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SPECTROSCOPIC CHARACTERISATION OF CELL-SURFACE CARBOHYDRATES: LACTONISATION OF KETODEOXYOCTONATE

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ABSTRACT

Ketodeoxyoctonate of bacterial cells shows close structural analogy to *N*-acetylneuraminic acid of animal cell-walls. In neutral solution, their circular dichroism (c.d.) and 300-MHz p.m.r. spectra show the expected similarities, although the proportion of the less-stable anomer (axial carboxyl) is appreciably higher in ketodeoxyoctonate. On acidification, however, ketodeoxyoctonate shows an unexpected reversal of sign in c.d., and the n.m.r. spectrum becomes complex, suggesting the co-existence of more than one molecular species. This inference was confirmed by thin-layer chromatography, which showed two distinct bands under acid conditions, and only one at neutral pH. On partial re-neutralisation (pH 5.5), reversal of the observed spectral changes was slow, indicative of a chemical process. I.r. spectra recorded at acid pH show bands consistent with the presence of an ester or lactone, in addition to the expected carboxyl bands. The magnitude of the c.d. changes on acidification argue against intermolecular ester formation, but are fully consistent with the radical change in sugar-ring geometry to be expected on lactonisation. I.r. and c.d. evidence suggests that the lactone formed spontaneously in acid solution is not the same as that previously identified on heating ketodeoxyoctonate at 95° in 0.1M HCl. An alternative 1,5-lactone structure is proposed, which explains the resistance of *N*-acetylneuraminic acid to lactonisation, since the relevant hydroxyl group is replaced by the acetamido substituent.

INTRODUCTION

In a recent paper[1], we demonstrated the scope of circular dichroism (c.d.) in the characterisation of the conformation and linkage pattern of *N*-acetylneuraminic acid (**1**), a widely occurring component of glycoproteins and glycolipids of animal cells and tissues, which is implicated in their control and recognition mechanisms[2]. We have now examined the c.d. behaviour of 3-deoxy-D-*manno*-2-octulosonic acid (ketodeoxyoctonate; **2**), which shares some of the unusual structural features of *N*-acetylneuraminic acid, and occurs in the outer structures of certain bacterial cells[3,4]. Comparison of structures **1** and **2** shows striking structural analogies between *N*-acetylneuraminic acid and ketodeoxyoctonate in the appropriate, stable chair-

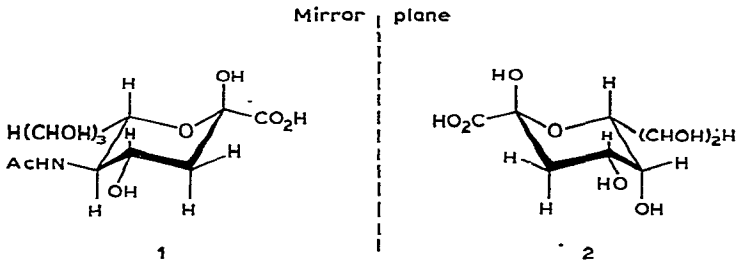
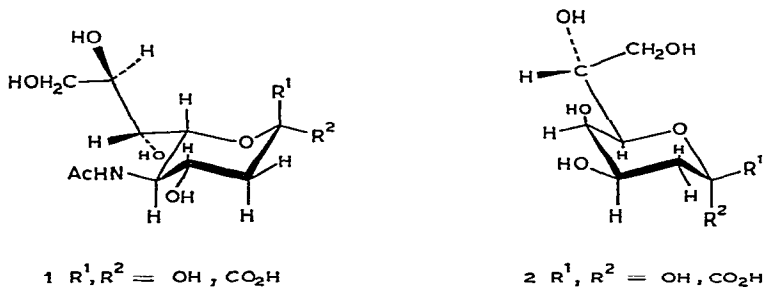


Fig. 1. Structural analogy between *N*-acetylneuraminic acid (**1**) and ketodeoxyoctonate (**2**). In their preferred, stable anomeric configurations (β and α, respectively), the molecules are almost mirror images, particularly around the carboxyl chromophore, and should therefore show approximately equal and opposite c.d. behaviour.

conformations, which may indicate a possible functional analogy in the organisation and behaviour of bacterial and animal cell-surfaces. Indeed, as shown in Fig. 1, the molecules are almost mirror images, and should therefore show c.d. transitions of approximately equal and opposite intensity[5]. However, unlike *N*-acetylneuraminic acid, ketodeoxyoctonate has only one accessible c.d. chromophore (the carboxyl group) and might therefore be expected to provide an analogous but less-complex molecule to further test and explore our previous[1] spectroscopic and conformational interpretations.



