphate:phosphate monoester	repeating unit phosphodiester,[b] %	chain length[c]	K_d[d]
1482	0.33	0.10	0.22	1.9	99.35	153	0.52
Eagen	1.15	0.31	<0.05[e]	>23	98.54	67	0.60
JC-17	0.65	0.17	0.52	1.6	98.66	74	0.57
Rab	1.33	0.62	<0.05[e]	>39	98.05	50	0.52
Madigan	2.06	0.50	<0.10[e]	>25	97.43	38	0.53

[a] See Experimental Section for spectral acquisition parameters. [b] Relative molar percentages; calculated from integrated signal intensities relative to the total value for all detectable end groups and the repeating unit phosphodiester linkage, except as noted. [c] See text and ref 12. [d] Partition coefficient for size-exclusion chromatography with Sepharose CL-4B. [e] Estimated upper limit; no signal was detected.

The 3.95 ppm signal (Figure 2) was assumed to arise from phosphate monoester end groups attached to D-ribose or D-ribitol or both of these positions. By analogy to the above results with EDAC, similar treatment of the polysaccharide could cause intramolecular cyclization of these putative end groups to afford their cyclic counterparts. The type b capsular polysaccharide was thus reacted at pH 7 with an excess of EDAC; comparative ^{31}P NMR data obtained for the starting material and the product are listed in Table I. The absence of a detectable signal at 3.95 ppm and the essentially unchanged total end-group content before and after EDAC treatment indicated complete conversion of phosphate monoester end groups into cyclophosphate end groups. The relative ratio of D-ribosyl:D-ribitol cyclophosphate signals was not significantly altered by reaction with EDAC, suggesting that the signal at 3.95 ppm in the starting material was due to a ca. 3:1 ratio of D-ribosyl 3-phosphate:D-ribitol 5-phosphate end groups.

Facile conversion of phosphate monoester to cyclophosphate end groups by reaction with EDAC at neutral pH implies that mechanistically analogous cyclization during isolation of the capsular polysaccharide could lead to artifacts regarding natural states of end-group composition. Scheme I illustrates catalytic cyclization that could accompany the phenolic NaOAc (pH 7) extraction, which is used to remove protein from the crude polysaccharide preparation. D-Ribitol 5-phosphate was thus subjected to the usual phenol–NaOAc protein extraction conditions; however, ^{31}P NMR analysis showed only the presence of unchanged starting material. In addition, ^{31}P NMR analysis of type b capsular polysaccharide before and after treatment with phenolic NaOAc showed no significant change in the end-group composition.

The availability of various strains (human disease isolates) of type b *H. influenzae* allowed for quantitative comparisons of end-group composition and chain length.[12] From the data summarized in Table II, the following observations can be made. (1) All five strains have capsular polysaccharide that contains both kinds of cyclophosphate end groups, and in each case D-ribosyl 2,3-cyclophosphate predominates to approximately the same extent, viz., 3.4 ± 0.5:1. This feature may be rationalized by invoking either a biosynthetic determinant or nonenzymatic thermodynamic factors. (2) The combined cyclophosphate content is always greater than that of the phosphate monoester. This is contrary to a priori expectations based upon the relatively high energy content ("ring strain") of five-membered cyclic phosphodiesters.[13,14] (3) The relative ratio of cyclophosphate to phosphate monoester end groups is variable, covering a range of ca. 2 to >40. (4) Chain lengths, which were calculated with the assumption of one phosphorus-containing end group per chain, do not correlate with polysaccharide partition coefficients (K_d) measured by size-exclusion chromatography (cf. Table II). While this observation can be attributed to aggregation of polysaccharide chains to form "networks" having roughly the same overall size characteristics, it should be noted that size-exclusion chromatography of a sample of type b capsular polysaccharide afforded three fractions that were analyzed by ^{31}P NMR and found to have chain lengths that were proportional to weighted K_d values (Figure 3).

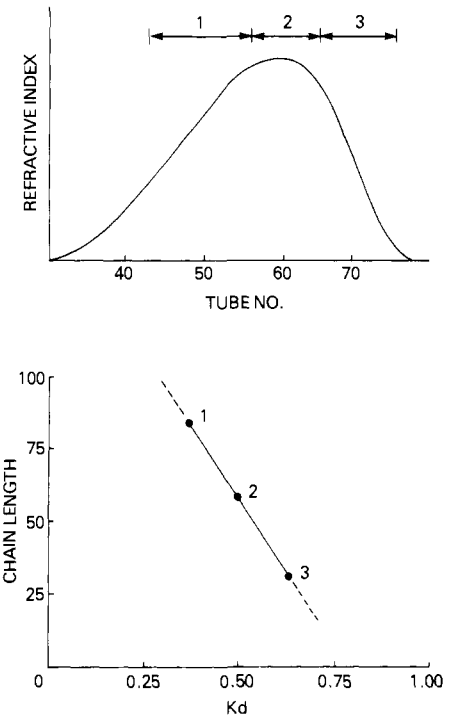
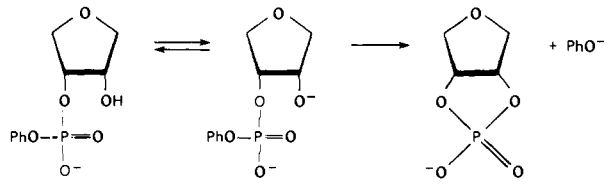


Figure 3. (Top) Elution profile for size-exclusion chromatography of *H. influenzae* type b capsular polysaccharide (strain Eagen, 300 mg) with a Sepharose CL-6B column (5 × 100 cm) and 0.2 M NaCl at 1 mL/min (200 drops/tube). Fraction numbers 1–3 indicate those tubes which were pooled for ^{31}P NMR determination of number-average chain length. (Bottom) Plot of ^{31}P NMR-derived number-average chain length for fractions 1–3 vs. their respective weighted K_d values.

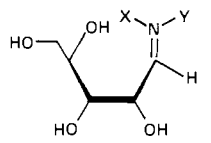
(12) For simplicity, the term "chain length" will be used throughout as the equivalent of number-average chain length. Determination of chain lengths for type b and related capsular polysaccharides by ^{31}P NMR spectroscopy requires, inter alia, accurate measurement of molar ratios for the repeating-unit phosphodiester linkage and phosphorus-containing end groups. Differential nuclear Overhauser enhancements were minimized by gated ^1H decoupling, and the inversion–recovery method was applied for ^{31}P T_1 measurements by using a *freshly prepared* solution of type b capsular polysaccharide in its usual Ca^{2+} form. At 108.7 MHz, 20 °C, a value of 1.2 s was found for the repeating unit phosphodiester linkage and for the D-ribitol 4,5-cyclophosphate end groups. On the other hand, a value of 5.6 s was obtained for the D-ribosyl 2,3-cyclophosphate termini. The broadness of the phosphate monoester end-group signal (cf. Figure 2) precluded reliable measurement of its T_1 value by this technique; however, it appeared to have a relaxation rate roughly comparable to that of the repeating unit phosphodiester linkage. A recovery time of 30 s was chosen for quantitative ^{31}P NMR measurements. The influence of sample "age" was gauged by studies with the Na^+ form of type b capsular polysaccharide, which showed that T_1 values for the repeating unit phosphodiester linkage decreased upon sample storage. For example, at a polysaccharide concentration of 15 mg/mL, the phosphodiester T_1 at 40.25 MHz (20 °C) changed from 3.6 s to 3.1 s after 7 days at 5 °C and further decreased to 2.1 s after an additional week at this temperature. Phosphorus relaxation mechanisms in 2′,3′-cAMP and 5′-AMP have been recently investigated (Nanda, R. K.; Ribeiro, A.; Jardetzky, T. S.; Jardetzky, O. *J. Magn. Reson.* **1980**, *39*, 119).

(13) Marsh, F. J.; Weiner, P.; Douglas, J. E.; Kollman, P. A.; Kenyon, G. L.; Gerlt, J. A. *J. Am. Chem. Soc.* **1980**, *102*, 1660.
(14) Haake, P. C.; Westheimer, F. H. *J. Am. Chem. Soc.* **1961**, *83*, 1102.

Scheme II



The corollary to assuming one phosphorus-containing end group per chain of type b polysaccharide is that the remaining end groups are D-ribosyl or D-ribitol moieties or both. A C_3-linked terminal D-ribosyl group (cf. Figure 1) allows for tautomerization between its cyclic hemiacetal and acylic aldehydic forms, the latter of which may be trapped and quantified by oxime formation. D-Ribose and type b polysaccharide (strain Eagen) were separately reacted with an excess of *O*-methylhydroxylamine hydrochloride (CH_3ONH_2·HCl). After 24 h at pH 7 ± 0.5, the recovered (88%) polymer showed no detectable ^1H NMR doublet for an imine proton of either the major or minor isomer of the D-ribosyl *O*-methyloxime model compound: *E* (78%), 7.57 ppm, $^3J_{HH}$ = 6.4 Hz; *Z* (22%), 6.90 ppm, $^3J_{HH}$ = 6.0 Hz. This novel method for



E; X = lone pair, Y = OCH_3
Z; X = OCH_3, Y = lone pair

end-group analysis, which indicated <10 aldehyde-forming D-ribose termini per 100 phosphorus-containing end groups, was further evaluated with type b polysaccharide that had been partially depolymerized in 1 M HOAc at 37 °C. The recovered material showed a low intensity doublet (J = 6.4 Hz, 7.61 ppm) for the (*E*)-oxime derivative of C_3-linked terminal D-ribosyl residues; ^{31}P NMR analysis indicated 5% hydrolysis of phosphodiester repeating-unit linkages (vide infra). Attempts to carry out periodate oxidation[15] of the type b capsular polysaccharide for quantitative colorimetric[15] determination of formaldehyde (from D-ribitol termini) were foiled by excessive interference due to color-forming side reactions. The biosynthesis of this capsular material may involve a chain-elongation step which proceeds by D-ribosyl C_3 hydroxyl displacement of lipid monophosphate from a lipid pyrophosphate carrier of the repeating unit;[16] however, other mechanisms akin to those proposed[16] for teichoic acids in Gram-positive bacteria are also possible.

Hydrolysis. A solution of the type b polysaccharide in 0.1 M glycine–NaOH buffer (pH 10) containing 0.1 M $CaCl_2$ was monitored at 50 °C by ^{31}P NMR spectroscopy, and kinetic profiles for the various phosphorus species were obtained from changes in their relative signal intensities as a function of time. The individual profiles during 85% hydrolysis of the repeating unit phosphodiester linkage are presented in Figure 4. It can be seen that as the phosphodiester repeating unit linkage (1.35 ppm) undergoes cleavage, there is a steady production of three phosphate monoester end groups (5.49, 4.96, and 4.66 ppm). The D-ribosyl 2,3-cyclophosphate end group (20.96 ppm) reaches a maximum concentration after ca. 4 h and then diminishes in intensity, while the D-ribitol 4,5-cyclophosphate end-group (18.98 ppm) concentration attains an apparent plateau. Figure 4 also shows a pseudo-first-order kinetic plot for disappearance of the repeating unit phosphodiester linkages. Deviation from linearity is apparent after ca. 5 h, which corresponds to approximately 60% hydrolysis. The initial linear portion of this plot affords a value of $\tau_{1/2}$ = 4.3 h (k' = 4.47 × 10^{-5} s^{-1}) for phosphodiester cleavage.

