The Acid and Enzymic Hydrolysis of *O*-Acetylated Sialic Acid Residues from Rabbit Tamm–Horsfall Glycoprotein

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Rabbit Tamm–Horsfall glycoprotein and bovine submaxillary glycoprotein were both found to contain sialic acid residues which are released at a slow rate by the standard conditions of acid hydrolysis. These residues are also resistant to neuraminidases from *Vibrio cholerae* and *Clostridium perfringens*. This behaviour was attributed to the presence of *O*-acetylated sialic acid, since the removal of *O*-acetyl groups by mild alkaline treatment normalized the subsequent release of sialic acid from rabbit Tamm–Horsfall glycoprotein by acid and by enzymic hydrolysis. Determination of the *O*-acetyl residues in rabbit Tamm–Horsfall glycoprotein indicated that on average two hydroxyl groups of sialic acid are *O*-acetylated, and these were located on the polyhydroxy side-chain of sialic acid or on C-4 and C-8. These findings confirm the assumption that certain *O*-acetylated forms of sialic acid are not substrates for bacterial neuraminidases. Several explanations have been suggested to explain the effect of *O*-acetylation of the side-chain on the rate of acid-catalysed hydrolysis of sialic acid residues.

Tamm–Horsfall (T–H) glycoproteins are found in urine and are believed to be formed in the kidney (McKenzie & McQueen, 1969). The T–H glycoproteins obtained from man and rabbits have different, but closely similar, chemical compositions (Fletcher *et al.*, 1970; Marr *et al.*, 1971). Both glycoproteins contain about 5% sialic acid; but, whereas all the sialic acid of human T–H glycoprotein could be released by hydrolysis in 0.05M-H₂SO₄ at 80°C within 1 h, the sialic acid of rabbit T–H glycoprotein was released at a much slower rate, and an apparent maximum was reached only after 5 h of hydrolysis (Marr *et al.*, 1971).

An incomplete release of sialic acid within 1 h by mild acid hydrolysis is not unique; thus, in the following cases, sialic acid was found to be incompletely released by hydrolysis under the standard conditions (0.05M-H₂SO₄, 80°C, 1 h): colominic acid (Aminoff, 1961), bovine submaxillary glycoprotein and bovine cervical glycoprotein (Gibbons, 1963). Further, neuraminidase from either *Vibrio cholerae* or *Clostridium perfringens* incompletely released sialic acid from colominic acid (McGuire & Binkley, 1964), bovine submaxillary glycoprotein (Gottschalk, 1957; Gibbons, 1963; Bertolini & Pigman, 1967), and bovine cervical glycoprotein (Gibbons, 1963). A form of sialic acid in bovine submaxillary glycoprotein was shown to be resistant to neuraminidase and also to be responsible for the anomalous acid-hydrolysis time-curve. This behaviour was attributed to the *O*-acetylated sialic acid residues known to be present in bovine submaxillary glycoprotein (Blix & Lindberg, 1960), since after de-*O*-acetylation by mild

alkaline treatment of the glycoprotein the anomalies in the acid hydrolysis time-curve disappeared and almost all the sialic acid could be released by neuraminidase (Gibbons, 1963).

In the present study a number of methods have been applied to determine both the content and structure of sialic acid in rabbit T–H glycoprotein, and an attempt has been made to account for its slow rate of acid hydrolysis. The presence of *O*-acetylated sialic acid residues has been established and the effect of de-*O*-acetylation by mild alkali on the subsequent release of sialic acid by acid and enzymic hydrolysis is described. Studies based on periodate oxidation of the glycoprotein have permitted certain conclusions to be reached as to the positions of substitution of the *O*-acetyl groups.

Experimental

Chemicals and enzymes

N-Acetyl neuraminic acid (synthetic type IV) and neuraminidase type VI (*Clostridium perfringens*) were obtained from Sigma (London) Chemical Co. Ltd. (London S.W.6, U.K.); 1 mg of this neuraminidase preparation liberated 1.3 μmol of *N*-acetylneuraminic acid/min from bovine submaxillary mucin at pH 5.0 at 37°C. *N*-Acetylglucosamine was from Koch–Light Laboratories Ltd., (Colnbrook, Bucks., U.K.) and was further purified by passage through Dowex-50 (H⁺ form) and recrystallization. Neuraminidase (*Vibrio cholerae*) was from Behringwerke A.G. (Hoechst U.K. Ltd., Hounslow, Middx., U.K.)

and contained 500 units/ml, where 1 unit releases 1 μ 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. Pronase P was obtained from Kaken Chemical Co. Ltd. (Tokyo, Japan). Bovine submaxillary glycoprotein (Gibbons, 1963) was kindly given by Dr. R. A. Gibbons and 1-*N*-acetyl-2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranosylamine, $[\alpha]_D^{24} + 18.4^\circ$ ($c = 2 \text{ g}/100 \text{ ml}$ in CHCl_3), m.p. 162–164°C, was kindly given by Mr. B. M. Austen.

Methods

Analytical methods. The total sialic acid content of T-H glycoprotein was determined by the resorcinol method (Svennerholm, 1957) and the periodate-resorcinol method (Jouradian *et al.*, 1971), without prior release of sialic acid by acid hydrolysis. Total sialic acid in acid hydrolysates was also measured by the periodate-resorcinol method. Both *N*-acetylneuraminic acid and *N*-acetyl-7-*O*-acetylneuraminic acid give similar extinction coefficients in the resorcinol method, since the method involves heating in strong mineral acid, which produces de-*O*-acetylation (Svennerholm, 1957). The periodate-resorcinol method, a recent modification of the resorcinol method, involves periodate oxidation of the polyhydroxy side chain of sialic acid, and in the subsequent resorcinol reaction the oxidized sialic acid produces a chromogen giving a greater extinction coefficient than that of unoxidized sialic acid. Thus sialic acid which is substituted on C-7 and/or C-8 will give a low value by this method (Jouradian *et al.*, 1971).

