Carbon-13 Nuclear Magnetic Resonance Spectroscopic Study of Teichuronic Acid from *Micrococcus luteus* Cell Walls. Comparison of the Polysaccharide Isolated from Cells with That Synthesized in Vitro†

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ABSTRACT: Teichuronic acid isolated from the cell walls of *Micrococcus luteus* has been examined by natural-abundance ^{13}C NMR spectroscopy. Proton-decoupled and proton-coupled spectra were obtained for native teichuronic acid and also after the teichuronic acid had been oxidized with periodate and reduced with borohydride. The spectra are consistent with the structure $[ManNAcUA_p\text{-}(\beta\text{-}1,6)\text{-}Glc_p\text{-}(\alpha\text{-}1,4)]_n$. Teichuronic acid synthesized in vitro from suitable substrates by the particulate enzyme fraction obtained from *M. luteus* yielded a ^{13}C NMR spectrum which is indistinguishable from that of the native teichuronic acid, indicating a structural identity of the teichuronic acid synthesized in vitro with that isolated from cell walls.

Cell walls of *Micrococcus luteus* (*Micrococcus lysodeikticus*) consist of peptidoglycan and a glucose-containing anionic polysaccharide (Czerkawski et al., 1963). This polysaccharide, which is called teichuronic acid, is composed of alternating residues of D-glucose and *N*-acetyl-D-mannosaminuronic acid (ManNAcUA)[1] (Perkins, 1963). Chemical degradation studies showed that teichuronic acid has the repeating sequence $[ManNAcUA_p\text{-}(\beta\text{-}1,6)\text{-}Glc_p\text{-}(\alpha\text{-}1,4)]_n$ (Hase & Matsushima, 1970, 1971, 1972). We have studied the biosynthesis in vitro of a polymeric material presumed to be teichuronic acid (Page & Anderson, 1972; Anderson et al., 1972; Rohr et al., 1977; Stark et al., 1977). We now report on the use of ^{13}C NMR spectroscopy to show that the teichuronic acid produced in vitro by a particulate enzyme fraction from *M. luteus* (in vitro teichuronic acid) has the same covalent structure as the teichuronic acid isolated from cell walls (native teichuronic acid). The spectra indicate that the structure of both native and in vitro teichuronic acid preparations is consistent with that reported previously (Hase & Matsushima, 1972).

Experimental Procedures

Isolation of the Particulate Enzyme Fraction and Native Teichuronic Acid. *Micrococcus luteus* (*M. lysodeikticus*) ATCC 4689 cells harvested from 30 to 40 L of late-exponential phase cultures grown in a medium of peptone (10 g/L) and sodium chloride (5 g/L) (Page & Anderson, 1972) were washed in 85 mM sodium chloride and resuspended in 25 mM Tris-HCl, pH 8.2, 0.5 mM magnesium acetate, 1.0 mM β-mercaptoethanol, and 42 mM sodium chloride to a final wet weight concentration of 0.2 g/mL. Lysozyme (125 μg/mL cell suspension), deoxyribonuclease (2 μg/mL), and ribonuclease (2 μg/mL) were incubated with the cell suspension for 120–150 min at room temperature. Undigested cells and debris were removed by centrifugation at 1000*g* for 5 min. The resulting supernatant fraction was centrifuged at 48000*g* for 1 h. The supernatant fraction was used for the purification of native teichuronic acid while the sedimented particulate enzyme fraction was washed and sedimented 2 more times in 50 mM Tris-HCl, pH 8.2, 1 mM magnesium acetate, and 2 mM β-mercaptoethanol (TMM buffer). The particulate enzyme fraction was resuspended in TMM buffer to give a protein concentration of 10 mg/mL and stored at −15 °C until used for the in vitro biosynthesis of teichuronic acid.

