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Stable Thiobarbituric Acid Chromophore with Dimethyl Sulphoxide

APPLICATION TO SIALIC ACID ASSAY IN ANALYTICAL DE-*O*-ACETYLATION

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With dimethyl sulphoxide instead of butanol in the thiobarbituric acid assay for sialic acid, a non-fading chromophore with $\lambda_{max.} = 549$nm was produced in a homogeneous solution, allowing dilution of the test mixture in case of high colour yield. This test adapted well to studies on alkaline de-*O*-acetylation. Bovine and rat submaxillary mucins, and rabbit Tamm–Horsfall urinary sialoproteins contain *O*-acetyl isomers of neuraminic acid that are resistant to the thiobarbituric acid assay. Alkaline de-*O*-acetylation converted resistant *O*-acetylneuraminic acid into thiobarbituric acid-reactive sialic acid, and such conversion paralleled de-*O*-acetylation as measured by the ferric hydroxamate method. The colour increment was similar when the alkaline treatment of bovine submaxillary mucin either preceded or followed the acid hydrolysis. Only alkaline pretreatment was effective with rat submaxillary mucin. By selecting optimal conditions for alkaline de-*O*-acetylation, *O*-acetyl isomers can be accurately assessed by the thiobarbituric acid assay.

Among the colorimetric methods in use for the quantification of sialic acid (*N*-acetylneuraminic acid) the thiobarbituric acid test gives the highest molar extinction coefficient and thus is the most sensitive [direct Ehrlich test, $\varepsilon = 2.2 \times 10^3$ (Werner & Odin, 1952; Pigman *et al.*, 1958); resorcinol, $\varepsilon = 8.9 \times 10^3$ (Svennerholm, 1957); periodate/thiobarbituric acid, $\varepsilon = 68.0 \times 10^3$ litre·mol^{-1}·cm^{-1} (Warren, 1959; Aminoff, 1961; Schauer, 1973)]. However, the fading character of the chromophore and the necessity to extract it with acidified butan-1-ol are inconvenient. In addition the thiobarbituric acid assay lacks the uniformity of colour development with the different sialic acid analogues (*N*-acetyl-, *N*-glycollyl-neuraminic acid and their *O*-acetyl-substituted forms). We investigated the effect of the water-miscible organic solvents ethylene glycol, dimethyl sulphoxide, dimethylformamide and methyl-Cellosolve on the thiobarbituric acid chromophore.

Although both the Warren (1959) and Aminoff (1961) procedures are able to measure 2–20μg of sialic acid with an accuracy of ± 1%, they react only with free sialic acid, which must therefore be released by acid hydrolysis or neuraminidase digestion. To extend the usefulness of the test, several scaled-down versions of the standard Warren (1959) assay were developed (Bretscher, 1971; Hahn *et al.*, 1974), which used 0.05–0.025 times the sample and reagent volumes. These techniques demand the same working concentration of sialic acid in the sample as in the original procedure and are not satisfactory for low concentrations. Although the fluorimetric assay of Hess & Rolde (1964) is capable of detecting submicrogram concentrations of sialic acid, it requires acid hydrolysis for 24h at 100°C for maximum efficiency. With our modified method we have reinvestigated the sensitivity of the assay and also report on the alkaline conditions necessary to convert thiobarbituric acid-resistant *O*-acetylated sialic acid variants into reactive neuraminates.

Materials and Methods

Materials

N-Acetylneuraminic acid was from General Biochemical Co., Chagrin Falls, OH, U.S.A. [lots 58673 (99% pure) and 47173 (95% pure)]. Glomerular basement membrane was prepared from calf kidneys, as described previously (Mohos & Skoza, 1969; Skoza & Mohos, 1974). Bovine and rat submaxillary mucins were gifts from Dr. A. Herp (Department of Biochemistry, New York Medical College, NY, U.S.A.). Their sialic acid content was 25% by the resorcinol method (Svennerholm, 1957). Bovine submaxillary mucin contained 0.85mol of *O*-acetyl groups/mol of sialic acid, and in rat submaxillary mucin, 1.75mol of *O*-acetyl groups/mol was present. All other reagents were of analytical grade.

