Kinetic Studies on the Acid Hydrolysis of the Methyl Ketoside of Unsubstituted and *O*-Acetylated *N*-Acetylneuraminic Acid

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The hydrolysis of the model compound 2-*O*-methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid by acid and neuraminidase (*Vibrio cholerae*) closely resembled that of the *O*-acetylated sialic acid residues of rabbit Tamm–Horsfall glycoprotein. This confirmed that *O*-acetylation was responsible for the unusually slow rate of acid hydrolysis of *O*-acetylated sialic acid residues observed in rabbit Tamm–Horsfall glycoprotein and their resistance to hydrolysis by neuraminidase. The first-order rate constant of hydrolysis of 2-methyl-*N*-acetyl- α -D-neuraminic acid by 0.05 M-H₂SO₄ was 56-fold greater than that of 2-*O*-methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid. Kinetic studies have shown that in the pH range 1.00–3.30, the observed rate of hydrolysis of 2-methyl-*N*-acetyl- α -D-neuraminic acid can be attributed to acid-catalysed hydrolysis of the negatively charged CO₂[−] form of the methyl ketoside.

The sialic acid residues in rabbit Tamm–Horsfall (T–H) glycoprotein are *O*-acetylated (on average 2 *O*-acetyl residues/sialic acid residue) and evidence was obtained indicating that the *O*-acetyl substituents are located on the polyhydroxy side-chain of sialic acid, possibly at C-7 and C-8 (Neuberger & Ratcliffe, 1972). Under conditions of acid hydrolysis (0.05 M-H₂SO₄, 80°C) that lead to quantitative release of sialic acid in 1 h from some glycoproteins, and which are often assumed to be universally applicable, these sugar residues in rabbit T–H glycoprotein are released at an unusually slow rate, and an apparent maximum amount was liberated after 5 h. The fraction of sialic acid that was released slowly on acid hydrolysis was also resistant to hydrolysis by bacterial neuraminidases. These unusual properties were attributed to *O*-acetylation of the sialic acid residues, since saponification of the *O*-acetyl substituents normalized the subsequent behaviour of the sialic acid residues in both acid and enzymic hydrolyses. Other glycoproteins containing *O*-acetylated sialic acid residues and which are relatively resistant to acid have been mentioned by Neuberger & Ratcliffe (1972).

In experiments designed to investigate this hypothesis, the model compound 2-*O*-methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid was prepared and its behaviour in colorimetric measurements for sialic acid and its hydrolysis characteristics in acid solutions were compared with those of 2-*O*-methyl-*N*-acetyl- α -D-neuraminic acid.

The ease of hydrolysis of sialic acid residues at low acid concentrations has been attributed, in part, to

the proximity of the CO₂[−] group (C-1) to the ketosidic O atom (Marshall & Neuberger, 1972). The proposed mechanism of hydrolysis of sialic acid residues has been investigated by studying the effect of the H⁺ concentration on the rate of hydrolysis of 2-*O*-methyl-*N*-acetyl- α -D-neuraminic acid.

Materials and Methods

N-Acetylneuraminic acid, m.p. 185°C (decomp.), [α]_D²⁰ = 36±1° (*c* 1 in water) was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. 2-*O*-Methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid was prepared as described by Meindl & Tuppy (1965) and recrystallized three times from methanol–ether. The melting point obtained (182–185°C) was similar to that found by Meindl & Tuppy (1965) (184–187°C). T.l.c. on silica gel G in butan-1-ol–propan-1-ol–0.1 M-HCl (1:2:1, by vol.) gave a single component of *R*_{NeuNAc} 3.50, where *R*_{NeuNAc} = (mobility of unknown)/(mobility of *N*-acetylneuraminic acid). 2-*O*-Methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid was saponified with a twofold molar excess of NaOH (relative to *O*-acetyl groups) at 4°C for 3 h. Cations were removed by the addition of Dowex-50 (H⁺ form) in excess, the resin was removed by filtration and the glycoside was recovered by freeze-drying the filtrate. 2-Methyl-*N*-acetyl- α -D-neuraminic acid was homogeneous in the t.l.c. system previously described (*R*_{NeuNAc} 1.6); [α]_D²² = −3.5° (*c* 1.0 in water).