For the isolation of the native teichuronic acid, the supernatant, prepared as described above, was adjusted to contain 10 mM magnesium ion and heated at 100 °C for 5 min. After removal of the heat-precipitated materials by centrifugation at 48000*g* for 20 min, the resulting supernatant solution was dialyzed against five changes of deionized water, after which phosphate buffer, pH 6, was added to a concentration of 10 mM. The solution was applied to an ECTEOLA-cellulose column (50 × 4.5 cm), and teichuronic acid was eluted from the column with a linear concentration gradient of sodium chloride (Hase & Matsushima, 1970). The gradient was developed with 3.5 L of 10 mM sodium phosphate buffer, pH 6, in the mixing chamber and 3.5 L of 10 mM sodium phosphate buffer, pH 6, and 0.8 M sodium chloride in the reservoir. Column fractions were assayed by the anthrone (Bitter & Muir, 1962) and the carbazole (Spiro, 1966) reactions. Appropriate fractions from the ECTEOLA-cellulose column were combined, lyophilized to dryness, resuspended in 40 mL of water, and passed through a Bio-Gel P-2 column (55 × 4.5 cm) with deionized water as the irrigating solvent. Teichuronic acid was located by the anthrone procedure. Appropriate fractions were combined and lyophilized to dryness. Yields of native teichuronic acid ranged from 600 to 700 mg from 100 g of wet weight cells.

Periodate Oxidation and Borohydride Reduction of Native Teichuronic Acid. Native teichuronic acid (500 mg) was dissolved in 250 mL of 50 mM sodium acetate buffer, pH 4.2, containing 2.7 g of $NaIO_4$ and allowed to react for 10 days in the dark at 4 °C. The reaction was stopped with the addition of 25 mL of ethylene glycol. The reaction mixture was dialyzed against five changes of deionized water and concentrated to dryness by lyophilization. To the lyophilized sample of teichuronic acid was added 1.1 mL of 2 mM NaOH for each milligram of dried polymer to make the solution 2.5 mM

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[1] Abbreviations used: ManNAcUA, *N*-acetyl-D-mannosaminuronic acid; Hepes, buffer solution of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and its sodium salt; TEABC, triethylammonium bicarbonate; TSP, sodium 3-(trimethylsilyl)-1-propanesulfonate; TMM, buffer solution of 50 mM Tris-HCl, pH 8.2, 1 mM magnesium acetate, and 2 mM β-mercaptoethanol; ECTEOLA-cellulose, the derivative of cellulose prepared by reaction with epichlorohydrin and triethanolamine (Cellex E purchased from Bio-Rad Laboratories).

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Table I: Carbon-13 Chemical Shifts of Teichuronic Acid[a]

	glucose						ManNAcUA							
preparation	C-1	C-2	C-3	C-4	C-5	C-6	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′	CH_3	C=O
native teichuronic acid	101.0	73.5	74.9 or 75.4	71.6	74.9 or 75.4	70.6 (t)	102.4	55.7	74.3	79.5	76.9	177.2 (s)	24.8 (q)	178.1 (s)
periodate-oxidized, borohydride-reduced teichuronic acid	106.5	65.6 (t)		64.2 (t)	80.2	72.0 (t)	102.1	55.7	74.4	80.2	78.4	177.9 (s)	24.9 (q)	177.9 (s)

[a] In parts per million downfield from external TSP. Splitting patterns in proton-coupled spectra were doublets except as noted by s, singlet; t, triplet; and q, quartet.

with respect to reducible aldehyde groups. For each milliliter of solution, 2 mg of $NaBH_4$ was added, making the reaction mixture 50 mM in $NaBH_4$. The reduction reaction mixture was incubated at room temperature for 20 h, at which time acetone (100 μL/mL) was added to stop the reaction. The reduced polymer was dialyzed against five changes of deionized water and lyophilized to dryness.

Substrates. UDP-glucose and UDP-GlcNAc were purchased from Sigma. UDP-[^{14}C]GlcNAc was purchased from Amersham or from New England Nuclear. UDP-ManNAcUA and UDP-[^{14}C]ManNAcUA were prepared from UDP-GlcNAc and UDP-[^{14}C]GlcNAc, respectively, by a crude enzyme preparation from *Escherichia coli* 014:K7:H$^-$ (stock culture kindly supplied by Drs. F. and I. Ørskov, Statens Seruminstitut, Copenhagen, Denmark) (Ichihara et al., 1974). Addition of fosfomycin [(1*R*,2*S*)-1,2-epoxypropylphosphonic acid] (kindly supplied by Drs. H. Brown and F. Kahan, Merck, Sharp & Dohme Research Laboratories, Rahway, NJ) to the reaction mixtures inhibited utilization of UDP-GlcNAc for synthesis of UDP-*N*-acetylmuramic acid (Hendlin et al., 1969; Venkateswaran & Wu, 1972) and thereby improved the conversion to UDP-ManNAcUA. Preparations of UDP-ManNAcUA were purified by ion-exchange chromatography on DEAE-cellulose, utilizing a gradient of triethylammonium bicarbonate (TEABC), pH 7.

