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Proton Magnetic Resonance Spectroscopy of *Klebsiella* Capsular Polysaccharides

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The presence of acetate and pyruvate groups in *Klebsiella* capsular polysaccharides may be demonstrated and estimated quantitatively by running the proton magnetic resonance spectrum of the polysaccharide (as sodium salt) in deuterium oxide at 95 C. Such spectra also permit an assessment to be made of the number of α- and β-linkages in the repeat unit of the polysaccharide structure.

Pyruvic acid is found covalently linked to a sugar residue in a variety of polysaccharides, especially those which form the capsule of *Klebsiella* bacteria (3). There is a lack, however, of a nondestructive method for the estimation of pyruvate. It has been shown recently (Y. M. Choy et al., Anal. Lett. 5:675, 1972) that certain capsular polysaccharides (molecular weights 5 to 9 $\times$ 10^5) from *Klebsiella*, after exchange with deuterium oxide (D_2O), give proton magnetic resonance (PMR) spectra in which the presence of a pyruvic acid ketal is clearly demonstrated by the signal at τ 8.5, characteristic of CH_3—C. Derivatives of these ketals, prepared during structural studies (Y. M. Choy et al., Anal. Lett. 5:675, 1972), also give signals at τ 8.5 to 8.6.

MATERIALS AND METHODS

In PMR spectra the chemical shift (i.e., position) of peaks is measured downfield from the signal given by tetramethylsilane (TMS) which is assigned a value of τ = 10. The integral gives the area beneath a peak which is proportional to the number of protons resonating at that particular frequency. Labile hydrogen atoms in poly- and oligosaccharides are deuterated by repeated exchange with D_2O. Further details may be found in many reviews (4, 5).

Some difficulty was experienced in making quantitative determination of the pyruvic acid content because of the large peak present due to partially deuterated water (HOD). This peak appeared at approximately τ 5 to 6, partially covering those regions of the spectrum associated with anomeric and ring protons. The magnitude of the HOD peak was largely due to the necessity of working with solutions of less than 2% concentration because of their viscosity. This same viscosity prevented the solutions from being cooled in order to move the HOD peak downfield. The HOD peak may be moved downfield by running the spectrum on a solution of polysaccharide in trifluoroacetic acid which permits integration of the pyruvic acid and ring proton signals (Y. M. Choy et al., Anal. Lett. 5:675, 1972). This is not very precise because of the disparity in size of the integrals being compared. The fact that the acid may degrade the polysaccharide is a further disadvantage. Conversely, the HOD signal is moved upfield when the polysaccharide (in free acid form) is dissolved in methyl sulfoxide-d_6 and the spectrum is run at 95 C. This method also suffers from the disadvantage of heating solutions of acidic polysaccharides (Y. M. Choy et al., Anal. Lett. 5:675, 1972).

We have now found that excellent PMR spectra may be obtained by dissolving the sodium salt of the polysaccharide, after exchange, in D_2O and running the spectra at 95 C. This is currently used as a routine screening process from which the polysaccharide samples may be recovered unchanged. Some representative results are discussed and the figures illustrate spectra obtained from *Klebsiella* capsular polysaccharides containing *O*-acetyl or pyruvate ketal groups or both. Polysaccharides were isolated as described elsewhere (1; Y. M. Choy and G. G. S. Dutton, Can. J. Chem., in press), converted to their sodium salts (It is convenient to use samples whose equivalent weight has been determined by titration.), and exchanged 2 or 3 times with D_2O. Spectra were run on solutions of approximately 2% concentration in D_2O at 95 C using a Varian XL100 instrument with tetramethylsilane as the external standard.

RESULTS

Figure 1A shows the spectrum of K21 polysaccharide and illustrates the sharp signal at τ 8.5 for the CH_3—C of the pyruvate ketal. The four signals in the range τ 4.5 to 5.5 are due to the anomeric protons and integration shows clearly that there are five anomeric protons to