Conditions employed for the acid and enzymic hydrolysis of sialic acid residues have been described (Neuberger & Ratcliffe, 1972). Neuraminidase (*Vibrio cholerae*) was from Behringwerke A. G. (Hoechst

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U.K. Ltd., Hounslow, Middx., U.K.) and contained 500 units/ml where 1 unit releases $1\mu\text{g}$ of *N*-acetylneuraminic acid from α_1 acid glycoprotein (human) in 15 min at 37°C in 0.05M-sodium acetate buffer, pH 5.5, containing 9 mg of NaCl/ml and 1 mg of CaCl_2 /ml. Total sialic acid was determined by the method of Jourdan *et al.* (1971). In the kinetic studies, at least six duplicate samples of the acid hydrolysates were removed at different measured time-intervals and immediately frozen, and the free sialic acid was determined later by the method of Warren (1959). First-order rate constants for the hydrolysis of ketosides were obtained from the slope of the linear regression of the logarithm of the ketoside concentration as a function of time. In all experiments the correlation coefficients of the regression lines were greater than 0.980. The energies of activation were calculated from the Arrhenius equation. The pH of hydrolysates of the methyl ketoside was calculated from the known H_2SO_4 concentrations, and no correction for the effect of ionic strength was made.

Results and Discussion

Hydrolysis of 2-O-methyl-4,7,8,9-tetra-O-acetyl-N-acetyl- α -D-neuraminic acid by 0.05M- H_2SO_4 at 80°C

The rates of hydrolysis of the ketosidic and ester linkages of 2-*O*-methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid in 0.05M- H_2SO_4 at 80°C are not too dissimilar (Neuberger & Ratcliffe, 1972). Thus the products of partial hydrolysis of 2-*O*-methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid consist of a mixture of unsubstituted and partially *O*-acetylated forms of free sialic acid. Since 7-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid is known to be unreactive in the thiobarbituric acid method of Aminoff (1961), and may also be unreactive in the thiobarbituric acid method of Warren (1959), *O*-acetyl substituents were removed by saponification before determination of the free sialic acid content of the hydrolysate by the method of Warren (1959). To determine whether free sialic acid was stable under the alkaline conditions necessary for the saponification of *O*-acetyl residues, *N*-acetylneuraminic acid ($9.8\mu\text{g}$) was left in various concentrations of NaOH (0.005–0.5M) at 4°C for 2 h. No destruction was obtained at NaOH concentrations of less than 0.25M, as determined by the extinction coefficient obtained when *N*-acetylneuraminic acid was assayed by the thiobarbituric acid method. Acid hydrolysates of 2-*O*-methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid were subsequently treated with 0.1M-NaOH at 4°C for 2 h to liberate *O*-acetyl substituents. The difference in the free sialic acid contents of the hydrolysate and alkali-treated hydrolysate should reflect the amount of free sialic acid in the hydrolysate that is *O*-acetylated at C-7 (see Fig. 1).

Fig. 1. *Hydrolysis of 2-O-methyl-4,7,8,9-tetra-O-acetyl-N-acetyl- α -D-neuraminic acid by 0.05M- H_2SO_4 at 80°C as determined by the method of Warren (1959)*

2-*O*-Methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid ($133.7\mu\text{g}/\text{ml}$) was hydrolysed in 0.05M- H_2SO_4 and samples (0.2 ml) were removed and the free sialic acid contents of the hydrolysates were determined by the method of Warren (1959), either before or after treatment of the hydrolysate with alkali to liberate *O*-acetyl groups (0.1M-NaOH, 4°C , 2 h). The free sialic contents of the hydrolysate and alkali-treated hydrolysate are represented by ○ and ● respectively.