Synthesis of in Vitro Teichuronic Acid. UDP-GlcNAc (0.4 mM), UDP-glucose (0.4 mM), and UDP-[^{14}C]ManNAcUA (0.2 mM, 5×10^5 dpm/μmol) were incubated with particulate enzyme fraction (8 mg of protein/mL) in 50 mM Hepes buffer, pH 8.2, 20 mM magnesium acetate, and 2 mM β-mercaptoethanol (total reaction volume of 100 mL) at 37 °C for 5 h to synthesize in vitro teichuronic acid (Page & Anderson, 1972). Radioactively labeled substrate was included to facilitate assay procedures for teichuronic acid during isolation and purification. The reaction mixture was centrifuged at 48000*g* for 60 min to separate the teichuronic acid carrier lipid–particulate enzyme complex from residual substrates. In vitro teichuronic acid was released from carrier lipid (Stark et al., 1977) by mild acid hydrolysis (20 mM HCl in 50% propanol at 90 °C for 30 min) followed by neutralization with NaOH. Greater than 90% of the in vitro teichuronic acid was solubilized by this method. Centrifugation at 48000*g* for 40 min separated solubilized polymer from membrane fragment debris. The aqueous propanol phase was decanted, concentrated to dryness in vacuo, and redissolved in 100 mL of deionized water. The solution was back-extracted with 100 mL of chloroform:methanol (2:1 v/v) to remove hydrophobic contaminants. The aqueous phase was made 50 mM with TEABC buffer, pH 6.5, and applied to an ECTEOLA-cellulose column (30 × 3 cm). After the column was washed with one bed volume (210 mL) of 50 mM TEABC buffer, pH 6.5, a linear gradient was developed with 600 mL of 50 mM TEABC buffer, pH 6.5, in the mixing chamber and 600 mL of 500 mM TEABC buffer, pH 6.5, in the reservoir. In vitro teichuronic acid eluted from the column was located by assay for radioactivity in the column fractions. Appropriate fractions were combined and lyophilized to dryness, which effectively removed all the TEABC buffer. Three such reaction preparations were pooled to provide sufficient material to determine the NMR spectrum.

To establish that the in vitro teichuronic acid preparation was indeed a biosynthetic product and not endogenous teichuronic acid in the particulate enzyme fraction, it was necessary that a control reaction mixture containing a comparable amount of particulate enzyme fraction be incubated without substrates and subjected to the same isolation and purification procedures. No teichuronic acid was detected (anthrone procedure) in the control experiment at any stage of the isolation procedure.

^{13}C NMR Spectroscopy. Teichuronic acid preparations to be used in NMR studies were dialyzed for 1 day against 1 mM EDTA and then for 3 days against several changes of deionized water. The samples were lyophilized to dryness and twice resuspended in 98% D_2O and lyophilized. The teichuronic acids were redissolved in D_2O to a concentration of approximately 100 mg/mL with concurrent adjustment to neutrality with NaOD.

Natural-abundance ^{13}C NMR spectra were obtained from a Varian XL-100 NMR spectrometer operated in the Fourier-transform mode at a frequency of 25.2 MHz. Spectra were obtained at ambient temperature (approximately 37 °C) and at 60 ± 5 °C. Sample volumes were either 0.5 mL (5-mm tube) or 3.0 mL (12-mm tube). The field was locked on the deuterium signal. The spectra were recorded either with proton noise decoupled or with the proton decoupler gated so that ^{1}H–^{13}C coupling could be observed without loss of the nuclear overhauser effect. Chemical shifts are reported in parts per million downfield from an external sodium 3-(trimethylsilyl)-1-propanesulfonate (TSP) reference solution (5% TSP in D_2O in a coaxial tube). Transients (20000–100000) were normally collected to obtain spectra with adequate signal to noise ratios. The spectral width was 5500 Hz. Pulses were between 22.5° and 90° with the acquisition time being 0.727 s.

