Studies on the Stability of Simple Derivatives of Sialic Acid*

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(Received for publication, May 8, 1963)

The sialic acids, whose chemistry and metabolism have been reviewed repeatedly in the recent past (1-5), are important constituents of many biological heteropolymers. It is quite clear that the multiple types of linkage in which a nonulosaminic acid can engage may influence the outcome of reactions designed to demonstrate its presence, estimate its quantity, or determine its modes of attachment, in different polymers. Even the pretreatment of the polymer, in the course of its purification, may not be without effect on the attachment of sialic acid to one or the other of the constituents through its various functional groups. This problem of stability was brought to our attention in the course of studies in this laboratory on the mucopolysaccharides of brain (6-13).

The literature is not abundant in information on this point. It is generally recognized that the free sialic acid is unstable towards acid and especially towards alkali, and that it is released readily, when linked as a glycoside, by very mild acid treatment and even by autohydrolysis. A qualitative study of the stability of sialic acids and their methyl esters at different pH values has been published (14), and occasional mention of various observations on this subject will be found in many papers dealing with other aspects of the chemistry of sialic acid. A detailed investigation, specifying the exact conditions under which the ester or the glycosidic link of sialic acid is ruptured, however, has not come to our attention. The present study describes experiments that may serve as a first step in this direction. Some of the findings have been mentioned very briefly in a preliminary form (15).

The stability of sialic acid and of three of its simplest functional derivatives was investigated under a variety of conditions, mainly by means of colorimetric techniques. The three derivatives represent models of the three principal ways in which sialic acid (I) may be linked to another molecule: (a) through an ester bond (sialic acid methyl ester, II); (b) through a glycosidic bond (methosyialic acid, III); and (c) through a combination of these links (methosyialic acid methyl ester, IV). The structures of the four compounds under investigation, in their currently accepted configurations (16), are shown in Fig. 1.

The majority of the experiments were carried out with a preparation of sialic acid isolated from ox serum proteins and composed of 37% N-acetyl- and 63% N-glycoly neuraminic acid. In addition, a few orienting studies were performed with a specimen of N-acetylneuraminic acid secured from human serum proteins. Experiments with sialic acid from sheep serum are omitted for the sake of brevity, as the results only duplicated those presented here.

EXPERIMENTAL PROCEDURE

Analytical Methods

The direct Ehrlich reaction was carried out as described in the literature (17), but the volumes were reduced so that a final reaction mixture of 3 ml resulted. The procedure for the thiobarbiturate reaction has been described before (18), as has been that for the resorcinol reaction (19); in the latter instance, heating at 100°C was applied. For the hydroxamic acid reaction, an arrangement developed for sugar esters (20) was followed; the reaction time was reduced to 30 minutes, as preliminary experiments showed that with the methyl esters of sialic acid and methosyialic acid the maximal absorptions were attained within a few minutes.

The oxidation experiments with periodate were performed in unbuffered aqueous 0.01 m solutions of NaIO₄ at room temperature in the dark. In samples removed after various intervals, the remaining periodate was determined, after dilution with an equal volume of phosphate buffer (12 g of Na₂HPO₄-12H₂O and 20 ml of H₂SO₄ in 100 ml), pH 6.5, by the addition of 1 drop of a concentrated KI solution and titration of the liberated iodine with a 0.01 m solution of sodium thiosulfate in the presence of starch.

Gilmont microburettes served for the removal of all samples and for the titrations. The melting points, reported without correction, were determined on an electrically heated stage (Fisher-Johns).

Materials

Sialic Acids (I)—Preparations were isolated from serum proteins of man, sheep, and ox by hydrolysis and ion exchange chromatography on Dowex 50 and Dowex 1-X8, essentially as described in the literature (21). The crude substances were recrystallized three times from water-methanol-ether. The melting point (with decomposition) of all three preparations was 184-185°C (uncorrected). The ratios of N-acetyl- to N-glycoly neuraminic acid, as found by microestimation of glycolic acid (22), were: bovine, 37:63; ovine, 87:13; in human sialic
acid, the reading for glycolic acid was essentially negative (less than 2%). These findings are in remarkable agreement with the literature (compare p. 33 of a recent monograph (3)).