The rate of hydrolysis of 2-*O*-methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid in 0.05M- H_2SO_4 at 80°C (Fig. 1) was similar to that of the sialic acid residues of rabbit T-H glycoprotein, in that an apparent maximum was reached after 5 h (Neuberger & Ratcliffe, 1972). Since free sialic acid is progressively destroyed under these conditions of acid hydrolysis (Gibbons, 1963), the sialic acid content attained in 5 h is an underestimate owing to destruction of free sialic acid. In the first 3.5 h of hydrolysis, sialic acid with an *O*-acetyl substituent at C-7 forms a significant proportion of the free sialic acid present (Fig. 1), indicating that hydrolysis of the *O*-acetyl substituent at C-7 is not an essential requirement for the subsequent hydrolysis of the ketosidic bond. The experiment also shows that the ketosidic bond and the *O*-acetyl substituent at C-7 are hydrolysed by acid at similar rates.

By contrast, 2-*O*-methyl-*N*-acetylneuraminic acid was hydrolysed by 0.05M- H_2SO_4 at 80°C at a much faster rate, as determined by the thiobarbituric acid method, and hydrolysis was essentially complete after 25 min.

In a recent modification of the resorcinol method of Svennerholm (1957) for the determination of sialic acid, an increased extinction coefficient was obtained in the resorcinol assay after periodate

oxidation (Jourdian *et al.*, 1971). The greater sensitivity of the periodate–resorcinol method is due to the formation of a number of resorcinol-reactive chromogens, the major chromogen being the aldehyde formed by cleavage between C-7 and C-8 of the side chain of sialic acid. The lower extinction coefficient of 2-*O*-methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid ($7400 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) in the periodate–resorcinol method, compared with that given by *N*-acetylneuraminic acid ($29000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) may be assumed to reflect the presence of acetyl substituents on C-7 and C-8. After saponification of 2-*O*-methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid by 0.1M-NaOH at 4°C for 2h, the resulting methyl ketoside of *N*-acetylneuraminic acid gave an extinction coefficient of $30500 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ in the periodate–resorcinol method.

The hydrolysis of 2-*O*-methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid by 0.05M- H_2SO_4 at 80°C was followed by the periodate–resorcinol method (Fig. 2). The behaviour of the *O*-acetylated ketoside closely resembled that of sialic acid residues of rabbit T-H glycoprotein (see Neuberger & Ratcliffe, 1972) in that an apparent maximum value for the total sialic acid content was obtained after hydrolysis for 6h in 0.05M- H_2SO_4 at 80°C. The increase obtained in total sialic acid content of the *O*-acetylated ketoside on hydrolysis in acid (Fig. 2)

Fig. 2. Hydrolysis of 2-*O*-methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid by 0.05M- H_2SO_4 at 80°C as determined by the periodate–resorcinol method (Jourdian *et al.*, 1971)

2-*O*-Methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid ($133.7 \mu\text{g/ml}$) was hydrolysed by 0.05M- H_2SO_4 and samples were removed for determination of the total sialic acid content by the periodate–resorcinol method, either before or after treatment of the hydrolysate with alkali to liberate *O*-acetyl groups (0.1M-NaOH, 4°C, 2h). The total sialic acid contents of the hydrolysate and alkali-treated hydrolysate are represented by ○ and ● respectively.

reflects the rate of hydrolysis of the *O*-acetyl substituents on C-7 and C-8, superimposed on the rate of destruction of free sialic acid. The rate of destruction of sialic acid under these conditions is reflected by the decrease in the total sialic acid content of the *O*-acetylated ketoside as determined by the periodate–resorcinol method after treatment of the hydrolysate with alkali to release *O*-acetyl substituents (Fig. 2).