EXPERIMENTAL

Spectroscopic methods. — C.d. spectra were recorded on a Cary 61 c.d. spectropolarimeter (1-mm path-length cell; 10-s integration period). Sample temperature was regulated by using a Haake thermocirculator and thermostatable cell-holder. I.r. spectra (KBr microdiscs) were recorded on a Perkin–Elmer 257 grating spectrophotometer, and the method of Bociek and Welti[6] was used to distinguish carboxylate salts from free acid, ester, or lactone. Samples were prepared by dissolving the carbohydrate (20 mg; free-acid form) in D_2O (1 ml) and adjusting the pH, if necessary, by dropwise addition of DCl (0.1M) or NaOD (0.1M), or by buffering[6] with NaH_2PO_4 and Na_2HPO_4. Reference blanks were prepared in the same manner, but without the carbohydrate. High-resolution p.m.r. spectra were recorded at 300 MHz on a Varian

HR-300 instrument equipped with a superconducting magnet and operating in the Fourier-transform mode. The instrument was used under contract at Toegepast Natuurwetenschappelijk Onderzoek, Delft, The Netherlands.

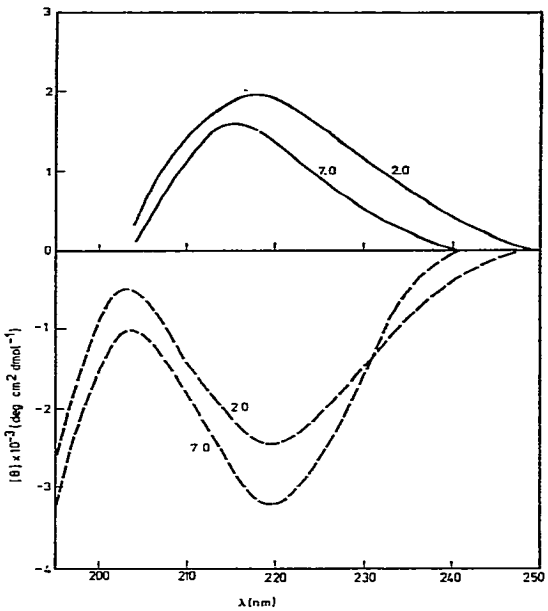
Thin-layer chromatography. — T.l.c. plates were prepared with Merck Silica Gel HF (type 60) and the solvent was butanone–methanol–M acetic acid (12:3:5). Mobilities (R_N) were measured relative to that of *N*-acetylneuraminic acid. Lactones were detected as hydroxamic acids by reaction with ferric nitrate[7], and amines with ninhydrin (2%) in ethanol. *N*-Acetylneuraminic acid and its derivatives were detected with a resorcinol reagent[8]; the plates were developed by heating at 130° for 15–20 min. For general detection, t.l.c. plates were sprayed with 10% sulphuric acid and then heated.

Lactonisation of ketodeoxyoctonate (**2**). — In a modification of the method of Yu and Ledeen[9], ketodeoxyoctonate (acid form; 22 mg) was dissolved in pyridine (5 ml) with dicyclohexylcarbodiimide (15.4 mg) under nitrogen at room temperature; after 44 h, the mixture was heated at 50° for 3 h. T.l.c. of the starting material showed components at R_N 1.76 (major) and 1.25. Only the major component gave a positive reaction with hydroxylamine–ferric nitrate. After the reaction, the minor component had been virtually removed, giving a single product having the same R_N value and behaviour with hydroxylamine–ferric nitrate as the initial, major component.

