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ULTRASONIC IRRADIATION OF BACTERIAL POLYSACCHARIDES. CHARACTERIZATION OF THE DEPOLYMERIZED PRODUCTS AND SOME APPLICATIONS OF THE PROCESS

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ABSTRACT

Ultrasonic irradiation (u.i.) has been used to depolymerize biopolymers including DNA, dextran, and the Vi capsular polysaccharide from *Citrobacter freundii*. Representative bacterial polysaccharides were subjected to u.i. and the effect of this energy upon their molecular weight and chemical structure was characterized. U.i. depolymerized a neutral polysaccharide (dextran) and acidic polysaccharides containing either a phosphoric diester linkage [*Haemophilus influenzae* type b (Hib) and pneumococcus types 6A and 6B] or a uronic acid moiety (pneumococcus type 9N). Prolonged u.i. depolymerized all the polysaccharides to a finite and similar molecular mass (~50 000 daltons). The rate of depolymerization induced by u.i. depended on the viscosity of the solvent and the concentration of the polysaccharide. ^{13}C-N.m.r. data of the native Hib polysaccharide and its depolymerized products indicated that u.i. did not alter the chemical structure of the repeating units. Determination of the monophosphate terminal residues by ^{31}P-n.m.r. spectroscopy and of the reducing end groups by the Park–Johnson reaction indicated that both the phosphoric diester and the glycosidic linkages were cleaved. The Vi polysaccharide, prepared as an investigational vaccine, could not be analyzed for its chemical structure by ^{13}C-n.m.r. spectroscopy owing to its high viscosity but depolymerization by u.i. permitted this analysis. The finite molecular weight of the products observed after prolonged u.i. is best explained by the postulation that the mechanical torque necessary to rupture the linkages is dependent upon the length of the polysaccharide. The method of u.i. for depolymerization is useful for the preparation of homogeneous, low-molecular-weight polysaccharides without alteration of the chemical structure of the repeating units.

INTRODUCTION

Many methods have been applied to depolymerize biopolymers into lower-molecular-weight fragments that retain biological activity. Such techniques as acid

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and alkaline hydrolysis, or thermal denaturation may, however, yield depolymerized products having modified chemical structures. Enzymic digestion involving the terminal monosaccharide unit is only effective for depolymerization of linear homopolymers. Enzymes that are specific for one linkage in copolymers have been used to prepare fragments of polysaccharides; this method for depolymerization usually provides heterogeneous products in comparatively low yields. Shearing stress, such as rotation in a shear flow rotor[1,2], ultrasonic irradiation (u.i.)[3-7], or passage through a syringe needle[7], have been shown to depolymerize macromolecules including polysaccharides. U.i., which can generate velocity gradients as high as 10^{-5} cm, has been used[4-7] to depolymerize polymers, such as polystyrene[8], polyvinyls[9], collagen[3], Vi polysaccharide[10], and DNA. It was proposed, in the case of DNA, that u.i.-induced cleavage occurred by a "random double-stranded scission"[5]. There is a lower limit to the molecular weight of the fragments produced by u.i.; the molecular-weight distribution of such products is relatively monodisperse. In spite of its long and successful applications in the study of DNA, no detailed understanding of the mechanism(s) of the depolymerization induced by u.i. is as yet available.

DNA and some bacterial capsular polysaccharides share common physicochemical properties: (a) both are linear polymers with negative or zero charge; (b) both exist in random-coil conformations and are difficult to crystallize; and (c) both form aggregates in low-ionic environments or in trivalent counter-ions solutions.

The molecular weight of polysaccharides has been related to their immunological properties[11]. Martin *et al.*[10] showed that u.i. treatment of the Vi capsular polysaccharide of *C. freundii* reduced both its immunogenicity and protective activity against *Salmonella typhi* infection in mice. The immunogenicity of the polysaccharide component of polysaccharide–protein conjugates may also be related to its molecular weight. Makela *et al.*[12] showed that dextran, M_r ~20 0000–40 000, covalently bound to serum albumin elicited higher levels of antibodies than conjugates prepared with dextrans of either higher- or lower-molecular weight. The ability to form conjugates with carrier proteins may also depend on the molecular weight of the polysaccharide. In addition, specimens of lower-molecular weight are preferable for n.m.r. analyses of polysaccharide structures and their interaction with small ligands, because the spin-lattice-relaxation time is shorter and signal-to-noise levels are improved[13].

We report herein studies on the effect of u.i. upon bacterial polysaccharides. Depolymerization of the polysaccharides was measured by molecular-weight estimations of the products subjected to gel filtration. The site of cleavage and chemical composition of u.i.-depolymerized polysaccharide were studied by ^{31}P,^{13}C-n.m.r. spectroscopy and by measurement of the molar ratio of the reducing sugars. Comparisons were made with other methods of depolymerization, *viz.*, acidic, alkaline, and thermal degradation. U.i. was shown to cause cleavage of the linkages between the repeating units of four representative bacterial polysaccharides.

