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Note

Interconversion rates of tautomers of 3-deoxy-D-*manno*-octulosonic acid (KDO) from a quantitative analysis of two-dimensional n.m.r. exchange data

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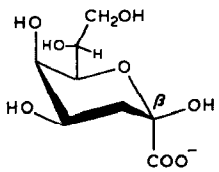
Monosaccharides generally exist in several interconverting, tautomeric forms in solution[1–3]. This complicates the interpretation of enzyme kinetic data involving sugars, as the reaction rates depend on the concentration of the tautomeric form utilized by the enzyme and, under some conditions, these rates may be limited by the interconversion rates between the different tautomers. Recently[4], we determined the anomeric specificity of CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidylyltransferase (CMP-KDO synthetase) by ^{13}C-n.m.r. spectroscopy. Addition of CMP-KDO synthetase to reaction mixtures containing either [1-^{13}C]- or [2-^{13}C]-labeled KDO resulted in a substantial decrease in the ^{13}C-enriched resonances of the β-pyranose form of KDO (see formula **1**) relative to the resonances of other KDO species in solution, demonstrating that the β-pyranose is the favoured substrate[4]. This finding, together with recent n.m.r. evidence[5–9] that KDO is in the α configuration when linked in lipopolysaccharides (LPS) suggested that the transfer of KDO to LPS occurs with inversion of configuration.



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We now describe ^{13}C-n.m.r. studies of the overall interconversion-rates for the different KDO tautomers made by using two-dimensional exchange spectroscopy[8]. This technique has been shown to be a useful method in the study of chemical-exchange processes in slowly ($\sim$1 s^{-1}) exchanging systems[9–11]. Unlike one-dimensional methods used to measure exchange rates (*e.g.*, saturation transfer[12]), the 2-D method does not require the selective irradiation of a resonance and is, therefore, the method of choice for spectra having n.m.r. signals that are close in chemical shift. In addition, because all of the exchange processes can be examined in a single experiment, 2D exchange spectroscopy is particularly valuable for the

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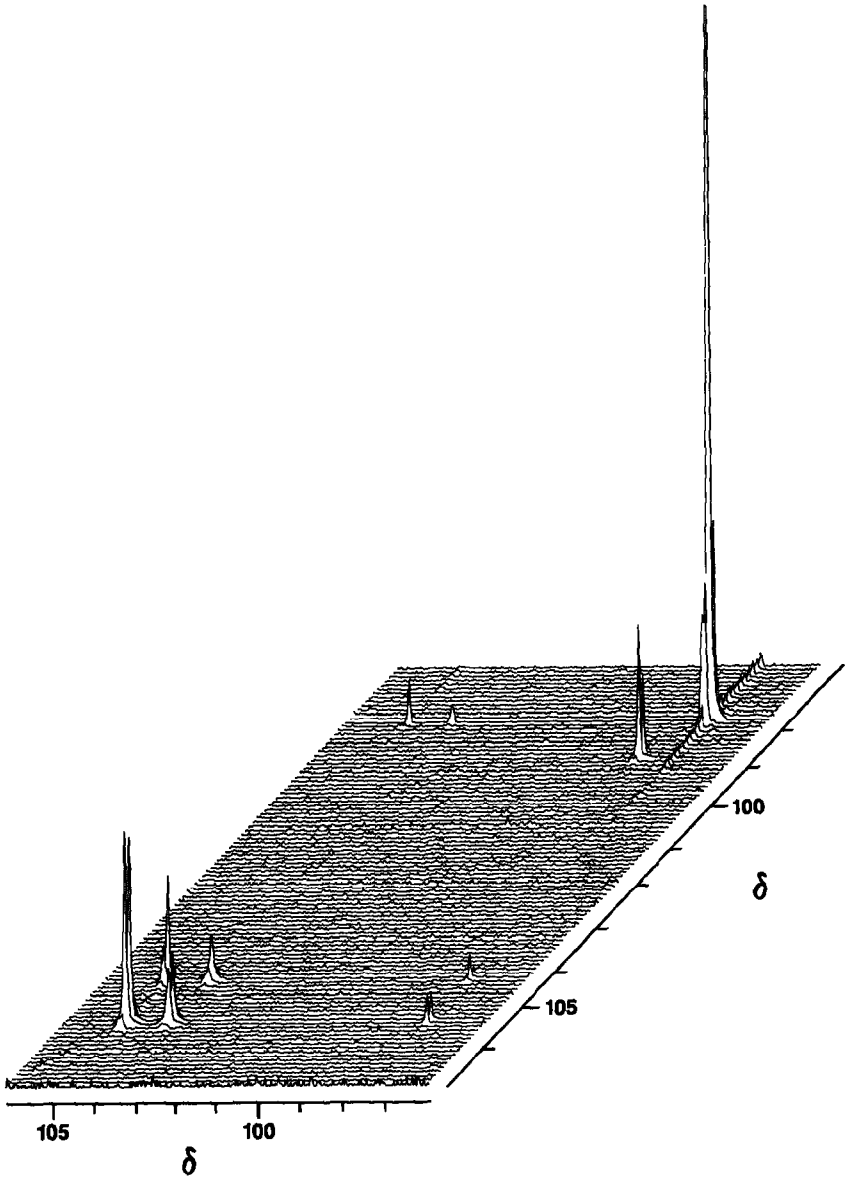

Fig. 1. Stacked plot of a pure absorption two-dimensional exchange experiment on a sample (30 mg) of [2-^{13}C]KDO in 0.25mM Hepes buffer (pH = 7.90). [2-^{13}C]KDO was prepared as previously described[3]. Data were acquired at 25° with an NT-360 Nicolet spectrometer operated at a frequency of 90.8 MHz. Free-induction decays (256 of 1024 points each) were collected with the carrier to one side of the spectrum, using a sweep width of 2 kHz and a delay of 3.01 s between acquisitions. The data were processed by using the F.t.-n.m.r. program of Dr. D. Hare by applying a complex Fourier transformation in t_2 after exponential multiplication (1 Hz) and zero filling, and a real Fourier transformation in t_1. The cross and diagonal peaks that were used in the calculation of exchange rates were integrated from the resulting matrix by summing all data points within a chosen radius.

analysis of exchange rates in systems with many sites[13]. Both of these factors suggest the importance of 2D exchange spectroscopy in exchange studies of sugars, most of which have several interconverting, tautomeric forms.