Materials. — *N*-Acetylneuraminic acid (**1**) was obtained from Koch–Light Laboratories, England. Ketodeoxyoctonate in the ammonium salt form was a gift from Dr. L. Rothfield. The ammonium ions were removed on Amberlite IR-120 (H^+) resin, and the resulting acid (**2**) was freeze-dried. *N*-Acetyl-α-neuraminyl-(2→3)-lactose (**3**) and *N*-acetyl-α-neuraminyl-(2→6)-lactose (**4**) were isolated from bovine colostrum, following the method of Schneir and Rafelson[10], except that final purification was by aqueous elution from a column of Sephadex G-10 rather than by ether–methanol precipitation.

RESULTS AND DISCUSSION

Circular dichroism. — Our earlier studies[1] have shown intense optical activity from the *N*-acetyl chromophore of *N*-acetylneuraminic acid, which virtually swamps the c.d. contribution of the more structurally and conformationally diagnostic carboxyl chromophore. On *N*-deacetylation, however, the chiroptical behaviour of the carboxyl group is unmasked, and, as shown in Fig. 2, is particularly sensitive to anomeric configuration and to pH. Comparison of the c.d. of ketodeoxyoctonate at neutral pH (Fig. 3) with that of neuraminic acid in the β configuration, which predominates in solution, shows the expected reversal of sign in the carboxyl $n\rightarrow\pi^*$ region around 215 nm, but a substantial decrease in magnitude. More spectacularly, acidification produces a complete reversal of sign. This behaviour has no parallel in the c.d. of neuraminic acid, and is at variance with the predictions of the planar rule, which was originally proposed by Listowsky[11] to rationalise the c.d. behaviour of α-substituted carboxylic acids, and which we have shown[1] to be applicable to



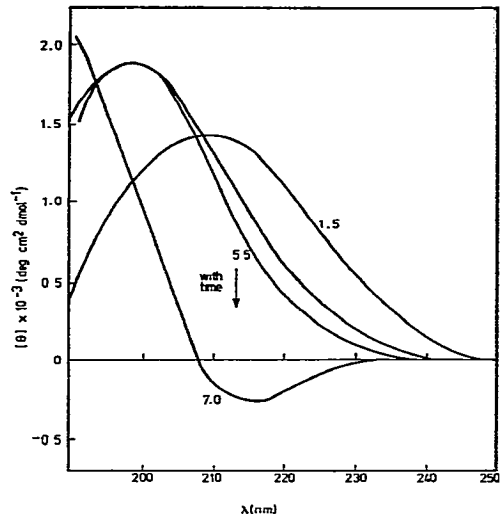


Fig. 2 (left). Effect of ionisation on the c.d. behaviour of α- (----) and β-glycosides (———) of neuraminic acid (pH values as shown; from ref. 1).

Fig. 3 (right). C.d. evidence for lactonisation of ketodeoxyoctonate (2) in acid solution. On acidification from pH 7.0 to 1.5, the spectral change is much greater than those observed for neuraminic acid derivatives (Fig. 2). On partial re-neutralisation of the acid solution to pH 5.5, the reverse spectral-change is slow.

carboxylic acids, esters, and lactones in general, and to neuraminic acid and its derivatives in particular. On partial neutralisation (pH 5.5) of the solution, the spectral change is not instantaneous, but shows a gradual shift towards the previously recorded spectrum for the salt. This behaviour clearly indicates a chemical process, such as intermolecular ester formation or lactonisation. To investigate this further, we have studied the pH-dependent behaviour of ketodeoxyoctonate by high-resolution n.m.r. and i.r. spectroscopy and by t.l.c.

High-resolution n.m.r. spectroscopy. — Fig. 4 shows the 300-MHz p.m.r. spectra of *N*-acetylneuraminic acid (**1**) and ketodeoxyoctonate (**2**) at neutral pH; in Fig. 5, the spectral region in which the structurally and conformationally diagnostic resonances of the deoxy protons occur is shown in detail for both compounds, at neutral and acid pH. The most intense peak in the spectra of **1** and its salt is the *N*-acetyl singlet at δ 2.04. This is flanked by two complex resonances (doublets of doublets) centred around δ 1.88 and δ 2.35, which are assigned, respectively[12], to the axial and equatorial deoxy-protons of the preponderant β anomer (eq CO_2H). The substantially smaller resonances around δ 2.7 can be attributed to the equatorial deoxy-proton of the less-stable α anomer, since the spectra of *N*-acetyl-α-neuraminyl-(2→3)-lactose (**3**) and *N*-acetyl-α-neuraminyl-(2→6)-lactose (**4**) show no resonances