Acid hydrolysis for the liberation of sialic acid

Samples of glycoprotein (0.2–1.0ml, with a sialic acid content of 0.3–20μg) were hydrolysed in 0.025

or 0.05M-H_2SO_4, the pH being rigorously maintained between 1.6 and 2.0. Hydrolysis was performed at 80°C for 60min.

Thiobarbituric acid assay for sialic acid

Our periodate/thiobarbituric acid assay is a modification of the Aminoff (1961) assay, and covers a range of 0.3–20μg of sialic acid. The final volume varies according to the sialic acid content of the test material.

If sialic acid content of the test material is low, as in glomerular sialoprotein or in γ-globulin (less than 2%), the acid-hydrolysed material, usually 1mg/ml, containing up to 3μg of sialic acid in sample volumes of 0.2ml, can be measured by the micro-method. The sample (0.2ml) is oxidized by addition of 0.05ml of 25μM-periodic acid/62.5mM-H_2SO_4 at 37°C for 30min. Oxidation is terminated by the addition of 0.05ml of 2% (w/v) sodium arsenite/0.5M-HCl. This is followed by the addition of 0.1ml of 6% (w/v) thiobarbituric acid, adjusted to pH9.0 with NaOH, to give a final concentration of at least 1%. The chromophore is developed by heating the reaction mixture in a boiling-water bath for 7.5min. The colour is intensified by the addition of an equal volume of dimethyl sulphoxide, bringing the total volume to 0.8ml.

For sialoproteins with high sialic acid content such as the sialomucins (20–25% sialic acid), the same method may be used, with the refinement that the volume used in spectrophotometry can be increased by the addition of more dimethyl sulphoxide (4ml or more; see Fig. 2, inset), to obtain a final range of 1–2μg of sialic acid/ml. In manipulations requiring several pH adjustments, as in alkaline de-*O*-acetylation, it is most convenient to use the macro-method. In this, 1.0ml batches of the processed material, 0.25ml of periodate, 0.25ml of arsenite, 0.5ml of thiobarbituric acid reagent and 2.5ml of dimethyl sulphoxide are used.

The E_{549} and E_{532} were measured. Other steps in the procedure, including preparation of the reagents, followed the original procedure of Aminoff (1961). The molar extinction coefficient of the chromophore, by using *N*-acetylneuraminic acid with the stated 95% purity, was 6.8×10^4 litre·mol^{-1}·cm^{-1}. By the same method, the molar extinction coefficient for deoxyribose at 532nm was 1.38×10^5 litre·mol^{-1}·cm^{-1}, as shown in the calibration graphs of Fig. 1.

Alkaline treatment for analytical de-O-acetylation

A sample of glycoprotein (1mg/ml) in water is mixed with an equal volume of 0.2M-NaOH and kept for 20min at room temperature (21°C). To terminate the reaction, the pH is adjusted to 1.6–2.0 with 0.1M-H_2SO_4. If 0.1M-NaOH is used (0.05M final concentration), the incubation period is extended to 60min (see Fig. 3). After alkaline treatment, the

