The Mechanism of the Periodate–Thiobarbituric Acid Reaction of Sialic Acids

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1. The chromogen formation from *N*-acetylneuraminic acid in the periodate–thiobarbituric acid reaction was investigated. Measurement of periodate consumption showed an uptake of approx. 3 moles/mole of substrate in neutral as well as in strongly acidic solution. Therefore the chromogen β-formylpyruvic acid is not a direct product of the periodate oxidation; it is presumed to be formed from the true oxidation product, a hexos-5-uluronic acid, by aldol splitting during the reaction in hot acidic solution with thiobarbituric acid. 2. Methyl (methyl β-L-*threo*-hexos-4-enepyranosid)uronate, an analogue of the pre-chromogen, has been shown to yield with thiobarbituric acid in acidic solution a pigment exhibiting an identical absorption spectrum and showing the same behaviour on paper chromatography as the pigment obtained from *N*-acetylneuraminic acid in the periodate–thiobarbituric acid assay. 3. The substitution at C-2 of methoxyneuraminic acid does not inhibit the periodate–thiobarbituric acid reaction. In neutral solution methoxyneuraminic acid is oxidized by periodate to a substance that reacts readily with thiobarbituric acid in acidic solution. When periodate oxidation is attempted in acidic solution, protonation of the amino group protects this group against oxidation, rendering methoxyneuraminic acid negative in the assay systems of Warren (1959*a*,*b*) and Aminoff (1959, 1961).

The periodate–thiobarbituric acid reaction is the most important assay for sialic acids because of its sensitivity and specificity. A special feature of this method is that only free sialic acids are estimated; the glycosides of sialic acid (sialosides) do not react. This reaction was introduced by Waravdekar & Saslaw (1957, 1959) for the determination of 2-deoxyribose. Modifications of this method for the assay of sialic acids have been described by Warren (1959*a*,*b*) and Aminoff (1959, 1961). Weissbach & Hurwitz (1959) applied this reaction to the determination of 3-deoxyaldulosonic acids. By analogy of the periodate–thiobarbituric acid assay of 2-deoxyribose (with $\lambda_{max.}$ at 532mμ), in which assay malonaldehyde is the chromogen (the substance that after condensation with thiobarbituric acid affords the pigment) (Waravdekar & Saslaw, 1957, 1959), it has been assumed that in the corresponding estimation of 3-deoxyaldulosonic acids and sialic acids β-formylpyruvic acid should be the chromogen, which results from the periodate oxidation and is subsequently condensed with thiobarbituric acid to the red pigment with $\lambda_{max.}$ at 549mμ.

This has been confirmed by Srinivasan & Sprinson (1959), who obtained the same pigment from the bis-2,4-dinitrophenylhydrazone of β-formylpyruvic acid, and by Kuhn & Lutz (1963). These authors prepared the red pigment in crystalline form from a derivative of β-formylpyruvic acid. The analysis agreed with a condensation product of 2mol. of thiobarbituric acid and 1mol. of β-formylpyruvic acid. Aminoff (1961) demonstrated the identity of the red pigments obtained from *N*-acetylneuraminic acid and 3-deoxy-D-*erythro*-hexulosonic acid (3-deoxy-2-keto-D-gluconic acid) by paper chromatography. This means that the same chromogen is produced from both compounds in the periodate–thiobarbituric acid reaction. The formation of β-formylpyruvic acid from the 3-deoxyaldulosonic acids by periodate oxidation is easily understood; with the sialic acids, however, because of the acylated amino group, the formation of this chromogen should not be possible. For this reason Warren (1959*b*) used a high concentration of phosphoric acid in his periodate reagent to hydrolyse the acyl group before the oxidation of the amino group. Hydrolysis of the acyl group, however, under these conditions is not probable. The acetamido bond is more stable to acid than is the glycosidic bond.

N-Acylated neuraminic acids can be prepared by hydrolysis of their glycosides with dilute acid at 80°. Sialosides, however, do not react in the assay systems of Warren (1959*a*,*b*) and Aminoff (1959, 1961). This means that under these conditions even the glyco-

sidic bond is not broken. Moreover, Aminoff (1961) showed that acidic conditions during the oxidation are not essential. Acylation of the amino group does not seem to protect *N*-acetylglucosamine against complete oxidation by periodate. This rapid and complete oxidation of *N*-acetylglucosamine is, however, due to the hydroxylation of the malonaldehyde derivative, which results from the oxidation of the C-3–C-4 bond of the pyranose ring (Cantley & Hough, 1963). The formation of an activated methylene group at C-5 is not possible in the sialic acids. For that reason the formation of β-formylpyruvic acid cannot be explained by this mechanism. As a reasonable explanation for the chromogen formation from the sialic acids in the periodate–thiobarbituric acid reaction was not forthcoming, we investigated this reaction.