In general sialic acid was released by mild acid hydrolysis of the glycoprotein (0.05M- H_2SO_4 , 80°C) and the free sialic acid was determined by the thiobarbituric acid method (Warren, 1959), or the alkali-Ehrlich method (Aminoff, 1961). An essential step in the thiobarbituric acid method is the formation of a carbonyl group at C-6 of sialic acid by periodate oxidation. Whereas *N*-acetyl-4-*O*-acetylneuraminic acid gives a positive reaction in the thiobarbituric acid method, *N*-acetyl-7-*O*-acetylneuraminic acid is unreactive, since the essential periodate-oxidation step is prevented (Aminoff, 1961). The alkali-Ehrlich method measures total free sialic acid, since de-*O*-acetylation occurs under the conditions of alkaline degradation of sialic acid (Aminoff, 1961). Thus a comparison of the results of the thiobarbituric acid and alkali-Ehrlich methods should reflect the *N*-acetyl-7-*O*-acetylneuraminic acid content of a hydrolysate.

The total glycolyl contents of the T-H glycoproteins were found by the method of Gibbons (1962). Hexose and amino sugars were determined as described by Fletcher *et al.* (1970).

T-H glycoprotein. The preparation of T-H glycoprotein from human and rabbit urine has been described (Fletcher *et al.*, 1970; Marr *et al.*, 1971), and these studies were carried out with two preparations of each of the glycoproteins.

Pronase digestion. Rabbit T-H glycoprotein (15 mg) and pronase (300 μ g) were dissolved in 5 ml of 0.01M-tris-HCl, pH 8.5, containing 15 mM- CaCl_2 and kept at 37°C for 24 h. The extent of proteolytic digestion was followed by means of the ninhydrin reagent of Rosen (1957).

Paper chromatography of acetyl hydroxamate. Acetyl hydroxamate was formed from rabbit T-H glycoprotein (20 mg) by treatment with 0.09M-hydroxylamine hydrochloride in 0.38M-NaOH (Ludowig & Dorfman, 1960), the solution was neutralized and dialysed, and the diffusate isolated by freeze-drying. Acetyl hydroxamate was separated from the salt by passage through a mixed-bed ion-exchange column (Amberlite MBI). Chromatography was performed on Whatman no. 1 paper in the solvent butan-1-ol-acetic acid-water (4:1:5, by vol.) (Thompson, 1951), which separates the hydroxamates of formic acid, acetic acid and propionic acid. Acyl hydroxamates were detected by a ferric chloride reagent (5% FeCl_3 in methanol-acetone, 4:3, v/v). Authentic acetyl hydroxamate was prepared from ethyl acetate by treatment with the alkaline hydroxylamine reagent.

Release of sialic acid by acid hydrolysis. T-H glycoprotein was hydrolysed in 0.05M- H_2SO_4 at 80°C at a concentration of about 1 mg/ml and bovine submaxillary glycoprotein was hydrolysed at a concentration of about 300 μ g/ml. Samples for sialic acid determination were removed at appropriate times and stored at -20°C until analysis. After adjustment of the pH of the hydrolysate to approx. 5.5, the free sialic acid was separated from the glycoprotein by extensive dialysis against water, and both the diffusate and glycoprotein fractions were recovered by freeze-drying.

Release of sialic acid by neuraminidase. T-H glycoprotein (about 1 mg/ml) was incubated at 37°C with 50–100 units of neuraminidase from *Vibrio cholerae* or with 20 μ g of neuraminidase from *Clostridium perfringens* in 0.05M-sodium acetate-acetic acid buffer, pH 5.5, containing 0.9% NaCl, 0.1% CaCl_2 and 0.02% NaN_3 ; samples were removed over a period of 24 h for determination of free sialic acid. After neuraminidase digestion the glycoprotein was recovered by freeze-drying, after dialysis against water.

Alkaline treatment of T-H glycoprotein. Rabbit T-H glycoprotein was treated with 0.5M-NaOH at 4°C for 2 days to determine whether there were any alkali-labile carbohydrate-protein linkages present in the glycoprotein. Subsequent hexose and amino sugar analyses were related to the dry weight of the

alkali-treated glycoprotein. To release *O*-acetyl groups T-H glycoprotein was also treated with (a) 0.05M- Na_2CO_3 at 100°C for 20min (Gibbons, 1963), (b) 0.2M- NaOH at 4°C for 2h or (c) 0.09M-hydroxylamine hydrochloride in 0.38M- NaOH for 30min at room temperature (Ludoweig & Dorfman, 1960). After alkaline treatment the glycoprotein was dialysed extensively against water and recovered by freeze-drying.

Determination of ester groups. Ester groups were determined by a modification of the Hestrin (1949) alkaline hydroxylamine method (Ludoweig & Dorfman, 1960) with ethyl acetate as a standard. To correct for turbidity arising from the presence of the glycoprotein, a blank determination was made by treating an identical weight of glycoprotein with an alkaline solution from which the hydroxylamine hydrochloride had been omitted. The diffusible acyl hydroxamates of T-H glycoprotein were determined by a method similar to that employed by Bertolini & Pigman (1967). After treatment of T-H glycoprotein with alkaline hydroxylamine, the pH of the reaction solution was adjusted to 5.5 and the solution was dialysed against three changes of water totalling about 300ml. The diffusates were combined and freeze-dried and the hydroxamates present were determined as described above.