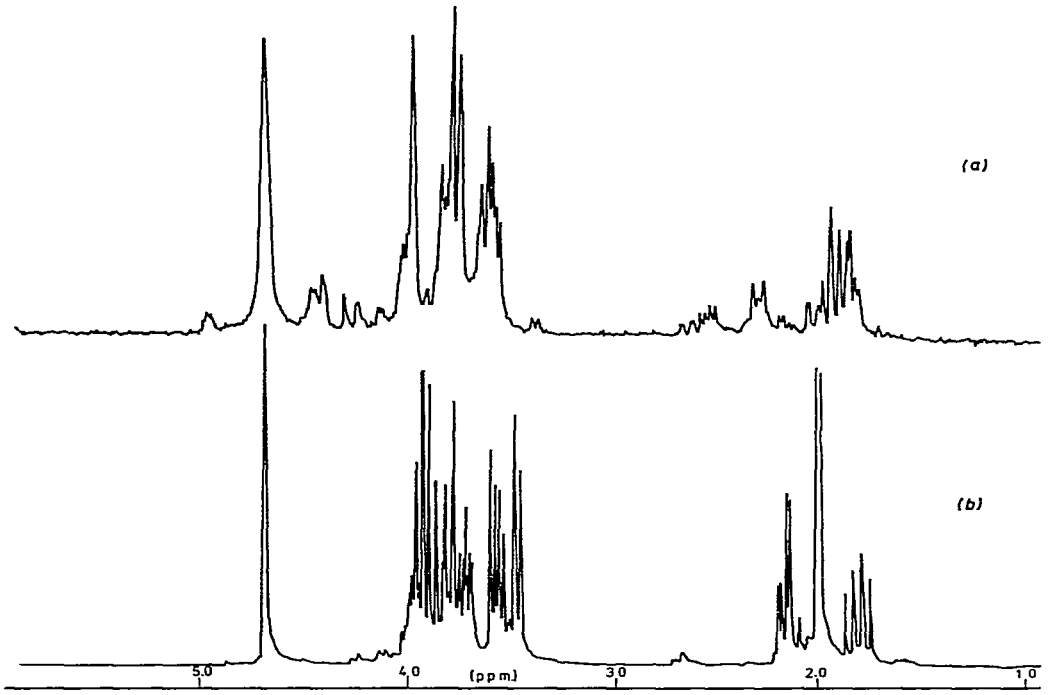
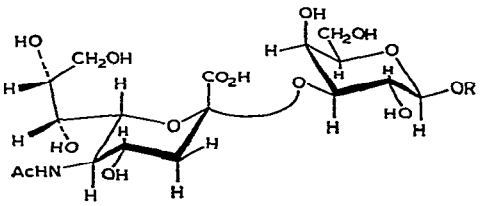


Fig. 4. High-resolution p.m.r. spectra (300 MHz) of (*a*) ketodeoxyoctonate and (*b*) *N*-acetylneuraminic acid at neutral pH.

around δ 2.35, but a doublet of doublets centred at δ ~2.7 (Fig. 6). For *N*-acetylneuraminic acid, the intensities of these bands show little pH-dependence, indicating no significant change in anomeric equilibrium.



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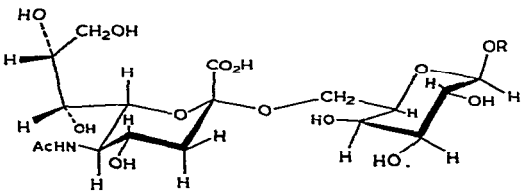

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R = (1→4)-linked D-glucopyranose