EXPERIMENTAL

Materials. — Pneumococcal capsular polysaccharides, types 6A (Institute Merieux, Lyon, France), 6B and 9N (Lederle Laboratories, Pearl River, New York), *Haemophilus influenzae* type b (Hib), and Dextran T-2000, T-110, T-50, and T-10 (Pharmacia Fine Chemicals, Piscataway, New Jersey), were used. The structures of these polysaccharides[14–19] are shown in Table I. The mol. wts. of the dextrans were determined by light scattering as specified by the manufacturer. Solutions of the polysaccharides (5.0 mg/mL), unless stated otherwise, were prepared in 0.2M NaCl. The pH of these polysaccharide solutions ranged from 6.0 to 7.5. D-Ribose (Sigma Chemical Co., St. Louis, MO) was used as a reference for the Park–Johnson reaction for reducing sugars. Chemicals used in these studies were of the highest quality available from commercial sources.

Ultrasonic irradiation (u.i.). — A Heat System Ultrasonic (Model W225R) sonicator with a tapered microprobe (0.3 mm diameter) and an Artek system (Model 300) sonicator with a 0.9-mm tapered probe were used. The temperature during u.i. was maintained at $+5\pm3°$. U.i. treatment of the polysaccharides was performed under a stream of N_2 to minimize oxidation. Samples were withdrawn during the u.i., passed through a 0.22-nm membrane (Millipore Corporation, Bedford, MA), and then subjected to gel filtration.

Gel filtration. — The molecular weight of the polysaccharides and their depolymerized products were estimated by gel filtration through Sepharose CL-6B with 0.2M NaCl as the eluent, at room temperature as described[20]. A column (1.5 × 30 cm) was calibrated with dextrans of different mol. wts. The polysaccharides (0.25 mL) were loaded on to the CL-6B Sepharose column at a concentration of 1.0 mg/mL.

Spectroscopic methods. — The polysaccharides were examined by n.m.r. spectroscopy as described[21]. Hib polysaccharide (20.0 mg/mL in 0.2M NaCl) was subjected to u.i. for 20 and 60 min. Vi polysaccharide was subjected to u.i. for 90 min. Both products were dialyzed against de-ionized water, freeze-dried, and reconstituted in 99.1% D_2O–0.2M NaCl for n.m.r. analysis.

^{13}C-N.m.r. spectra were recorded at 37° for analysis of Hib and at 60° for analysis of Vi polysaccharide using a 5-mm sample tube and a Nicolet 270 spectrometer operating at 67.89 MHz in the pulse F.t. mode with complete proton decoupling and quadrature-phase detection. A 4-Hz line broadening was applied to the signal prior to F.t. to enhance the signal-to-noise ratio. Chemical shifts are positive for signals downfield from ^{13}C-enriched acetonitrile which was the internal reference signal.

The solutions were adjusted to pH 7.7 with NaOH for the ^{31}P-n.m.r. analysis to ensure that signals for the monophosphate end-group would be shifted downfield relative to the signals for repeating-unit phosphoric diester linkage[21]. ^{31}P-N.m.r. spectra at 121.47 MHz were recorded as described[20], and the molar ratio (R) of repeating-units to monophosphate termini was obtained from the relative

TABLE I

STRUCTURES OF THE BACTERIAL POLYSACCHARIDES

Polysaccharide	*Structure of repeating unit*	*Ref.*
Dextran	(1→6)-D-Glc*p*	14
Pneumococcus type 6A	→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-D-Ribitol-5-(PO$_4^-$→	15
Pneumococcus type 6B	→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→4)-D-Ribitol-5-(PO$_4^-$→	16
Pneumococcus type 9N	→4)-α-D-Glc*p*A-(1→3)-α-D-Glc*p*-(1→3)-β-D-Man*p*NAc-(1→4)-α-D-Glc*p*-(1→4)-β-D-Glc*p*NAc-(1→	17
H. influenzae type b	→3)-β-D-Rib*f*-(1→1)-D-Ribitol-5-(PO$_4^-$→	18
Vi	→4)-α-D-Gal*p*NAcA-(1→	19

signal intensities (peak-heights) of the phosphoric diester and monophosphate signals measured at δ −2.87 and 0.4, respectively, relative to external trimethyl phosphate as a chemical-shift reference.

Reducing end-group analysis. — The concentration of D-ribofuranose (PO_2H→3-*b*-D-Rib*f*) and D-ribofuranosyl[*b*-D-Rib*f*-(1→1)-D-Ribol→] termini were determined by a modified Park–Johnson reaction for reducing sugars[22]. The extinction coefficient for D-ribose in this reaction was 19.1/mM/cm. Solutions of Hib polysaccharide (0.5 mg/mL) were analyzed before and after u.i. treatment. The molar amount of reducing sugar components induced by u.i. was compared with the theoretical value for complete depolymerization of Hib polysaccharide. The ratio of these values was used to calculate the percentage of glycoside bonds cleaved by u.i.