The sialic acid from ox serum served for the preparation of the ester and methoxy derivatives investigated in this study.

N-Acetylneuraminic acid:

\[ \text{C}_9\text{H}_18\text{NO}_5 \] (309.3)

Calculated: C 42.9, H 6.62, N 4.43

N-Glycolyneuraminic acid:

\[ \text{C}_9\text{H}_15\text{NO}_5 \] (325.3)

Calculated: C 40.5, H 5.87, N 4.31

Calculated for ox serum sialic acid: C 41.4, H 6.00, N 4.30

Found (bovine): C 40.80, H 6.00, N (Dumas) 4.69

Found (human): C 42.62, H 5.99, N (Dumas) 4.28

Sialic Acid Methyl Ester (II)—This derivative was prepared by heating, under reflux for 30 minutes, sialic acid in dry methanol in the presence of a small amount of Dowex 50 which had previously been twice refluxed with dry methanol for 1 hour. The filtrate from the resin then was passed through a column of Dowex 50 (H+ form) and evaporated in a vacuum. Attempts at crystallization having failed, a semicrystalline powder was obtained by precipitation with ether from aqueous methanol. This product was then recrystallized twice from methanol-ether, melted with decomposition at 169-171° (uncorrected). The N-acetyl to N-glycolyl ratio was 37:63.

Methoxysialic Acid Methyl Ester (IV)—This compound was prepared according to Blix et al. (23) by heating sialic acid, under a reflux, with three changes of dry methanol in the presence of Dowex 50 for a total of 15 hours. The product, recrystallized twice from methanol-ether-petroleum ether, melted at 195° (uncorrected). The N-acetyl to N-glycolyl ratio was 36:64.

Methoxy-N-acetylneuraminic acid methyl ester:

\[ \text{C}_{13}\text{H}_{26}\text{NO}_6 \] (337.3)

Calculated: C 46.3, H 6.87, N 4.15

Methoxy-N-glycolyneuraminic acid methyl ester:

\[ \text{C}_{13}\text{H}_{24}\text{NO}_6 \] (353.3)

Calculated: C 44.2, H 6.56, N 3.97

Found: C 45.0, H 6.75, N (Dumas) 4.11

Methoxysialic Acid (III)—This derivative was prepared from methoxysialic acid methyl ester by mild alkaline hydrolysis. An aqueous solution of the ester (IV) was maintained at pH 10 and room temperature for 4 hours by means of an automatic titrator (Radiometer, Copenhagen), when 1 molar equivalent of NaOH had been consumed and the hydroxamic acid reaction for ester had become negligible. The solution was passed through a column of Dowex 50 (H+ form) and evaporated in the frozen state in a vacuum. Attempts at crystallization having failed, a semicrystalline powder was obtained by precipitation with ether from aqueous methanol. This product gave almost no color in the thiobarbiturate reaction; however, after hydrolysis in citrate buffer, pH 3, at 100° for 45 minutes, the absorbance was the same as that shown by free sialic acid. No hydroxamic acid reaction was observed. The extent of the resorcinol test was the same as that afforded by free sialic acid. The N-acetyl to N-glycolyl ratio was 37:63.

Methoxy-N-acetylneuraminic acid:

\[ \text{C}_{13}\text{H}_{26}\text{NO}_6 \] (323.3)

Calculated: C 44.6, H 6.55, N 4.33

Methoxy-N-glycolyneuraminic acid:

\[ \text{C}_{13}\text{H}_{24}\text{NO}_6 \] (339.3)

Calculated: C 42.5, H 6.32, N 4.13

Found: C 43.5, H 6.32, N 3.80

RESULTS AND DISCUSSION

Color Reactions

The molar absorptions of the sialic acids (human, sheep, ox) and of the three derivatives of sialic acid from ox serum under investigation in the direct Ehrlich, resorcinol, and thiobarbiturate reactions, performed by the techniques mentioned before, are presented in Table I.

