

Acid-Catalyzed Hydrolysis and Lactonization of α 2,8-Linked Oligosialic Acids

Yi-Ping Yu,[†] Mou-Chi Cheng,[‡] Heng-Ru Lin,[‡]
Chun-Hung Lin,^{†,§} and Shih-Hsiung Wu^{*,†,§}

Graduate Institute of Life Sciences, National Defense Medical Center; Institute of Biological Chemistry, Academia Sinica; and Graduate Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan

shwu@gate.sinica.edu.tw

Received February 19, 2001

Polysialic acids (PSA) exist mainly on the surface of mammalian cells and certain bacteria and are involved in many important biological roles.¹ For example, the α 2,8-PSA chain is expressed in neural cell adhesion molecules (N-CAM) in a developmentally regulated manner. The homopolymers of *N*-acetylneuraminic acids have been implicated in altering the shape and movement of cells because PSA can attenuate adhesion forces and modulate overall cell surface interaction.² There are two distinct features of PSA in relation to its critical physiological roles: intramolecular lactonization and the lability of glycosidic linkages. The former influences the charge distribution and tertiary structure of PSA, and the latter varies the size of PSA-containing biopolymers.

Of all the glycosidic linkages found in oligosaccharides, those of polysialic acids are remarkably labile.³ α -2,8-Polysialic acid has been noted for its degradation in prolonged freezer storage⁴ and in the preparation of N-CAM for SDS gel analysis.⁵ Acid-catalyzed cleavage of the α 2,8-linkage in oligo- and polysialic acids (OSA/PSA) was reported to occur preferentially at the linkage between two internal sialic acid residues. The cleavage of the internal α 2,8-linkages in PSA is much easier as a

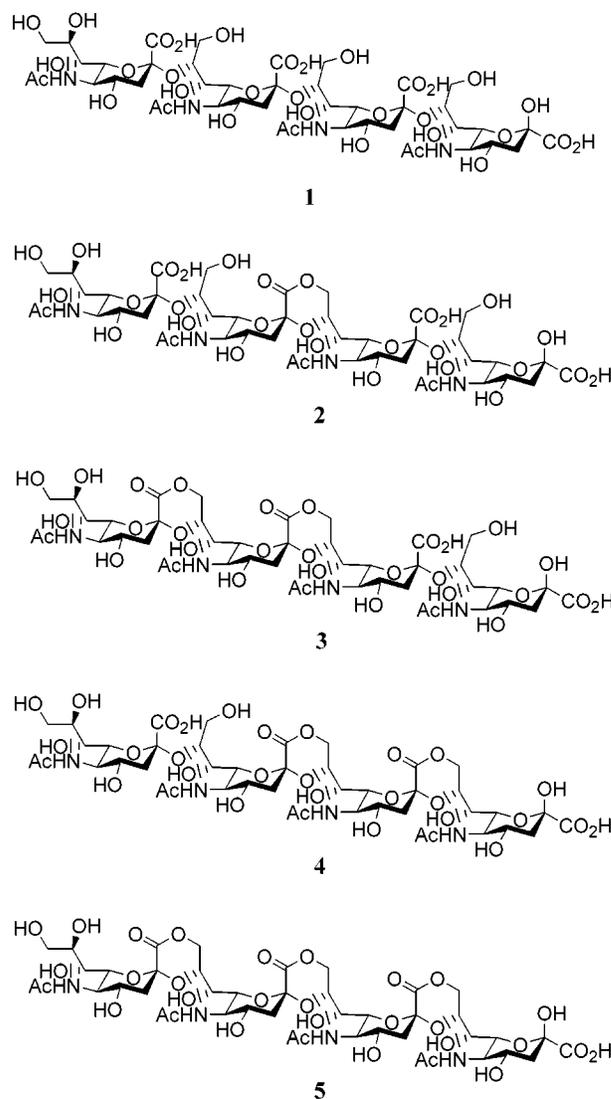


Figure 1. Structures of α 2,8-linked sialic acid tetramer **1** and partially and fully lactonized tetramer **5**.

result of the intramolecular catalysis of general acid from the adjacent carboxyl groups that have high pK_a 's as proton donors.⁴ Intramolecular lactonization of OSA/PSA (Figure 1) was also easily catalyzed by acid and occurred preferentially at the linkage between two internal sialic acid residues. The lactonization process displays three discrete stages from limited lactonization to full lactonization.⁶ Therefore, lactonization and hydrolysis are competitive under acidic conditions. We have previously shown that lactonized species of OSA/PSA, generated from the hydrolysis of colominic acid under acidic conditions, severely interfere with their separation in capillary electrophoresis, but their existence could be detected by mass spectrometry⁷ or high performance anion-exchange

* Fax: +886-2-2653-9142.