Determination of total acetyl content (*N*-acetyl plus *O*-acetyl). Total acetyl groups were determined by a

method involving methanolysis and distillation of the resulting volatile methyl ester, which was determined as the acyl hydroxamate (Ludoweig & Dorfman, 1960).

Determination of *O*-acetyl content. The method of *O*-acetyl determination involved transesterification in 0.1M-sodium methoxide at 0°C for 30min (see Whistler & Jeanes, 1943), followed by distillation of the resulting methyl acetate, which was determined by the method of Ludoweig & Dorfman (1960).

Release of formaldehyde by periodate oxidation. T-H glycoprotein (approx. 1–2mg/ml) was treated with 10mM-sodium periodate at 0°C in the dark and samples (0.5ml) were removed at various times. The excess of periodate in the sample was destroyed by the addition of 0.1 ml of 0.5M-KI followed by 0.1 ml of 0.5M- Na_2SO_3 and the formaldehyde was determined by the method of Nash (1953).

Paper chromatography of sialic acid. Glycoproteins were hydrolysed at pH 2.3 in 5mM- H_2SO_4 at 70°C for 1h and then the pH of the hydrolysate was adjusted to approx. 4 by the addition of NaOH . The hydrolysate was applied to a Dowex-1 (acetate form) column (0.5cm $\times$ 4.0cm), and the column was washed with 20ml of water. Sialic acid was eluted with 0.5M-formic acid and recovered by freeze-drying. Descending paper chromatography was carried out on Whatman no. 1 paper with the solvent butan-1-ol-pyridine-water (6:4:3, by vol.) (Blix *et al.*, 1956).

Table 1. Sialic acid content of human and rabbit T-H glycoproteins determined by various methods

The glycoproteins were hydrolysed in 0.05M- H_2SO_4 at 80°C. For full details see the text.

Method	Human T-H glycoprotein		Rabbit T-H glycoprotein	
	Acid or enzymic hydrolysis*	Sialic acid content (% dry wt.)	Acid or enzymic hydrolysis*	Sialic acid content (% dry wt.)
Thiobarbituric acid (Warren, 1959)	1	5.6	5	4.2
Alkali-Ehrlich (Aminoff, 1961)	1	4.8	4–5	4.0
Resorcinol (Svennerholm, 1957)	—	6.7	—	5.4
Periodate-resorcinol (Jourdan <i>et al.</i> , 1971)				
1. Total sialic acid	—	6.1	—	1.5
2. Glycosidic sialic acid	—	5.8	—	1.6
3. Total sialic acid	1	6.1	6	3.9
Thiobarbituric acid (Warren, 1959)	<i>Vibrio cholerae</i>	4.8	<i>Vibrio cholerae</i>	0.8
	<i>Clostridium perfringens</i>	5.3	<i>Clostridium perfringens</i>	1.0

* Where sialic acid was released by acid hydrolysis, the time (h) which liberated a maximum amount of sialic acid is given; and where the sialic acid was released by enzymic digestion for 24h, the source of the enzyme is given. These analyses were performed on one preparation of rabbit T-H glycoprotein.

Sialic acids were detected by an Ehrlich spray reagent (Svennerholm & Svennerholm, 1958).

Results

Sialic acid content of T-H glycoprotein

N-Acetylneuraminic acid was used as a standard in all methods of analysis of sialic acid, and the sialic acid contents obtained are expressed as a percentage of the dry weight of the glycoprotein. No glycollic acid could be detected in approx. 10mg of human or rabbit T-H glycoprotein by the method of Gibbons (1962), although recoveries of standard glycollate of approx. 80% were obtained.

Human T-H glycoprotein behaved as a typical glycoprotein in that a maximum amount of sialic acid was liberated within 1 h by mild acid hydrolysis (0.05M-H₂SO₄, 80°C) when the free sialic acid was determined by the thiobarbituric acid and alkali-Ehrlich methods (Table 1, Fig. 1). The two preparations of human T-H glycoprotein used in this study contained 5.6 and 5.4% sialic acid respectively as determined by the method of Warren (1959) after liberation of the sialic acid by acid hydrolysis for 1 h. Closely similar total sialic acid contents were obtained by the resorcinol and periodate-resorcinol methods (Table 1).

In contrast, acid hydrolysis of rabbit T-H glyco-

protein released sialic acid at a very slow rate, and an apparent maximum value was obtained after 5 h by the thiobarbituric acid method and in 4–5 h by the alkali-Ehrlich method (Fig. 1). Although the amount of free sialic acid destroyed in 1 h under these conditions of acid hydrolysis is slight (about 7%), progressive destruction occurs on further hydrolysis and 30% of the sialic acid is destroyed in 5–6 h (Gibbons, 1963). Thus the values of sialic acid attained after 5 h of hydrolysis are likely to underestimate the true sialic acid content of rabbit T-H glycoprotein. The periodate-resorcinol method gave a much lower value for the total sialic acid content of rabbit T-H glycoprotein than the resorcinol method (Table 1). After acid hydrolysis to liberate sialic acid, increasing values for the total sialic acid content were obtained by the periodate-resorcinol method, and these reached a maximum at 6 h (Fig. 1). These findings suggested the presence of a substituent on the hydroxyl group of either or both of carbon atoms 7 and 8, which was released slowly during acid hydrolysis for 6 h.