The thiobarbiturate reaction is by far the most sensitive reaction for sialic acids, as long as the hydroxyl on the anomeric carbon is unsubstituted. Its usefulness for glycosidically bound sialic acid is limited, however, because of the dependence on an effective hydrolysis. As the results of the experiments described below indicate, the conditions for such a hydrolysis must be chosen carefully, with the application of correction factors for destruction, according to the type of binding of sialic acid in the particular compound—information not always easily secured.

The resorcinol reaction is quite sensitive and very reproducible. Our results also indicate that, in contrast to the findings with the direct Ehrlich reaction, the absorbance is independent of the presence of substituents on the carboxyl group.

1 These analyses were performed by Micro-Tech Laboratories, Skokie, Illinois.

2 These analyses were performed by Schwarzkopf Micronanalytical Laboratory, Woodside, New York.
The interference by many substances, and especially by galactose, however, renders its use rather difficult in the case of natural materials.

In the direct Ehrlich reaction, on the other hand, which appears to be the most specific reaction in the case of complex mixtures (compare p. 60 of (3)), the extent of absorbance was found to depend on the substitution of the hemiacetalic hydroxyl. Thus, whereas sialic acid methyl ester exhibited the same absorbance as the free sialic acid, the molar absorbances of methoxysialic acid and its methyl ester were 85 and 80%, respectively, of the absorbance of the free acid.

The results of a time study of the color development in the direct Ehrlich reaction are illustrated in Fig. 2. As can be seen, the production of color increases linearly with time, with slopes almost identical for the four compounds, after a characteristic initial period of retardation. The linear rise in extinction continues much beyond the period shown in Fig. 2.

In this reaction, cyclization to yield a pyrrole compound has been postulated as the first event (3, 14, 24, 25). The differences observed in the shapes of the curves in Fig. 2 do not contradict such a mechanism. The free carbonyl required for the cyclization is present in sialic acid and its methyl ester, but must first be produced in methoxysialic acid and methoxysialic acid methyl ester by the removal of the glycosidic methoxyl, an event occurring more easily in methoxysialic acid than in its methyl ester, as will be shown below.

The curves in Fig. 2 are constructed in terms of the optical density. If the molar absorbances recorded in this experiment after a treatment of 30 minutes, the time required by the standard technique (17), were computed, they would be found to be appreciably lower than those listed in Table I; moreover, the differences in color development between the free carbonyl compounds, sialic acid and its methyl ester, and the glycosides, methoxysialic acid and its methyl ester, are more pronounced. These discrepancies are obviously due to the different experimental arrangement required by the time study: the size and density. If the molar absorbances recorded in this experiment was determined spectrophotometrically at 565 μm. Δ, sialic acid; ▲, sialic acid methyl ester; ○, methoxysialic acid; ●, methoxysialic acid methyl ester.

Table I

<table>
<thead>
<tr>
<th>Sialic acid source and derivative</th>
<th>N-Acyl</th>
<th>Glycol</th>
<th>Molar absorbances (X 10⁻³) at specified wave lengths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Direct Ehrlich</td>
</tr>
<tr>
<td>Human serum</td>
<td>100</td>
<td>0</td>
<td>2.20</td>
</tr>
<tr>
<td>Sheep serum</td>
<td>87.5</td>
<td>12.5</td>
<td>2.24</td>
</tr>
<tr>
<td>Ox serum</td>
<td>37</td>
<td>63</td>
<td>2.35</td>
</tr>
<tr>
<td>Sialic acid methyl ester</td>
<td>35</td>
<td>65</td>
<td>2.33</td>
</tr>
<tr>
<td>Methoxysialic acid</td>
<td>37</td>
<td>63</td>
<td>2.01</td>
</tr>
<tr>
<td>Methoxysialic acid methyl ester</td>
<td>36</td>
<td>64</td>
<td>1.90</td>
</tr>
<tr>
<td>Methoxysialic acid, after hydrolysate</td>
<td>36</td>
<td>64</td>
<td>2.30</td>
</tr>
</tbody>
</table>

Average deviation: 0.15 0.1 1.8

* These readings are, in view of the average deviation, not significant.
† At pH 3 and 100° for 45 minutes.