[†] National Defense Medical Center.

[‡] Academia Sinica.

[§] National Taiwan University.

(1) (a) Troy, F. A., II. *Glycobiology* **1992**, *2*, 5–23. (b) Rutishauser, U.; Acheson, A.; Hall, A. K.; Mann, D. M.; Sunshine, J. *Science* **1988**, *240*, 53–57. (c) Kitajima, K.; Inoue, S.; Inoue, Y.; Troy, F. A., II. *J. Biol. Chem.* **1988**, *263*, 18269–18276. (d) Zuber, C.; Lackie, P. M.; Caterall, W. A.; Roth, J. *J. Biol. Chem.* **1992**, *267*, 9965–9971. (e) Inoue, S.; Iwasaki, M. *Biochem. Biophys. Res. Commun.* **1978**, *83*, 1018–1023. (f) Robbin, J. B.; McCracken, G. H., Jr.; Gotschlich, E. C.; Ørskov, F.; Ørskov, I.; Hanson, L. A. *N. Engl. J. Med.* **1974**, *290*, 1216–1220. (g) Schiffer, M. S.; Oliverira, E.; Glode, M. P.; McCracken, G. H., Jr.; Sarff, L. M.; Robbin, J. B. *Pediatr. Res.* **1976**, *10*, 82–87. (h) Baumann, H.; Brisson, J. R.; Michon, F.; Pon, R.; Jennings, H. J. *Biochemistry* **1993**, *32*, 4007–4013. (i) Hayrinen, J.; Jennings, H. J.; Raff, H. V.; Rougon, G.; Hanai, N.; Gerardy-Schahn, R.; Finne, J. *J. Infect. Dis.* **1995**, *171*, 1481–1490.

(2) (a) Rutishauser, U.; Landmesser, L. *Trends Neurosci.* **1996**, *19*, 422–427. (b) Kiss, J. Z.; Rougon, G. *Curr. Opin. Neurobiol.* **1997**, *7*, 640–646. (c) Rutishauser, U. *Curr. Opin. Cell Biol.* **1996**, *8*, 679–684. (d) Shen, H.; Watanabe, M.; Tomasiewicz, H.; Rutishauser, U.; Magnuson, T.; Glass, J. D. *J. Neurosci.* **1997**, *17*, 5221–5229.

(3) Schauer, R. In *Sialic Acids: Chemistry, Metabolism and Function, Cell Biology Monographs*; Springer-Verlag: New York, 1982; Vol. 10.

(4) Manzi, A. E.; Higa, H. H.; Diaz, S.; Varki, A. *J. Biol. Chem.* **1994**, *269*, 23617–23624.

(5) (a) Hoffman, S.; Sorkin, B. C.; White, P. C.; Brakenbury, R.; Mailhammer, R.; Rutishauser, U.; Cunningham, B. A.; Edelman, G. M. *J. Biol. Chem.* **1982**, *257*, 7720–7729. (b) Key, B.; Akeson, R. B. *J. Cell Biol.* **1990**, *110*, 1729–1743.

(6) (a) Zhang, Y.; Lee, Y. C. *J. Biol. Chem.* **1999**, *274*, 6183–6189. (b) Cheng, M. C.; Lin, C. H.; Wang, H. Y.; Lin, H. R.; Wu, S. H. *Angew. Chem.* **2000**, *112*, 788–792; *Angew. Chem., Int. Ed.* **2000**, *39*, 772–776.