Enzymic release of sialic acid

Although the two neuraminidase preparations released at least 86% of the sialic acid of human T-H glycoprotein in 24 h, less than 20% of the total sialic acid of rabbit T-H glycoprotein was released under the same conditions (Table 2, Fig. 2). Digestion of

Fig. 1. Release of sialic acid from T-H glycoprotein by acid hydrolysis (0.05M-H₂SO₄, 80°C)

Free sialic acid was determined by the alkali-Ehrlich method (●, ○) and the thiobarbituric acid method (▲, △); total sialic acid was determined by the periodate-resorcinol method (■, □). Human and rabbit T-H glycoproteins are represented by filled and open symbols respectively.

Table 2. Release of sialic acid from rabbit T-H glycoprotein by acid hydrolysis and by neuraminidase

Free sialic acid was determined by the method of Warren (1959). For full details see the text. These analyses were performed on one preparation of rabbit T-H glycoprotein.

Substrate for hydrolysis	Acid hydrolysis or neuraminidase digestion for time shown	Sialic acid released (% dry wt.)
Rabbit T-H glycoprotein	0.05M-H ₂ SO ₄ , 80°C, 5h	4.2
Rabbit T-H glycoprotein	0.05M-H ₂ SO ₄ , 80°C, 1h	2.0
Rabbit T-H glycoprotein after acid hydrolysis (0.05M-H ₂ SO ₄ , 80°C, 1h)	0.05M-H ₂ SO ₄ , 80°C, 5h	1.6
Rabbit T-H glycoprotein	Neuraminidase (<i>Vibrio cholerae</i>), 24h	0.8
Rabbit T-H glycoprotein after neuraminidase treatment (<i>Vibrio cholerae</i>)	0.05M-H ₂ SO ₄ , 80°C, 5h	2.8
Rabbit T-H glycoprotein after acid hydrolysis (0.05M-H ₂ SO ₄ , 80°C, 1h)	Neuraminidase (<i>Vibrio cholerae</i>), 24h	<0.1

Fig. 2. Effect of alkaline pretreatment of rabbit T-H glycoprotein on the release of sialic acid by acid and enzymic hydrolysis

Free sialic acid was determined by the thiobarbituric acid method. Rabbit T-H glycoprotein was treated with 0.09M-hydroxylamine hydrochloride in 0.38M-NaOH (see the Experimental section). The sialic acid was released by acid hydrolysis (0.05M-H₂SO₄, 80°C) of rabbit T-H glycoprotein (○) and alkali-treated glycoprotein (●) or by neuraminidase (*Vibrio cholerae*) digestion of rabbit T-H glycoprotein (△) and alkali-treated rabbit T-H glycoprotein (▲).

rabbit T-H glycoprotein with neuraminidase from *Vibrio cholerae* for 2 days released 16% of the total sialic acid present as determined by the resorcinol method; incubation for 4 days with an additional 100 units of neuraminidase released a further 7% of the total sialic acid. Thus the sialic acid residues of rabbit T-H glycoprotein may be classified according

to whether they are released readily by neuraminidase (Fig. 2), released at a slow rate over several days, or are completely resistant to the action of the enzyme. Those sialic acid residues of rabbit T-H glycoprotein that were resistant to digestion with neuraminidase for 24h were also found to be released at a slow rate by acid hydrolysis, whereas the sialic acid residues

liberated within 1 h by acid hydrolysis were also substrates for both neuraminidase preparations (Table 2). Thus rabbit T-H glycoprotein appeared to contain at least two different forms of sialic acid residues, one of which was a substrate for neuraminidase and was released within 1 h by 0.05 M-H₂SO₄ at 80°C; another form was resistant to neuraminidase and also gave an anomalous acid-hydrolysis time-curve.

Acid hydrolysis of bovine submaxillary glycoprotein in 0.05 M-H₂SO₄ at 80°C released a maximum amount of sialic acid (19.8%) in 2.5 h as determined by the thiobarbituric acid method, which confirmed the findings of Gibbons (1963). Under the same hydrolysis conditions, 14.7% of sialic acid was released in 1 h and the glycoprotein was recovered after dialysis to remove free sialic acid; it was then re-hydrolysed in 0.05 M-H₂SO₄ at 80°C, which released a further 2.7% of sialic acid after 2.5 h. Neuraminidase digestion of bovine submaxillary glycoprotein, which had been hydrolysed in 0.05 M-H₂SO₄ at 80°C for 1 h, released only 0.9% of sialic acid after 24 h, indicating that some of the sialic acid released at a slow rate by acid hydrolysis was also resistant to neuraminidase. These findings indicate that the anomalous acid-hydrolysis time-curve of sialic acid in bovine submaxillary glycoprotein, as in rabbit T-H glycoprotein, can be attributed, at least in part, to a form of sialic acid that is released at a slow rate by acid hydrolysis and is also resistant to neuraminidase.

Rate of hydrolysis of the glycosidic bond of sialic acid in rabbit T-H glycoprotein

N-Acetyl-7-O-acetylneuraminic acid was unreactive in the thiobarbituric acid procedure of Aminoff (1961) in which the periodate oxidation step is carried out in 0.021 M-H₂SO₄. In contrast, the periodate-oxidation step in the procedure of Warren (1959) is carried out in 3 M-phosphoric acid and it is uncertain whether de-O-acetylation might occur under these conditions, resulting in N-acetyl-7-O-acetylneuraminic acid being chromogenic in this procedure. If it is assumed that N-acetyl-7-O-acetylneuraminic acid is not chromogenic in the thiobarbituric acid method of Warren (1959), then the following experimental findings suggest that the anomalous acid-hydrolysis time-curve given by rabbit T-H glycoprotein, under the standard conditions of acid hydrolysis, may be explained by the slow rate of hydrolysis of the glycosidic bond, rather than the release within 1 h of sialic acid substituted on C-7, which is not chromogenic in the thiobarbituric acid assay but is converted into a reactive species by further hydrolysis. When sialic acid was determined by the thiobarbituric acid and alkali-Ehrlich methods, similar acid-hydrolysis time-curves were obtained (Fig. 1), which suggested that the free acid in the