Fig. 2. Direct Ehrlich reaction: color development by sialic acid (from ox serum) and its derivatives. A solution of 10 μmoles of the compound in 60 ml of 0.1 HCl containing 0.83% of p-dimethylaminobenzaldehyde was heated in a boiling water bath. At the times indicated, 3 ml samples were withdrawn, cooled in ice water, and brought to room temperature, and their absorbance was determined spectrophotometrically at 565 μm. Δ, sialic acid; ▲, sialic acid methyl ester; ○, methoxysialic acid; ●, methoxysialic acid methyl ester.

Fig. 3. Oxidation of sialic acid (from ox serum) and derivatives by sodium metaperiodate. To a solution of 20 μmoles of the compound in 10 ml of water, the same volume of a 0.01 M aqueous solution of NaIO₄ was added. Equal samples (1 ml) were removed at different intervals and mixed with 0.3 ml of phosphate buffer, pH 6.5, and 1 drop of a concentrated KI solution. Five minutes later, the liberated iodine was titrated with 0.01 M Na₂S₂O₃ solution in the presence of starch. Δ, sialic acid; ▲, sialic acid methyl ester; ○, methoxysialic acid; ●, methoxysialic acid methyl ester.

Type of the vessels and the heating bath used, the volumes of the assay mixture, the rate of temperature equilibration, etc., all of which are not without influence on the color value.

Periodate Oxidation

Uptake of Oxidant—The periodate oxidation of sialic acid (10, 23, 26) and of methoxysialic acid methyl ester (23) has been investigated before. We show, in Fig. 3, a reinvestigation of these compounds together with sialic acid methyl ester and methoxysialic acid under uniform conditions. An initial very rapid uptake of 2 moles of the oxidant per mole of substance is observed with all compounds. In the case of the glycosidically substituted derivatives, methoxysialic acid and methoxysialic acid methyl ester, the curves level off at this point, with
four compounds studied here have until now not been success-
ful. The reactivity of the crude oxidation products in the
color reactions normally applied to sialic acid and its derivatives
could, however, be examined. The compounds were subjected
to the action of NaIO₄ under the conditions outlined above,
and the excess of the oxidant was reduced by means of ethylene
glycol to iodate, which was precipitated by the addition of the
required quantity of barium acetate. The filtrates were assayed
directly. Because of the sensitivity of color tests to admixtures,
control solutions were prepared in which the periodate reagent
was, with the omission of the substrate, put through the opera-
tions mentioned here (reduction, precipitation, etc.); the resul-
ting solution then served as the solvent for the untreated sialic
acid derivative, whose absorbance was compared with that of
the corresponding oxidation product.

The following observations were made on the oxidation pro-
ducts. (a) The absorbance in the thiobarbiturate reaction was
not altered. This was to be expected, as periodate oxidation
actually is the first step in this reaction. (b) The absorbance
of sialic acid in the resorcinol reaction was almost doubled,
with a shift of the absorption maximum from 580 to 600 mμ.
(c) The absorbance in the direct Ehrlich reaction decreased
by about 65% for methoxysialic acid and its methyl ester and
by about 80% for sialic acid and its methyl ester.

**Alkaline Hydrolysis of Ester Link**

The ester link of sialic acid is known to be cleaved under
very mild alkaline conditions (21, 23). In the course of the
present studies, the hydrolysis, at constant pH, of the ester
bond in sialic acid methyl ester was compared with that of
methoxysialic acid methyl ester. Aqueous solutions of the
esters were kept at a constant pH by means of an automatic
titrator. The equivalents of alkali consumed in these exper-
iments are plotted against time in Fig. 4. A parallel set of ex-
periments, in which the gradual disappearance of the ester was
followed at constant pH by means of the hydroxamic acid
reaction, confirmed that the alkali had actually been consumed
by the saponification process.