Results and Discussion

^{13}C NMR Spectrum of Native Teichuronic Acid. The natural-abundance ^{13}C NMR spectrum of native teichuronic acid obtained at 60 °C shows 14 resonance peaks (Figure 1B). There are 14 different carbon atoms in the repeating unit of teichuronic acid. The correspondence of the number of resonance peaks with the number of resonance peaks with the number of carbon atoms in the repeating unit is indicative of the homogeneity of the teichuronic acid preparation with respect to constituent residues. A contaminant of 10% or more should be detectable. The rationale for the resonance assignments is discussed below, and the assignments are summarized in Table I.

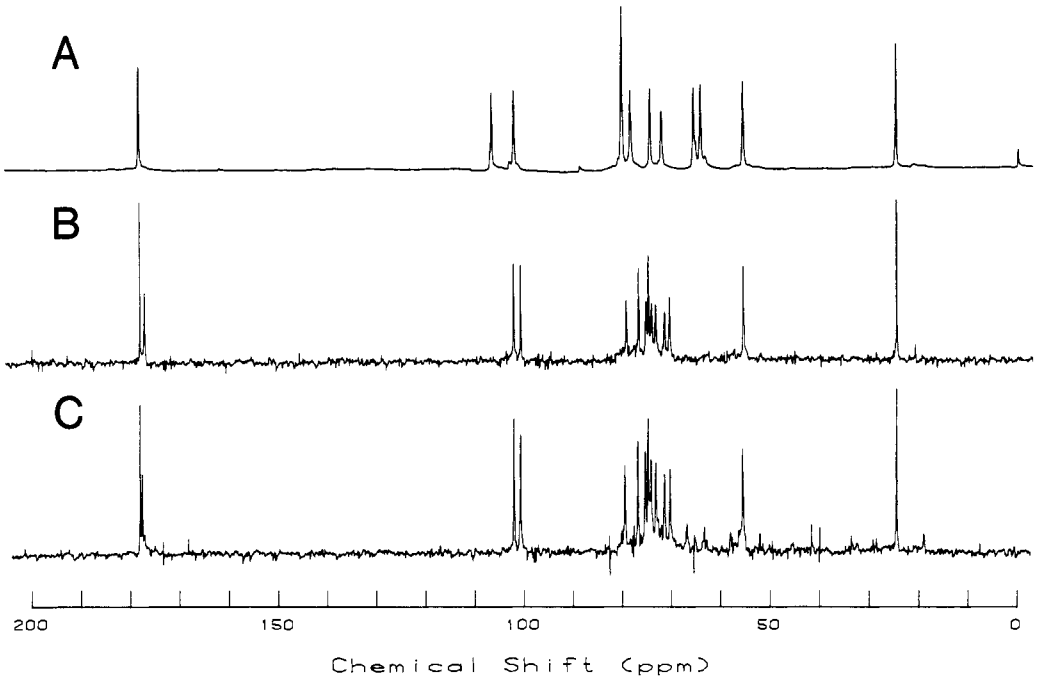

FIGURE 1: ^{13}C NMR spectra of teichuronic acid. (A) Periodate-oxidized, borohydride-reduced native teichuronic acid; 78 400 transients, 37 °C. (B) Native teichuronic acid; 69 600 transients, 60 °C. (C) In vitro teichuronic acid; 88 400 transients, 60 °C. Chemical shifts are expressed relative to TSP which was present as a coaxial reference sample in (A).

The presence of only two signals in the anomeric carbon region (101.0 and 102.4 ppm) is indicative of a highly repetitive structure, as would be the case for a polysaccharide having a sequence of alternating residues of ManNAcUA and glucose. One of the anomeric carbon resonances observed is characteristic of a pyranose with a glycosidic linkage in the α configuration whereas the other is characteristic of a pyranose of the β configuration. Typically, the signals for the α anomers are in the range 99–102 ppm (Nunez et al., 1977; Usui et al., 1973; Colson et al., 1974) and β anomers in the range 102–105 ppm (Nunez et al., 1977; Usui et al., 1973; Inoue & Chujo, 1978). Single bond carbon–hydrogen coupling constants verify this interpretation. In the proton-coupled spectrum, the two doublets centered at 101.0 and 102.4 ppm had coupling constants ($^1J_{C-H}$) of 171.9 and 160.4 Hz, respectively. Coupling constants of about 170 Hz are displayed by α-pyranosides whereas β-pyranosides display constants of about 160 Hz (Bock & Pedersen, 1974, 1975; Walker et al., 1976). Thus, the signal at 101.0 ppm is indicative of an α-pyranosidic linkage whereas that at 102.4 ppm is a β-pyranosidic linkage. Resonances characteristic of reducing end residues (90–100 ppm) (Nunez et al., 1977; Inoue & Chujo, 1978) were not detected.