Comparison (Fig. 5) of the relative magnitudes of the complex resonances around δ 2.35 and 2.7 for ketodeoxyoctonate at neutral pH suggests a more even split between the two possible anomeric configurations. This evidence offers a simple rationalisation of the low c.d. intensity in the $n \rightarrow \pi^*$ spectral region at neutral pH, since, by analogy with neuraminic acid (Fig. 2), the two anomeric forms would be expected to show c.d. behaviour of opposite sign. In marked contrast to *N*-acetyl-

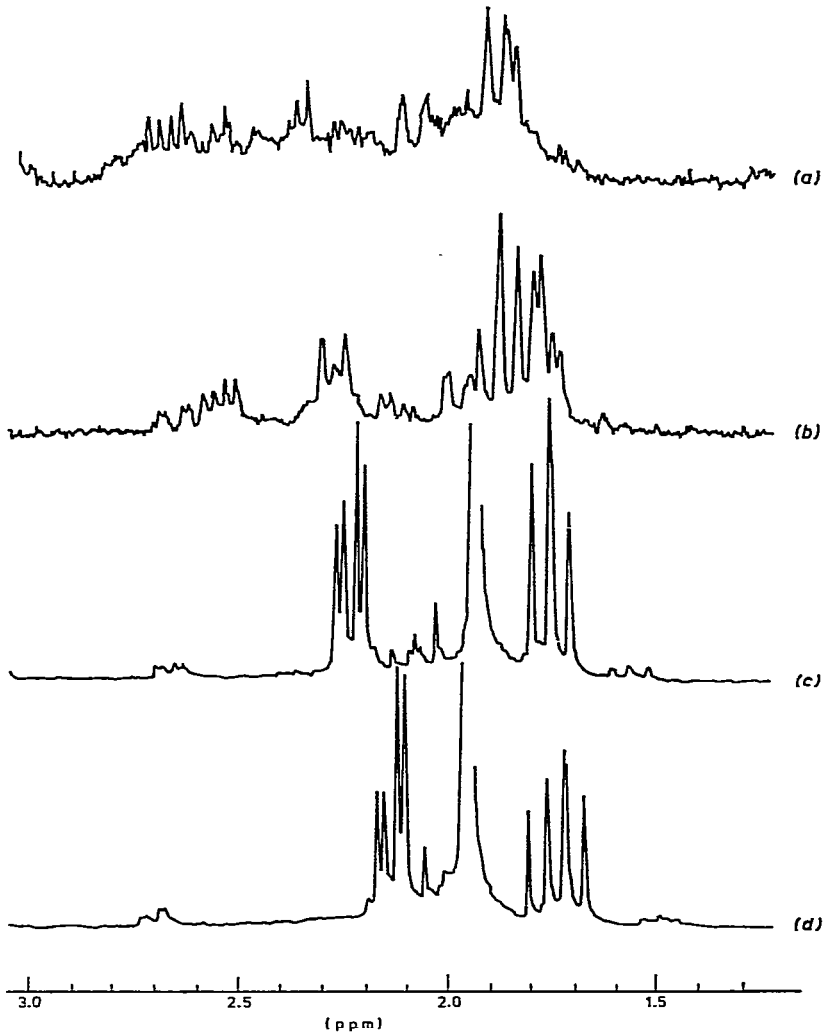


Fig. 5. Deoxy-proton n.m.r. spectra (300 MHz) of ketodeoxyoctonate (2) at pH 2.0 (*a*) and 7.0 (*b*), and of *N*-acetylneuraminic acid (1) at pH 2.0 (*c*) and 7.0 (*d*).

neuraminic acid, ketodeoxyoctonate shows large changes in n.m.r. behaviour on acidification (Fig. 5). In particular, the entire spectrum becomes far more complex than at neutral pH, consistent with the presence of several molecular species.