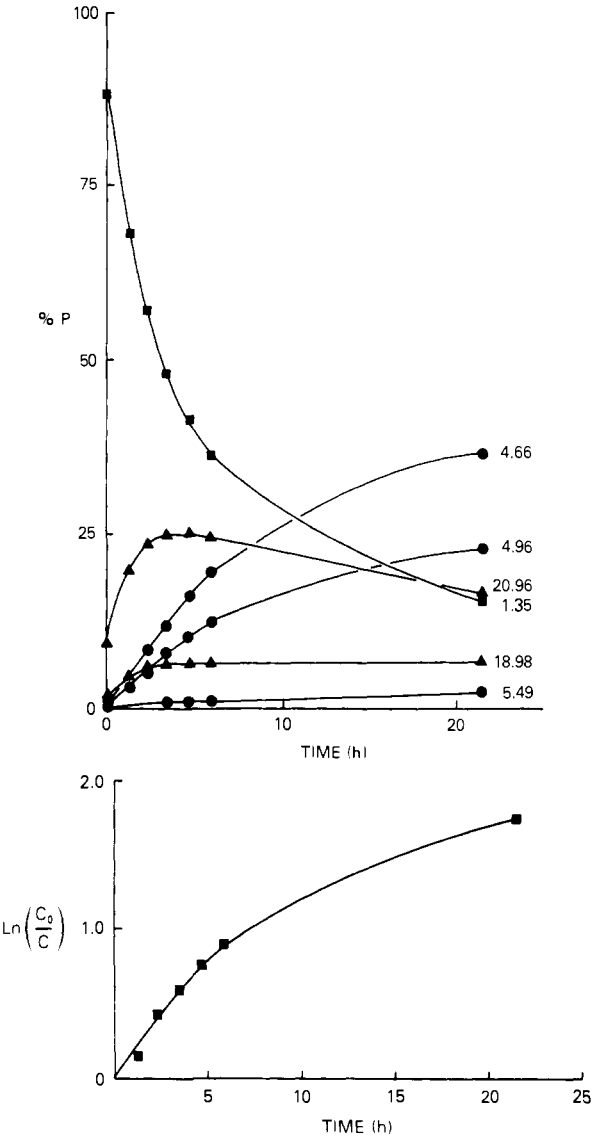
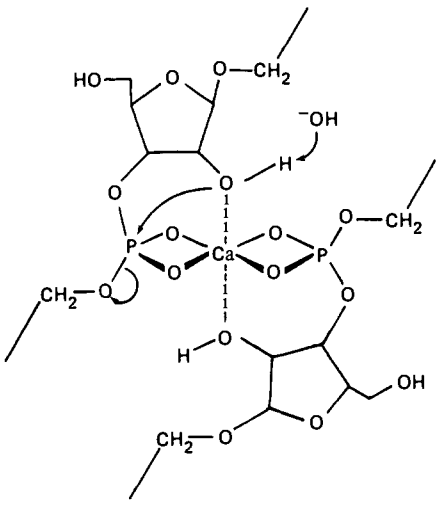


Figure 4. (Top) Plot of relative concentration (% P, relative ^{31}P NMR signal intensity) vs. time for phosphorus-containing moieties in *H. influenzae* type b capsular polysaccharide (strain 1482, 4 mg/1.5 mL) at 50 °C during depolymerization at pH 10 in 0.1 M glycine–NaOH buffer containing 0.1 M $CaCl_2$; repeating unit phosphodiester linkages (squares) at 1.35 ppm, phosphate monoester end groups (circles) at 4.66, 4.96, and 5.49 ppm, D-ribosyl 2,3-cyclophosphate end groups (triangles) at 20.96 ppm, and D-ribitol 4,5-cyclophosphate end groups (triangles) at 18.98 ppm. (Bottom) Pseudo-first-order kinetic plot for the disappearance of repeating unit phosphodiester linkages; concentration values C_0 and C correspond to % P initial and at time t, respectively.

The kinetics for hydrolysis of the phenyl ester of *cis*-4-hydroxytetrahydrofuran 3-phosphate have been studied[17] in detail as models for the elementary steps of ribonuclease action, and it has been suggested[17] that the mechanism involves an "in-line" S_N2-type displacement of phenoxide ion by the neighboring alkoxide to form the cyclophosphate shown in Scheme II. For comparison of the relative reactivity of this compound and the type b polysaccharide, the reported[17] pseudo-first-order hydrolytic rate constant at pH 9.7/50 °C (1.58 × 10^{-3} s^{-1}) can be corrected for replacement of PhO with $HOCH_2CH_2O$.[18] This gives a value

(15) Speck, J. C., Jr. "Methods in Carbohydrate Chemistry"; Whistler, R. L., Wolfrom, M. L., Eds.; Academic Press: New York, 1962; Vol. 1, pp 441–445.
(16) Baddiley, J. *Acc. Chem. Res.* **1970**, *3*, 98.
(17) Usher, D. A.; Richardson, D. I., Jr.; Oakenfull, D. G. *J. Am. Chem. Soc.* **1970**, *92*, 4699.
(18) Hydrolysis of esters of 2-hydroxypropyl phosphate occurs by neighboring group participation to give 1-methylglycerol cyclophosphate (Brown, D. M.; Usher, D. A. *J. Chem. Soc.* **1965**, 6547). For hydrolysis of the PhO and $HOCH_2CH_2$ esters in 1 N NaOH at 80 °C, k' = 7.40 × 10^{-6} and 2.35 × 10^{-8} s^{-1}, respectively (Brown, D. M.; Usher, D. A. *J. Chem. Soc.* **1965**, 6558).

Scheme III



(5.02×10^{-6} s^{-1}) only 9 times less than that measured for the type b polysaccharide under comparable reaction conditions. The close correspondence in hydrolytic reactivity and the appearance of cyclophosphate end groups during depolymerization of the polysaccharide are persuasive evidence for analogous mechanisms of phosphodiester bond cleavage. Scheme III is a representation of neighboring group participation by D-ribosyl C_2-alkoxide at an interchain cross-link in the Ca^{2+} form of type b polysaccharide. The dual role envisioned for Ca^{2+} as a stereochemical template and Lewis acid catalyst is analogous to various M^{2+}-dependent enzymatic hydrolyses of phosphoric acid esters.[19] Since divalent metal ion catalyzed hydrolysis of carboxylic esters having relatively poor leaving groups, such as alkoxides, may involve weak metal ion binding to the reactant and strong binding in the transition state,[20] which facilitates departure of the nucleofuge, the essential features of Scheme III could equally apply to the activated reaction complex. NMR spectroscopy was thus used to probe the extent of binding between the metal ion and polysaccharide in the reactant stage.

The chemical shifts of corresponding resonances in the ^{13}C NMR spectra of the Ca^{2+} and Na^+ salts of the type b polysaccharide were, to within 0.1 ppm, the same, as were the ^{31}P chemical shifts of the cyclophosphate end groups and the phosphodiester repeating unit resonances. These spectroscopic observations indicate that there is no substantial difference in the mode of cation association with the polymer. By inference, the association is not an intimate one; i.e., it does not appear that there is a substantial fraction of condensed Na^+ or Ca^{2+} ions, as is often found with other polyelectrolytes.[21] The line width at half-height, $W_{1/2}$, for the ^{23}Na resonance of the sodium salt of the type b polysaccharide was ca. 8 Hz, which is the same value found for an equimolar NaCl solution; if a significant fraction of Na^+ was intimately associated with the polysaccharide, the ^{23}Na resonance would be broad, relative to the ^{23}Na signal of a pure NaCl solution (as found, for example, with solutions of the sodium salts of DNA or poly(methyl methacrylate)[21]). Finally, we note that $W_{1/2}$ for corresponding ^{13}C resonances of the Na^+ and Ca^{2+} salts of the type b polysaccharide were, to within experimental error, the same and, moreover, were equivalent to those found for the $(n\text{-Bu})_4N^+$ salt. If Ca^{2+} ions were to cross-link and partially immobilize the type b polymer, we would not expect $W_{1/2}$ for the ^{13}C resonances to be unaltered. Nonetheless, replacement of Ca^{2+} with Na^+ led to a ca. 25-fold diminution in the rate of phosphodiester cleavage ($\tau_{1/2}$ = 98.3 h at pH 10/50 °C), and when a stoichiometric amount of Ca^{2+} was added to a solution of type b polysaccharide in its Na^+ form, the hydrolytic reactivity was restored to ca. 75% of the value initially found for the Ca^{2+} form. Similar kinetic measurements with added Mg^{2+} indicated that this divalent cation was approximately one-half as effective as Ca^{2+} in restoring hydrolytic reactivity to the Na^+ form, while substitution of $(CH_3)_4N^+$ for Ca^{2+} in the type b capsular polysaccharide afforded essentially the same kinetic results as did substitution with Na^+. The contrast between hydrolytic behavior and spectroscopic characteristics of the polysaccharide is consistent with transition-state stabilization by divalent metal ions during alkaline hydrolysis of the phosphodiester linkages; however, further experiments are needed to test this hypothesis.

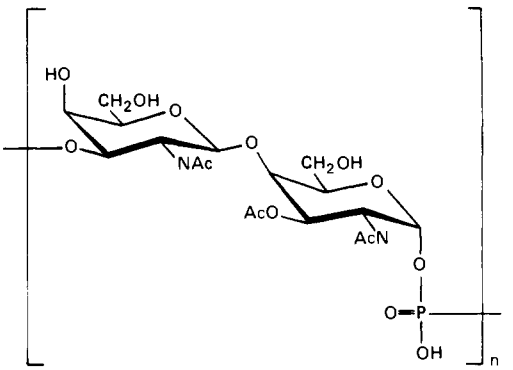


Figure 5. Repeating unit structure of *H. influenzae* type f capsular polysaccharide shown in its protonated form: →3)-β-D-GalNAc-(1→4)-α-D-GalNAc-1-(PO_4→.

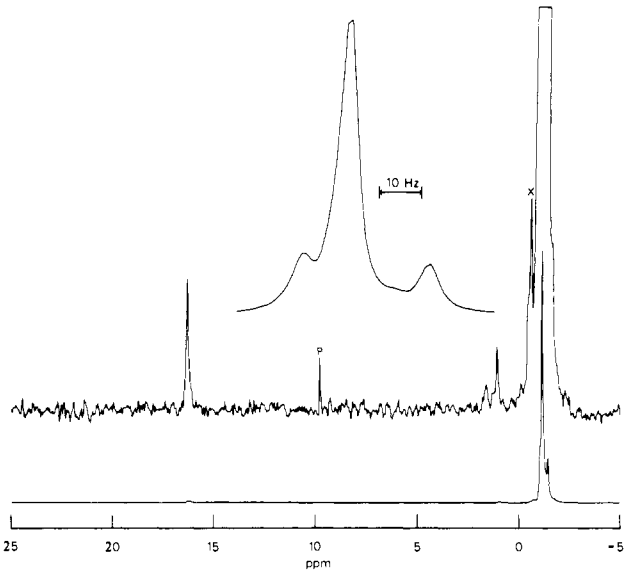


Figure 6. ^1H-decoupled 40.25-MHz ^{31}P NMR spectrum of *H. influenzae* type f (strain 644) capsular polysaccharide (25 mg/1.5 mL); $\pi/4$ pulse, 3-s repetition time, and 22 128 accumulations. The upper trace is an expanded and offset display of the repeating unit phosphodiester linkages centered at −1.18 ppm. The middle trace was recorded at 50 times the amplitude of the lower trace; P refers to the pulse position and X refers to a spinning sideband.