Teichuronic acid has two carbonyl groups (acetamido and uronic acid). The two signals at 177.2 and 178.1 ppm are not split by proton coupling and are thus due to carbonyl functions. Although no attempt was made to verify the assignment of the carbonyl functions within the pair of resonance peaks by a titration study, the resonance peak at 178.1 ppm showed no variation in chemical shift from sample to sample whereas the other resonance peak in this region showed small changes in chemical shift, presumably due to differences in the pD of various samples. On this basis, the resonance at 178.1 ppm in Figure 1B is probably due to the acetamido carbonyl group, and that at 177.2 ppm is the resonance peak of the uronic acid carboxyl group. The methyl carbon of the acetamido group is assigned to the resonance at 24.8 ppm which appears as a quartet in the proton-coupled spectrum resulting from direct ^{1}H–^{13}C coupling with three equivalent protons. The endocyclic carbon of ManNAcUA which bears the acetamido group (C-2′) is assigned to the resonance at 55.7 ppm. This resonance appears as a doublet in the proton-coupled spectrum. All of these assignments are in accord with similar assignments made for uronic acids (Hamer & Perlin, 1976; Shaskov et al., 1978) and 2-acetamido-2-deoxy-D-hexoses and their glycosides (Hamer & Perlin, 1976; Bundle et al., 1973; Perkins et al., 1977).

By comparison with reported chemical shifts for glucose and glucose-containing oligo- and polysaccharides, one other assignment can be made. Unsubstituted hydroxymethyl groups at C-6 of glucose residues have a signal at 61–63 ppm (Usui et al., 1973; Colson et al., 1974; Inoue & Chujo, 1978). There is no resonance peak in this region of the spectrum of native teichuronic acid. However, a glycosidic substitution of the C-6 of the glucose residue causes a 9-ppm downfield shift of the resonance (Usui et al., 1973; Hamer & Perlin, 1976; Shashkov et al., 1978; Dorman & Roberts, 1970; Colson & King, 1976). The peak at 70.6 ppm, which has a triplet splitting pattern in the proton-coupled spectrum, has been assigned to C-6 of the glucose residue which is glycosidically substituted at position 6.

One aspect of the teichuronic acid structure which remains to be deduced is the assignment of α and β configurations to specific residues. The ^{13}C NMR spectrum of periodate-oxidized, borohydride-reduced native teichuronic acid was used to make these assignments. In view of the involvement of C-1 and C-6 of glucopyranosyl residues in glycosidic linkages, the glucose residue is susceptible to periodate oxidation between the vicinal hydroxyl groups at C-2, -3, and -4. C-3 is lost as formic acid in the oxidation which yields aldehydes at C-2 and C-4 which are subsequently reduced by borohydride to hydroxymethyl groups. In contrast, the ManNAcUA residues are resistant to periodate since C-1′ and one of the two hydroxyl groups at C-3′ and -4′ are involved in glycosidic linkages. The proton-decoupled spectrum of the oxidized and reduced teichuronic acid shows 11 resonance peaks (resonance peaks at 80.2 and 177.9 ppm each represent a pair of carbon nuclei with overlapping resonances) (Figure 1A). Of the two

anomeric carbon resonance peaks, the one at 102.4 ppm is essentially unchanged, but the other is shifted downfield from 101.0 to 106.5 ppm. The resonance at 102.1 ppm, assigned to the undegraded ManNAcUA residue, has a coupling constant of 163 Hz, which is consistent with the β-anomeric configuration. Therefore, glucose is the residue with the α configuration, and the ManNAcUA residue has the β configuration.