Theoretical models of u.i. depolymerization. — The generally accepted theory for u.i.-induced depolymerization is that the sound waves induce transient cavitation. In the vicinity of these u.i.-induced collapsing cavities, the rapid motion of the solvent generates shearing forces that break chemical bonds within the polymer. Polymers subjected to u.i., therefore, have a reduction in their mol. wt. Two commonly observed phenomena induced by u.i. are (a) the occurrence of a limiting degree of depolymerization (P_L) and (b) the mol. wt. distribution of the depolymerized products follows the most probable Schutz distribution[4,23]. An expression (*1*) for the rate equation that is P_L dependent was formulated by Ovenall *et al.*[24], where B is the number of bonds broken in a unit volume, *Pi* is the degrees of

$$\frac{dB}{dt} = \sum_{i=L}^{m} K_i(Pi - Ni) \qquad Pi > P_L \qquad (1)$$

$$\frac{dB}{dt} = 0 \qquad Pi < P_L$$

polymerization at time *t*, and P_L is the limit degree of polymerization, respectively. The concentration of molecules with the degree of polymerization *Pi* is *Ni*. The first-order rate constant, *k*, is a function of the experimental conditions, such as the irradiation intensity and frequency, temperature, the presence or absence of free radicals, viscosity, pressure, etc. This expression and other similar equations have been used to interpret the effects of u.i.-induced depolymerization. These formulas imply that the rate of bond rupture induced by u.i. during the early stages is greater than that at the later stages. The shearing force is related to the length of the chain on either side of a given linkage. These expressions, however, do not provide an explanation for the narrowing of the mol. wt. distribution of the depolymerized products. The asymmetric bell-shaped distribution of mol. wts. in the early stage of u.i. and the Schutz distribution in the later stage suggest that the cleavage is random[24]. We investigated the validity of these ideas by use of representative bacterial polysaccharides. Comparisons were made between the mol. wts. of the depolymerized products and those expected from the rate equation *1*. We also investigated the chemical nature of the cleavage process in order to further understand the action of u.i. upon polysaccharides.

RESULTS AND DISCUSSION

Effect of ultrasonic irradiation (u.i.) upon the molecular weight of bacterial polysaccharides. — Dextran T2000 was used as a model to study the effect of u.i. upon the molecular weight of neutral polysaccharides. Fig. 1 shows the gel filtration profiles of Dextran T2000 through CL-6B Sepharose before and after exposure to u.i. The untreated Dextran T2000 showed a comparatively polydisperse gel-filtration profile and was largely excluded from the gel. U.i. of Dextran T2000 for 5 min reduced its molecular weight to ~142 000; treatment for 15, 30, and 60 min reduced the molecular weight to ~100 000, 79 000, and 50 000, respectively (Table II). Further u.i. had less pronounced effect upon the molecular weight of the dextran T2000 (data not shown). U.i. also resulted in a narrower molecular-weight distribution of the depolymerized Dextran T2000 as revealed by the decreased width of the peaks in the gel-filtration profiles of the treated samples. The rate of depolymerization became progressively lower as the molecular weight of the dextran approached a limiting value of 50 000. U.i. of the other five polysaccharides exerted a similar effect to that obtained with Dextran T2000; there was both a reduction and a narrowing of the molecular-weight distribution of the depolymerized products. All the polysaccharides had a limiting molecular weight of ~50 000 and a progressive decrease in the rate of depolymerization after prolonged u.i. (Table II). The decrease in the rate of depolymerization of the Dextran T2000 (neutral polysaccharide) and the five other polysaccharides (acidic polysaccharides) following prolonged u.i. was consistent with equation *1* where ($\mathrm{d}B/\mathrm{d}t$) is greater for higher-

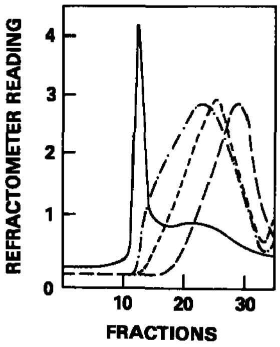

Fig. 1. Gel filtration profiles on Sepharose 6B-Cl of Dextran T2000 exposed to u.i. for various times: (——) untreated, (—·—) 15 min, (-----) 30 min, and (– – –) 60 min. The polysaccharides subjected to u.i. have both lower molecular weights and narrower molecular weight distributions than the parent molecules.