Type f. End-Group Studies. Previous NMR investigations of *H. influenzae* type f capsular polysaccharide found a single ^{31}P signal for the phosphodiester linkage in a homopolymer having the repeating unit structure pictured in Figure 5.[9] In the present work with purified type f material (strain 644), the ^{31}P NMR spectrum (Figure 6) showed three overlapped phosphodiester signals at −1.05, −1.18, and −1.44 ppm in a relative ratio of ca. 10:80:10, respectively, suggesting structural heterogeneity. The ^1H NMR spectral region for the acyl methyl groups (Figure 7) featured two *N*-Ac signals at 2.18 and 2.11 ppm, and an *O*-Ac signal at 2.02 ppm, in accord with earlier data reported[9] for the GalNAc and 3-(OAc)GalNAc components in the type f polysaccharide.[22] The additional low-intensity singlets seen (Figure

(19) Gray, C. J. "Enzyme-Catalyzed Reactions"; Van Nostrand Reinhold: London, 1971; Chapter 5.
(20) Fife, T. H.; Przystas, T. J. *J. Am. Chem. Soc.* **1980**, *102*, 7297.
(21) Manning, G. S. *Acc. Chem. Res.* **1979**, *12*, 443 and references cited therein.

Table III. ^{31}P NMR-Derived[a] Structural Data for Samples of Capsular Polysaccharide from *H. influenzae* Type f

sample	end group composition,[b] % C_3,C_4-cyclophosphate	C_1-phosphate monoester	C_3-phosphate monoester	cyclophosphate: phosphate monoester	repeating unit phosphodiester,[b] %	chain length[c]
644, run 1, fraction A[d]	<0.05[e]	0.11	0.10	<0.24	99.75	475
644, run 1, fraction B[f]	0.92	<0.10[e]	0.44	>1.70	98.64	73
644, run 2, fraction A[g]	<0.10	0.27	1.00	<0.08	98.73	78
686	1.01	<0.20	0.51	>1.42	98.48	65
B095	*h*	*h*	*h*	*h*	>99.38[i]	>160

[a] See Experimental Section for spectral acquisition parameters. [b] Relative molar percentages; calculated from integrated signal intensities relative to the total value for all detectable end groups and the repeating-unit phosphodiester linkage, except as noted; see text for end-group locations. [c] See text and ref 12. [d] Isolated by $CaCl_2$ precipitation from water. [e] Estimated upper limit; no signal was detected. [f] Isolated by $CaCl_2$ precipitation from aqueous EtOH. [g] A second preparation using $CaCl_2$ precipitation from water. [h] No signal was detected. [i] Estimated lower limit.

7) at 2.08 and 2.06 ppm were suggestive of *N*-Ac groups that reside in O-deacetylated regions of the polymer. This interpretation was tested by treatment of the ^1H NMR sample with ND_4OD at pH 10 ± 1 to achieve selective hydrolysis of the *O*-Ac groups.[9] As expected, there was a gradual disappearance of the signals at 2.18, 2.11, and 2.02 ppm and the production of acetate ion at 1.93 ppm (Figure 7). After complete reaction, ^{31}P NMR spectroscopy showed the presence of one major phosphodiester signal that had the same chemical shift (−1.44 ppm) as the minor upfield signal seen prior to treatment with ND_4OD. The original group of phosphodiester signals thus arose from partial (ca. 90%) O-acetylation leading to the three possible classes of phosphodiester linkages that are nonequivalent by virtue of their associated disaccharide residues.[23] Interestingly, ^1H NMR analysis of freshly prepared samples of the type f capsular polysaccharide obtained from strains 644, 686, and B095 indicated that each was ca. 98% O-acetylated, which suggests that strict control of the bacterial growth conditions may be necessary for isolation of completely O-acetylated polymer. Similar observations have been made in other bacterial systems.[24]

^{31}P NMR analysis of ca. 90% O-acetylated type f polysaccharide showed (Figure 6) the presence of low-intensity signals at 16.79, 1.68, and 1.09 ppm. A five-membered cyclophosphate end group indicated by the 16.79 ppm signal can be accommodated at the C_3 and C_4 positions of GalNAc (cf. Figure 5), while phosphate monoester termini located at C_3 of GalNAc and C_1 of 3-(OAc)GalNAc are likely candidates for the latter pair of upfield signals, which underwent the expected chemical shift change (ca. 4 ppm) upon increasing the pH from 6 to 10. Reaction of the sample with EDAC at pH 7 caused disappearance of the 1.09 ppm signal and a corresponding increase in signal intensity at 16.79 ppm, which confirms the aforementioned cyclophosphate identification and identifies the monophosphate end group attached to C_3 of GalNAc. By inference, the phosphate monoester observed at 1.68 ppm is attached to C_1 of 3-(OAc)GalNAc.

Quantitative ^{31}P NMR analysis of various samples of type f capsular polysaccharide gave the composition and chain length data summarized in Table III. Unlike the type b material (cf. Table II), the relative amount of cyclophosphate end groups does not uniformly predominate over that of the phosphate monoester end groups. The proportion of C_3-attached phosphate monoester generally exceeds that of the C_1-attached phosphate monoester; however, there is substantial variability in average chain length, as found for the type b capsular polysaccharide.

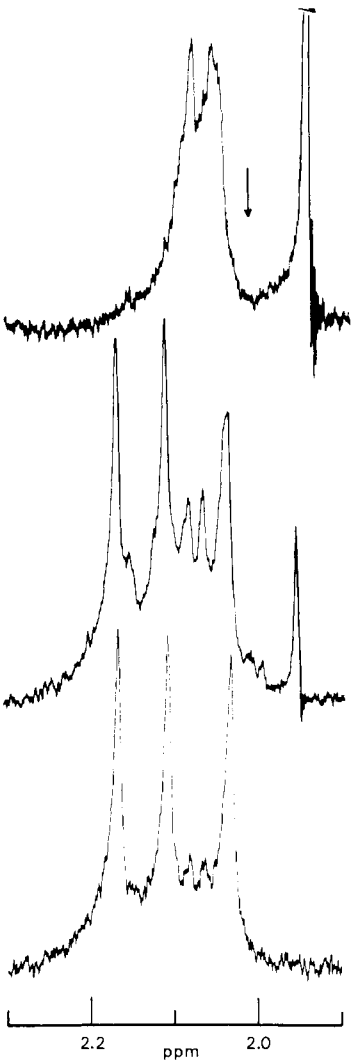


Figure 7. The acyl CH_3 region for 220-MHz ^1H NMR spectra of *H. influenzae* type f (strain 644) capsular polysaccharide (25 mg/2 mL) at pH 10 ± 1 (ND_4OD). The lower trace was recorded immediately after sample preparation. The middle trace was recorded after 15 h at 5 °C. The upper trace was recorded after an additional 24 h at 25 °C. See text for signal assignments; the arrow indicates the chemical shift measured for acetamide, which was added after recording the upper trace; the downfield acetate signal was identified by addition of NaOAc.

Hydrolysis. In 0.1 M glycine–NaOH buffer (pH 10, 0.1 M $CaCl_2$), the type f polysaccharide (strain 644, ca. 90% O-acetylated) gave a ^{31}P NMR spectrum in which the cyclophosphate and two phosphate monoester end-group signals appeared at 16.68, 5.51, and 3.78 ppm, and the signals for the repeating unit phosphodiester linkages appeared at −1.18, −1.30 (major signal), and −1.60 ppm. After 5 h at 60 °C, additional cyclophosphate, phosphate monoester, and repeating unit phosphodiester signals

(22) Abbreviations for carbohydrate residues are as follows: GalNAc = 2-acetamido-2-deoxy-D-galactose, 3-(OAc)GalNAc = 2-acetamido-2-deoxy-3-acetyl-D-galactose, GlcNAc = 2-acetamido-2-deoxy-D-glucose, Gal = D-galactose, and Glc = D-glucose.

(23) The major phosphodiester signal at −1.18 ppm is attributed to partial structure I. O-Deacetylation of a single 3-(OAc)GalNAc residue leads to formation of two nonequivalent phosphodiester linkages represented by partial structures II and III, which account for the equal intensity ^{31}P NMR signals at −1.05 and 1.44 ppm, respectively: [GalNAc][3-(OAc)GalNAc]PO_4H-[GalNAc][3-(OAc)GalNAc], I; [GalNAc][3-(OAc)GalNAc]PO_4H[GalNAc][GalNAc], II; [GalNAc][GalNAc]PO_4H[GalNAc][3-(OAc)GalNAc], III.

(24) Jennings, H. J.; Bhattacharjee, A. K.; Bundle, D. R.; Kenny, C. P.; Martin, A.; Smith, I. C. P. *J. Infect. Dis.* **1977**, *136* (*Suppl.*), S78.

Table IV. Kinetic Data for Hydrolytic Depolymerization *H. influenzae* Capsular Polysaccharides at pH 10[a]

type (strain)	temp, °C	k', s^{-1}	$\tau_{1/2}$, h	rate ratio[b]
a (Harding)	100	1.81×10^{-5}	10.6	0.0052
	90	7.62×10^{-6}	25.3	
b (1482)	50	4.47×10^{-5}	4.3	1
c (Ruggerio)	100	2.30×10^{-5}	8.4	0.0066
f (644)	60	1.53×10^{-5}	12.6	0.125
	50	5.70×10^{-6}	33.8	

[a] 0.1 M glycine–NaOH buffer containing 0.1 M $CaCl_2$; kinetic parameters k' and $\tau_{1/2}$ refer to pseudo-first-order hydrolysis of the repeating-unit phosphodiester linkage as determined by ^{31}P NMR (see text for details) and linear least-squares analysis. [b] Ratio of k' at 50 °C relative to that of type b polysaccharide; a rate deceleration factor of 2.4 per 10 °C decrease in temperature was applied to the type a and type c polysaccharides: $1.81 \times 10^{-5}/7.62 \times 10^{-6} = 2.4$.

were detected at 16.57, 3.65, and −1.48 ppm, respectively. The sets of cyclophosphate and phosphate monoester end groups represented 16 and 19% of the total ^{31}P signal intensity, respectively, indicating 35% depolymerization. Additional heating (19 h, 60 °C) led to complete disappearance of the original cyclophosphate signal at 16.68 ppm while the newly formed cyclophosphate termini seen at 16.57 ppm accounted for 17% of the total ^{31}P signal intensity. The phosphate monoester signal originally seen at 3.78 ppm was likewise not detectable, whereas its counterparts at 5.51 and 3.65 ppm were present in approximately equal amounts (40:60) and comprised 52% of the total ^{31}P signal intensity. The extent of depolymerization at this stage was 69%. The remaining phosphodiester linkages were seen as one predominant signal at −1.48 ppm. After complete cleavage of the phosphodiester linkages was effected by further heating (60 °C), a relatively small amount of residual cyclophosphate was detected (16.57 ppm) in addition to the aforementioned pair of phosphate monoester groups, which retained their ca. 40:60 ratio. A control experiment with completely O-deacetylated type f polysaccharide established that the galactosyl C_3–C_4 cyclophosphate signal has the same chemical shift (16.57 ppm) as the new cyclophosphate signal. Base-catalyzed O-deacetylation concurrent with depolymerization also accounts for the resonance signals seen at 3.65 and −1.48 ppm.

The comparatively mild conditions needed for hydrolysis of the phosphodiester linkages in the type f polysaccharide and the accumulation of transient cyclophosphate end groups are prima facie evidence for a neighboring group participation mechanism analogous to that suggested for the type b capsular polysaccharide: attack at phosphorus by C_4 alkoxide in GalNAc. An alternative participation mechanism bypassing cyclophosphate formation involves C_4-alkoxide attack at the C_3 position in GalNAc, leading to epoxide formation and expulsion of a phosphate monoester leaving group; however, stereochemical constraints to backside displacement at C_3 disfavor this mode of phosphodiester cleavage.