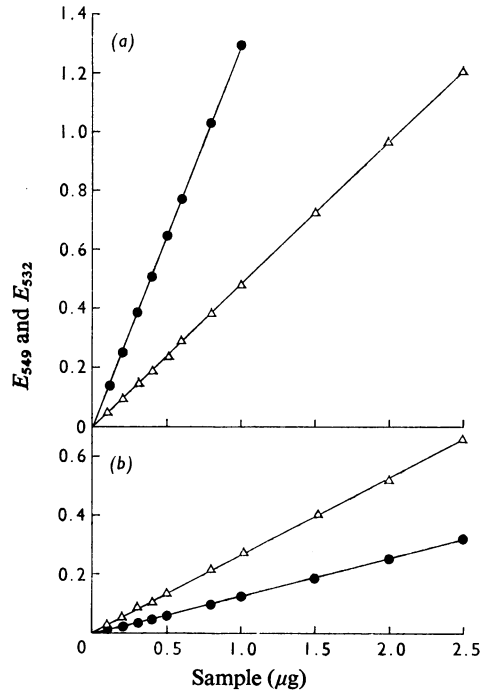

Fig. 1. *Calibration plot of deoxyribose (a) and of N-acetylneuraminic acid (b) by our modified thiobarbituric acid micro-assay*

For this 0.2ml of sample (0–2.5μg of test material), 0.05ml of periodic acid, 0.05ml of sodium arsenite, 0.1ml of thiobarbituric acid reagent and 0.4ml of dimethyl sulphoxide were used. Each test mixture was read at the absorption maximum for sialic acid (549nm) (△) and for deoxyribose (532nm) (●). The extinction coefficients are as follows: deoxyribose, $\varepsilon_{549}=5.2\times10^4$, $\varepsilon_{532}=1.38\times10^5$; *N*-acetylneuraminic acid, $\varepsilon_{549}=6.8\times10^4$, $\varepsilon_{532}=3.1\times10^4$ litre·mol^{-1}·cm^{-1}.

sialic acid of the sample is liberated by acid hydrolysis at 80°C for 1h. Samples of the thus processed material, containing approx. 1.75 or 8.8μg of sialic acid, are used in our micro- or macro-modification of the thiobarbituric acid assay. Alkaline treatment after the acid hydrolysis is similar, but requires initial neutralization of the acid and adjustment to acidic pH for the assay.

Test for acetyl ester groupings

For the determination of *O*-acetyl content, Hestrin's (1949) modification of the ferric hydroxamate procedure of Lipmann & Tuttle (1945) was applied as scaled down by a factor of five by Messer (1974). Glucose penta-acetate served as standard.

For all the spectrophotometric measurements, a

Zeiss PMQ II spectrophotometer was used, with 1-cm-path-length cuvettes.

Results and Discussion

The Aminoff (1961) procedure suffers from serious shortcomings: the necessity for centrifugation to separate the butanol extract of the red chromophore and any precipitate that forms during cooling of the test mixture, the rapid fading of the chromophore and the lack of uniformity of colour development of variously acylated neuraminic acids. These shortcomings have been largely overcome.

Application of water-miscible organic solvents

To circumvent the precipitation and the necessity for partitioning, and to stabilize the chromophore, attempts were made to use the following water-miscible organic solvents: ethylene glycol, dimethyl sulphoxide, dimethylformamide and methyl-Cellosolve. Fig. 2 indicates that the above solvents can substitute successfully for acidified butanol, since nearly identical absorption spectra are obtained. Methyl-Cellosolve has been applied for the extraction of the chromophore by Saifer & Gerstenfeld (1962); however, we found it inferior to the other listed solvents, because it yields some precipitate, and thus centrifugation is still necessary.

In most of the subsequent tests, we used dimethyl sulphoxide, as outlined in the Materials and Methods section. This reagent is an excellent solvent of most glycoproteins and also of the reagents used in the