(7) (a) Cheng, M. C.; Lin, S. L.; Wu, S. H.; Inoue, S.; Inoue, Y. *Anal. Biochem.* **1998**, *260*, 154–159. (b) Cheng, M. C.; Wang, K. T.; Inoue, S.; Inoue, Y.; Khoo, K. H.; Wu, S. H. *Anal. Biochem.* **1999**, *267*, 287–293.

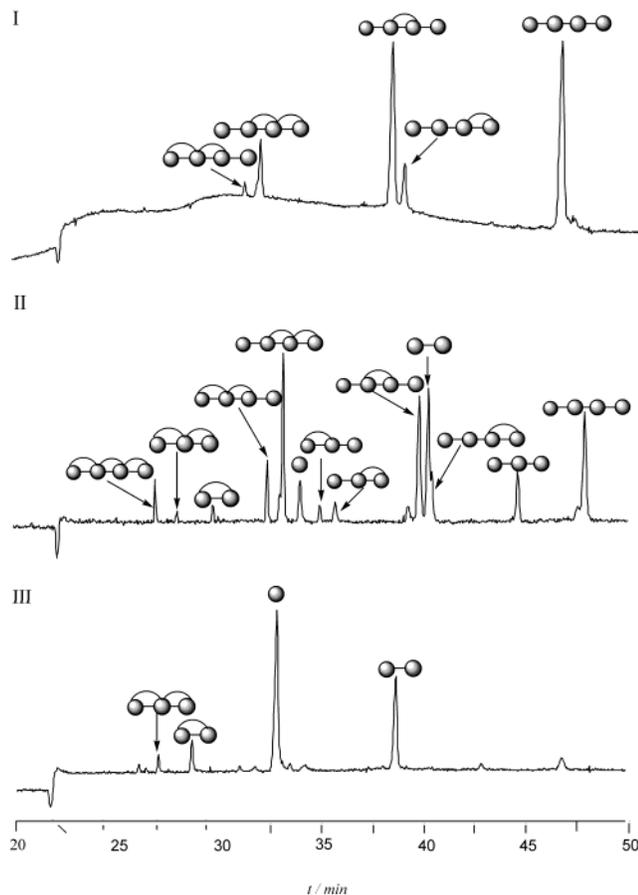


Figure 2. CE spectra of the lactonization and hydrolysis of $\alpha,2,8$ -linked sialic acid tetramer in 0.1 N acetic acid for 8 h at different reaction temperatures (I, 10 °C; II, 37 °C; III, 60 °C). The circles represent sialic acid residues on which the reducing termini are at the right side. Sites of lactone rings are marked by the curves between circles. The signals were identified by the co-injection with the authentic samples, as demonstrated in our previous reports.^{6b,9}

chromatography.^{6a} However, until now, lactonization and hydrolysis of oligosialic acids (OSA) were studied separately. To study simultaneously the lactonization and hydrolysis of OSA as the dimer, trimer, and tetramer, we determined the conditions that allow both of the reactions to proceed. The product mixtures were analyzed by capillary electrophoresis (CE).

The reaction temperature is the key factor that determines whether two reactions will proceed simultaneously (Figure 2). Lactonization occurred solely at low temperature (≤ 10 °C), whereas both hydrolysis and lactonization occurred at high temperature. Figure 3 shows that the $\alpha,2,8$ -dimer in 0.1 N acetic acid at 60 °C was converted to the lactonized product in high yield during the initial 10 min. Since lactonization is a reversible reaction, the lactonized dimer would be hydrolyzed back to the dimer. The monomer generated from hydrolysis of the dimer appeared after incubation for 30 min. Therefore, lactonization in OSA/PSA is much faster than hydrolysis, because (a) lactonization can reduce the tension of negative charge repulsion in OSA/PSA, (b) lactonization is a kinetic intramolecular reaction, and (c) the configurative orientation of the C-2 carboxylic acid of one residue is very suitable for condensing with the C-9 hydroxyl group of an adjacent residue to form a six-membered lactone ring. Even after 8 h of incubation, a significant