As shown in Fig. 4, the ester link of sialic acid methyl ester is
broken at room temperature and pH 8 within 20 minutes,
whereas methoxysialic acid methyl ester requires a higher pH
and a much longer time for complete saponification. The fol-
lowing reaction rate constants were found for the conditions
specified in Fig. 4: sialic acid methyl ester, \( k = 2.7 \times 10^{-3} \) 
\( \text{sec}^{-1} \); methoxysialic acid methyl ester, \( k = 3.1 \times 10^{-4} \) 
\( \text{sec}^{-1} \). The substitution of the neighboring hemiacetalic
hydroxyl thus appears to render the ester link more resistant to alkali.
Although steric hindrance could be invoked, it is more likely that
another factor is of importance. In contrast to free sialic acid,
the methyl ester has been reported to exhibit, in weakly alkaline
solution, a transitory absorption in the ultraviolet region (maxi-
mum at 265 μm) which has been attributed (14) to the enol
form of the open chain structure (11 B in Fig. 5), in analogy to
observations on ethyl acetoacetate (28). In the open structure,
sialic acid methyl ester would be expected to behave as an α-
ketoc acid ester, whereas methoxysialic acid methyl ester, owing
to the stabilization of the pyranose ring (11 B in Fig. 5) by the
substitution of the hemiacetalic hydroxyl, would be expected to
show the reactivity of an α-methoxy acid. Esters of α-keto
acids are known to be saponified by alkali at rates much higher
than those exhibited by their aliphatic counterparts or by the
substituted α-hydroxy acids: the rate of alkaline hydrolysis of

Fig. 4. Alkaline hydrolysis of the ester link of sialic acid methyl
ester (A) and methoxysialic acid methyl ester (B). Solutions of 50
μmoles of compound in 10 ml of CO₂-free water were used to de-
determine the required equivalents of alkali, added in the form of
0.01 N NaOH, in order to maintain the indicated pH. An auto-
matic titrator (Radiometer, Copenhagen) was employed.

![Fig. 4](http://www.jbc.org/)

Fig. 5. Tautomeric structures of sialic acid methyl ester

![Fig. 5](http://www.jbc.org/)
ethyl pyruvate was found to be 18,000 times higher than that of ethyl propionate and 840 times higher than that of methoxyglycolic acid ethyl ester (29).

**Treatment with Acid**

*Free Sialic Acid*—This substance is known to be decarboxylated when heated in strong acid (23) but has been considered, on the basis of the resorcinol reaction, to be relatively stable under less drastic conditions (30). A similar apparent stability of sialic acid was observed in the present study when the direct Ehrlich reaction was employed (Fig. 6). The same figure, however, shows that, under identical experimental conditions, the color values recorded with the thiobarbiturate reaction decrease considerably in a linear fashion, approximately one-half of the initial absorbance being found at the end of 1 hour. A similar observation has been reported recently (31). As is also shown in Fig. 6, N-acetyleneuraminic acid (from human serum) gave identical results; this excludes the possibility that the two different N-acyl substituents in the sialic acid from ox serum contributed in different manners to the behavior of the latter in the color reactions.

It is obvious that the action, even of weak acid, on sialic acid has complex consequences (14, 32), but the products cannot yet be identified conclusively. That some of the degradation products react with both the Ehrlich and the resorcinol reagents has been pointed out before (14). The treatment with acid obviously results in a modification of the molecule that depresses the formation of the thiobarbiturate chromogen, but does not interfere materially with the other color reactions.

Decarboxylation does not seem to play a role, as shown by the behavior of the degradation product towards the periodate-thiobarbiturate reagent. The treatment of sialic acid with HIO₄ in a strongly acidic environment presumably affords β-formylpyruvic acid, in analogy to the 2-keto-3-deoxy sugar acids (33, 34), whereas previous decarboxylation would lead to the formation of malondialdehyde (35). These two nitriles form, in the reaction with thiobarbituric acid, colored compounds whose absorption spectra can be distinguished through the position of the centers of absorption and the respective molar absorbances: the product formed with P-formylpyruvic acid has a maximum at 549 μm, and that formed with malondialdehyde, at 532 μm; the molar extinction shown by the latter product is about twice as high as that of the first (34). As we have observed, sialic acid heated in 0.1 N HCl at 100° for 1 hour (Fig. 6) exhibits no shift in the position of the absorption maximum (549 μm), and this, together with the decrease in the molar absorbance, speaks against the occurrence of decarboxylation.