^{31}P NMR spectroscopy was used to determine the pseudo-first-order rate constant (Table IV) for alkaline hydrolysis of the repeating unit phosphodiester linkage, and it was found that the type f capsular polysaccharide is 8 times less reactive than the corresponding type b material. This reactivity difference may result from the staggered orientation of C_4–OH and C_3–OPO_3R substituents in GalNAc, as opposed to the eclipsed orientation of C_2–OH and C_3–OPO_3R substituents in the ribosyl component of type b polysaccharide. In addition, the phosphodiester linkages in the type f polymer are flanked by only one participating group, whereas two participating groups are available in the type b capsular polysaccharide, viz., the β-hydroxyl substituents of ribosyl and ribitol moieties.

Type c. End-Group Studies. *H. influenzae* type c capsular polysaccharide has been shown[9] to have the repeating unit structure pictured in Figure 8. The ^{31}P NMR spectrum (Figure 9) of this polysaccharide featured three extensively overlapped signals at −0.94, −1.05 (major), and −1.12 ppm for the repeating unit phosphodiester linkages. ^1H and ^{13}C NMR analyses[9] have

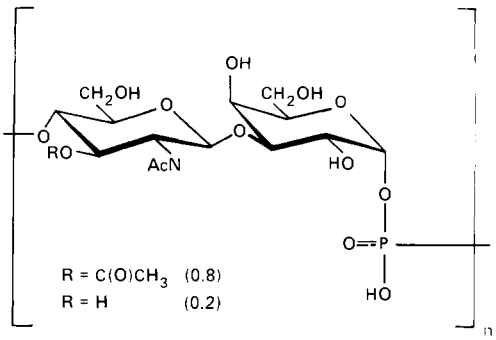


Figure 8. Repeating unit structure of *H. influenzae* type c capsular polysaccharide shown in its protonated form: →4)-β-D-GlcNAc-(1→3)-α-D-Gal-1-(PO_4→ with 80% *O*-acetylation at C-3 of the GlcNAc residue.

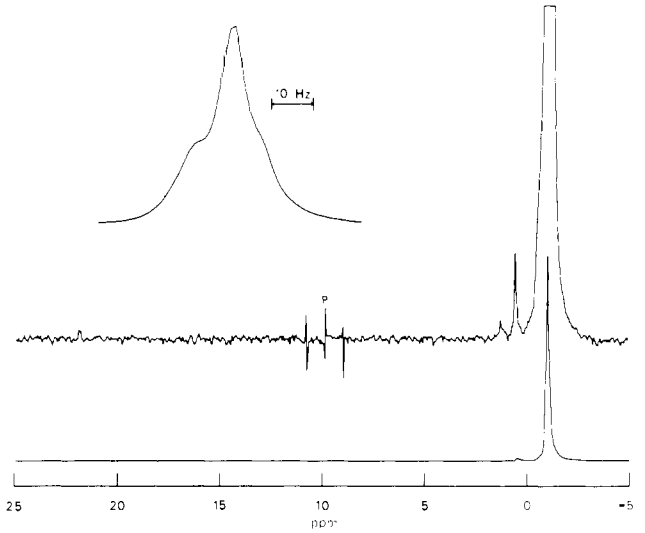


Figure 9. ^1H-decoupled 40.25-MHz ^{31}P NMR spectrum of *H. influenzae* type c (strain Ruggerio) capsular polysaccharide (18 mg/1.5 mL D_2O); $\pi/4$ pulse, 2-s repetition time, and 25 000 accumulations. The upper trace is an expanded and offset display of the repeating-unit phosphodiester linkages centered at −1.05 ppm. The middle trace was recorded at 25 times the amplitude of the lower trace; P refers to the pulse position. The small "peak" at 21.93 ppm was shown to be a spectrometer artifact.

indicated ca. 80% O-acetylation of the C_3 position in GlcNAc, which suggested that the ^{31}P signals result from phosphodiester linkages rendered nonequivalent by differences in their attached carbohydrate residues, as discussed above for the type f polysaccharide. Two phosphate monoester signals with a ca. 20:80 ratio were also seen (Figure 9) at 1.27 and 0.57 ppm, respectively; the correspondence between the extent of O-acetylation and this 20:80 ratio indicated that the signals are due to end groups attached to the C_4 position in nonacetylated and O-acetylated GlcNAc residues, respectively. These spectral interpretations were first tested by ^{31}P NMR spectroscopy to monitor the effects of O-deacetylation[9] with ND_4OD at 25 °C. After extensive hydrolysis of the *O*-Ac functionality, the major phosphodiester signal appeared at −0.94 ppm and is now identifiable with phosphodiester linkages connecting nonacetylated carbohydrate residues. As anticipated, the phosphate monoester signal at 1.27 ppm was the predominant end group seen in this O-deacetylated sample. The identities of the phosphate monoester termini in the original type c polysaccharide were further established by determining that neither underwent reaction with EDAC, which is consistent with transoid stereochemistry for C_4-OPO_3H_2 and C_3-OH in O-deacetylated GlcNAc terminal residues and argues against the existence of cisoid C_1–OPO_3H_2 and C_2–OH in galactosyl terminal residues (cf. Figure 8). End-group composition and average chain length data are summarized in Table V.

Hydrolysis. Sealed ampules containing a solution of type c capsular polysaccharide in 0.1 M glycine–NaOH buffer (pH 10,

Table V. ^{31}P NMR-Derived[a] Structural Data for Samples of Capsular Polysaccharide from *H. influenzae* Types a and c

type (strain)	phosphate monoester end groups,[b] %	repeating unit phospho-diester,[b] %	chain length[c]
a (Fin-35)	1.09[d]	98.91	91
a (Harding)	<4.00[d,e]	>96.00	>25
c (Ruggerio)	1.10[f]	98.90	90
c (Ruggerio)[g]	0.25[f]	99.75	399

[a] See Experimental Section for spectral acquisition parameters. [b] Relative molar percentages calculated from integrated signal intensities relative to the total value for all detectable end groups and the repeating unit phosphodiester linkage, except as noted. [c] See text and ref 12. [d] Attached to either D-Glc C_4 or D-ribitol C_5 or both of these positions; see text. [e] Line broadening, possibly due to nucleic acid contamination, led to a relatively high estimated upper limit; no signal was detected. [f] Attached to the C_4 position in partially O-acetylated D-GlcNAc; see text. [g] Different preparation from the previous entry.

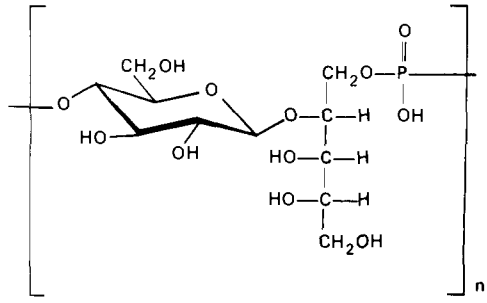


Figure 10. Repeating unit structure of *H. influenzae* type a capsular polysaccharide shown in its protonated form: →4)-β-D-Glc-(1→4)-D-ribitol-5-(PO_4→.

0.1 M $CaCl_2$) were heated at 100 °C for various times. ^{31}P NMR analysis of the hydrolysates showed that initial depolymerization (<20%) was accompanied by the gradual appearance of a monophosphate signal at 4.91 ppm and that two overlapping phosphodiester signals centered at −1.18 ppm maintained a relative ratio of roughly 20:80; no cyclophosphate signals were detectable. Reaction of partially depolymerized type c polysaccharide with EDAC led to disappearance of the phosphate monoester signal at 4.91 ppm and formation of a cyclophosphate signal at 16.38 ppm. The unchanged ratio of phosphodiester signals indicates that O-deacetylation is relatively slow under these hydrolysis conditions. With the assumption that the stereochemically preferred neighboring group participation by Gal C_2–OH leads to cleavage of phosphodiester linkages, the resultant Gal C_1–C_2 cyclophosphates do not accumulate but rather undergo comparatively facile ring opening to give monophosphate end groups seen at 4.91 ppm.

^{31}P NMR kinetic measurements gave the pseudo-first-order rate data listed in Table IV. The ca. 20-fold slower depolymerization rate for type c relative to type f polysaccharide suggests that subtle structural differences can influence the efficiency of neighboring group participation. In each of these polymers, torsion about a carbon–carbon bond is required to achieve a transition state wherein the participating hydroxyl group and the adjacent phosphodiester bond are syn-periplanar, and the relative energetics for such motion may be more favorable in the case of type f. Additional factors which may account for this rate difference include binding to Ca^{2+} and neighboring hydroxyl group pK_a.[17]

Type a. End-Group Studies. The repeating unit structure previously established[9] for the type a capsular polysaccharide is shown in Figure 10. This material gave a ^{31}P NMR spectrum (Figure 11) consisting of a sharp ($W_{1/2}$ = 5 Hz) resonance signal at 0.81 ppm for the phosphodiester linkage and a somewhat broadened ($W_{1/2}$ = 20 Hz) signal at 3.70 ppm. In addition, there was a broader ($W_{1/2}$ = 50 Hz) signal at −0.85 ppm, which is attributable to phospholipid contamination that was also present,

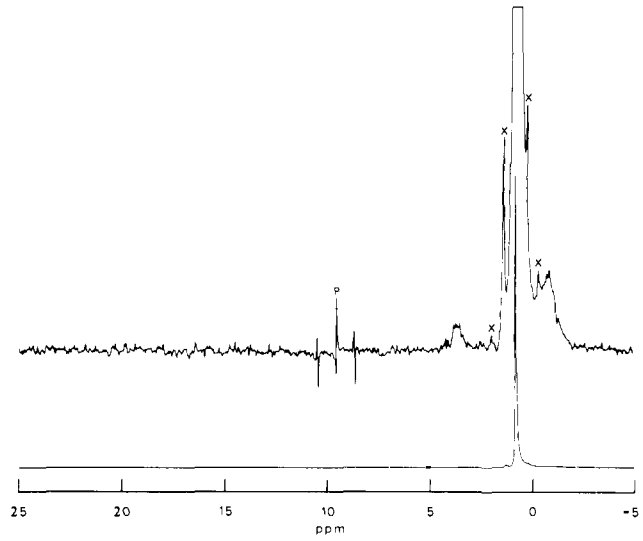


Figure 11. ^1H-decoupled 40.25-MHz ^{31}P NMR spectrum of *H. influenzae* type a (strain Fin-35) capsular polysaccharide (25 mg/1.5 mL); π/4 pulse, 2-s repetition time, and 22440 accumulations. The upper trace was recorded at 60 times the amplitude of the lower trace; P refers to the pulse position and X refers to a spinning sideband.