TABLE II

THE EFFECT OF ULTRASONIC IRRADIATION UPON THE MOLECULAR WEIGHT OF BACTERIAL POLYSACCHARIDES[a]

Polysaccharide	*Molecular weight (× 10^{-3}) after ultrasonic irradiation for*			
	0 min	*5 min*	*30 min*	*60 min*
Dextran T2000	100→2000	142	79	50
Pneumococcus type 6A	100→2000	180	80	[b]
Pneumococcus type 6B	>1000	140	92	50
Pneumococcus type 9N	>1000	144	93	52
H. influenzae type b	>1000	170	105	51
Vi Polysaccharide	>4000	[b]	[b]	78

[a]The mol. wt. of bacterial polysaccharides subjected to u.i. for various intervals was measured by gel filtration through Sepharose 6B-Cl equilibrated in 0.2M NaCl. [b]Not performed.

molecular-weight polymers. The decrease in the molecular weight and narrowing of the molecular-weight distribution were similar to the pattern observed[6] for DNA exposed to u.i. The limiting molecular weight of ~50 000 was also similar to that observed for DNA and other polymers exposed to prolonged u.i.

The effect of u.i. upon the structure of polysaccharides. — Depolymerization caused by u.i. can be accompanied by secondary side reactions and result in structural modifications. For example, the process of depolymerization results in an increased concentration of terminal groups; these newly formed termini may be relatively reactive compared to the parent polysaccharide. An increase in the concentration of reducing end groups (aldehydes) or monophosphoric esters could result in chemical modification of the polysaccharide during u.i.[25]. Trace metal ions, such as Cu^{2+}, or additives with high-binding properties, have been shown to alter the chemical structure of DNA during u.i.[5]. The presence of molecular oxygen in the reaction mixture could result in chemical changes to biopolymers during u.i. We maintained a stream of nitrogen over the reaction cell during the u.i. to avoid this complication. Martin *et al.*[10] reported that u.i. of Vi polysaccharide did not affect the *O*-acetyl or *N*-acetyl content of the depolymerized products. These groups are often removed by other methods of hydrolysis.

To investigate these points, the chemical structure of Hib polysaccharide was examined by ^{13}C-n.m.r. spectroscopy before and after prolonged u.i.; the spectral data of the Hib were unchanged before and after u.i. for 60 min (Fig. 2). Since the nuclear Overhauser enhancement was not suppressed during the data-acquisition period, the peak intensities were not quantitatively analyzed. Collectively, these data suggested that the repeating-unit structure of polysaccharides is not altered during the depolymerization induced by u.i.

Selectivity of bond cleavage. — It has not been established whether the depolymerization of biopolymers induced by u.i. is selective or random. Most investigators have proposed that the cleavage of biopolymer linkages is random, based upon the Schutz distribution of molecular weights following prolonged u.i.[4,5,26]. A "random", *i.e.*, chemically nonselective cleavage of linkages, is consistent with our finding that polysaccharides of diverse structure are depolymerized by u.i. at a similar rate and to a similar finite size (Table II).

The terminal groups in the depolymerized products of Hib polysaccharide were characterized to further elucidate the nature of the u.i.-induced cleavage. Hib polysaccharide contains a comparatively acid-labile phosphoric diester and a glycoside linkage. Theoretically, depolymerization of this polysaccharide could generate three types of end groups or nine different combinations of end groups[27]. Depolymerization leading to reducing-type ribosyl residues can be quantified by the Park–Johnson method[22]. The molar ratio of the repeating units to the reducing groups could not be measured accurately for the native Hib polysaccharide. It was 294 after 20 min of u.i. (120 000 daltons) and 217 after 60 min of u.i. (50 000 daltons), indicating that u.i. treatment had increased the amount of reducing terminal groups in the depolymerized Hib polysaccharide (Table III). The proportions of phosphoric diester bonds within the repeating unit and terminal monophosphoric ester groups (either D-ribofuranoside 3-monophosphate or D-ribitol 5-monophosphate) were determined by ^{13}P-n.m.r. spectroscopy (Fig. 3). The amount of terminal monophosphoric ester groups for the native Hib polysaccharide was too small to be measured. The molar ratios of the repeating unit to terminal monophosphoric ester groups were 470 and 290 for Hib polysaccharide after u.i. for 20 and 60 min, respectively. Combining the data for the reducing end and the terminal monophosphoric ester groups, we calculated that there were 0.34 ribosyl and 0.22