hydrolysates was unsubstituted on C-7 (Aminoff, 1961). If rabbit T-H glycoprotein was hydrolysed by acid under the standard conditions for 1 h and the diffusate was isolated, then the amount of sialic acid present was unchanged by hydrolysis for a further 3 h, under the same conditions, as determined by the thiobarbituric acid method. During 1 h of hydrolysis by 0.05 M-H₂SO₄ at 80°C, rabbit T-H glycoprotein released 2.0% of sialic acid, and when the glycoprotein from the hydrolysate was recovered and re-hydrolysed, a further 1.6% of sialic acid was released after 5 h (Table 2). Thus, 3.6% of an expected 4.2% was accounted for in this experiment, suggesting that only a small fraction of the free N-acetylneuraminic acid in the hydrolysate might be unreactive in the thiobarbituric acid reaction owing to a substituent on C-7.

These results may indicate that the sialic acid in rabbit T-H glycoprotein is not O-acetylated at C-7, although paper chromatography of the sialic acids released from rabbit T-H glycoprotein by mild acid and enzymic hydrolysis indicated the presence of N-acetyl-7-O-acetylneuraminic acid. Thus the apparent absence of N-acetyl-7-O-acetylneuraminic acid after hydrolysis of rabbit T-H glycoprotein under the standard conditions is evidence that de-O-acetylation of C-7 occurs at a rate that is similar to or faster than the rate of hydrolysis of the glycosidic bond.

Effect of Pronase digestion of rabbit T-H glycoprotein on the release of sialic acid

Pronase digestion of rabbit T-H glycoprotein cleaved 55% of the peptide bonds. The acid-hydrolysis time-curve of the Pronase digest closely resembled that of the intact glycoprotein and 3.9% of sialic acid was released in 5 h as determined by the thiobarbituric acid method. Further, neuraminidase (*Vibrio cholerae*) released only 0.7% of sialic acid in 24 h. Thus the unusual properties of sialic acid in rabbit T-H glycoprotein cannot be explained by either the conformation of the glycoprotein or its high molecular weight. This is noteworthy in view of the suggestion which has been made that the incomplete release of sialic acid by neuraminidase from certain high-molecular-weight glycoproteins, including human T-H glycoprotein, may be attributed to steric hindrance (Gottschalk, 1966).

Stability of the carbohydrate-protein linkages to alkali

Rabbit T-H glycoprotein was treated with 0.5 M-NaOH at 4°C for 2 days to determine whether alkali-labile O-glycosidic carbohydrate-protein linkages were present (Anderson *et al.*, 1964). The hexose and amino sugar contents expressed as a percentage of the dry weight of the glycoprotein (15.5 ± 1.1 [4] and 12.6 ± 0.2 [6]) were not significantly different from

those of the alkali-treated glycoprotein (16.8 ± 1.2 [4] and 12.8 ± 0.8 [6]). (The standard deviations of the mean were calculated from the number of experiments given in square brackets.) This indicates that the predominant type of carbohydrate-protein linkages present are stable under the conditions known to hydrolyse the *O*-glycosidic bonds to serine and threonine residues which occur in certain glycoproteins and therefore are probably of the *N*-glycosylamine type. Thus alkaline treatment of the glycoprotein to release *O*-acetyl residues is unlikely to result in cleavage of carbohydrate-protein linkages.

Effect of alkaline treatment of rabbit T-H glycoprotein on the subsequent release of sialic acid

After alkaline treatment to release *O*-acetyl groups (see the Experimental section), acid hydrolysis (0.05 M-H₂SO₄, 80°C) of rabbit T-H glycoprotein liberated a maximum amount of sialic acid within 1 h, and neuraminidase released a similar amount of sialic acid within 24 h (Fig. 2). Thus pretreatment of rabbit T-H glycoprotein with mild alkali normalized the release of sialic acid by acid hydrolysis and by neuraminidase. The sialic acid content of rabbit T-H glycoprotein was taken to be that given by the thio-barbituric acid method after release of the sialic acid from alkali-treated glycoprotein by neuraminidase digestion, since this method consistently yielded a slightly higher value than acid hydrolysis (Fig. 2, Table 3). Thus the two preparations of rabbit T-H glycoprotein used in these studies contained 5.7 and 5.1 % of sialic acid. Treatment of human T-H glycoprotein with alkali did not significantly affect the amount of sialic acid subsequently released by acid hydrolysis or by neuraminidase.

Total acetyl groups

Recoveries of 97 and 99 % of the theoretical contents of *N*-acetyl groups were obtained for *N*-acetylglucosamine and *N*-acetylneuraminic acid. The total

acetyl-group content obtained for human T-H glycoprotein can be accounted for by the *N*-acetyl groups present in *N*-acetylhexosamine and *N*-acetylneuraminic acid (Table 4). With rabbit T-H glycoprotein 29 acetyl residues/100 000 g were liberated by treatment with alkaline hydroxylamine and the remaining acetyl content corresponded closely to the *N*-acetylhexosamine and *N*-acetylneuraminic acid contents of the glycoprotein. These results also provided additional evidence that the neuraminic acid is present in human and rabbit T-H glycoproteins as the *N*-acetyl rather than the *N*-glycolyl derivative. The *O*-acetyl content of rabbit T-H glycoprotein (29 residues/100 000 g) may be somewhat inaccurate, since this value is the difference in two total acetyl contents and the errors involved are accumulative (see Table 4).