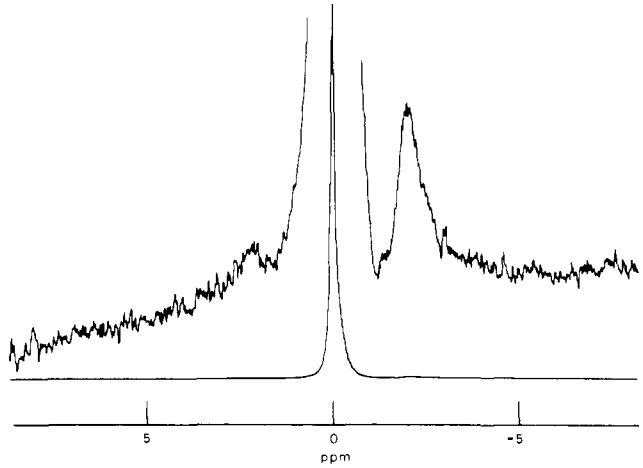
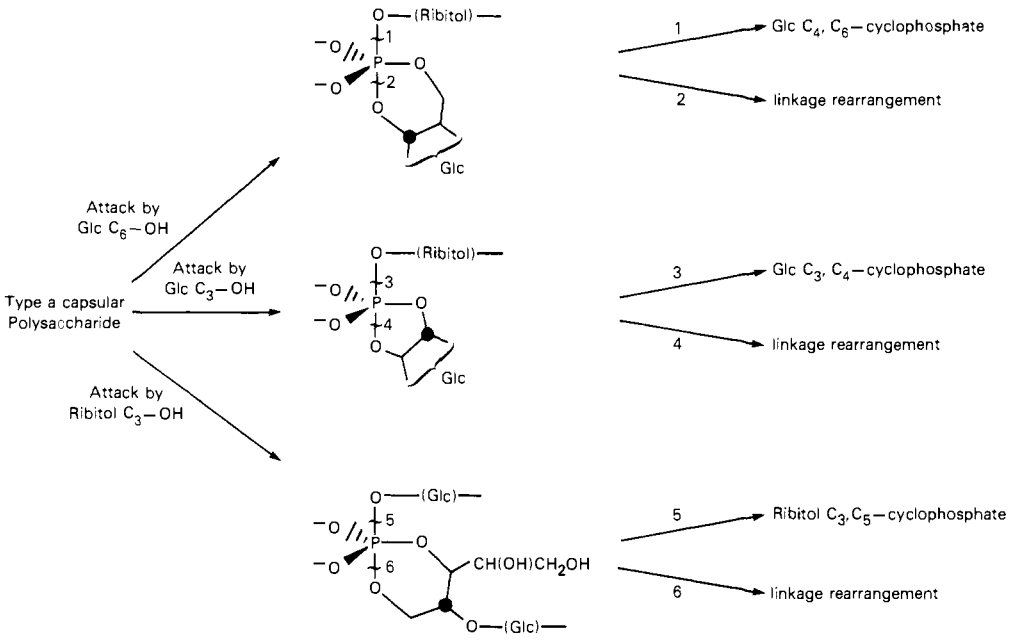


Figure 12. ^1H-decoupled 121.5-MHz ^{31}P NMR spectrum of *H. influenzae* type a (strain Fin-35) capsular polysaccharide (100 mg/1.5 mL); π/2 pulse, 2-s repetition time, and 5000 accumulations.

in various amounts, in other samples of type a and type f capsular polysaccharides. The signal at 3.70 ppm underwent chemical shift changes to 4.20 and 0.70 ppm at pH 8.4 and 3.5, respectively, indicative of a monophosphate. By analogy to the preceding results for other serotypes, phosphate monoester end groups in type a polysaccharide would be attached to either Glc C_4 or D-ribitol C_5 or both positions, which can, in principle, be differentiated by ^{31}P NMR spectroscopy using ^1H-coupled and selective ^1H irradiation modes. Unfortunately, such measurements were precluded by the excessive line width of the 3.70 ppm signal under various conditions: higher field strength (121.5 MHz, Figure 12), elevated temperature (70 °C), pH 3.5–8.4, Na^+ for Ca^{2+} exchange, and added EDTA. Reaction of the capsular polysaccharide with EDAC had no effect upon the phosphate monoester signal, which is consistent with both of the aforementioned end-group locations. A monophosphate group which is attached to Glc C_4 and is transoid to C_3–OH cannot form a stable C_3–C_4 cyclophosphate, while attachment to the D-ribitol C_5 position excludes the possibility of five-membered ring cyclophosphate formation. Table V lists end-group compositions and average chain lengths found for two strains of the type a capsular polysaccharide.

Hydrolysis. Base-catalyzed depolymerization of the type a polysaccharide was studied in the same manner as type c. After 19 h at 100 °C, the original phosphodiester signal at 1.19 ppm

Scheme IV

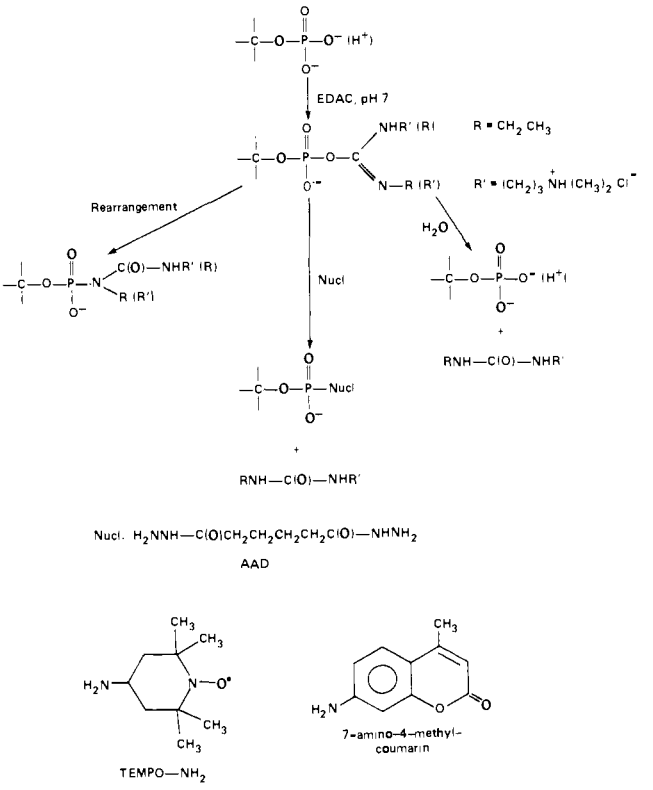


accounted for only 27% of the total ^{31}P NMR signal intensity. Five partially overlapped phosphate monoester signals at 4.54–5.46 ppm collectively represented 37% of the hydrolysate's phosphorus content, while the remaining 36% was accounted for by two new phosphodiester signals at −1.73 and −1.79 ppm. This hydrolysate was examined over a pH range of 2.5–10 to confirm the identity of all new signals as either phosphate monoester or phosphodiester. The original phosphodiester linkage accounted for 6% of the total ^{31}P NMR signal intensity after 46 h of heating; at this point the phosphate monoester and new phosphodiester signals each represented 47% of the phosphorus content.

The new phosphodiester signals at −1.73 and −1.79 ppm are indicative of linkage–rearrangement resulting from neighboring group participation. Scheme IV depicts participation by Glc C_4, Glc C_6, and D-ribitol C_3 alkoxide groups with subsequent P–O bond cleavage to afford either phosphodiester linkage–rearrangement or cyclophosphate precursors to the observed phosphate monoester end groups. For stereochemical reasons, each of these participation pathways is expected to be less favorable than those suggested for the previously discussed capsular polysaccharides and thus accounts for the comparatively slow depolymerization of the type a polymer (cf. Table IV). The linkage–rearrangement, which is unique to the type a polysaccharide, may be rationalized by invoking a pentacoordinate intermediate, as opposed to a pentacoordinate transition state for an "in-line" S_N2 displacement that provides no option for rearrangement. The latter mechanism is applicable to the types b, c, and f polysaccharides since in each of these cases the phosphodiester reaction center and participating alkoxide group(s) can adopt cisoid five-membered cyclic rearrangements that lend themselves (more or less favorably) to the "in-line" stereochemistry for the departing monophosphate moieties and incoming alkoxide ions.

End-Group Derivatization. The aforementioned inefficiency of intramolecular hydroxyl group attack at EDAC-activated phosphate monoester termini in type a capsular polysaccharide provided an opportunity for trapping the reactive *O*-phosphorylisourea intermediate[25,26] (Scheme V) with nucleophilic reagents to achieve *selective* derivatization of end groups in this polymer.[27] Such chemical modifications could provide useful

Scheme V



products and also serve as an independent method for verification of the ^{31}P NMR-derived end-group composition data. Under aqueous reaction conditions, bimolecular trapping of the *O*-phosphorylisourea moiety with added nucleophiles must compete

(25) Khorana, H. G.; Todd, A. R. *J. Chem. Soc.* **1953**, 2259. Khorana, H. G. *Chem. Rev.* **1953**, *53*, 145.

(26) Reaction of *O,O*-dialkylphosphorothio(seleno)ic acids with carbodiimides has been shown (Mikolajczyk, M.; Kielbasinski, P.; Goszczynska, Z. *J. Org. Chem.* **1977**, *42*, 3629) to give unstable *S(Se)*-phosphorylisothio-(seleno)ureas.

(27) Reaction of acetate ion and EDAC to form an *O*-acetylisourea occurs by protonation at nitrogen with concerted attack of acetate (Ibrahim, I.; Williams, A. *J. Chem. Soc., Chem. Commun.* **1980**, 25). By extension of this mechanism, it is expected that at pH 7 phosphate monoester end groups (pK_a^2 = ca. 6.5) would be much more reactive toward EDAC than the repeating unit phosphodiester linkages (pK_a = ca. 1.5). Reactions between the carbohydrate hydroxyl groups and EDAC to give electrophilic *O*-alkylisourea moieties are conceivable; however, catalysts such as Cu^+ are generally required for such transformations (Mathia, L. J. *Synthesis* **1979**, 561 and references therein).

with hydrolysis, which regenerates a phosphate monoester terminus but consumes EDAC (Scheme V). This competition could be minimized by using either a large excess of the trapping agent or an organic solvent with a soluble form of the polysaccharide, e.g., R_4N^+ salts. Another side reaction to consider is rearrangement[28] of the *O*-phosphorylisourea to a "blocked" ureido end group (Scheme V); however, in a control reaction with the type a polysaccharide and a 6-fold molar excess of EDAC for 24 h at pH 7/25 °C, it was found (^{31}P and ^1H NMR) that the recovered polymer was free of detectable ureido end groups.

Adipic acid dihydrazide (AAD) has been recently employed in the synthesis of polysaccharide–protein conjugates by reaction of cyanogen bromide activated *H. influenzae* capsular polysaccharides with AAD-functionalized proteins.[8] These conjugates have been found to have altered immunogenic properties;[8] however, the employed methodology affords a structurally complex matrix of polysaccharide and protein molecules. Attachment of protein to AAD-functionalized polysaccharide termini would, by contrast, provide relatively well-defined conjugates. Type a capsular polysaccharide (average chain length 91) was thus reacted (pH 7, 24 h, 25 °C) with 6 molar equiv of EDAC in the presence of 10 molar equiv of AAD, which respectively represent 546- and 910-fold molar excesses of these reagents relative to the phosphate monoester end groups. Following thorough dialysis of the reaction mixture against water, 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay for AAD incorporation indicated ca. 20 times the theoretical amount expected for covalent bonding of AAD to each phosphate monoester end group. Ion-exchange chromatography at pH 7 using the Na^+ form of a Dowex resin was employed for removal of electrostatically attached AAD from the product, and the recovered material was found to have incorporated 52% of the theoretical amount of AAD. A duplicate preparation and multiple TNBS assays gave an average coupling yield of 55 ± 11%. ^{31}P NMR analysis of the product showed a signal at 4.61 ppm in buffered solution at pH values of pH 8.4 *and* 5.0, which was assigned to the phosphoramidic end groups. The fact that no pH-dependent signal for residual phosphate monoester end groups was detectable suggests that the yield for AAD coupling may have been substantially greater than the TNBS-derived value of ca. 55%. Resolution of this discrepancy by ^1H NMR measurement of incorporated AAD was not possible due to interfering phospholipid signals (vide supra).