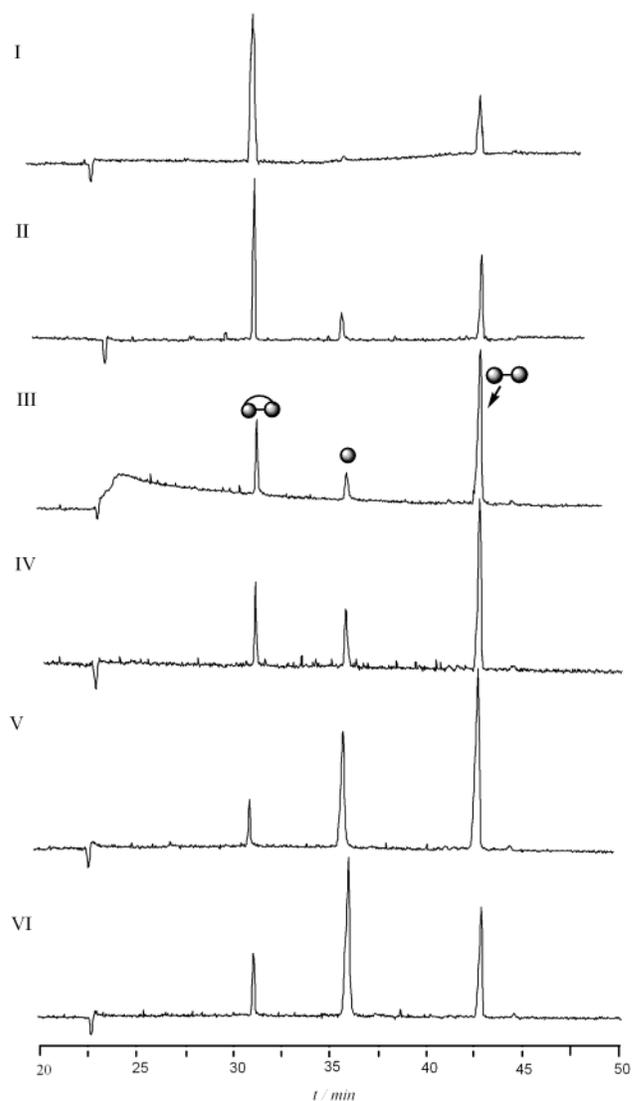


Figure 3. CE spectra of the lactonization and hydrolysis of $\alpha,2,8$ -linked sialic acid dimer in 0.1 N acetic acid, 60 °C, at different reaction times (I, 10 min; II, 30 min; III, 1 h; IV, 2 h; V, 4 h; VI, 8 h).

quantity of the dimer still remained intact. This clearly indicates that the $\alpha,2,8$ -linkage of the dimer is very stable as a result of the formation of a lactone ring.

The same conditions were used to study the lactonization and hydrolysis of the trimer and tetramer (Figures 4 and 5). Figure 4 shows that the monomer, dimer, and two monolactones were formed from the trimer during the first 10 min of incubation. Only a small quantity of the trimer dilactone was formed after incubation for 30 min. This indicates that it is difficult for the two monolactones to be further lactonized into the trimer dilactone and implies that it is easier to hydrolyze the free $\alpha,2,8$ -glycosidic bond of the monolactone trimers than to lactonize them further. Thus, monomers and dimers were produced by direct hydrolysis of the trimer or hydrolysis of two monolactones followed by the reversible delactonization of the lactonized dimer. The twisted conformation of the dilactone trimer may explain the difficulty of the formation of two consecutive lactone rings in the trimer.⁸

Studies of the lactonization of the $\alpha,2,8$ -tetramer has shown that initial lactonization takes place in the