Content of ester groups

The ester group content of T-H glycoprotein (44.2 ± 2.6 [4] residues/100 000 g) was found to be very similar to the diffusible acyl hydroxamate content (42.1 ± 3.8 [4] residues/100 000 g), indicating that the acyl group involved in the ester linkage is of low molecular weight. No ester groups were detected in human T-H glycoprotein or in rabbit T-H glycoprotein after alkaline hydroxylamine treatment.

O-Acetyl content

Analysis of the *O*-acetyl contents of ethyl acetate and 1-*N*-acetyl-2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranosylamine gave 104 % and 107 % of the theoretical *O*-acetyl contents. The *O*-acetyl content of rabbit T-H glycoprotein (34.9 ± 2.3 [5] residues/100 000 g) compared reasonably well with the value obtained from the total acetyl content, although it was lower than the ester content of the glycoprotein. No *O*-acetyl groups were found to be present in human T-H glycoprotein or in rabbit T-H glycoprotein after mild alkaline treatment.

Table 3. *Sialic acid content of alkali-treated T-H glycoprotein*

The glycoprotein was first treated with alkali as shown in the Table and after acid hydrolysis of the glycoprotein (0.05 M-H₂SO₄, 80°C for 1 h) or neuraminidase digestion (*Vibrio cholerae*), free sialic acid was determined by the method of Warren (1959).

Alkaline treatment	T-H glycoprotein	Sialic acid content (% dry wt.)	
		Acid hydrolysis (1 h)	Neuraminidase (24 h)
0.05 M-Na ₂ CO ₃ , 100°C, 20 min (Gibbons, 1963)	Human	5.7	4.8
	Rabbit	5.6	5.8
0.2 M-NaOH, 4°C, 2 h	Rabbit	5.1	5.7
0.09 M-Hydroxylamine hydrochloride in 0.38 M-NaOH (Ludowig & Dorfman, 1960)	Rabbit	5.0	5.6

Table 4. Total acetyl contents of human and rabbit T-H glycoprotein as determined by the method of Ludowig & Dorfman (1960)

The acetyl contents are expressed in terms of the dry weight of the glycoprotein recovered at each stage. Results are expressed as mean $\pm$ s.d. of the numbers of experiments in parentheses.

Glycoproteins	Acetyl content (residues/100000 g)	Δ	Amino sugar content (residues/100000 g)	Sialic acid content (residues/100000 g)
Rabbit T-H glycoprotein	118 $\pm$ 5 (5)	29	70	18
Rabbit T-H glycoprotein pretreated with alkaline hydroxylamine	89 $\pm$ 3 (3)			
Rabbit T-H glycoprotein, neuraminidase-digested after alkaline hydroxylamine treatment	68 $\pm$ 3 (3)			
Human T-H glycoprotein	77 $\pm$ 2 (3)	23	51	18
Human T-H glycoprotein after alkaline pretreatment (0.2M-NaOH, 4°C, 2h)	78 $\pm$ 3 (2)			
Human T-H glycoprotein, neuraminidase-digested	54 $\pm$ 4 (3)

Fig. 3. Release of formaldehyde by periodate oxidation

N-Acetylneuraminic acid (○), human T-H glycoprotein (●), rabbit T-H glycoprotein (▲) and rabbit T-H glycoprotein treated with 0.09M-hydroxylamine hydrochloride in 0.38M-NaOH (△) were oxidized with 10mM-sodium periodate at 0°C, and formaldehyde was determined by the method of Nash (1953). The sialic acid contents of the glycoproteins were determined by the method of Warren (1959) after hydrolysis in 0.05M-H₂SO₄ at 80°C for 1 h. Rabbit T-H glycoprotein had been treated under alkaline conditions to release *O*-acetyl groups before acid hydrolysis. For full details see the text.

Acetyl hydroxamate resulting from treatment of rabbit T-H glycoprotein with alkaline hydroxylamine was readily identified by paper chromatography and could be co-chromatographed with authentic acetyl hydroxamate.

Release of formaldehyde from sialic acid by periodate oxidation

The formaldehyde liberated by periodate oxidation of sialoglycoproteins is derived only from C-9 of sialic acid, provided that the other sugar residues present are in the pyranose rather than the furanose ring form. Periodate oxidation of *N*-acetylneuraminic acid and human T-H glycoproteins under the conditions described released 0.90 and 0.97 mol of formaldehyde/mol of sialic acid within 10–15 min (Fig. 3). Alkaline pretreatment of rabbit T-H glycoprotein increased the formaldehyde liberated by periodate oxidation from 0.1 to 0.9 mol of formaldehyde/mol of sialic acid, evidence that in the native glycoprotein, C-8 and/or C-9 of sialic acid are *O*-acetylated. These findings provide good evidence that the formaldehyde liberated by periodate oxidation of T-H glycoprotein is indeed derived from C-9 of sialic acid.

After acid hydrolysis of human T-H glycoprotein (0.05M-H₂SO₄, 80°C) for up to 8 h, periodate oxidation released approx. 1 mol of formaldehyde/mol of sialic acid, whereas increasing amounts of formaldehyde were released by oxidation of rabbit T-H glycoprotein after increasing times of acid hydrolysis, and

Fig. 4. Liberation of formaldehyde by periodate oxidation after hydrolysis of T-H glycoprotein in 0.05M- H_2SO_4 at 80°C

Rabbit T-H glycoprotein (●) and human T-H glycoprotein (○) were hydrolysed in 0.05M- H_2SO_4 at 80°C, and samples were removed at intervals, neutralized by the addition of an equal volume of 0.1M-NaOH, then oxidized by 10mM-periodate at 0°C for 20min in the dark. The formaldehyde liberated was determined by the method of Nash (1953). The sialic acid contents of the glycoproteins were determined by the method of Warren (1959) after hydrolysis in 0.05M- H_2SO_4 at 80°C for 1h. Rabbit T-H glycoprotein had been treated under alkaline conditions to release *O*-acetyl groups before acid hydrolysis. For full details see the text.

reached a maximum (1.17mol of formaldehyde/mol of sialic acid) after 10h of acid hydrolysis (Fig. 4). The increasing amounts of formaldehyde released by periodate oxidation reflect the rate of acid hydrolysis of the *O*-acetyl residues substituted on C-8 and/or C-9 of sialic acid.