Extension of the above procedure to type f capsular polysaccharide (average chain length 73) afforded a product which was found by NMR analysis to be void of both phosphate monoester end groups and AAD methylene proton signals; however, there was an increase in cyclophosphate content. These results indicate that intramolecular trapping of the *O*-phosphorylisourea intermediate by an adjacent hydroxyl group was (on the basis of molar ratios) roughly 5000 times faster than intermolecular capture by a hydrazide moiety in AAD.

Type b capsular polysaccharide (strain Eagen) was partially hydrolyzed (14%) at pH 10 to create a measurable amount of phosphate monoester end groups (1.1%), and its tetrabutylammonium salt was then reacted with 6 molar equiv of EDAC and 30 molar equiv of AAD in anhydrous dimethyl sulfoxide (Me_2SO) as solvent. After dialysis and ion-exchange chromatography to remove excess AAD, TNBS assay indicated 57% of the theoretical amount of AAD incorporation. This finding supports the utility of nonaqueous reaction media for nucleophilic trapping of *O*-phosphorylisourea groups without significant interference by competing cyclophosphate formation, as was found for the type f polysaccharide in water.

The 4-amino-2,2,6,6-tetramethylpiperidinyl-1-oxy free radical (Tempo-NH_2, Scheme V) and related stable nitroxides have been used in a wide variety of biophysical spin-labeling studies,[29] and its reaction with the type a polymer was selected for synthesis of a prototype spin-labeled capsular polysaccharide. Tempo-NH_2, EDAC, and the type a polymer (average chain length 91) were reacted according to the procedure outlined for AAD, and this reaction afforded a product which exhibited a triplet ESR spectrum characteristic of the piperidinyl-1-oxy moiety. Comparison of the peak heights and line widths of the triplet pattern from this sample with those obtained for Tempo-NH_2 in a solution of the Na^+ form of type a polymer revealed a slightly motionally restricted (covalently bonded) spin label in the former case: assuming isotropic reorientation, the calculated rotational correlation times (τ_c) were ca. 1.55×10^{-10} and 9.56×10^{-11} s, respectively. Reaction of the spin-labeled polysaccharide with type a antiserum afforded a precipitate of antigen–antibody complex that showed a very broad triplet ESR spectrum (suspension in phosphate–saline buffer, pH 7.4), which is consistent with further immobilization of the spin-label. Comparison of the relative ESR signal intensities for the spin-labeled polysaccharide sample and the control sample of Tempo-NH_2 in a solution of the type a polymer indicated that 30% of the phosphate monoester end groups had reacted with Tempo-NH_2. Independent verification of this yield by ^{31}P and ^1H NMR was not possible because of paramagnetic line-broadening.

Fluorescence labeling was briefly investigated with type a polysaccharide, 7-amino-4-methylcoumarin (Scheme V), and reaction conditions analogous to those employed with AAD. The extent of covalent labeling was assessed by ^1H NMR in D_2O by using the relative integrated signal intensities for the coumarin CH_3 substituent (2.37 ppm) and all nonexchangeable carbohydrate protons; the 65% yield found by this method agreed reasonably well with a value of 46% that was determined from UV absorbance.

In concluding this section, it is worthwhile to note that various attempts were made to functionalize the type b polysaccharide by selective ring opening of the "strained" cyclophosphate end groups in this polymer. Acid-catalyzed hydrolysis with either HCl (0.1 M, 50 °C) or HOAc (1 M, 37 °C) was monitored by ^{31}P NMR spectroscopy, and it was found that cyclophosphate ring opening was not significantly faster than cleavage of the phosphodiester linkages, which presumably involves neighboring group participation. An alternative strategy involved reacting the tetramethylammonium form of type b polysaccharide with excess AAD and either NaF, CsF, or CaF_2 in 1:1 anhydrous Me_2SO–formamide. It was hoped that fluoride ion ring opening of the cyclophosphate end group would be followed by AAD trapping of the resultant fluorophosphate; however, ^{31}P NMR spectra of the reaction mixtures showed that at 50 °C there was gradual conversion of phosphodiester linkages into cyclophosphate termini without formation of detectable signals for either fluorophosphate or phosphoramidic end groups.

Conclusions

The present studies have demonstrated that ^{31}P NMR spectra of phosphodiester-linked capsular polysaccharides isolated from *H. influenzae* can be used to quantitatively measure the phosphate monoester and cyclophosphate end-group compositions of these bacterial polymers, which in turn leads to direct measurement of number-average chain lengths. For type b polysaccharide fractions obtained by size-exclusion chromatography, the number-average chain lengths correlated linearly with K_d values, indicating that the ^{31}P NMR method can be applied to analogous polymers in order to translate a relative molecular size parameter into number-average molecular weight.

Information regarding the location and stereochemical configuration of phosphate monoester attachment sites was deduced from ^{31}P NMR spectra following reaction of the capsular polysaccharide with EDAC, which causes intramolecular cyclization of phosphate monoester termini and adjacent cisoid hydroxyl groups to give cis-fused, five-membered ring, cyclophosphate residues having characteristic downfield chemical shifts in the range of ca. 15–20 ppm. In cases where such closure is precluded by O-acetylation of the terminal carbohydrate residue, NH_4OH can be used to selectively deblock the hydroxyl group for subsequent cyclization with EDAC. The phosphorus-containing end groups for each of the capsular polysaccharides reported herein

(28) Dekker, C. A.; Khorana, H. G. *J. Am. Chem. Soc.* **1954**, *76*, 3522.
(29) For reviews of recent studies in this area, see: "Spin Labeling Theory and Applications"; Berliner, L. J., Ed.; Academic Press: New York, 1976; pp 1–560.

appear to be exclusively attached to carbohydrate positions which are linked by the phosphodiester moiety in their respective repeating unit structures, and furthermore, the stereochemistry at these phosphorylated carbohydrate positions is the same as that in the repeating unit structure. These findings are consistent with biosynthetic proposals advanced for related bacterial cell-wall components[16] and also require that any enzymatic or chemical hydrolysis of the phosphodiester linkages in *H. influenzae* capsular polysaccharides occur with retention of configuration at the phosphodiester linkage. The cyclophosphate end groups detected in the types b and f capsular polysaccharides may be vestiges of such enzymatic processes. Factors which underlie the marked differences in end-group composition and number-average chain length between various strains of a given *H. influenzae* serotype are unclear at this time and deserve further attention.

A salient finding concerning the base-catalyzed hydrolytic depolymerization of the capsular polysaccharides is that the relative rates and initial products of cleavage of the repeating unit phosphodiester linkages can be rationalized by neighboring group participation mechanisms that consider stereochemical and statistical factors within the repeating unit structure. A predictable relationship between hydrolytic reactivity and repeating unit structure may be a useful criterion for distinguishing between alternative formulations that cannot be differentiated by conventional spectroscopic or chemical methods. Insight regarding capsular polysaccharide stability can also be of importance in the preparation and storage of vaccine products.

The utility of EDAC as a reagent for selective modification of phosphate monoester end groups in phosphodiester-linked polysaccharides has been demonstrated with diverse nucleophilic trapping agents. More importantly, the products can be applied to novel biological and biophysical studies to further elucidate the detailed nature of these bacterial capsular polysaccharides and their role as immunochemical determinants. Our work in these areas will be reported in the future.

Experimental Section

D-Ribitol 5-phosphate barium salt was obtained from Dr. Darrell Liu (FDA, Bureau of Biologics). Commercially available materials used as received are as follows: 2',3'-cAMP sodium salt (Aldrich), 3',5'-cAMP (Aldrich), EDAC (Bio-Rad), D-ribose 5-phosphate disodium salt (Sigma), Tempo-NH_2 (Aldrich), TNBS (Eastman Organic), 7-amino-4-methylcoumarin (Eastman Organic), tetramethylammonium hydroxide (Sigma), tetrabutylammonium hydroxide (Aldrich), CH_3ONH_2·HCl (Pierce Chemical), $NaBD_4$ (98 atom % D, Aldrich), Dowex AG 50W-X8 ion-exchange resin [200–400 mesh, H^+ form, 1.7 mequiv/mL resin bed (Bio-Rad)], CL-4B Sepharose (Pharmacia), and Tris (Bethesda Research Laboratories). AAD (Eastman Organic) was purified by decolorization with activated charcoal and then recrystallization (5 g) from hot 95% EtOH–H_2O (35:15, 50 mL). Dialysis tubing was obtained from Union Carbide (10 000 molecular weight cutoff) and Spectrum Medical Industries (3500 molecular weight cutoff); dialyses were generally performed at 5 °C. In all cases, "water" refers to deionized water.

Strains of the various *H. influenzae* serotypes were obtained from the following sources: type b—strain 1482, Dr. J. W. McReynolds, National Naval Medical Center, Bethesda, MD; strains Eagen and Madigan, Dr. P. Anderson, Children's Hospital Medical Center, Harvard Medical School, Boston, MA; strain JC-17, Dr. J. C. Parke, Charlotte Memorial Hospital, Charlotte, NC; strain Rab, Dr. H. Alexander, Babies Hospital, Columbia University, New York, through the courtesy of G. Leidy; type f—strain 644, Bureau of Biologics/NIH, ATCC No. 9833, Bethesda, MD; strain 686, L. Wetterlow, Massachusetts Department of Public Health, Jamaica Plain, MA; B095, Dr. W. L. Albritton, University of Manitoba, Winnipeg, Canada; type c—strain Ruggerio, L. Wetterlow; type a—strain Fin-35, Dr. H. Makela, Helsinki, Finland; strain Harding, L. Harding, Children's Hospital Medical Center, Harvard Medical School, Boston, MA. The capsular polysaccharides were isolated as previously described,[30] and determinations of protein, phosphorus, nucleic acid, endotoxin, moisture, *O*-acetyl, and molecular size were carried out according to published methods.[31]

Absorbance (A) and ESR measurements were obtained with Cary 15 and Varian E9 instruments, respectively. Atomic absorption analyses for Ca^{2+} were kindly performed by Norene Sullivan (National Institute of Dental Research, NIH, Bethesda, MD).

Nuclear Magnetic Resonance Spectroscopy. ^{31}P NMR spectra were recorded by quadrature phase detection at either 40.25 MHz on a JEOL FX-100 spectrometer or 121.5 MHz on a Bruker WM-300 spectrometer. Samples were dissolved in 90:10 H_2O–D_2O (1.5 mL) and placed in 10-mm NMR tubes. The sampling conditions at 40.25 MHz were as follows: 13 μs $\pi/2$ pulse; 5-kHz spectral window; 8192 data points. Sampling conditions at 121.5 MHz were as follows: 30 μs $\pi/2$ pulse; 10-kHz spectral window; 8192 data points. Prior to Fourier transformation, spectra were zero-filled with 8192 data points and exponentially multiplied so as to result in an additional 1-Hz line broadening in the frequency domain spectra. Low-power (to minimize dielectric heating of the samples), continuous-wave ^1H decoupling was routinely employed. Chemical shifts are given in parts per million and are relative to external 25% H_3PO_4. Individualized spectral details are presented in the appropriate figure legends. For more quantitative measurements, the possible nuclear Overhauser effect was suppressed by a gated-decoupling technique; the delay time between pulse sequences was 30 s.