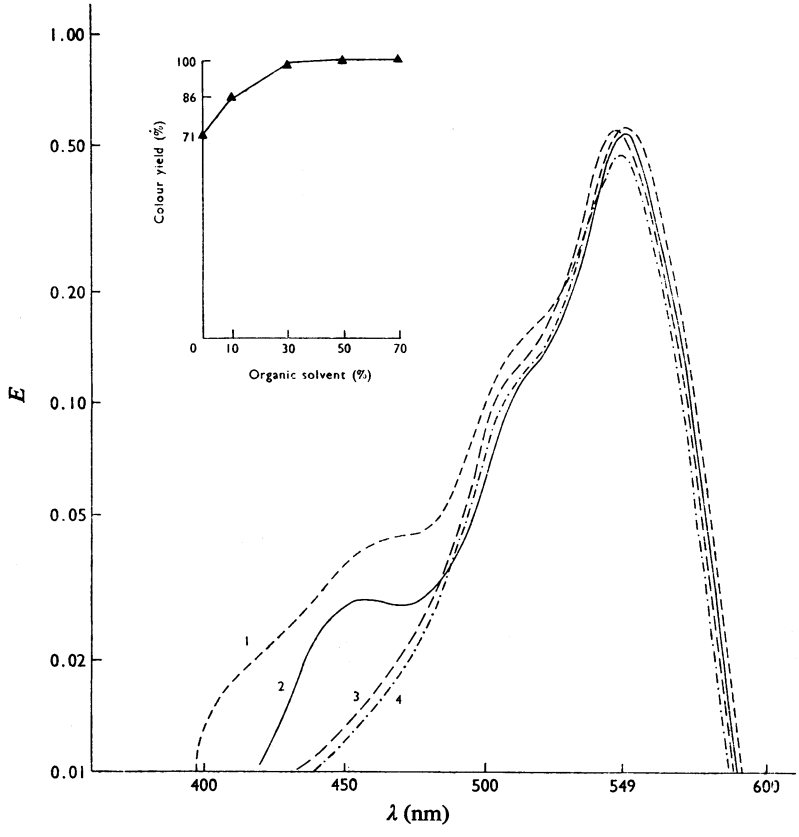

Fig. 2. *Effect of organic solvents on the spectrum of thiobarbituric acid chromophore of* 12.5 *μg of N-acetylneuraminic acid*

1, Ethylene glycol; 2, dimethyl sulphoxide; 3, dimethylformamide (1–3 all water-miscible solvents); 4, acidified butanol ('water-immiscible' solvent). Solvent volume exceeded that of the sample mixture by a factor of 10, because at this proportion butanol becomes miscible with water and does not give phase separation, allowing direct comparison of spectra. *N*-Acetylneuraminic acid (100 *μg*) in 2 ml of water reacted with 0.5 ml of periodate, 0.5 ml of arsenite and 1.0 ml of thiobarbituric acid reagents; 0.5 ml of this test mixture was added to 5 ml of the respective organic solvents. The inset shows yield of chromophore as a function of the percentage of solvent/total test volume. Molar extinction coefficient (6.8×10^4 litre·mol^{-1}·cm^{-1}) is reached at 40% organic solvent.

thiobarbituric acid assay. Because there is no phase separation or precipitation, no centrifugation is needed. Since the chromophore is distributed in the total reaction volume, the concentration of dye is only 50% of that obtained with an equal volume of acidified butanol. The inset in Fig. 2 indicates that for optimal colour yield with dimethyl sulphoxide at least 50% of the total volume must consist of the organic solvent. However, a great advantage is that the colour is stable for days. This eliminates the uncertainties associated with the fading character of the chromophore of acidified butanol extraction and allows dilution with additional dimethyl sulphoxide and accurate measurement if a particular sample gives too high absorbance. By using the micro-technique in the range of 0.3–2.5 μg of sialic acid, the mean molar extinction coefficient was 68000 litre·mol^{-1}·cm^{-1} and the coefficient of variation 4.1%.