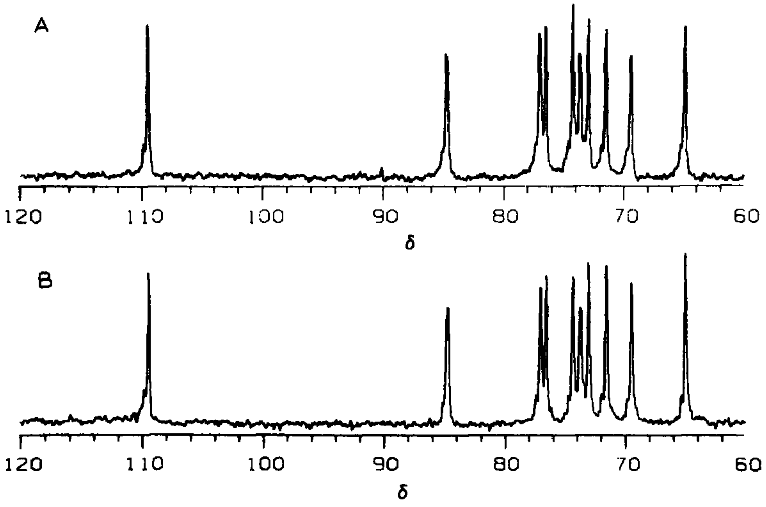

Fig. 2. ^{13}C-N.m.r. chemical shift of Hib polysaccharide before (A) and after (B) u.i. for 60 min with ^{13}C-enriched acetonitrile as an internal standard. The spectra were recorded for a solution in a 5-mm tube at 37°, with a Nicolet 270 spectrometer operating at 67.89 MHz in pulse F.t. mode with complete proton-decoupling and quadrature-phase detector. The data were taken at 38 000 accumulations with nuclear-Overhauser enhancement.

monophosphate end groups per 100 residues after 20 min of u.i. Treatment with u.i. for 60 min yielded 0.46 ribosyl and 0.34 monophosphate end groups per 100 residues. U.i. of Hib polysaccharide, therefore, resulted in a progressive increase in both the expected end groups, which is consistent with the proposal that u.i. induces cleavage of all the linkages in the repeating unit structure. Frenkel[28] has shown that the force necessary for cleavage of a rigid-rod polymer was proportional to the square of its length. Polysaccharides, such as Hib, are considered to have comparatively flexible structures. The force necessary for cleavage of polysaccharides by u.i. is proportional to their length. When the length of the Hib polysaccharide was reduced to a limiting value, the bending moment induced by u.i. was not sufficient to cause further cleavage.

Viscosity effects. — The viscosity of biopolymers is lowered upon depolymerization by u.i.[1,4,10], and the extent of u.i.-induced depolymerization can be monitored by measurement of the intrinsic viscosity of the reaction mixture. We studied the effect of solvent viscosity upon u.i.-induced depolymerization of Dextran T2000 by adding, to the reaction mixture, glycerol[29] to a final concentration of either 10 or 20% w/w. The constant increase in solvent viscosity, unaffected by the u.i., that resulted from the addition of glycerol was much greater than the decrease in the intrinsic viscosity of Dextran T2000 induced by prolonged u.i. (data not shown). The addition of glycerol resulted in an increase in the rate of depolymerization of Dextran T2000 induced by u.i. and in a lowered limiting molecular weight (Fig. 4). These two effects of glycerol upon the depolymerization induced by u.i. were greater for the 20% than those for the 10% glycerol, indicating that the mechanism of u.i. cleavage of Dextran T2000 was related to the viscosity of the solvent; this relation may be described by Eq. *2*, where v is the shear-flow velocity from the cavitation generated by u.i. and F the viscosity force exerted on the polymer. Thus,

$$\vec{F} = -\eta \operatorname{grad} \vec{v} \qquad (2)$$

TABLE III

END-GROUP COMPOSITION[a] OF *Haemophilus influenzae* TYPE b POLYSACCHARIDE SUBJECTED TO ULTRASONIC IRRADIATION

End groups	*Duration of treatment (min)*		
	0	*20*	*60*
D-Ribose	<0.01	0.34	0.46
PO_4	<0.01	0.22	0.34
D-Ribitol	<0.001	0.11[b]	0.15[b]
Estimated mol. wt.[c]	>100 000	103 500	69 000

[a]End groups/100 repeating units. [b]Estimated from end-group distribution by acid hydrolysis[21]. [c]Mol. wt. calculated, based on the molar ratio of the sum of all three end groups to the repeating units.