^{13}C NMR spectra were recorded at 75.47 MHz on a Bruker WM-300 spectrometer equipped with quadrature phase detection; spectral conditions: a 30 μs $\pi/2$ pulse, 20-kHz spectral window, 16 384 data points, 1.5-s pulse repetition rate. Prior to Fourier transformation, spectra were zero-filled with 16 384 data points and exponentially multiplied so as to result in an additional 0.5-Hz line broadening in the frequency domain spectra. Broad-band ^1H decoupling was employed. NMR spectra of the Na^+ and Ca^{2+} forms of *H. influenzae* type b polysaccharide were determined as 25 mg/1.5 mL solutions in D_2O, and the $(n\text{-Bu})_4N^+$ salt as a 40 mg/1.5 mL solution in D_2O, using 10-mm NMR tubes. Spectra were determined at pH ~7.0, 25 °C. Chemical shifts were measured relative to the hydroxymethyl carbon for comparing the Na^+ and $(n\text{-Bu})_4N^+$ type b salts, otherwise to internal TSP.

^1H NMR spectra were recorded as D_2O solutions in 5-mm NMR tubes, using either the continuous-wave mode with a Varian HR-220 spectrometer (220 MHz) or the Fourier transform mode with either a JEOL FX-100 spectrometer (99.55 MHz), a Bruker WM-300 spectrometer (300.153 MHz), or a Nicolet Technology NT-360 spectrometer (360 MHz). Conditions for the pulse Fourier transform spectra include the following: a 10-ppm spectral window; 8192 data points (zero-filled to 16 384, 32 768, and 32 768 at 100, 300, and 360 MHz, respectively); $\pi/2$ pulses of 18 μs, 6 μs, and 6 μs at 100, 300, and 360 MHz, respectively. Prior to Fourier transformation, spectra were exponentially multiplied so as to result in an additional 0.25-Hz line broadening in the frequency domain spectrum. Chemical shifts were measured relative to internal TSP.

^{23}Na NMR spectra were recorded on a Bruker WM-300 spectrometer at 79.388 MHz, using 10-mm NMR tubes and ca. 35 mg of sample in 1.5 mL of D_2O. Spectral conditions were as follows: 2048 data points, 10-kHz spectral window, 30-μs $\pi/2$ pulse, and pulse recycle time of 0.65 s. Prior to Fourier transformation, the free induction decay signal was zero-filled to 8192 data points and then exponentially multiplied so as to result in an additional 3-Hz line broadening in the frequency domain spectrum.

Temperatures of NMR kinetic samples were obtained by immersion of a copper–constantan thermocouple connected to a precalibrated Doric Trendicator 400 type T/C digital readout temperature meter. Measurement of pH utilized a precalibrated Radiometer PHM 64 instrument equipped with an Ingold combination electrode that could be inserted directly into the NMR tube. Values of pH recorded for solutions containing D_2O refer to observed readings and have not been corrected for isotope effects.[32]

Ion Exchange. General Procedure. Dowex ion-exchange resin was sequentially washed with 6 N HCl, water, 1 N MOH [$M^+ = Na^+$, H_4N^+, $(CH_3)_4N^+$, or $(n\text{-Bu})_4N^+$], and water to afford the desired cation form of the resin. A 100-mL plastic syringe was filled with 50 mL of freshly prepared resin for loading ca. 50–150-mg samples of capsular polysaccharide dissolved in 2–5 mL of water. Elution with 150 mL of water followed by lyophilization gave >90% yields of the expected material. In the case of R_4N^+ exchange, the initial Ca^{2+}-containing polysaccharide was first converted to its Na^+ form (>99.9% Ca^{2+} removal, atomic absorption); ^1H NMR (220 MHz) confirmed the expected 1:1 repeating unit: R_4N^+ stoichiometry (integrated signal intensities for nonexchangeable carbohydrate vs. alkyl proton absorptions).

D-Ribitol 4,5-Cyclophosphate. A suspension of the Ba^{2+} salt of D-ribitol 5-phosphate (60 mg, 0.6 mmol) in water (5 mL) at 5 °C was

(30) Gotschlich, E. C.; Rey, M.; Etienne, C.; Sanborn, W. R.; Trian, R.; Cvetanovic, B. *Prog. Immunol. Stand.* **1972**, *5*, 485.

(31) Wong, K. H.; Barrera, O.; Sutton, A.; May, J.; Hochstein, D.; Robbins, J. D.; Robbins, J. B.; Parkman, P. D.; Seligmann, E. B., Jr. *J. Biol. Stand.* **1977**, *5*, 197.

(32) Lumry, R.; Smith, E. L.; Glantz, R. R. *J. Am. Chem. Soc.* **1951**, *73*, 4330.

brought to pH 2.5 with 1 N H_2SO_4, and after 5 min $BaSO_4$ was removed by centrifugation. Lyophilization of the supernatant gave D-ribitol 5-phosphate as a hygroscopic white powder (35 mg, 92%), which was identified by ^{13}C NMR.[11] A sample of D-ribitol 5-phosphate (20 mg, 0.086 mmol) in D_2O (1.5 mL) at pH 4.7 was combined with EDAC (15 mg, 0.78 mmol) and then monitored at 25 °C by ^{31}P NMR spectroscopy; starting material signal at 3.12 ppm, product peak at 19.06 ppm. After 1 h the pH (2.7) was adjusted to 5.3 with 1 N NaOH and more EDAC (20 mg, 0.10 mmol) was added. The reaction was complete after a total of 2 h. Ion exchange of the reaction mixture using Dowex resin (5 mL) in the H_4N^+ form gave, following lyophilization, the H_4N^+ salt of D-ribitol 4,5-cyclophosphate (80% yield) as a white powder, which was further characterized by ^{13}C NMR spectroscopy.[11]

D-Ribitol-*1-d* 5-Phosphate. The disodium salt of D-ribose 5-phosphate (1 g, 3.42 mmol) and $NaBD_4$ (0.8 g, 20 mmol) were reacted in water (10 mL) for 1 h at 25 °C. Portions of Dowex resin (H^+ form) were added with vigorous stirring to the diluted (10 mL) reaction mixture until H_2 evolution ceased, and the filtered solution was then concentrated on a rotary evaporator with added methanol. The residue was dissolved in water (10 mL), treated with saturated aqueous $Ba(OH)_2$ (30 mL), and after adjustment to pH 7 with glacial HOAc, an equal volume of EtOH was added to precipitate the product, which was collected by centrifugation and washed with water (10 mL). Conversion to the free acid (97% yield) with H_2SO_4 and cyclization with EDAC were carried out as described above for D-ribitol 5-phosphate.

Reaction of Capsular Polysaccharides with EDAC. General Procedure. The following details for *H. influenzae* type b are representative. After the ^{31}P NMR spectrum of the capsular polysaccharide (25 mg, 0.068 mmol) was recorded in 9:1 H_2O–D_2O (1.5 mL), the pH was maintained at 7 ± 0.5 with 1 N NaOH during the gradual addition of a solution of EDAC (25–75 mg, 0.13–0.39 mmol) in water (0.2 mL). The ^{31}P NMR spectrum was recorded after the mixture was stirred for 5–15 h at 25 °C. Isolation of the polysaccharide product (>90% yield) was achieved by dialysis against NaCl (1 L, 0.2 M, 2 times) and then water (1 L, 2 times) followed by ion exchange with Dowex resin in the Na^+ form as described above.

Reactions with *O*-Methylhydroxylamine. D-Ribose. A solution of CH_3ONH_2·HCl (42 mg, 0.50 mmol) in D_2O (3 mL) was adjusted to pH 7 with 1 N NaOD in D_2O and was then stirred with D-ribose (60 mg, 0.40 mmol) for 2 h at 25 °C, keeping the pH at 7 ± 0.5. The reaction mixture was saturated with NaCl and then extracted with EtOAc (3 × 3 mL). Removal of the solvent under reduced pressure gave the expected[33] *E* and *Z* isomers of D-ribose *O*-methyloxime, which were identified by ^1H NMR spectroscopy (360 MHz); see text for pertinent data.

***H. influenzae* Type b.** A sample of the capsular polysaccharide (strain Eagen, 25 mg, 0.068 mmol) in water (1.5 mL) was reacted with CH_3O-NH_2·HCl (5.1 mg, 0.061 mmol) according to the above procedure. The reaction mixture was dialyzed against $CaCl_2$ (5 L, 0.2 M) and then water (5 L) before lyophilization to afford a sample (88% yield) which had no detectable $HC{=}NOCH_3$ signals (6–8 ppm) in its ^1H NMR (360 MHz) spectrum. Repetition of this experiment with exclusion of the dialysis steps gave a similar result. An identical sample of the capsular polysaccharide starting material was heated at 37 °C in 1 M HOAc for 24 h and then neutralized with 1 N NaOH. ^{31}P NMR signal intensities for the monophosphate end groups (ca. 4 ppm) and remaining phosphodiester linkages (ca. 1 ppm) had a relative ratio of 5:95. The hydrolysate was dialyzed against water (5 L, 3 times), and the retained material was reacted with CH_3ONH_2·HCl (5.5 mg, 0.066 mmol) at pH 7 ± 0.5 for 15 h at 25 °C. The reaction mixture was dialyzed against $CaCl_2$ (5 L, 0.2 M) and then water (5 L) before lyophilization to give the product (25 mg, 100% recovery); ^1H NMR (360 MHz): 7.61 ppm, d, $^3J_{HH}$ = 6.4 Hz. The extent of D-ribosyl *O*-methyloxime formation was not quantified.

Reactions with PhOH/NaOAc. A solution of the type b capsular polysaccharide (strain 1482, 28 mg) in water (5 mL) was vigorously shaken for 4 h at 37 °C with an equal volume of PhOH/NaOAc solution (prepared from 454 g of PhOH in 9:1 water-saturated aqueous NaOAc, pH 7). After centrifugation, the upper layer was dialyzed against $CaCl_2$ (5 L, 0.1 M, 2 times) and then water (5 L, 2 times). Lyophilization gave recovered material (88%) that had a ^{31}P NMR spectrum identical with that of the starting material (Table II). Repetition of this experiment using D-ribitol 5-phosphate (15 mg) and excluding the dialysis steps afforded (100%) unchanged starting material (^{31}P NMR).

O-Deacetylation of Capsular Polysaccharide Types f and c. The following details are representative. A solution of the type f polysaccharide (strain 644, 25 mg) in D_2O (2 mL) was adjusted to pH 11 with 30% ND_4OD–D_2O. A ^1H NMR (220 MHz) spectrum was recorded after 15 h at 5 °C (see Figure 7), and the pH was increased from 9.5 to 11. After an additional 24 h at 25 °C, the ^1H NMR spectrum was again recorded (see Figure 7). ^{31}P NMR (40.25 MHz) at pH 10: repeating unit phosphodiester, 0 ppm (relative chemical shift), 62.5% (relative intensity); phosphate monoester, 5.06 ppm, 11.3%, and 6.96 ppm, 5.1%; cyclophosphate, 17.92 ppm, 21.3%.