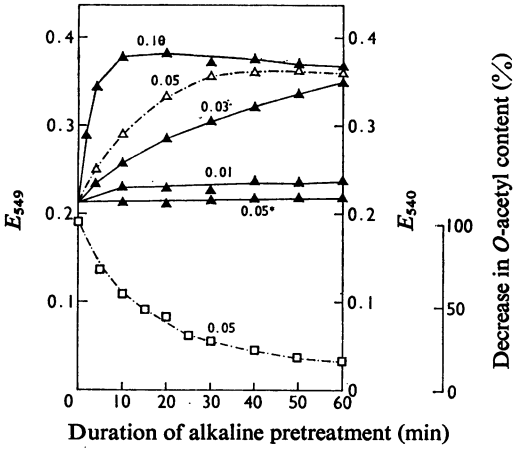

Fig. 3. *Progress curve of de-O-acetylation under different alkaline conditions*

Bovine submaxillary mucin was used with approx. 25% sialic acid content. To 5mg of bovine submaxillary mucin in 5.0ml of water 0.25ml of 1M-NaOH was added (final concn. approx. 0.05M), incubated at room temperature, and at the specified time-intervals 0.4ml (400 μg of bovine submaxillary mucin) batches were removed and assayed for *O*-acetyl content by the ferric hydroxamate reaction (E_{540}, □), or to terminate the alkaline treatment and to prepare for acid hydrolysis (E_{549}, △) 0.035ml (35 μg of bovine submaxillary mucin) portions of the incubation mixture, containing 8.8 μg of sialic acid, were adjusted to pH1.6 by the addition of 1.0ml of 0.05M-H_2SO_4. The test mixtures also contained 0.25ml of periodic acid, 0.25ml of arsenite, 0.5ml of thiobarbituric acid reagents and 2.5ml of dimethyl sulphoxide. De-*O*-acetylation in the other alkaline milieus was performed in a similar manner (▲). Note the maximum colour increment was with 0.1M-NaOH in 20 min. The numbers beside the curves are the concentrations of NaOH (M) used (except for *, which was Na_2CO_3).

Analytical aspects of alkaline de-O-acetylation by using our dimethyl sulphoxide/thiobarbituric acid method

Another deficiency of the routine thiobarbituric acid spectrophotometric assay is its lack of uniformity of colour yield with the different sialic acids (*N*-acetyl-, *N*-glycollyl-neuraminic acid and their *O*-acetyl-substituted forms) (Karkas & Chargaff, 1964; Pepper, 1968; Schauer, 1973). The *O*-acetyl groups of *N*-acetyl-*O*-acetylneuraminic acids are cleaved under very mild alkaline conditions (Neuberger & Ratcliffe, 1972), converting these sialic acids into their thiobarbituric acid-sensitive *N*-acetyl forms.

To establish the optimal analytical conditions for alkaline de-*O*-acetylation, we studied the kinetics of the reaction on bovine submaxillary mucin with a known *O*-acetylneuraminic acid content (approx. 60%), the rest being *N*-acetyl- and *N*-glycollyl-neuraminic acids (Schauer, 1973; Buscher *et al.*, 1974). Fig. 3 illustrates the progress of alkaline de-*O*-acetylation at various concentrations of NaOH, followed by acid hydrolysis and thiobarbituric acid reaction. As Fig. 3 indicates, without alkaline treatment, only 56% of the sialic acid was reactive in the thiobarbituric acid assay, whereas at completion of the alkaline reaction (20min for 0.1M-NaOH or 50min for 0.05M-NaOH), the de-*O*-acetylated neuraminic acid reacted in the thiobarbituric acid assay with an efficiency equal to that of the *N*-acetyl form, resulting in 100% recovery. Total recovery was confirmed by the resorcinol assay, a test capable of detecting both the *N*-acetyl and the *O*-acetyl forms of both free and bound sialic acids. The gradual disappearance of the *O*-acetyl content of bovine submaxillary mucin on alkaline saponification was followed by the hydroxamic acid method of Hestrin (1949) for acetyl ester groups. The decrease of acetyl esters was proportional to the thiobarbituric acid-colour increment. Both reactions followed first-order kinetics. With 0.05M-NaOH, both reactions reached one-half completion in 12.5min.