The partial lactonization of sialic acid (32) could account for the lack, or the retardation, of the response to periodic acid and for the diminution of the color value in the thiobarbiturate reaction. Dimerization through the formation of a glycosidic bond between the carbonyl group of one molecule and a hydroxyl of another could also be invoked. No direct evidence of either step, however, is available.

The cyclization to a pyrrole structure, as discussed before, would also block the oxidative formation of formylpyruvic acid. There is, in fact, some evidence that such an event takes place: we have observed that after sialic acid has been heated in 0.1 N HCl at 100° for 30 minutes, the solution forms a purple color in the cold when mixed with the p-dimethylaminobenzaldehyde reagent; this is characteristic of pyrroles, whereas untreated sialic acid develops the color only after being heated with the reagent for some time.

It is not unlikely, therefore, that several events occur simultaneously: lactonization, pyrrole formation, and further degradation; the extent to which each contributes to the final result, however, cannot yet be assessed.

**Ester Link of Sialic and Methoxysialic Acids**—The effect of the treatment of the methyl esters of these compounds with 0.1 N HCl at 100° for 1 hour is illustrated in Fig. 7. Under these conditions, the ester link of sialic acid methyl ester is cleaved rapidly (Curve 3, Fig. 7A); the results of the direct Ehrlich and thiobarbiturate reactions resemble, as expected, those obtained with sialic acid (Fig. 6). The ester link of methoxysialic acid methyl ester, on the other hand, appears to be much more stable (Curve 3, Fig. 7D). The hydroxamic acid values observed in the course of 1 hour diminish only little. The thiobarbiturate reaction, negative for methoxysialic acid methyl ester in the beginning of the treatment, increases with time, owing no doubt to the liberation of the carbonyl group. For the same reason a slight increase in the direct Ehrlich values is recorded (compare Table 1). That the absorbance values in the thiobarbiturate test increase more steeply than the hydroxamate values drop (Fig. 7B) could be taken to indicate that the removal of the glycosidic methoxyl precedes that of the ester methoxyl.

**Glycosidic Link of Methoxysialic Acid and Its Methyl Ester**—The acid lability of the glycosidic link of sialic acid was recognized very early; the first isolation of the compound made use of the
completed in a relatively short time, without the degradation of the liberated sialic acid. At higher degrees of acidity, cleavage of the glycosidic link seems to be at pH 3, since the rupture is completed at pH 4. The optimal condition for the hydrolysis of the glycosidic link is at pH 3, since the rupture is completed in a relatively short time, without the degradation of the liberated sialic acid. At higher degrees of acidity, cleavage occurs more rapidly, but the free sialic acid evidently is degraded further.

The stability of the glycosidic link towards acid is heightened by the substitution of the neighboring carboxyl. No cleavage of methoxysialic acid methyl ester is apparent at pH values as low as 3. The compound is hydrolyzed slowly in 0.1 N HCl and more rapidly in 0.5 N HCl, but the liberation of sialic acid is overtaken by its destruction. Hence, the complete hydrolysis and quantitative recovery of the liberated sialic acid appear unattainable if it is doubly linked through its hemiacetalic hydroxy and carboxy groups.

The hydrogen of the carboxyl plays an important role in the removal of the glycosidic methoxyl. This intramolecular catalytic effect suggests the formation of a bond between this hydrogen and the glycosidic oxygen, such hydrogen bonds, resulting in the formation of a 5-membered ring, are not as strong as those of their 6-membered counterparts, but are still capable of influencing the rate of chemical reactions (37).

The enzyme was prepared from culture filtrates of Vibrio cholerae and used in the purification stage referred to as Stage 6 in a previous publication (9). When methoxysialic acid or its methyl ester, in concentrations of 10 or 100 μg per ml of 0.01 M Tris-acetate buffer, pH 6.6 (4 mM with respect to Ca++, containing 100 sialidase units (9), was incubated for 24 hours at 37°C, no liberation of sialic acid was observed. Under the same conditions of enzymic assay, ox brain mucolipid (8) and sialyllactose+ (38) showed considerable cleavage even after 1 hour of incubation. The liberation of sialic acid was followed by means of the thiobarbiturate test. It is likely that the resistance of methoxysialic acid and its methyl ester to attack by sialidase, when compared to the susceptibility of the naturally occurring sialic acid glycosylates, is due to the aglycone rather than to differences in the stereochemistry around carbon 2.