Alkaline Hydrolysis Kinetics. The 0.1 M glycine–NaOH buffer[34] (pH 10) was stored at 5 °C and was freshly prepared after 30-day periods. Samples contained 4 mg of capsular polysaccharide dissolved in 1.4 mL of buffer and 0.1 mL of aqueous 1.5 M $CaCl_2$. Kinetic runs with the type b and type f materials were carried in tightly capped NMR tubes (10 mm), using the JEOL FX-100 variable temperature controller and the selective ^1H-decoupling mode. Aliquots (1.5 mL) of the type a and type c capsular polysaccharide solutions were sealed in standard glass ampules and submerged in a controlled-temperature water bath. After samples (six) were heated for a given kinetic run, they were frozen and stored for batchwise ^{31}P NMR analysis; see text for results. A $\pi/2$ pulse, 2-s recovery time, and nuclear Overhauser enhancement (NOE) were used in all cases, which gave accurate pseudo-first-order kinetic data (Table IV) but only approximate product ratios, due to NOE and T_1 differences. Values of k' were derived from linear least-squares fits (<±10% slope errors) of ln (C_0/C_t) vs. time, where C_0 = (repeating unit phosphodiester signal intensity)/(total signal intensity), at zero time, and C_t is similarly defined for aliquots heated for time t.

Reaction of Type a Capsular Polysaccharide with AAD/EDAC. The polysaccharide (strain Fin-35, 52 mg, 0.13 mmol) was dissolved in water (10 mL) and the pH was adjusted to 7 by a pH stat with 0.1 N HCl titrant. A solution of AAD (225 mg, 1.3 mmol) in water (2 mL) was added at 25 °C followed by gradual (5 min) addition of a solution of EDAC (150 mg, 0.78 mmol) in water (1 mL). After 2 h of stirring, the reaction mixture was kept at 5 °C for 16 h and was then dialyzed against $CaCl_2$ (5 L, 0.2 M, 2 times) and then water (5 L, 2 times). Lyophilization gave recovered material (50 mg) that was ion exchanged (2 times) with a 25-mL bed of Dowex resin in the Na^+ form and 100 mL of water as the eluent; ^{31}P NMR at pH 8.4 (Tris) and pH 5.0 (HOAc/NaOAc): end-group signal at 4.61 ppm. TNBS assay (vide infra) for AAD incorporation: 2.40 μg of AAD/mg of product, 52% yield; duplicate, 42% yield; repeat reaction, 71% yield; control reaction (no EDAC), 0% yield.

TNBS Assay. Stock solutions were freshly prepared as follows: 5×10^{-5} M AAD, 3 g of TNBS/100 mL of water, and water saturated at room temperature with sodium borate decahydrate. Various aliquots (10–200 μL) of the AAD stock solution were each diluted with water to a volume of 0.5 mL, and each sample was then combined with 0.5 mL of the borate solution and 0.15 mL of the TNBS reagent. After exactly 10 min, absorbance (A) values were quickly measured at 500 nm and were then plotted as A vs. ng of AAD. Aliquots (50, 100, and 200 μL) of the polysaccharide samples were then processed as described above; the average value of ng AAD/mg polysaccharide was corrected by a factor of 2.

Reaction of Type b Capsular Polysaccharide with AAD/EDAC. The polysaccharide (strain Eagen, 100 mg) was dissolved in 0.1 M glycine–NaOH buffer (37.5 mL, pH 10, 0.1 M $CaCl_2$) and then heated at 60 °C for 30 min. The reaction mixture was neutralized, dialyzed against water (5 L, 2 times), and then lyophilized (75 mg); ^{31}P NMR: 14% hydrolysis of phosphodiester linkages, 1.1% phosphate monoester end groups. Ion exchange of this product with Dowex resin in the $(n\text{-}Bu)_4N^+$ form gave material (90 mg, 0.15 mmol) that was dissolved in anhydrous Me_2SO (6 mL) and then reacted with AAD (783 mg, 4.5 mmol) and EDAC (172 mg, 0.9 mmol) for 24 h at 25 °C. Undissolved AAD was removed by filtration through a plug of glass wool, and the filtrate was added to Tris buffer (pH 7.5, 6 mL) before dialysis against $CaCl_2$ (1 L, 0.1 M, 2 times) and then against water (1 L, 2 times). The dialyzed product was ion exchanged with Dowex resin in the Na^+ form and then lyophilized (70 mg); TNBS assay: 2.86 μg of AAD/mg (57% yield).

Reaction of Type a Capsular Polysaccharide with Tempo-NH_2/EDAC. The polysaccharide (strain Fin-35, 52 mg, 0.13 mmol) was dissolved in water (10 mL) and was reacted with Tempo-NH_2 (250 mg, 1.46 mmol in 2 mL of wter) and EDAC (150 mg, 0.76 mmol in 1 mL of water) for 24 h at 25 °C. After dialysis and ion exchange (once) as described in the preceding paragraph, a portion of the lyophilized product was prepared to a concentration of 46 mg of polysaccharide/2 mL of water; a comparative sample of 45 mg of starting polysaccharide (Na^+ form)/2 mL of water contained 10^{-4} M Tempo-NH_2; relative ESR signal intensities (peak height times width at half-height) 1.00:0.52 (1.86×10^{-4} M spin-label in product sample; 30% yield).

Type a antiserum (50 mL, FDA burrow no. 255) containing 0.25 mL of 20% aqueous NaN_3 was centrifuged at 10000 rpm for 30 min at 5 °C, and the cold supernatant was added to a solution of the spin-labeled

(33) Laine, R. A.; Sweeley, C. C. *Carbohydr. Res.* **1973**, *27*, 199.

(34) Williams, C. A.; Chase, M. W., Eds. "Methods in Immunology and Immunochemistry"; Academic Press: New York, 1968; Vol. II, p 395.

polysaccharide (1.25 mg) in water (0.8 mL). After incubation for 1 h at 37 °C and then storage for 15 h at 5 °C, the antigen-antibody complex was pelleted by centrifugation (10 000 rpm).

Reaction of Type a Capsular Polysaccharide with 7-Amino-4-methylcoumarin/EDAC. The polysaccharide (strain Fin-35, 50 mg, 0.13 mmol) was dissolved in water (5 mL) and then reacted with 7-amino-4-methylcoumarin (126 mg, 0.7 mmol in 2 mL of water) and EDAC (230 mg, 1.2 mmol in 2 mL of water) for 24 h at 25 °C. After filtration of the undissolved coumarin and processing as described in the preceding section, the UV absorbance of the lyophilized product (2.95×10^{-3} M repeating unit, $A_{354} = 0.30$) was compared with that of a 7-amino-4-methylcoumarin (1.50×10^{-6} M) plus type a polysaccharide (2.95×10^{-3} M repeating unit) standard sample ($A_{354} = 0.03$); 46% yield. ^{1}H NMR (100 MHz): (normalized CH_3 signal intensity)/(normalized carbohydrate signal intensity) = 0.0071; 65% yield.

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Registry No. EDAC, 25952-53-8; CH_3ONH_2·HCl, 593-56-6; GalNAc C_3 monophosphate, 81158-42-1; 3-(OAc)GalNAc C_1 monophosphate, 81158-43-2; GlcNAc C_4 monophosphate, 81158-44-3; 4-(OPD_3H_2)Glc NAcO acetylated, 81158-45-4; AAD, 1071-93-8; Tempo-NH_2, 14691-88-4; D-ribosyl 2,3-cyclophosphate, 81158-46-5; D-ribitol 4,5-cyclophosphate, 81203-22-7; D-ribosyl 3-phosphate, 81158-47-6; D-ribitol 5-phosphate, 35320-17-3; D-ribose, 50-69-1; (*E*)-D-ribosyl *O*-methyl oxime, 81203-23-8; (*Z*)-D-ribosyl *O*-methyl oxime, 81203-24-9; galactosyl C_3,C_4-cyclophosphate, 81158-48-7; 7-amino-4-methylcoumarin, 26093-31-2.

Proton-Nuclear Spin Relaxation and Molecular Dynamics in the Lysozyme-Water System

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Abstract: ^{1}H NMR relaxation measurements are reported for lyophilized lysozyme rehydrated through the gas phase to 0, 13, and 21 g of water/100 g of protein. Protein proton and water proton magnetizations were monitored simultaneously, and the applicability of the cross-relaxation model decribing exchange of magnetization between these two spin baths at high temperatures is supported in detail. At the lowest temperatures, water motion becomes sufficiently slow that the separation between water and protein baths is no longer observed; in this region the water protons simply add to the relaxation load of the solid which is dominated by the rotating methyl groups. Analysis of the relaxation parameters derived from the cross-relaxation analysis requires inclusion of the effects of anisotropic water molecule motion in order that the activation parameters for water reorientation are reasonable when compared with thermodynamic measures of the water-protein interaction.

Introduction

Water is a crucial component of living systems. Understanding water in the complex soup of cytosol is complicated by its interaction with many structures of diverse composition ranging from small solute ions to macroscopic surfaces. The present study is part of an attempt to understand better the dynamical properties of water in the water-protein interface. To simplify the physical problem, we have chosen to immobilize the protein, i.e., to eliminate bulk rotational motion of the protein molecules by studying lyophilized powders rehydrated through the gas phase to various water contents. The possibility that the relatively dry state of the proteins studied here offers a nonnative and possibly inactive conformation is not of great concern because we are confident that the major features of the water-protein interaction are preserved despite minor protein conformation changes.

Nuclear magnetic resonance relaxation is among the useful and direct methods of studying the dynamics of the water-protein interaction.[1-3] Many NMR studies of the water on surface systems have been incomplete because proper account of magnetic interactions between water protons and protein protons has not been included. Though several have taken this magnetic cross-relaxation into account,[4-9] to date there has not been a data set sufficiently complete to test all aspects of the relaxation models over wide temperature ranges. We have, therefore, measured ^{1}H NMR relaxation for protein protons and water protons simultaneously over a wide temperature range for lysozyme powder systems at several water contents and isotopic compositions. The results provide a basis for a critical evaluation of the several models used to interpret such data.

In particular we monitor the protein proton and water proton magnetization simultaneously and check their initial amplitudes and time dependence against the predictions of the cross-relaxation model most clearly formulated by Edzes and Samulski.[7] From careful analysis of the response to selective excitation, we extract the cross-relaxation-model parameters characterizing the two spin systems, water and protein, and the transfer of magnetization between them. Analysis of the data using approximate strategies is evaluated, and the water relaxation parameters are further analyzed critically in order to draw conclusions about the nature of water-molecule motion at the protein surface. This analysis

(1) Pfeifer, H. In "NMR, Basic Principles and Progress"; Diehl, P., Fluck, E., Kosfeld, R., Eds.; Springer-Verlag: New York, 1972; Vol. 7, pp 53-153.
(2) Cooke, R.; Kuntz, I. D. *Ann. Rev. Biophys. Bioeng.* **1974**, *3*, 95-126.
(3) Bryant, R. G. *Annu. Rev. Phys. Chem.* **1978**, *29*, 167-188.
(4) Hilton, B. D.; Hsi, E.; Bryant, R. G. *J. Am. Chem. Soc.* **1977**, *99*, 8483-8490.
(5) Bryant, R. G.; Shirley, W. M. "Water in Polymers"; Rowland, S. P., Ed.; American Chemical Society, Washington, D.C., 1980; *ACS Symp. Ser.* 127, 147-156.
(6) Kruger, G. J., presented at Fourth International Symposium on Magnetic Resonance, Rehovot, Israel, 1971.
(7) Edzes, H. T.; Samulski, E. T. *J. Magn. Reson.* **1978**, *31*, 207-229.
(8) Koenig, S. H.; Bryant, R. G.; Hallenga, K.; Jacob, G. S. *Biochemistry* **1978**, *17*, 4348-4358.
(9) Fung, B. M.; McGaughy, T. W. *J. Magn. Reson.* **1980**, *39*, 413-420.

0002-7863/82/1504-2910$01.25/0