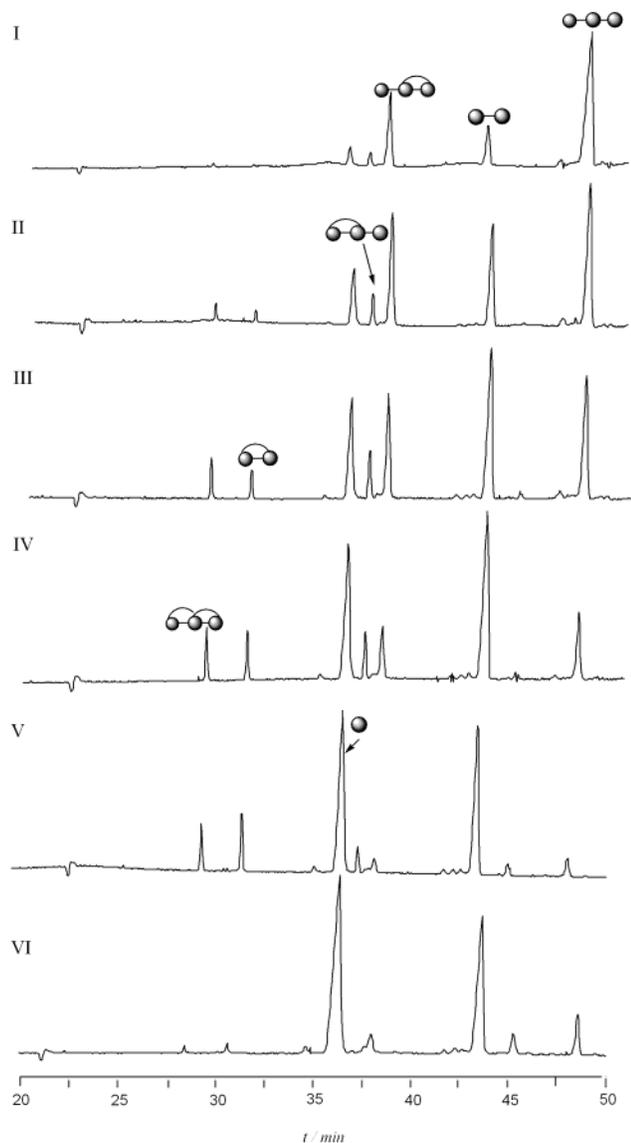


Figure 4. CE spectra of the lactonization and hydrolysis of α 2,8-linked sialic acid trimer in 0.1 N acetic acid, 60 °C, at different reaction times (I, 10 min; II, 30 min; III, 1 h; IV, 2 h; V, 4 h; VI, 8 h).

internal position (middle).⁶ It has also been reported that in mildly acidic conditions the tetramer was hydrolyzed mainly to the dimer with just a little trimer and monomer because the middle α 2,8-linkage of the tetramer is more labile than linkages at either end.⁴ However, in the initial 10-min incubation of α 2,8-tetramer in 0.1 N acetic acid at 60 °C, the monolactone tetramer (**2**) was the major product, whereas the monomer, dimer, trimer, and dilactone tetramer (**3** and **4**) were the minor products (Figure 4). This implied that the hydrolysis and lactonization of the tetramer proceeded simultaneously in the middle α 2,8-linkage but that lactonization was faster than hydrolysis. The dimer was generated by cleaving the middle α 2,8-linkage of the tetramer. The trimer might be generated by direct hydrolysis of the tetramer or by hydrolysis of the monolactone tetramer (**2**) and