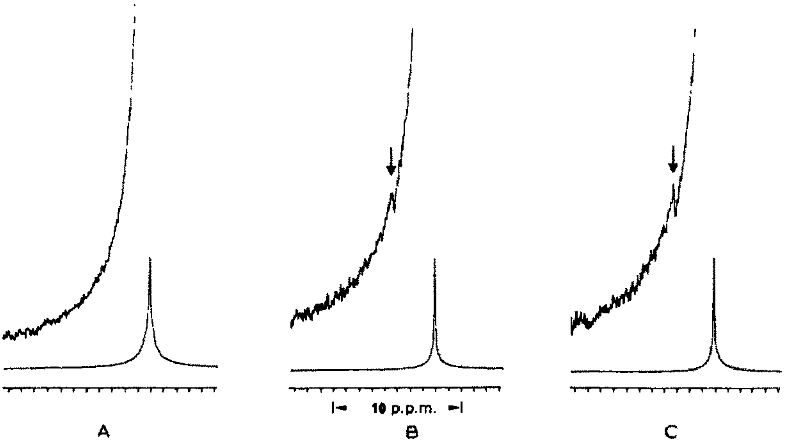

Fig. 3. ^{31}P-N.m.r. spectra recorded at 121.47 MHz for aqueous samples of Hib polysaccharide before (A), and after 20 (B) and 60 min (C) of exposure to u.i. In each spectrum, the relatively intense signal at δ −2.87 of the repeating-unit phosphoric diester linkage was on-scale at an amplitude setting of ×1. The relatively weak signal at δ 0.40 for the monophosphate end group, indicated by the arrow, was recorded at an amplitude setting of ×100 for the u.i.-treated material, and at ×25 for the starting material, due to the greater width-at-half-height for the phosphoric diester signal. Chemical shifts were measured relative to an external standard of trimethyl phosphate.

the addition of glycerol may be used to increase the viscosity force of the solvent and obtain a lower limiting molecular weight of the depolymerized dextran, as immobilization of the dextran by solvents of high viscosity enhances the u.i.-induced bending force.

Concentration effects. — A direct relation between the concentration of the polysaccharide and the rate of depolymerization induced by u.i. was observed, and this relation is illustrated by the depolymerization rates for two concentrations (5.0 and 20.0 mg/mL) of Hib polysaccharide (Fig. 5). The time of u.i. required to reach the limiting molecular weight was proportional to the concentration of Hib polysaccharide. This concentration dependence of the rate of depolymerization induced by u.i. has been observed for other polymers. In his study of the relation between the concentration of poly(methyl methacrylate) and u.i.-induced depolymerization in benzene, Henglein[30] found that the rate of depolymerization increased as the concentration of the polymer increased between 0.2 to 40 μg/mL. The rate of depolymerization was maximal at 40.0 μg/mL, and then progressively decreased as the concentration increased. These differences in the effect of the concentration of the polymer upon the rate of u.i.-induced depolymerization originated from an interplay of several variables, such as the total number of bonds to be cleaved, the intensity of the cavitation generated by u.i., and the viscosity force due to the concentration of the polymer.

Comparison of u.i. with other methods of depolymerization. — The depolymerizations induced by thermal energy and by u.i. of the two constitutionally related pneumococcal type 6A and type 6B polysaccharides (Table I) were

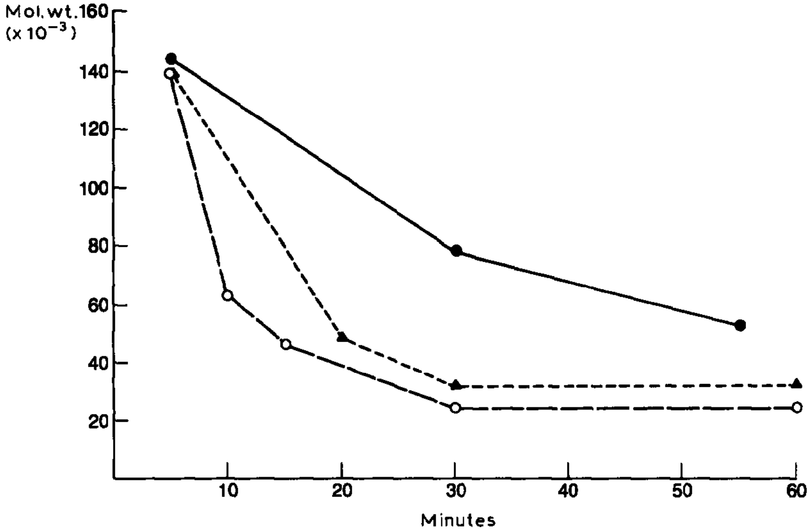

Fig. 4. The effect of addition of glycerol to Dextran T2000 upon the rate and extent of depolymerization induced by u.i. The viscosity of the solutions is expressed as η: (—●—●—, η 1.55) Dextran T2000 alone, (--▲--▲--, η 2.01) plus 10% glycerol, and (—○—○—, η 3.85) plus 20% glycerol.

compared. Thermal depolymerization of type 6B occurred by random cleavage of various linkages within the repeating unit[31], whereas that of type 6A polysaccharide was largely due to cleavage of the phosphoric diester bonds[31]. The difference in the lability of the phosphoric diester bond affected the rate of depolymerization considerably ($>10^3$ faster for type 6A). Extensive thermal depolymerization reduced both types of molecules to oligosaccharides. U.i., in contrast, had an indiscriminate effect upon the depolymerization of type 6A and type 6B polysaccharides and resulted in a limiting molecular weight of several hundred monomer units for each (Table II).