**Treatment with Sialidase**

The enzyme was prepared from culture filtrates of Vibrio cholerae and used in the purification stage referred to as Stage 6 in a previous publication (9). When methoxysialic acid or its methyl ester, in concentrations of 10 or 100 μg per ml of 0.01 M Tris-acetate buffer, pH 6.6 (4 mM with respect to Ca++), containing 100 sialidase units (9), was incubated for 24 hours at 37°C, no liberation of sialic acid was observed. Under the same conditions of enzymic assay, ox brain mucolipid (8) and sialyllactose+ (38) showed considerable cleavage even after 1 hour of incubation. The liberation of sialic acid was followed by means of the thiobarbiturate test. It is likely that the resistance of methoxysialic acid and its methyl ester to attack by sialidase, when compared to the susceptibility of the naturally occurring sialic acid glycosylates, is due to the aglycone rather than to differences in the stereochemistry around carbon 2.

**Treatment with Glacial Acetic Acid and Pyridine**

Mucolipids and related derivatives of sialic acid are, in the course of their preparation, often exposed to solvents such as pyridine or glacial acetic acid. It was of some interest to examine the stability of the simple derivatives studied here to treatment with these solvents.

Solutions of the four compounds in either pyridine or acetic acid were heated at 100°C. The samples were tested by the direct Ehrlich and thiobarbituric acid reactions. The experimental conditions and results are summarized in Figs. 9 and 10.

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4 We are very grateful to Dr. Karl Meyer for a sample of this compound.
The treatment with glacial acetic acid results in the rapid degradation of sialic acid (Fig. 9A), as shown by both the direct Ehrlich and the thiobarbiturate reactions. In the case of methoxysialic acid (Fig. 9C), considerable degradation is shown by the direct Ehrlich reaction, while the thiobarbiturate values fail to indicate an accumulation of free sialic acid. To what extent the glycosidic bond is broken in this case cannot be assessed, as most of the sialic acid liberated would be further degraded. The methyl ester of sialic acid (Fig. 9B) is degraded slowly; it will be noticed that there is an initial, as yet unexplained, increase of the absorbance in the thiobarbiturate reaction. N-Acetyleneuraminic acid has a higher molar absorbance than N-glycolyneuraminic acid (18), but a transamidation, effecting the replacement of N-glycolyl by N-acetyl groups, is not likely under the experimental conditions. Methoxysialic acid methyl ester (Fig. 9D) appears to be rather stable; the small initial increase of absorbance in both color reactions could indicate some cleavage of the glycosidic link.

It should be noted that the most severe degradation is suffered by the two compounds possessing a free carboxyl group, sialic acid and methoxysialic acid. Whether the degradation is caused by a specific action of the acetic acid or is due in part to the acidity of sialic acid itself cannot be decided without further experimentation.

The treatment with pyridine results in a rapid degradation of sialic acid methyl ester (Fig. 10B), which was confirmed by both color reactions. With sialic acid (Fig. 10A) and methoxysialic acid (Fig. 10C), on the other hand, a disagreement between the two color tests was observed, similar to that mentioned before in the treatment with HCl: absorbance in the direct Ehrlich reaction remained practically unchanged whereas the thiobarbiturate reaction indicated a marked degradation. Pyrole formation, which is more pronounced under alkaline conditions, could account for this discrepancy, as discussed before. Methoxysialic acid methyl ester (Fig. 10D) also appears stable by the direct Ehrlich test; the comparison, however, of the results of the hydroxamic acid reaction before and after the treatment (3 hours) indicated a drop in the ester content of 50%.