By using 0.05M-H_2SO_4 the time-curve of acid hydrolysis of glycoproteins with *N*-acetylneuraminic acid alone indicates that the amount of *N*-acetylneuraminic acid liberated from the glycoprotein is maximal within 30min and declines thereafter (Gibbons, 1963). Acid hydrolysis of bovine submaxillary mucin under the same conditions gave a thiobarbituric acid maximum only after 2h, at a time when a substantial portion of free *N*-acetylneuraminic acid, used as control, was already destroyed, as indicated by the *N*-acetylneuraminic acid-decay curve in Fig. 4. Alkaline treatment with 0.1M-NaOH before the acid hydrolysis of bovine submaxillary

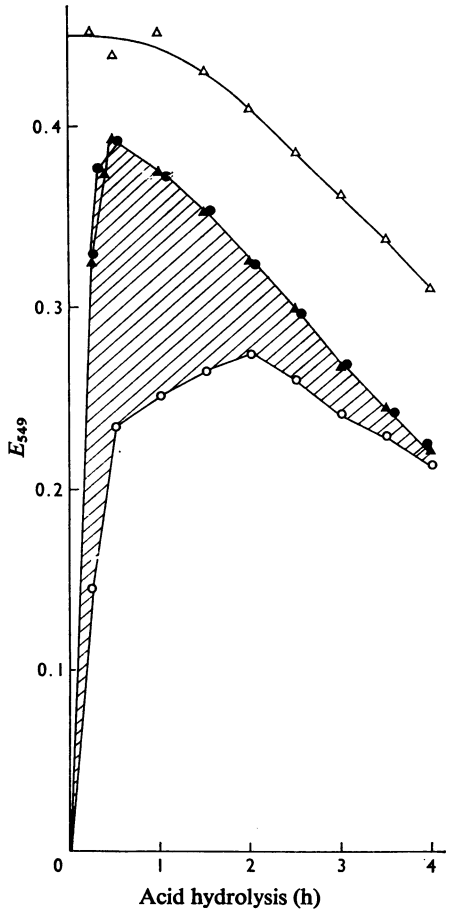

Fig. 4. *Release of sialic acid by acid hydrolysis* (0.05M-H_2SO_4) *of bovine submaxillary mucin and the effect of preceding or subsequent alkaline treatment*

Bovine submaxillary mucin (35μg; approx. 8.8μg of sialic acid) was used per test sample of 1.0ml. Total reaction volume with dimethyl sulphoxide was 4.5ml. Acid hydrolysis alone (○): note rapid increment of thiobarbituric acid-reactive sialic acid within 30min, followed by a slow rise to maximum at 2h. Alkaline treatment preceding (▲) or following (●) acid hydrolysis maximized colour yield within 30min. The subsequent decline of these curves reflects the normal decay of *N*-acetylneuraminic acid under acid conditions (△). The shaded area is the increment on account of the de-*O*-acetylated sialic acids. To detect decay, 100μg of *N*-acetylneuraminic acid was hydrolysed in 10.0ml of 0.05M-H_2SO_4, and the content determined on 1.0ml samples by the macro-assay at specified intervals (△). For acid hydrolysis of bovine submaxillary mucin 350μg was used in 10.0ml of 0.05M-H_2SO_4, and 1.0ml samples were tested by the macro-method. For alkaline treatment before acid hydrolysis, 350μg of bovine submaxillary mucin in 2ml of water was combined with 2ml of 0.2M-NaOH (pH13–14) and incubated at room temperature for 20min. This was followed by pH adjustment to 1.6 by 0.1M-H_2SO_4, volume adjustment to 10.0ml by water, and hydrolysis at 80°C. Samples (1.0ml) were taken at specified intervals for the macro-assay. For alkaline treatment after acid hydrolysis, 350μg of bovine submaxillary mucin in 2ml of water was combined with 2.0ml of 0.05M-H_2SO_4. During acid hydrolysis at 80°C, 0.4ml samples were removed, pH was adjusted to 13–14 with 0.2M-NaOH, the mixture incubated for 20min, and pH re-adjusted to 1.6 for the assay. Samples were made up to 1.0ml with water for macro-assay.