Infrared studies. — The i.r. spectra of a solution of ketodeoxyoctonic acid in D_2O at pH 1.65 and 2.6 show an asymmetric peak at 1750 cm^{-1}, with a shoulder at 1720 cm^{-1}, suggesting overlap of the C=O stretching bands for a carboxylic acid and for an ester or lactone[6]. At pH 5.8, two symmetric peaks are observed at 1755 and 1610 cm^{-1}, which can be assigned to a lactone C=O stretching band and an antisymmetric, COO^- stretching band, respectively. At pH 10.0, only a carboxylate

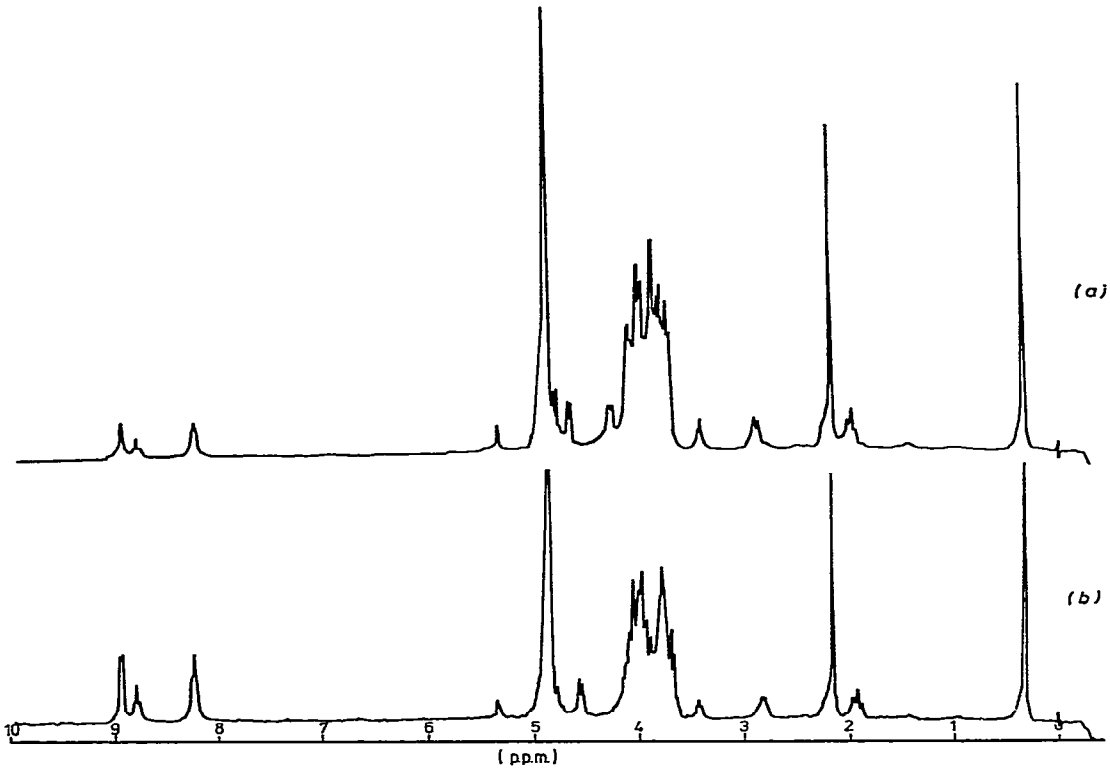


Fig. 6. High-resolution p.m.r. spectra (300 MHz) of (*a*) *N*-acetyl-α-neuraminyl-(2→3)-lactose (3) and (*b*) *N*-acetyl-α-neuraminyl-(2→6)-lactose (4).

band at 1610 cm^{-1} is observed. These results are consistent with the existence of significant amounts of lactone in solutions of ketodeoxyoctonate at acid pH. On partial re-neutralisation of an acid solution to pH 5.5, the ratio of the peak heights at 1755 and 1610 cm^{-1} decreased continuously during several days, although the most rapid change occurred during the first few hours. Furthermore, the decrease in height of the 1755-cm^{-1} band was matched by a concomitant increase in height of that at 1610 cm^{-1}.

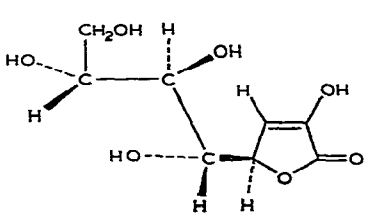
The band at 1755 cm^{-1} observed at pH 5.8 is consistent with a δ-lactone or larger ring, rather than a γ-lactone[13,14]. Intermolecular ester formation is less likely, since the c.d. is so dramatically different from that of the free acid. Previous studies have established[5] that, in general, the c.d. of esters is closely similar to that of the corresponding un-ionised carboxylic acid. However, adoption of a lactone structure in which the ring shape is fundamentally changed would be fully consistent with the large changes in c.d. which we have observed (Fig. 3).