Resonance peaks due to the acetamido methyl group, the C-2′ of ManNAcUA, and the acetamido carbonyl group are essentially unchanged in the spectrum of the periodate-oxidized, borohydride-reduced teichuronic acid. The small shift of the resonance peak of the uronic acid carboxyl group so that it overlaps with the resonance peak of the acetamido carbonyl group is probably due to differences of sample pD and/or the temperature at which the spectra were obtained (37 °C vs. 60 °C). The resonance peaks at 64.2 and 65.6 ppm have chemical shifts appropriate for the newly formed hydroxymethyl groups originally derived from C-2 and -4 of glucose residues. In the proton-coupled spectrum, both of these signals appear as triplets. The signal at 72.0 ppm is that of C-6 of glucose, which is shifted only 1.4 ppm downfield from its resonance in native teichuronic acid. This identification is verified by the triplet splitting pattern in the proton-coupled spectrum.

A second aspect of the teichuronic acid structure which can now be deduced is the position of the linkage of glucose to ManNAcUA. Chemical shifts for ManNAcUA have not been reported. However, examination of the chemical shift values for carbons 3, 4, and 5 of *N*-acetyl-α- and -β-D-mannosamine and methyl (methyl α-D-mannopyranosid)uronate reveals that they vary only by 1–2 ppm from those of the corresponding anomers of D-mannose (Bundle et al., 1973; Gorin & Mazurek, 1975). Thus, one would predict the β anomer of ManNAcUA to have resonance peaks for C-3′ at 73–75 ppm, C-4′ at 67–69 ppm, and C-5′ at 77–78 ppm. Substitution of either C-3′ or C-4′ would be expected to introduce a downfield shift of 9–10 ppm for the nucleus at the position substituted and an upfield shift of 1 ppm for the adjacent carbon nucleus due to the β effect (Usui et al., 1973; Hamer & Perlin, 1976; Shashkov et al., 1978; Dorman & Roberts, 1970). Thus, if the linkage were to C-4′ of ManNAcUA, the spectrum would be expected to have signals at 72–74, 76–79, and 76–77 ppm for C-3′, -4′ and -5′, respectively, whereas if the linkage were to C-3′, the signals would be expected at 81–84, 66–68, and 77–78 ppm for C-3′, -4′, and -5′, respectively. Similar patterns of chemical shifts were reported for the β-1,4- and β-1,3-linked mannose residues of a mannan (Gorin, 1975). The spectrum of periodate-oxidized, borohydride-reduced native teichuronic acid has resonance peaks at 74.4, 78.4, and 80.2 ppm. This pattern of resonances corresponds closely to the pattern expected for a glycosidic linkage to C-4′. Therefore, the linkage of the glucose residue is to C-4′ of ManNAcUA, and the signals in the periodate-oxidized, borohydride-reduced native teichuronic acid are assigned as follows: C-3′, 74.4 ppm; C-4′, 80.2 ppm; C-5′, 78.4 ppm. The resonance at 80.2 ppm corresponds to two nuclei. The C-5 of the modified glucose residue in the oxidized, reduced teichuronic acid also resonates at 80.2 ppm.

In native teichuronic acid, the chemical shifts of the carbon nuclei of the ManNAcUA residue are essentially the same as those of the periodate-oxidized, borohydride-reduced native teichuronic acid. The chemical shifts for C-2, -3, -4, and -5 of the glucose residue of native teichuronic acid are tentatively assigned by comparison with reported chemical shifts for methyl β-gentiobioside (Usui et al., 1973) and (1–6)-β-D-glucan (Bassieux et al., 1977). The resonance assignments are summarized in Table I.

^{13}C NMR Spectrum of in Vitro Teichuronic Acid. The major objective of this work was to show that teichuronic acid formed in vitro has the same structure as that isolated from cell walls. For the accomplishment of this objective, the ^{13}C NMR spectrum of in vitro teichuronic acid (Figure 1C) was compared to that obtained from native teichuronic acid (Figure 1B). The spectra are essentially indistinguishable, indicating that the structural characteristics correlated with the spectrum for native teichuronic acid apply equally well to the spectrum for in vitro teichuronic acid. Of particular relevance are the spectroscopic evidence for (i) the C-6 substitution of the glucose residues (resonance peak at 70.6 ppm and no evidence of any resonance at 61–63 ppm), (ii) the regular alternation of residues (only two resonance peaks corresponding to anomeric carbon atoms), (iii) the presence of one α- and one β-glycosidic linkage (anomeric carbon resonances at 101.0 and 102.4 ppm), and (iv) the C-4′ substitution of the ManNAcUA residues (C-4′ resonance peak at 79.5 ppm). The very nearly identical spectra leave no doubt that the teichuronic acid synthesized in vitro has the same covalent structure as the teichuronic acid isolated from cell walls.