As mentioned before, the stability of the glycosidic link to acetic acid or pyridine could not be determined by treatment of the simple derivatives, owing to further degradation. For this reason, an orienting experiment with a preparation of ox brain mucolipid may be of interest; it shows that the pretreatment accorded these complex and delicate substances may not be without consequence. A specimen of mucolipid from ox brain purified to "Stage III" (8) was found, by means of the direct Ehrlich reaction with our preparation of crystalline sialic acid as the standard, to contain 32.4% of sialic acid. Portions

4 The preparation of ox brain mucolipid investigated previously in detail (8) had a sialic acid content of 26.0%. Since that time, we have encountered numerous instances of preparations of the same degree of purity that contained considerably more sialic acid. For instance, in as yet unpublished studies in collaboration with Drs. O. W. Garrigan and N. Z. Stanacev, 10 preparations were examined whose sialic acid content ranged from 27.3 to 32.4%, with a mean of 30.1% and a standard deviation of 1.4.
The polymer. Some of these polymers, in whose structure are delicate and labile instances of macromolecules found in nature. Acid participates, probably are to be counted among the most fully known, and the hydrolysis method must be in keeping with the type or types of linkage in which sialic acid occurs in to this sort of lability having been overlooked. What this study standard must be chosen, the history of the sample must be likely that many discrepancies in the literature are attributable to the choice of suitable means of release and determination of sialic acid and of the standards to be used in a given case.

The great lability of the ester link in a sialic acid molecule that is not stabilized by glycoside formation (sialic acid methyl ester) is not enganged. Since the investigation of the polymer will have to make empirical use of reactions that mostly esterification of the neighboring carboxyl, as in methoxysialic acid methyl ester, rendered the ester bond much more resistant to hydrolysis both by acid and by alkali. Treatment with 0.1 N HCl at 100° was found to cause a modification of the molecule of sialic acid, resulting in a loss of reactivity in the thiobarbiturate, but not in the direct Ehrlich reaction. The hydrolysis of the glycosidic link of methoxysialic acid was investigated at various pH values; pH 3 was found to be the most suitable for a rapid hydrolysis without further degradation of the liberated sialic acid. Here again, the same effect of double substitution was observed: esterification of the neighboring carboxyl, as in methoxysialic acid methyl ester, increased markedly the resistance of the glycosidic link to acid hydrolysis.

The heteropolymers occurring in biological material are usually determined through the quantitative estimation of one or more of their characteristic monomeric constituents. This requires, among other things, the availability of procedures permitting the complete release and recovery of the intact monomer. For the proteins and the nucleic acids these requirements can, on the whole, be met, but this is less true of the lipids and the polysaccharides. The increasing interest in the biological function of various derivatives of sialic acid occurring in nature prompted the present study, which deals with the simplest representatives of the various types of links in which sialic acid is able to engage. Since the investigation of the quantity and the manner in which sialic acid is integrated into a polymer will have to make empirical use of reactions that mostly are not entirely specific, the results reported here may serve to place certain limitations on the interpretation of such studies.

Another cautionary reservation which may accrue from the present observations regards conclusions as to structure drawn from experiments on the action of periodate on polymers containing sialic acid, such as were attempted in a previous study from this laboratory (10). The finding that the products of the action of periodate on the sialic acid derivatives studied here still give some direct Ehrlich reaction makes difficult the quantitative interpretation of oxidation experiments performed on an intact polymer.

The present studies also emphasize the necessity of gaining an insight into the types of links of sialic acid prevailing in the polymer to be investigated before the results of quantitative estimations can be evaluated properly. They may contribute to the choice of suitable means of release and determination of sialic acid and of the standards to be used in a given case. The great lability of the ester link in a sialic acid molecule that is not stabilized by glycoside formation (sialic acid methyl ester is completely hydrolyzed at pH 8 and 25° within 20 minutes) makes it evident how difficult the isolation of an intact macromolecule may be under certain circumstances. It is not unlikely that many discrepancies in the literature are attributable to this sort of lability having been overlooked. What this study underlines is that for results to be meaningful the appropriate standard must be chosen, the history of the sample must be fully known, and the hydrolysis method must be in keeping with the type or types of linkage in which sialic acid occurs in the polymer. Some of these polymers, in whose structure sialic acid participates, probably are to be counted among the most delicate and labile instances of macromolecules found in nature.

Concluding Remarks

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Studies on the Stability of Simple Derivatives of Sialic Acid
John D. Karkas and Erwin Chargaff


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