Release of *O*-acetylated sialic acid by mild acid hydrolysis

The *O*-acetylated sialic acid of submaxillary-gland glycoproteins has been isolated after its release by mild acid hydrolysis (Blix & Lindberg, 1960; Schauer, 1970). Hydrolysis of bovine submaxillary glycoprotein and human and rabbit T-H glycoproteins under similar conditions (5mM- H_2SO_4 , 70°C, 1h at pH2.3) released respectively 38, 54 and 7% of the total sialic acid of these glycoproteins as determined by the thiobarbituric acid method, or 34, 54 and 8% of the

sialic acid as determined by the resorcinol method. Since mild acid hydrolysis of rabbit T-H glycoprotein released only a small proportion of the sialic acid present, this method is unsuitable for the release of the *O*-acetylated sialic acid on a preparative scale.

Paper chromatography of sialic acids

Paper chromatography of the sialic acid released from human T-H glycoprotein by mild acid hydrolysis (5mM- H_2SO_4 , 70°C, 1h) gave a single component possessing the same mobility as standard *N*-acetylneuraminic acid. Paper chromatography of the sialic acid released from rabbit T-H glycoprotein by mild acid hydrolysis gave 3 components of R_{NeuNAc} 1.0, 2.2 and 3.7 where R_{NeuNAc} = (mobility of unknown)/(mobility of *N*-acetylneuraminic acid). Neuraminidase digestion for 24h released 16% of the total sialic acid of rabbit T-H glycoprotein, and paper chromatography of this sialic acid fraction gave two components of R_{NeuNAc} 1.0 and 2.3. These mobilities are somewhat similar to those of the *O*-acetylated sialic acids of bovine submaxillary glycoprotein (Blix *et al.*, 1956; Blix & Lindberg, 1960) and paper chromatography of a sialic acid fraction derived from bovine submaxillary glycoprotein by mild acid hydrolysis gave two main components of R_{NeuNAc} 1.0 and 2.1, the latter identified from its mobility as *N*-acetyl-7-*O*-acetylneuraminic acid. This suggests that the *N*-acetylneuraminic acid present in rabbit T-H glycoprotein is acetylated at C-7.

Discussion

Preparations of T-H glycoproteins from man and rabbits were found to contain similar amounts of *N*-acetylneuraminic acid (5.5 and 5.4% respectively). This corresponds to approx. 18 residues/100 000 g. No *O*-acetyl residues were found in human T-H glycoprotein, whereas rabbit T-H glycoprotein contains approx. 35 *O*-acetyl residues/100 000 g, indicating that on average two hydroxyl groups of sialic acid are *O*-acetylated. The methods of acetyl-group analysis employed in these studies, which involved either methanolysis or transesterification to form the volatile methyl ester, are not specific for acetyl groups, but the acetyl hydroxamate formed by treatment of rabbit T-H glycoprotein with alkaline hydroxylamine was identified chromatographically. Although the *O*-acetylated sialic acids have not been fully characterized, the effect of mild alkaline treatment of T-H glycoprotein on the subsequent release of sialic acid by acid or enzymic hydrolysis is good evidence that the sialic acid residues present are indeed *O*-acetylated.

Methods involving periodate oxidation of rabbit

T-H glycoprotein have indicated that the polyhydroxy side-chains of the sialic acid residues are *O*-acetylated. These experimental findings could be explained by *O*-acetylation of C-8 only; but the glycoprotein contained an average two *O*-acetyl residues/mol of sialic acid, and *O*-acetylation of C-7 and C-8, C-7 and C-9 or C-8 and C-9 could explain the findings. Since the hydroxyl group of C-4 may also be acetylated, the experimental results are also compatible with *O*-acetylation of C-4 and C-8. Paper chromatography of the sialic acids released by acid and enzymic hydrolysis gave a component tentatively identified as *N*-acetyl-7-*O*-acetylneuraminic acid, providing evidence that C-7 may be one of the positions of *O*-acetylation. To explain the low yield of formaldehyde obtained by periodate oxidation of the glycoprotein, the sialic acid residues must also be *O*-acetylated at either C-8 or C-9. An *N*-acetylneuraminic acid *O*-acetyltransferase specific for both C-7 and C-8 has been demonstrated in the submaxillary gland of the ox (Schauer, 1970) and the occurrence of *N*-acetyl-9-*O*-acetylneuraminic acid has not so far been reported. Therefore it is suggested that C-7 and C-8 of sialic acid may be *O*-acetylated in rabbit T-H glycoprotein.

Methods involving periodate oxidation also indicated that the rate of de-*O*-acetylation under the standard conditions of acid hydrolysis was similar to the rate of hydrolysis of sialic acid, and that de-*O*-acetylation was essentially complete after approx. 6 h of hydrolysis.