Thin-layer chromatography. — T.l.c. of an aqueous solution containing ketodeoxyoctonate in the free-acid form showed at least two components with R_N 1.25 and 1.76. The latter reacted strongly with hydroxylamine–ferric nitrate[7], indicating

an ester or lactone. When the sugar acid was treated with dicyclohexylcarbodiimide[9] in a manner expected to cause lactonisation, only the product having R_N 1.76 was observed. This gave a strong reaction with hydroxylamine–ferric nitrate, and corresponded to the major component detected in the equilibrium mixture present in solution at acid pH.

Comparisons with N-*acetylneuraminic acid.* — Because ketodeoxyoctonate (**2**) spontaneously forms a lactone, it might be anticipated that *N*-acetylneuraminic acid (**1**), with a similar structure, would also do so. However, no indication of lactones in solutions of *N*-acetylneuraminic acid could be found by c.d., t.l.c., or i.r. The i.r. spectra of *N*-acetylneuraminic acid at pH 1.9 and 1.5 exhibited bands at 1730 and 1630 cm^{-1} (interpreted as carboxylic C=O stretching and amide stretching, respectively) and, at pH 5.5, a band at 1615 cm^{-1} with a shoulder at 1640 cm^{-1} (interpreted as antisymmetric COO^- stretching and amide stretching, respectively). Likewise, i.r. spectroscopy at intermediate pH values of 3.5 and 4.5 gave no evidence for lactone. Furthermore, the high-resolution n.m.r. spectra of *N*-acetylneuraminic acid at pH 1.5, 2.6, and 7.0 gave no indication of the shifts to be expected from lactone formation. It appears that ester or lactone formation by *N*-acetylneuraminic acid or its derivatives occurs only by reaction with a carbodiimide[6,9] or by use of vigorous acidic conditions (glacial acetic acid for 5 days)[15].

Lactone structure. — The present evidence does not permit unambiguous identification of the ketodeoxyoctonate lactone formed spontaneously in solution. Molecular models show that, for steric reasons, intramolecular bicyclic structures may be ruled out. Charon and Szabo[16] showed that ketodeoxyoctonate forms an enolic 1,4-lactone (**5**) when heated at 95° in 0.1M HCl. We have observed bands at 1750 and 1650 cm^{-1} for ketodeoxyoctonic acid dispersed in KBr, which at first suggested formation of lactone **5**. However, when the ketodeoxyoctonate was first freeze-dried in D_2O, and care was taken to exclude moisture while preparing the KBr disc, a substantial decrease was observed in the height of the 1650-cm^{-1} peak, with no change in the height of other peaks in the spectrum. This indicated that the 1650-cm^{-1} band in our compound is not due to C=C stretching, but rather to the presence of water. In addition, the enolic 1,4-lactone **5** would be expected to give a negative peak in c.d., whereas a positive peak is observed. Furthermore, the high-resolution n.m.r. spectrum of ketodeoxyoctonate in the free-acid form, dissolved in D_2O, showed no signal in the region of δ 6.2 which would be expected for a vinylic



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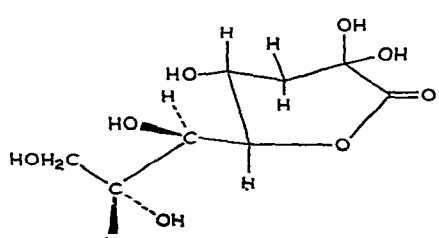

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proton of an enolic lactone. Thus, there is no evidence that the lactone observed when ketodeoxyoctonate is allowed to stand in aqueous solution is the same as that formed[16] on heating in hydrochloric acid.

On steric grounds, the most likely possibility seems to be a 1,5-lactone (**6**) in which the keto group exists as the hydrate, especially since no carbonyl c.d. is observed. A lactone of this type would not be possible for *N*-acetylneuraminic acid, because the acetamido group replaces the relevant hydroxyl group, thus offering a possible interpretation of the resistance to lactonisation.

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