The NMR spectra provide only limited information about the number of repeating disaccharide units in the polymer chain of either native or in vitro teichuronic acid. The absence of any signal which might correspond to either the reducing end residue or the linkage region by which the teichuronic acid is attached to peptidoglycan (Stark et al., 1977; Hase & Matsushima, 1977) indicates that such residues must constitute less than about 10% of the total sample, thus suggesting a polymer length of at least five disaccharide repeating units. Other experimental approaches are necessary to establish the degree of polymerization more precisely.

The spectroscopic data presented above indicate clearly that both native and in vitro teichuronic acid have the structure $[\text{ManNAcUA}_p\text{-}(\beta\text{-}1,6)\text{-Glc-}(\alpha\text{-}1,4)]_n$, in complete agreement with the results obtained by chemical degradation procedures (Hase & Matsushima, 1970, 1971, 1972). The identity of the covalent structure of native and in vitro teichuronic acid establishes that the process of in vitro utilization of UDP-glucose and UDP-ManNAcUA by the particulate enzyme preparation from *M. luteus* (Page & Anderson, 1972; Rohr et al., 1977; Stark et al., 1977) yields a polymeric product which possesses the correct functional groups and the correct linkage positions as well as the correct anomeric configurations and therefore is authentic *M. luteus* teichuronic acid. Prior to this report, there was little definitive experimental evidence that the reaction product produced in vitro was structurally identical with the teichuronic acid present in the cell walls of *M. luteus*.

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References

Anderson, J. S., Page, R. L., & Salo, W. L. (1972) *J. Biol. Chem. 247*, 2480–2485.

Bassieux, D., Gagnaire, D., & Vignon, M. (1977) *Carbohydr. Res. 56*, 19–33.

Bitter, T., & Muir, H. M. (1962) *Anal. Biochem. 4*, 330–334.

Bock, K., & Pederson, C. (1974) *J. Chem. Soc., Perkin Trans. 2*, 293–297.