Bacterial neuraminidases are specific for sialic acid with an α -ketosidic linkage (Yu & Ledeen, 1969) and a free carboxyl group at C-1 (Karkas & Chargaff, 1964). There is some evidence that sialic acid residues with a modified or substituted polyhydroxy side chain are poor substrates, whereas those substituted on the ring are no longer substrates for these enzymes. Thus, sialic acid which is *O*-acetylated on C-7 or C-8 is released at a slow rate by neuraminidase (Schauer & Faillard, 1968) and the seven carbon chain C-7 analogue of *N*-acetylneuraminic acid (5-acetamido-3,5-dideoxy-D-galacto-heptulosonic acid) is cleaved at 5–10% the rate of *N*-acetylneuraminic acid (Suttajit & Winzler, 1971). The presence of a bulky *N*-substituent confers resistance towards neuraminidase (Hakomori & Saito, 1969) as does *O*-acetylation

at C-4 (Pepper, 1968; Schauer & Faillard, 1968). The effects of *O*-acetylation of sialic acid at two or more positions are not known, but it may be assumed these are additive. The demonstration that all the acyl hydroxamates derived from rabbit T-H glycoprotein were diffusible on dialysis excludes the possibility that the carboxyl group (C-1) of sialic acid is involved in an ester or lactone linkage, such as occurs in colominic acid (McGuire & Binkley, 1964). Further, proteolytic digestion of the glycoprotein had no effect on the subsequent release of sialic acid by acid or enzymic hydrolysis, so that steric hindrance of the enzyme by the peptide moiety of the protein is unlikely. The resistance of the sialic acid residues to enzymic hydrolysis was abolished by prior treatment of the glycoprotein with mild alkali and this is good evidence that *O*-acetylation of sialic acid, possibly at C-7 and C-8, confers resistance to bacterial neuraminidases.

The longer period of acid hydrolysis required to release a maximum amount of sialic acid from T-H glycoprotein compared with bovine submaxillary glycoprotein (5 h compared with 2.5 h), and the resistance to neuraminidase of a greater proportion of the sialic acid of T-H glycoprotein compared with that of bovine submaxillary glycoprotein (at least 80% compared with 50%) may reflect a difference in the position or degree of *O*-acetylation of the sialic acid residues of the two glycoproteins. Indeed, the *O*-acetyl content of rabbit T-H glycoprotein (approx. 2 residues/mol of sialic acid) is considerably higher than that of bovine submaxillary glycoprotein, 0.82 (ester) residue/mol of sialic acid (Bertolini & Pigman, 1967).

The *N*-acetylneuraminic acid residues present in glycoproteins are readily hydrolysed in 1 h by 0.05 M-H₂SO₄ at 80°C, for which several explanations have been suggested. First, *N*-acetylneuraminic acid is a 2-deoxy sugar and it is known that the glycosides of 2-deoxy sugars are more readily hydrolysed than the corresponding glycoside of glucose (Overend *et al.*, 1962). The ease of hydrolysis by acid may also be explained by the ketosidic nature of the linkage, since fructopyranosides are hydrolysed more than 1000 times faster than most aldopyranosides (Heidt & Purves, 1944). The generally accepted mechanism for the acid hydrolysis of sialic acid shown in Scheme 1 involves protonation of the ketosidic oxygen atom

Scheme 1. Possible mechanism for the acid hydrolysis of sialic acid residues (I $\rightarrow$ III)

For explanation see the Discussion section.

followed by a rate-determining decomposition to form an alcohol and a resonance-stabilized carbonium-oxonium ion, which probably exists in the half-chair conformation with C-3, C-2, O and C-6 in a plane. The carbonium ion then reacts with water forming the free sugar. The carboxyl group (C-1) of sialic acid (I) has a pK of about 2.6 and protonation of the glycosidic oxygen may be facilitated by the proximity of the ionized carboxyl group (II), which may also stabilize the carbonium ion of the postulated intermediate in the hydrolysis (III) (Neuberger & Marshall, 1966). Thus hydrolysis of the ketosidic linkage in sialic acid may involve specific catalysis by the ionized carboxyl group (C-1).

The question arises why *O*-acetylation of the side chain of sialic acid decreases the rate of acid hydrolysis of the glycosidic bond. A possible explanation may be that hydrogen-bond formation occurs between the hydrogen atom of the un-ionized carboxyl group and the oxygen atom of the carbonyl group of an acetyl substituent in position C-7, C-8 or C-9. A Dreiding molecular model indicates that such a hydrogen bond could be most readily formed if the acetate was at position C-8. The hydrogen bond would have the effect of increasing the pK of the carboxyl group, greatly favouring the un-ionized form and thus decreasing the rate of hydrolysis by the mechanism suggested here. This hypothesis would be capable of experimental verification.

Another possible explanation is that electronic and steric effects arising from *O*-acetylation of the ring or side chain may decrease either the rate of protonation of the glycosidic oxygen atom, or the stability of the carbonium-ion intermediate. Steric effects arising from *O*-acetylation of C-7 may decrease the rate of hydrolysis by hindering the conformational change from chair to half-chair form which requires rotation about the C-3-C-4 and C-5-C-6 bonds of sialic acid (Edward, 1955). Also, the rates of hydrolysis of pyranosides are known to be related, at least in part, to the inductive effect of the substituent at C-5 of the pyranose ring (Dyer *et al.*, 1962; Timell *et al.*, 1965). Since an acetoxy substituent produces a greater inductive effect than an hydroxyl group (McDaniel & Brown, 1958), the slower rate of hydrolysis might be explained by the increased inductive effect arising from *O*-acetylation of C-7, resulting in the decreased stability of the carbonium ion intermediate. Acetoxy substituents at C-8 and C-9 of sialic acid may be too far from the carbonium ion for this effect to operate.

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