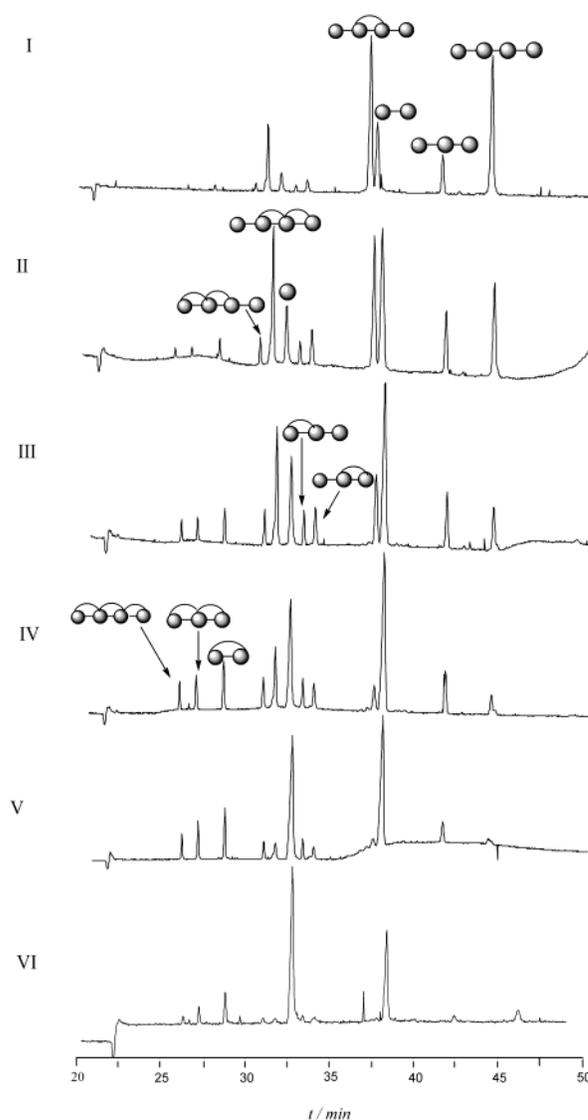


Figure 5. CE spectra of the lactonization and hydrolysis of α 2,8-linked sialic acid tetramer in 0.1 N acetic acid, 60 °C, at different reaction times (I, 10 min; II, 30 min; III, 1 h; IV, 2 h; V, 4 h; VI, 8 h).

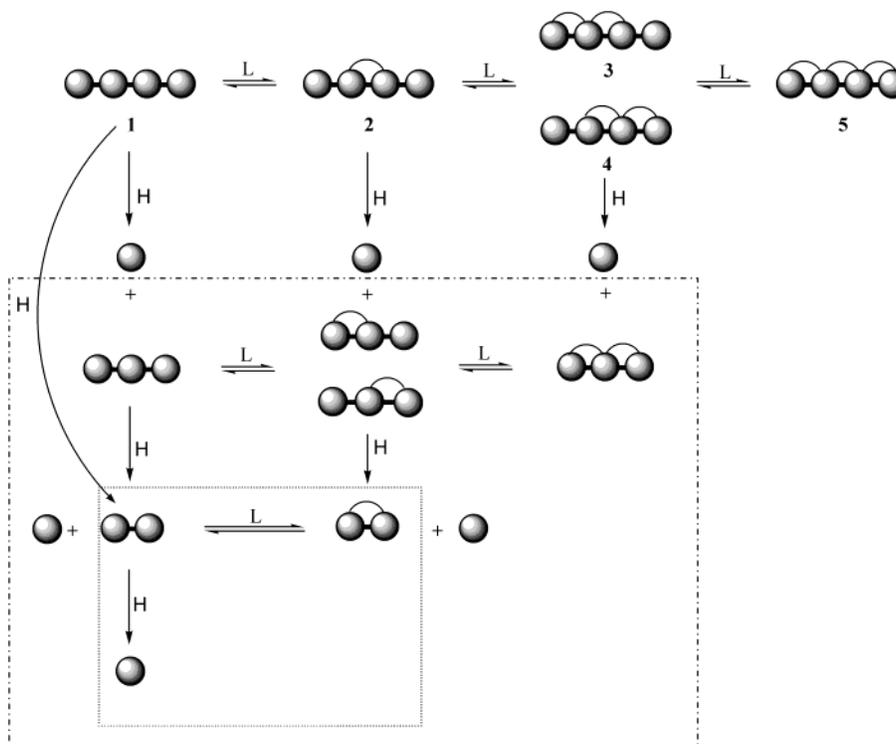
dilactone tetramer (**3** and **4**) followed by reversible delactonization of the monolactone trimer and dilactone trimer. It has been shown that, under acidic condition, the tetramer underwent selective cleavage of its middle linkage, resulting in the accumulation, probably because of the limitations of the analytical method and the prolonged incubation without examination of the samples at shorter intervals. The whole picture of lactonization and hydrolysis of dimer, trimer, and tetramer are summarized in Scheme 1. The products from hydrolysis and lactonization of the tetramer shown in Figure 5 were all separated and identified by capillary electrophoresis. The pathways of lactonization of the trimer and tetramer in 0.1 N acetic acid at 60 °C are the same as those in glacial acetic acid.^{6b,9}

In conclusion, lactonization and hydrolysis of oligosialic acids are competitive under acidic conditions at higher temperature (Scheme 1 and Figures 3–5). The process

(8) Lin, C. H.; Cheng, M. C.; Khoo, K. H.; Wu, S. H. In *Sialobiology and Other Novel Forms of Glycosylation*; Inoue, Y.; Lee, Y. C.; Troy, Y. F. A., II., Eds.; Gakushin Publishing Company: Osaka, 1999; pp 45–50.