The close similarity of the rates of hydrolysis of sialyl residues in rabbit T-H glycoprotein and 2-*O*-methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid by 0.05M- H_2SO_4 at 80°C as determined by both colorimetric methods, confirms that *O*-acetylated sialic acid residues in rabbit T-H glycoprotein are responsible for the unusual hydrolysis characteristics observed. Further evidence is that in both rabbit T-H glycoprotein and the model compound the acetyl substituents and the ketosidic bond are hydrolysed at similar rates. 4-*O*-Acetyl-*N*-acetylneuraminic acid occurs in a glycoprotein of horse serum, and the optimum conditions for its release (0.0125M- H_2SO_4 , 80°C, 1h) are similar to those of other glycoproteins containing unsubstituted *N*-acetylneuraminic acid (Pepper, 1968). Since *O*-acetylation at C-4 has no effect on the rate of hydrolysis of sialyl residues, substitution of the polyhydroxy side chain must be responsible for the slow rate of hydrolysis of sialyl residues in rabbit T-H glycoprotein and the model compound. Several explanations have been suggested to explain the effect of *O*-acetylation of the side chain on the rate of acid-catalysed hydrolysis, including steric and inductive effects arising from *O*-acetylation of C-7 (Neuberger & Ratcliffe, 1972). The presence of significant amounts of 7-*O*-acetylsialic acid in hydrolysates of the model compound suggests that the hydrolysis of an acetyl substituent at C-7 is not a prerequisite for the hydrolysis of the ketosidic bond.

Effect of neuraminidase (Vibrio cholerae) on 2-O-methyl-4,7,8,9-tetra-O-acetyl-N-acetyl- α -D-neuraminic acid

2-*O*-Methyl-*N*-acetylneuraminic acid was incubated with 100 units of neuraminidase (*Vibrio cholerae*) for 24h at 37°C. In this time 98% of the theoretical amount of sialic acid was liberated, confirming that virtually all the ketoside present was in the form of the α -anomer (Yu & Ledeen, 1969).

When 2-*O*-methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid ($346 \mu\text{g}$) was incubated with 100 units of neuraminidase for 24h at 37°C, no free sialic acid could be detected in the digest by the thiobarbituric method, even after treatment of the digest with 0.1M-NaOH at 4°C for 2h to liberate *O*-acetyl residues. This confirms that *O*-acetylation of *N*-acetylneuraminyl residues confers resistance to hydrolysis by neuraminidase.

Table 1. Comparison of the first-order rate constants of hydrolysis of 2-*O*-methyl-*N*-acetyl- α -D-neuraminic acid and 2-*O*-methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid in 0.05M-H₂SO₄

The free sialic acid contents of the hydrolysates were determined by the method of Warren (1959); with 2-*O*-methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid this was after treatment of the hydrolysate with 0.1M-NaOH at 4°C for 2h to liberate *O*-acetyl residues. The rate constants given in parentheses were obtained by extrapolation of the slope of the plot of $1/T$ versus $\log k$, assuming that the activation energy is independent of the temperature. The kinetic constants for the *O*-acetylated ketoside describe the analytical results but are not true kinetic constants (see the text).

Compound	$10^5 \times k \text{ (s}^{-1}\text{)}$			$10^5 \times k \text{ (s}^{-1}\text{)}$			$E \text{ (kJ} \cdot \text{mol}^{-1}\text{)}$
	45°C	50°C	55°C	75°C	80°C	85°C	
2- <i>O</i> -Methyl- <i>N</i> -acetyl- α -D-neuraminic acid	6.60	12.6	21.2	(186)	(306)	(498)	102
2- <i>O</i> -Methyl-4,7,8,9-tetra- <i>O</i> -acetyl- <i>N</i> -acetyl- α -D-neuraminic acid	(0.112)	(0.205)	(0.372)	3.55	5.27	9.90	105

Table 2. Effect of H⁺ concentration on the first-order rate constants of hydrolysis of 2-*O*-methyl-*N*-acetyl- α -D-neuraminic acid at 50°C

2-*O*-Methyl-*N*-acetyl- α -D-neuraminic acid was hydrolysed at 50°C in various H₂SO₄ concentrations as described in the text and the free sialic acid liberated was determined by the method of Warren (1959).