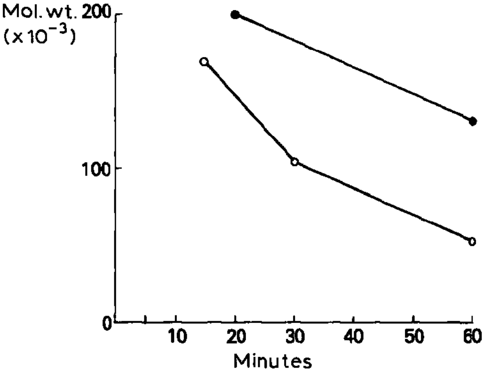

Fig. 5. The effect of Hib polysaccharide concentration upon the depolymerization induced by u.i.: (●—●) conc. 20 mg/mL and (○—○) conc. 5.0 mg/mL.

Acid and alkaline hydrolysis of polysaccharides also lead to the formation of oligosaccharides and the mode of cleavage is relatively selective. For instance, when Hib polysaccharide was subjected to hydrochloric acid or to alkaline hydrolysis, only 70% (on a molar basis) of the products had terminal groups as monophosphoric esters[27]. By contrast, exposure to 68% hydrofluoric acid promoted, almost exclusively, phosphoric diester cleavage. Acid and thermal hydrolysis of dextran, which is composed only of glycoside linkages[14], occurred in a progressive manner and gave D-glucose or oligosaccharides[32]. U.i.-induced cleavage of the glycoside bonds is less selective, and the rate of depolymerization is directly related to the molecular weight of the dextran. The molecular weight of the final product of u.i.-treated dextran was ~50 000.

N.m.r. studies of polysaccharide structure, conformation, and interactions with ligands can be facilitated by partially depolymerized polysaccharides. For example, the molecular weight of the native Vi polysaccharide (>4 × 10^6, Table II) was too high for resolution of the polymer by chromatography on Sepharose 2B, and the linewidth of the ^{13}C-n.m.r. signals were broad (~25 Hz, Fig. 6). After u.i. treatment, the mol. wt. of the Vi polysaccharide was reduced to 78 000. It was, therefore, possible to prepare a solution of the depolymerized Vi polysaccharide at a higher concentration (20 mg/mL) than with the native preparation, which facili-

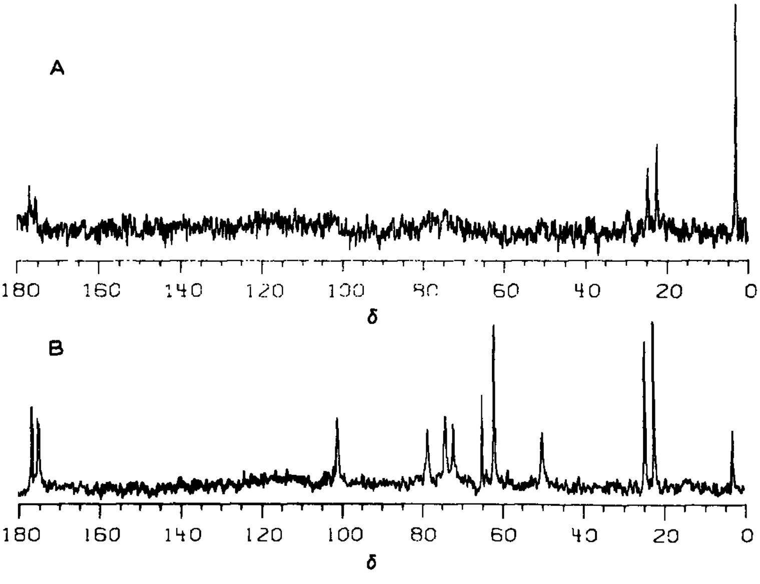

Fig. 6. ^{13}C-N.m.r. chemical shift of: (A) the native Vi polysaccharide (mol. wt. >4 000 000, conc. 10 mg/mL) and (B) Vi polysaccharide depolymerized by u.i. (mol. wt. 48 000, conc. 20 mg/mL), downfield from ^{13}C-enriched acetonitrile used as an internal standard. The spectra were recorded for a solution in a 5-mm tube with a Nicolet 270 spectrometer operating at 67.89 MHz in pulse F.t. mode with complete proton-decoupling and quadrature-phase detector. The data were accumulated for 50 000 scans with nuclear-Overhauser enhancement.

tated accumulation of ^{13}C-n.m.r. signals that showed a linewidth reduced to ~14 Hz and a greatly improved signal-to-noise level (Fig. 6).

It is worthwhile to emphasize (a) that u.i. is a reproducible and convenient method for obtaining lower-molecular-weight fragments of the same repeating unit as the parent molecule without loss of material; (b) that there is no need for further purification to remove the products of adventitious side-reactions, such as take place in the acid-catalyzed rearrangement of pneumococcus type 6A polysaccharide[31]; and (c) that the potential application of this depolymerization technique is the preparation of polysaccharide components for covalent binding to T-dependent proteins (conjugates) that are more immunogenic in this configuration than the native polysaccharides[12,33].