Fig. 1 depicts a stacked plot of a ^{13}C-n.m.r., pure absorption, 2D exchange experiment acquired with a [D5-90°-t_1-90°-τ_m-90°-acquire]$_n$ pulse sequence for [2-^{13}C]-labeled KDO. The diagonal peaks correspond to the C-2 resonances of KDO, and had been assigned[14] (α-furanose, 105.0; β-furanose, 103.8; β-pyranose, 98.2; and α-pyranose, 97.2 p.p.m.). Chemical exchange between the different KDO species in solution is indicated in the 2D experiment by the presence of off-diagonal elements, *i.e.*, peaks having different frequencies in the two dimensions (cross peaks). Cross peaks are observed when magnetization evolving in the t_1 period with a resonance frequency corresponding to one species precesses at a different frequency[8] in the t_2 period after exchange has occurred during τ_m. From the cross and diagonal peak-volumes, V_{ij} (τ_m), measured from the 2D data, exchange rates can be calculated from the equation[8]

$$V_{ij}(\tau_m) = (e^{-R_{\tau_m}})_{ij} V_j^o,$$

where V_j^o is the equilibrium magnetization of the nuclei for species j, and R is the rate matrix with off-diagonal elements, $-k_{ji}$, corresponding to first-order rate-constants for chemical exchange from site j to site i. To quantitate the exchange rates between the different KDO tautomers, a set of 2D spectra was collected with different mixing-times (0.1, 0.25, 0.4, 0.6, and 1.0 s). Fig. 2 depicts the dependence of the cross-peak volumes as a function of increasing mixing-time for the different KDO forms. As shown, an excellent agreement was obtained by using a multisite

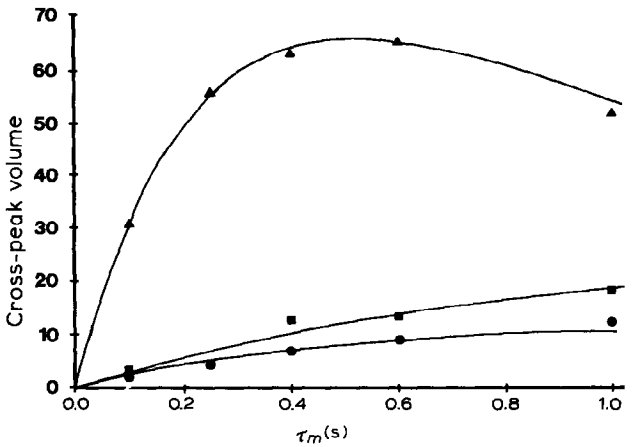

Fig. 2. Cross-peak volumes as a function of mixing time for the different interconverting forms of KDO (▲, α-furanose–β-furanose; ■, α-furanose–α-pyranose; and ●, β-furanose–α-pyranose). The solid lines are simulations based on the rate constants in Table I.

TABLE I

KINETICS OF INTERCONVERSIONS BETWEEN THE TAUTOMERIC FORMS OF KDO AT 25°, pH 7.90[a]

Starting tautomer	*Rate constants[b] (s^{-1}) for conversion of the starting tautomer into*		
	α-Furanose	*β-Furanose*	*α-Pyranose*
α-Furanose	—	0.97 ±0.06	0.07 ±0.02
β-Furanose	2.02 ±0.12	—	0.15 ±0.03
α-Pyranose	0.02 ±0.005	0.02 ±0.006	—

[a]For experimental details, see caption to Fig. 1. [b]The estimated errors represent the standard deviation of the measured rate-constants for the five data-sets.

fit to the experimental data. From the cross and diagonal peak-volumes measured at the different mixing-times, the overall exchange-rates between different KDO tautomers were calculated (see Table I). These rates are a function of the microscopic rate-constants for ring opening and closing and the level of the acyclic form of KDO, which is presumably an intermediate in the exchange process.

The interconversion rates between the α- and β-furanose forms were found to be at least 10-fold faster than any of the other exchange processes. This trend had been observed[15] for aldoses, and attributed to the greater ring-strain present for the furanose forms. Due to the low intensity of the signal for the β-pyranose tautomer of KDO no direct measure of interconversion rates involving this tautomer could be obtained. However, based on the cross-peak intensity that was measurable under the experimental conditions employed, an upper limit of 0.04 s^{-1} can be placed on the exchange rate between the β-furanose and the β-pyranose. All of the other exchange rates involving the β-pyranose must be <0.01 s^{-1}.

The slow exchange into the β-pyranose is interesting, in that this form of KDO, although a minor form in solution, is the actual substrate for the CMP-KDO synthetase[4]. This low rate of interconversion suggests that the rate of KDO incorporation into LPS may be limited by the rate of formation of the β-pyranose.

In summary, two-dimensional exchange spectroscopy has allowed us to determine the interconversion rates between the different KDO tautomers. This methodology should prove very helpful in defining important aspects of sugar metabolism, as well as aiding in the characterization of the enzymic reactions involved in sugar production and utilization.

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