Bock, K., & Pedersen, C. (1975) *Acta Chem. Scand., Ser. B B29*, 258–264.
Bundle, D. R., Jennings, H. J., & Smith, I. C. P. (1973) *Can. J. Chem. 51*, 3812–3819.
Colson, P., & King, R. R. (1976) *Carbohydr. Res. 47*, 1–13.
Colson, P., Jennings, H. J., & Smith, I. C. P. (1974) *J. Am. Chem. Soc. 96*, 8081–8087.
Czerkawski, J. W., Perkins, H. R., & Rogers, H. J. (1963) *Biochem. J. 86*, 468–474.
Dorman, D. E., & Roberts, J. D. (1970) *J. Am. Chem. Soc. 92*, 1355–1361.
Gorin, P. A. J. (1975) *Carbohydr. Res. 39*, 3–10.
Gorin, P. A. J., & Mazurek, M. (1975) *Can. J. Chem. 53*, 1212–1223.
Hamer, G. K., & Perlin, A. S. (1976) *Carbohydr. Res. 49*, 37–48.
Hase, S., & Matsushima, Y. (1970) *J. Biochem. (Tokyo) 68*, 723–730.
Hase, S., & Matsushima, Y. (1971) *J. Biochem. (Tokyo) 69*, 559–565.
Hase, S., & Matsushima, Y. (1972) *J. Biochem. (Tokyo) 72*, 1117–1128.
Hase, S., & Matsushima, Y. (1977) *J. Biochem. (Tokyo) 81*, 1181–1186.
Hendlin, D., Stapley, E. O., Jackson, M., Wallick, H., Miller, A. K., Wolf, F. J., Miller, T. W., Chaiet, L., Kahan, F. M., Foltz, E. L., Woodruff, H. B., Mata, J. M., Hernandez, S., & Mochales, S. (1969) *Science (Washington, D.C.) 166*, 122–123.
Ichihara, N., Ishimoto, N., & Ito, E. (1974) *FEBS Lett. 39*, 46–48.
Inoue, Y., & Chujo, R. (1978) *Carbohydr. Res. 60*, 367–370.
Nunez, H. A., Walker, T. E., Fuentes, R., O'Connor, J., Serianni, A., & Barker, R. (1977) *J. Supramol. Struct. 6*, 535–550.
Page, R. L., & Anderson, J. S. (1972) *J. Biol. Chem. 247*, 2471–2479.
Perkins, H. R. (1963) *Biochem. J. 86*, 475–483.
Perkins, S. J., Johnson, L. N., Phillips, D. C., & Dwek, R. A. (1977) *Carbohydr. Res. 59*, 19–34.
Rohr, T. E., Levy, G. N., Stark, N. J., & Anderson, J. S. (1977) *J. Biol. Chem. 252*, 3460–3465.
Shashkov, A. S., Sviridov, A. F., Chizhov, O. S., & Kovac, P. (1978) *Carbohydr. Res. 62*, 11–17.
Spiro, R. G. (1966) *Methods Enzymol. 8*, 3–26.
Stark, N. J., Levy, G. N., Rohr, T. E., & Anderson, J. S. (1977) *J. Biol. Chem. 252*, 3466–3472.
Usui, T., Yamaoka, N., Matsuda, K., Tuzimura, K., Sugiyama, H., & Seto, S. (1973) *J. Chem. Soc., Perkin Trans. 1*, 2425–2432.
Venkateswaran, P. S., & Wu, H. C. (1972) *J. Bacteriol. 110*, 935–944.
Walker, T. E., London, R. E., Whaley, T. W., Barker, R., & Matwiyoff, N. A. (1976) *J. Am. Chem. Soc. 98*, 5807–5813.

Hydrogen-Bonded Structure of the Complex N-Linked Fetuin Glycopeptide†

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ABSTRACT: The conformation of the N-linked complex glycopeptide of fetuin was examined with hydrogen-exchange techniques. The glycopeptide molecule contains eight acetamido hydrogens stemming from five *N*-acetylglucosamine residues and three *N*-acetylneuraminic acid residues and also one from the remaining sugar–peptide linkage. The hydrogen-exchange rates of these secondary amides were compared with small molecule model compounds having identical primary structures at their exchangeable hydrogen sites. Differences between the model rates and glycopeptide rates therefore cannot be accounted for by primary structure effects but reflect conformational features of the glycopeptide. Two glycopeptide hydrogens exhibit significantly hindered exchange; the rest exchange at the model rates. Removal of the three *N*-acetylneuraminic acid residues from terminal positions on the three branches of the glycopeptide removes the slowed hydrogens. The remaining ones continue to exchange at the model rate. These results indicate that two of the eight sugar acetamido hydrogens are involved in intramolecular hydrogen bonds. A likely structure includes two hydrogen bonds between the three *N*-acetylneuraminic acid residues. These two hydrogens, slowed to a moderate degree, reflect a preferred conformation stabilized by about 1 kcal/mol in free energy. The solution conformation of the glycopeptide suggested by these results is one that is partially ordered and can be easily modulated, owing to the relatively small amount of energy stabilizing the preferred conformation.

The structures of the oligosaccharide prosthetic groups of glycoproteins have become of great interest in recent years largely as a result of their probable role in cell-surface recognition phenomena (Ashwell & Morell, 1974; Hughes, 1976; Humphreys et al., 1977; Burger & Jumblatt, 1977; Lash & Burger, 1977; Frazier & Glaser, 1979). Knowledge of the primary structures of glycoprotein carbohydrate chains, aided by the development of new techniques for their determination (Spiro, 1973; Kornfeld & Kornfeld, 1976), has grown in concert with increasing interest in their function. Most chains can be categorized as one of two types: those attached to the polypeptide backbone by an O-glycosidic bond to serine or threonine, and those attached by an N-glycosidic bond to asparagine. The O-glycosidically linked chains are frequently the smaller of the two types and as a class exhibit fewer general

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