(9) Cheng, M. C.; Lin, C. H.; Khoo, K. H.; Wu, S. H. *Angew. Chem.* **1999**, *111*, 743–746; *Angew. Chem. Int. Ed.* **1999**, *38*, 686–689.

Scheme 1. Simplified Representation of Dimer, Trimer, Tetramer, and Lactonized Species in the Pathways of Lactonization and Hydrolysis^a



^a This represents the pathway of lactonization and hydrolysis of the tetramer; the dot-dashed square is for the trimer, and the dotted square is for the dimer. The upper-case letters L and H represent lactonization and hydrolysis, respectively

of acidic degradation reveals three stages. In the initial stage, the formation of the lactone ring is a kinetic intramolecular reaction, so lactonization is much faster than hydrolysis, even in aqueous solution. In the middle stage, the lactonized species may directly hydrolyze to lower-DP lactonized species, or they may be equilibrated into their nonlactonized counterparts, hydrolyzed to lower-DP oligomers, and in the final stage, completely cleaved to dimer and monomer. The concentration of remaining reactants as well as the concentration of dimer and monomer could be used as indicators for the progress of the two reactions. At lower temperature (<10 °C), only lactonization can proceed under the acidic conditions tested and the whole processes of the reactions may stop in the initial stage. At higher temperature and more acidic condition, the rate of lactonization and hydrolysis will be accelerated to produce all dimers and monomers. The α 2,8-linked glycosidic bonds in oligosialic acids are protected if the bonds are in the lactone ring, especially in the dimer. This can explain why the dimer is the major product in the hydrolysis of oligo/polysialic acids under acidic conditions. The present study describes the relationship between lactonization and hydrolysis of OSA under acidic conditions (0.1 N acetic acid), a relationship that has not been previously reported.

Experimental Section

Materials. *N*-Acetylneuraminic acid dimer ($[\rightarrow 8\text{Neu}5\text{Ac}\alpha 2\rightarrow]_2$), trimer ($[\rightarrow 8\text{Neu}5\text{Ac}\alpha 2\rightarrow]_3$), and tetramer ($[\rightarrow 8\text{Neu}5\text{Ac}\alpha 2\rightarrow]_4$)

were purchased from Nacalai tesque (Japan). Neuraminidase from *Arthrobacter ureafaciens* was purchased from Nacalai tesque (Japan). All other reagents for reactions and high performance capillary electrophoresis (HPCE) were of the highest grade commercially available.

Reaction for the Dimer, Trimer, and Tetramer of α 2 \rightarrow 8 *N*-Acetylneuraminic Acid. Free dimer, trimer, and tetramer of α 2 \rightarrow 8 *N*-Acetylneuraminic acid (25 μ g) were incubated in 1 mL of 0.1 N acetic acid at 60 °C. For studies of temperature effect, the tetramer was incubated at 10, 37, and 60 °C. The reactions were stopped, frozen with liquid nitrogen, and then dried immediately in a Speed Vac (Heto, Denmark) to remove acetic acid. Dried samples were dissolved in double distilled water, and an aliquot (20 μ L) of the mixture was analyzed by HPCE.

Chromatographic Conditions. Capillary electrophoresis (CE) was performed on a Beckman capillary electrophoresis system (P/ACE 2100) using a fused silica capillary (107 cm \times 75 μ m (i.d.)) and carried out by applying 20 kV at 25 °C. Phosphate buffer (50 mM, pH 7.0) was used as the running buffer. The UV absorption at 200 nm was monitored. Samples were injected into the capillary under a high pressure of nitrogen (1.3 bar) for 5 s. The capillary was regenerated by washing with double distilled water for 3 min and then 0.1 N NaOH for 5 min.

Acknowledgment. Financial support of this work was provided by the National Science Council of Taiwan and Academia Sinica, Taipei, Taiwan. The authors thank Professor Y. Inoue for helpful discussion.

JO015580C