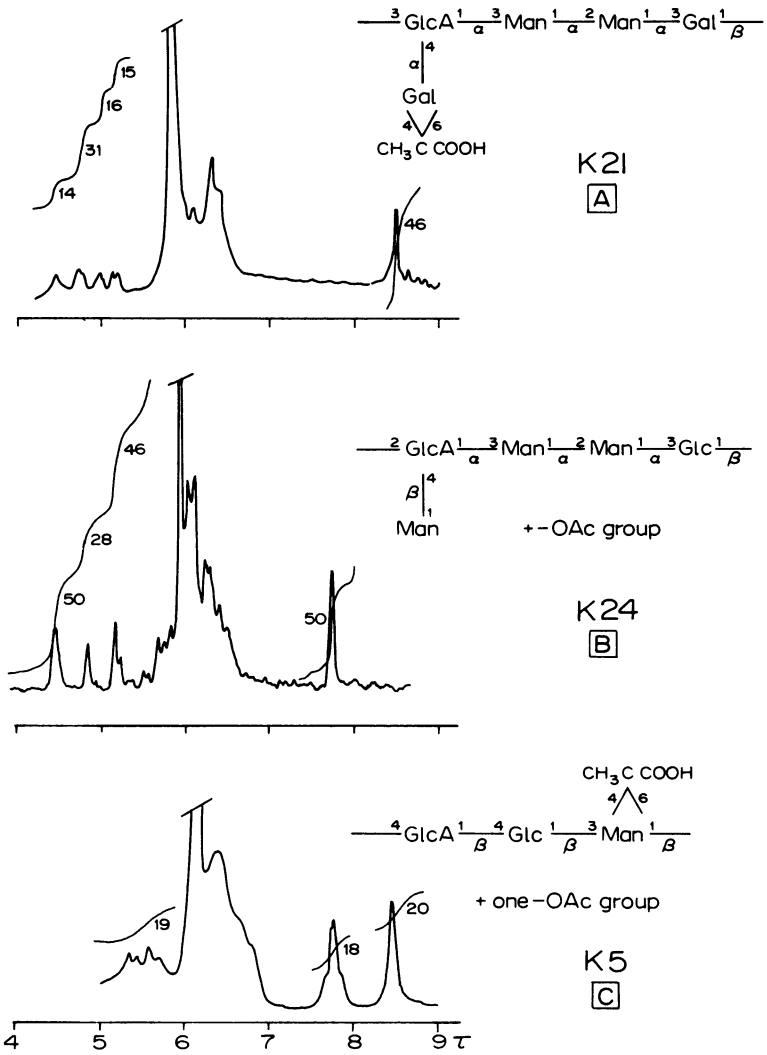

FIG. 1. *PMR spectra (100 MHz) run in D_2O at 95 C of capsular polysaccharides from Klebsiella: A, K21; B, K24; C K5. The numbers beside the integrals represent the areas under each peak in arbitrary units. Thus, in A the signal at τ 8.5 is due to the methyl group of the pyruvic acid ketal, and therefore represents three protons; hence one proton ≡ 46/3 units ~ 15 units. It is then clear that the peaks around τ 5 correspond to 1 + 2 + 1 + 1, or five protons. The signals around τ 5 are those given by the anomeric protons, and therefore there are five sugar units and one pyruvic acid ketal per repeat unit. In C the signals at τ 7.8 and τ 8.5 each correspond to 3 protons (CH_3— of acetate and pyruvate, respectively); thus, taking the average, 3 protons ≡ 19 units and therefore the integral of the signals at τ 5.5 shows that there are three anomeric protons (i.e., 3 sugar residues) per repeat unit. Case B is less precise but suggests 1-O-acetyl group (τ 7.8, 3H ≡ 50 units) for about 7 sugar residues.*

each pyruvate ketal. Furthermore, an anomeric signal above τ 5.0 is indicative of a hexose unit linked in the β-D-configuration (6). The PMR spectrum therefore suggests that in this K21 polysaccharide, only one of the five sugar units in the repeat unit has a β-D-linkage. This polysaccharide contains D-galactose and D-mannose, in addition to D-glucuronic acid. The splitting of 7 Hz at the signal of τ 5.15 shows that the protons on C-1 and C-2 are trans-diaxial; i.e. they have a dihedral angle of 180°. This signal therefore cannot be due to a D-mannose unit, since in this sugar the protons on C-1 and C-2 are either trans-diequatorial (α-D-) or cis-axial-equatorial (β-D-) and in each case have a splitting (coupling constant) of 2-3 Hz (4).

This splitting is not distinct when the spectrum is run in methyl sulfoxide-d_6. Detailed examination of K21 polysaccharide has subsequently confirmed that the structure has a repeat unit of five sugar residues, of which one D-galactose unit is β-linked (Y. M. Choy and G. G. S. Dutton, Can. J. Chem., in press).

Figure 1B is the spectrum for K24 polysaccharide which shows the presence of an *O*-acetyl group (τ 7.8) and the absence of pyruvate. The anomeric signals integrate for five protons, but in this case, the chemical shifts suggest that three sugar units are α-D-linked and 2 are β-D-linked. The acetate content corresponds to one *O*-acetyl group per seven or eight sugar units. This indicates a certain degree of random character in the acetate substitution or loss of *O*-acetyl during the isolation of the polysaccharide. The latter is unlikely in view of the mild procedures used.

Figure 1C shows the spectrum for K5 polysaccharide and demonstrates that there are one *O*-acetyl group and one pyruvate ketal to every repeat unit of three sugars, each of which is linked by a β-D-bond. This is in accord with the chemical structure as subsequently determined (2).

In a similar manner, the ratio of pyruvate to L-fucose in K6 has been shown to be 1:1; K7 has one pyruvate to 8 to 9 sugar residues; K18 has neither *O*-acetyl nor pyruvate. In K32, the ratio of pyruvate to L-rhamnose is 1:4, and in K56 the ratio is 1:1, with the L-rhamnose being one member of a five-sugar repeat unit.

The sharpness of the signals due to the acetate and pyruvate groups, as well as those of the anomeric protons, may be taken to indicate that such structural features all have a constant environment in these bacterial polysaccharides. In other words, the nature of these signals is further evidence that *Klebsiella* capsular polysaccharides are indeed composed of true repeat units.

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