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REFERENCES

1 R. E. ADAM AND B. H. ZIMM, *Nucleic Acids Res.*, 4 (1977) 1513–1537.
2 R. E. HARRINGTON AND B. H. ZIMM, *J. Phys. Chem.*, 69 (1965) 161–175.
3 I. E. EL'PINER, *Ultrasound: Physical, Chemical and Biological Effects*, Consultants Bureau, New York, 1961.
4 A. R. PEACOOKE AND N. J. PRITCHARD, *Prog. Biophys. Mol. Biol.*, 18 (1968) 185–208.
5 A. W. DAVIS AND D. R. PHILLIPS, *Biochem. J.*, 173 (1978) 179–183.
6 C.-W. CHEN, J. S. COHEN, AND A. ZADAR, *J. Biochem. Biophys. Methods*, 5 (1981) 293–295.
7 D. C. RICHARDS AND P. D. BOYER, *J. Mol. Biol.*, 11 (1965) 327–340.
8 N. H. LANGTON AND P. VAUGHAN, *Br. J. Appl. Phys.*, 8 (1957) 289–292.
9 N. SATO, H. OKAYAMA, AND K. CHINJO, *Kolloid-Z.*, (1951) 46.
10 D. G. MARTIN, F. G. JARVIS, AND K. C. MILNER, *J. Bacteriol.*, 94 (1967) 1411–1416.
11 E. A. KABAT AND A. E. BEZER, *Arch. Biochem. Biophys.*, 78 (1958) 306–310.
12 O. MAKELA, F. PETERFLY, J. G. OUTSCHOORN, A. W. RICHTER, AND I. SEPPALA, *Scand. J. Immunol.*, 19 (1984) 541–550.
13 D. R. KEARNS, *Annu. Rev. Biophys. Bioeng.*, 6 (1977) 477–523.
14 F. R. SEYMOUR, R. D. KNAPP, AND S. H. STEPHEN, *Carbohydr. Res.*, 51 (1976) 179–194.
15 P. A. REBERS AND M. HEIDELBERG, *J. Am. Chem. Soc.*, 83 (1961) 3056–3059.
16 L. KENNE, B. LINDBERG, AND J. K. MADDEN, *Carbohydr. Res.*, 73 (1979) 175–182.
17 K.-G. ROSELL AND H. J. JENNINGS, *Can. J. Biochem. Cell Biol.*, 61 (1983) 1102–1107.
18 R. M. CRISEL, R. S. BAKER, AND D. E. DORMAN, *J. Biol. Chem.*, 250 (1975) 4926–4933.
19 K. HEYNS, G. KIESSLING, W. LINDENBERG, H. PAULSEN, AND M. E. WEBSTER, *Chem. Ber.*, 92 (1959) 2435–2437.
20 K. H. WONG, O. BARRERA, A. SUTTON, J. MAY, D. H. HOCHSTEIN, J. D. ROBBINS, J. B. ROBBINS, P. D. PARKHAM, AND E. B. SELIGMANN, JR., *J. Biol. Stand.*, 5 (1977) 197–215.
21 W. EGAN, R. SCHNEERSON, K. E. WERNER, AND G. ZON, *J. Am. Chem. Soc.*, 104 (1982) 2898–2910.
22 M. PARRO, S. VITI, G. ANTONI, AND P. NERI, *Anal. Biochem.*, 118 (1981) 301–306.
23 R. A. GIBBONS, S. N. DIXON, AND D. H. POCOCK, *Biochem. J.*, 135 (1973) 649–655.
24 D. W. OVENALL, G. W. HASTINGS, AND P. E. M. ALLEN, *J. Polym. Sci.*, 33 (1958) 207–212.

25 M. A. K. MOSTAFA, *J. Polym. Sci.*, 33 (1958) 295–316.
26 P. ALEXANDER AND M. FOX, *J. Polym. Sci.*, 12 (1954) 533–541.
27 G. ZON AND J. D. ROBBINS, *Carbohydr. Res.*, 114 (1983) 103–121.
28 J. FRENKEL, *Acta Physicochim. URSS*, 14 (1944) 51–76.
29 M. T. RECORD, JR., C. P. WOODBURY, AND R. B. INMAN, *Biopolymers*, 14 (1975) 393–408.
30 A. HENGLEIN, *Makromol. Chem.*, 15 (1955) 188–210.
31 G. ZON, S. C. ZSU, W. EGAN, J. D. ROBBINS, AND J. B. ROBBINS, *Infect. Immun.*, 37 (1982) 89–103.
32 M. STACEY, *Research (London)*, 4 (1951) 48.
33 R. SCHNEERSON, O. BARRERA, A. SUTTON, AND J. B. ROBBINS, *J. Exp. Med.*, 141 (1980) 140–154.