H ₂ SO ₄ (mM)	$10^5 \times k \text{ (s}^{-1}\text{)}$
50	12.65
25	12.75
12.5	11.07
5.0	8.73
2.5	5.68
1.25	3.25
0.5	2.33
0.25	1.24

Kinetic studies

The hydrolysis of 2-*O*-methyl-*N*-acetyl- α -D-neuraminic acid by 0.05M-H₂SO₄ was studied at 45–55°C, whereas a higher temperature range (75–85°C) was required for the hydrolysis of the *O*-acetylated ketoside. Since no corrections could be applied for the destruction of free sialic acid, which is known to occur in 0.05M-H₂SO₄ at 80°C (see Gibbons, 1963), in kinetic studies involving the *O*-acetylated ketoside all measurements were made within 1h to minimize this effect. Hydrolysates of the *O*-acetylated ketoside were treated with 0.1M-NaOH at 4°C for 2h to liberate *O*-acetyl residues, followed by determination of the free sialic acid content by the thiobarbituric acid method. The rates of hydrolysis of 2-*O*-methyl-*N*-acetyl- α -D-neuraminic acid and 2-*O*-methyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl- α -D-neuraminic acid

were found to be first-order with respect to the concentration of ketoside in the hydrolysate. By assuming that the activation energies of hydrolysis were independent of temperature, the plots of $1/T$ against $\log k$ were extrapolated to allow a comparison of the rates of hydrolysis of both compounds in the two temperature ranges studied (Table 1). The first-order rate constants of hydrolysis of the methyl ketoside in 0.05M-H₂SO₄ were on average 56-fold greater than the observed first-order rate constants of hydrolysis of the *O*-acetylated ketoside. Partial hydrolysates of the *O*-acetylated ketoside contain a mixture of fully *O*-acetylated, partially *O*-acetylated ketosides of sialic acid and also sialic acid itself. Although no quantitative values can be given, it appears that the rates of the glycosidic splitting and those of de-acetylation of the *O*-acetylated ketoside are closely similar. If this is so, the rates of hydrolysis of the ketosidic bond of the fully *O*-acetylated compound would be almost half those given in Table 1 and the ratio between the rate constants of the hydrolysis of the two ketosides would be about 100 and not 56, as given above.

The possible mechanisms of acid-catalysed hydrolysis of sialic acid residues have been recently reviewed (Marshall & Neuberger, 1972). The ease of hydrolysis of sialic acid residues by acid has been attributed, in part, to the proximity of the CO₂H group (C-1) to the ketosidic O atom. A possible mechanism involves specific H⁺-catalysed hydrolysis of the negatively charged species, where the presence of the CO₂[−] group facilitates protonation of the ketosidic O atom and also stabilizes the postulated carbonium ion intermediate (Marshall & Neuberger, 1972). Another possible mechanism involves intramolecular catalysis by protonation of the ketosidic O atom by the H⁺ of the un-ionized carboxyl group (Karkas & Chargaff, 1964).

In general, for acid hydrolysis of glycosides a direct relationship exists between the rate of hydrolysis and the acidity (Bunton *et al.*, 1955). For 2-*O*-methyl-*N*-acetyl- α -D-neuraminic acid the relationship between the first-order rate constant and the H^+ concentration was found to be non-linear, and in the pH range studied, the rate constant varied only 10-fold for a 200-fold change in the H^+ concentration (Table 2). The rate of hydrolysis of the methyl ketoside may be written as the sum of two terms where the first term refers to the acid-catalysed hydrolysis of the carboxylate form and the second term refers to acid-catalysed hydrolysis of the un-ionized form. The rate observed $= k_1[H^+][RCO_2^-] + k_2[H^+][RCO_2H] = k_1K[RCO_2H] + k_2[H^+][RCO_2H]$, where k_1 and k_2 are the rate constants of hydrolysis of the ionized and un-ionized species respectively and K , the dissociation constant of the carboxyl group, $= ([H^+][RCO_2^-])/[RCO_2H] = K^*[RCO_2H] + k_2[H^+][RCO_2H]$, where $K^* = k_1K$.