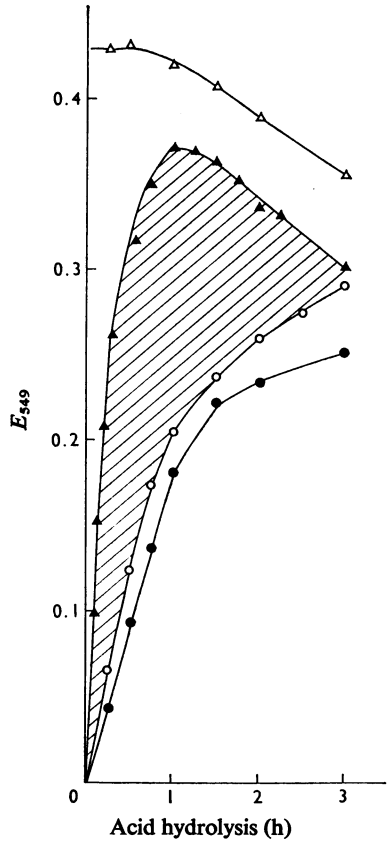

Fig. 5. *Progress curve of acid hydrolysis* (0.025M-H_2SO_4) *of rat submaxillary mucin and the effect of preceding and subsequent alkaline treatment*

The processed sample (0.3ml) contained approx. 1.75μg of sialic acid. Abnormal acid hydrolysis of rat submaxillary mucin (○), with delayed maximum at 3h. Preceding alkaline treatment normalized acid hydrolysis, reaching maximum at 1h (▲), yet subsequent alkaline treatment failed to do so (●). The increase in yield represents the *O*-acetyl component (shaded area). Alkaline treatment and acid hydrolysis were performed as with bovine submaxillary mucin, but on a micro-scale. Total reaction volume with dimethyl sulphoxide was 1ml.

mucin allows all the sialic acid to react and peak efficiency to be achieved in 30min, when destruction of free *N*-acetylneuraminic acid is still negligible. Nearly complete recovery of all sialic acids was confirmed by the resorcinol method. Alkaline treatment after acid hydrolysis also yielded the same alkaline increment, suggesting that the acid-hydrolysed fraction already contained the non-chromogenic *O*-acetylneuraminic acid in a free form that became fully chromogenic (de-*O*-acetylated) on the subsequent alkaline treatment. Alkaline treatment preceding acid hydrolysis de-*O*-acetylates *in situ* and provides more *N*-acetylneuraminic acid for acid hydrolysis and direct thiobarbituric acid reaction. Thus the 'alkali' curves in Fig. 4 are a composite of release of *N*-acetylneuraminic acid, release of the *O*-acetylated (alkaline post-treatment) or de-*O*-acetylated (alkaline pre-treatment) neuraminic acid and of decay. Results with rat submaxillary mucin under identical conditions indicated that liberation of *O*-acetylneuraminic acid by acid hydrolysis is not universal. Rat submaxillary mucin also yielded an abnormal hydrolysis curve, which could be corrected by preceding alkaline treatment, probably removing the *O*-acetyl group while the sialic acid is still glycoprotein bound. However, alkaline treatment after acid hydrolysis did not give the increment (Fig. 5). This aberrant behaviour suggests that the *O*-acetyl derivative of neuraminic acid in rat salivary mucin is not liberated by acid hydrolysis.

In contrast with our short and mild alkaline treatment, de-*O*-acetylation for extended time or at elevated temperature (L. Skoza, & S. Mohos, unpublished work) may lead to a false thiobarbituric acid-positive reaction, unrelated to sialic acid. Gibbons (1963) used 0.05M-Na_2CO_3 at 100°C for 20min. Under these harsher conditions alkaline treatment produces isosaccharinic acid from 1–4-linked aldoses (such as lactose or maltose) if present. These, like sialic acid, yield formylpyruvic acid on periodate oxidation and give the same chromophore ($\lambda_{max.}$ = 549nm) with thiobarbituric acid (Barker *et al.*, 1967). However, under the mild alkaline conditions used in our tests, these sugars did not give the red chromophore and could not lead to false results.

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