In the pH range 1.00–3.30, the pH–rate profile of hydrolysis of the methyl ketoside is sigmoid in shape and closely resembles the theoretical dissociation curve of an acid with a pK of 2.15 (Fig. 3). Thus the rate of hydrolysis of the ketoside is directly proportional to the concentration of the un-ionized species as referred to in the first term in the above equation. Since the experimental findings can be explained in terms of the first term in the equation, it follows that the second term can be neglected in the pH range studied, although it may be of considerable importance at much higher H^+ concentrations. Thus the experimental findings have indicated that in the pH range 1.00–3.30, the observed rate of the hydrolysis of sialyl residues may be almost entirely due to acid-catalysed hydrolysis of the CO_2^- form. Specific H^+ catalysis of the CO_2^- form cannot be distinguished by kinetic methods from intramolecular catalysis by an un-ionized CO_2H group.

Other glycosides are known where the rate of hydrolysis is influenced by the proximity of a CO_2H group to the glycosidic O atom. For 2-naphthyl β -D-glucuronide, the rate of hydrolysis in the pH range 0–5 was shown to be related to the ionization of the CO_2H group at C-6 (Capon & Gosh, 1965). The ready hydrolysis of *O*-carboxyphenyl β -D-glucoside in the pH range 1–5 is an example of intramolecular catalysis by a CO_2H substituent on the aglycone (Capon, 1963).

O-Acetylation of the sialic acid side-chain at C-7, C-8 and C-9 decreased the observed rate constant of hydrolysis by 0.05M- H_2SO_4 about 56-fold. A possible explanation may be that inductive and steric effects arising from *O*-acetylation of the side chain (and in particular C-7) lower the pK of the carboxyl group. According to the proposed mechanism of hydrolysis, this would cause a decrease in the observed rate of acid-catalysed hydrolysis at the acidities employed.

Fig. 3. pH–rate profile of the hydrolysis of 2-*O*-methyl-*N*-acetyl- α -D-neuraminic acid at 50°C

The solid line represents the theoretical curve for the dissociation of an acid of pK 2.15. The experimental points, represented by ●, are the first-order rate constants for the hydrolysis of 2-*O*-methyl-*N*-acetyl- α -D-neuraminic acid at 50°C (Table 2).

Alternatively, the hydrolysis of *O*-acetylated sialyl residues might proceed by an entirely different mechanism from that of the unsubstituted residues.

Titration studies have indicated that the pK of the CO_2H group of *N*-acetylneuraminic acid is of the order of 2.6 at 25°C (Bettelheim, 1963), although several forms of sialic acid possessing pK values in the range 2.0–2.8 have been claimed to occur in bovine submaxillary mucins (Scheinthal & Bettelheim, 1968). Our kinetic studies have indicated that the pK of the carboxyl group of *N*-acetylneuraminic acid at 50°C is 2.15, which is somewhat lower than the generally accepted value of 2.6, although the conditions employed in the kinetic and equilibrium measurements, especially the temperatures, were not identical.

The conditions that are generally employed for the release of sialic acid residues from glycoproteins are 0.05M- H_2SO_4 at 80°C for 1 h, and the destruction of free sialic acid may be as high as 7% (Gibbons, 1963). The present studies suggest that lower concentrations of acid (e.g. 5mM- H_2SO_4) might prove sufficient for the complete release of *N*-acetylneuraminic acid, and the rate of destruction of free sialic acid under these conditions might be correspondingly less.

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