MATERIALS AND METHODS

Materials. 3-Deoxy-L-*erythro*-hexulosonic acid was the crystalline acid prepared in this Laboratory (Paerels, 1961). *N*-Acetylneuraminic acid was isolated from ovine submaxillary mucin and crystallized from 90% (v/v) acetic acid. This material contained 1·0% of *NO*-diacetylneuraminic acid and 0·9% of *N*-glycolylneuraminic acid. The equiv.wt. found was 305·1 (calc. 309·6). Methoxyneuraminic acid was prepared from *N*-acetylneuraminic acid according to the procedure of Weygand & Rinno (1957*a*). The optical rotation $[\alpha]_D^{24} - 54{\cdot}5°$ is in agreement with reported values. *N*-Benzoylmethoxyneuraminic acid was obtained from methoxyneuraminic acid by benzoylation with benzoyl chloride and $KHCO_3$ in water. The compound was chromatographically pure. (Methyl 5-amino-3,5-dideoxy-L-*arabino*-heptulosid)onic acid (methoxyneuraminic acid minus two CH·OH groups) was prepared according to the method of Weygand & Rinno (1957*b*). This compound is referred to below as 'C_7-methoxyneuraminic acid'. *N*-Benzoyl-C_7-methoxyneuraminic acid was obtained by the same procedure as the corresponding C_9 compound. Methyl (methyl β-L-*threo*-hexos-4-enepyranosid)uronate was synthesized by following the description of Heim & Neukom (1962). The syrup obtained had $\lambda_{max.}$ at 237 mμ (literature: 235 mμ). The starting material for this compound was methyl (methyl-α-D-galactopyranosid)uronate (anhydrous) prepared from D-galacturonic acid according to the method of Jones & Stacey (1947).

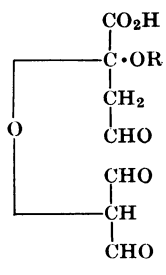
Determination of periodate consumption. This was performed by the method of Malaprade (1928) by the addition of KI in acid solution and titration of the liberated I_2 with $Na_2S_2O_3$. A favourable feature of this method is that, owing to the presence of HI, oxidation products with activated hydrogen (e.g. β-formylpyruvic acid) do not react with I_2 (Schwarz, 1954).

Estimation of chromogen formation. To a sample (0·2 ml.) of the reaction mixture was added 1 ml. of arsenite reagent (solution of 4% $NaAsO_2$ and 7% Na_2SO_4 in N-HCl) to destroy the excess of periodate. After the addition of 3 ml. of thiobarbituric acid reagent (0·6% thiobarbituric acid solution in 0·5 M-Na_2SO_4) the mixture was heated at 100° for 15 min. After rapid cooling in ice–water 5 ml. of cyclohexanone was added, and the mixture was shaken well and centrifuged for about 3 min. The extinction of the coloured upper layer was measured at 549 mμ.

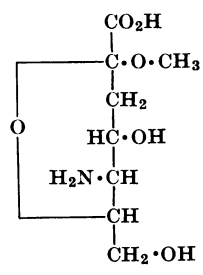
Procedure of experiments. To relate the colour formation to the periodate uptake, a solution of the substance to be investigated was oxidized with periodate (concentrations of substrate and periodate are given in each experiment). At distinct time-intervals samples were taken and periodate consumption and chromogen formation determined.

RESULTS

Behaviour of sialosides and methoxyneuraminic acid in the periodate–thiobarbituric acid reaction. From the fact that sialosides do not react in this assay, it may be concluded that the pyranoside ring is not attacked by periodate. If a cleavage of the bond between C-4 and C-5 by periodate were to occur, a ketal of β-formylpyruvic acid (I) would result. It might be expected that this ketal would readily yield β-formylpyruvic acid by hydrolysis in the subsequent reaction with thiobarbituric acid, which is performed by heating at 100° in acidic solution. This hydrolysis to the chromogen is indeed found during the reaction of an acetal of malonaldehyde (tetraethoxypropane) with thiobarbituric acid (Sinnhuber, Yu & Yu, 1958; Schmidt, 1959). Malonaldehyde is also formed by hydrolysis of 2-aminopyrimidine and sulphadiazine in the reaction of these substances with thiobarbituric acid (Shepherd, 1948). We found that, in accordance with this, methyl 2-deoxyglucoside gives the same colour intensity as the free sugar in the periodate–thiobarbituric acid assay. In methoxyneuraminic acid the amino group is not acylated. Oxidation with periodate should therefore result in the formation of the ketal (I). Indications for this postulate can be found in the studies of Klenk, Faillard, Weygand & Schöne (1956), Blix, Lindberg, Odin & Werner (1956) and Yamakawa & Suzuki (1952). These authors reported that methoxyneuraminic acid rapidly consumed 3 moles of periodate/mole. Moreover, 70–85% of the calculated amount of ammonia was found. Because of the above-mentioned reasons it may be expected that



(I)



(II)

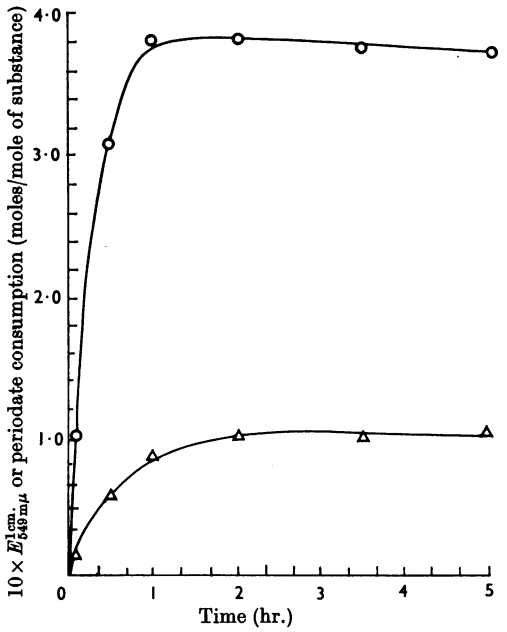


Fig. 1. Periodate consumption (△) and colour formation (○) of C_7-methoxyneuraminic acid (0·2mM) in neutral solution at room temperature. The initial concentration of $NaIO_4$ was 1·2mM.

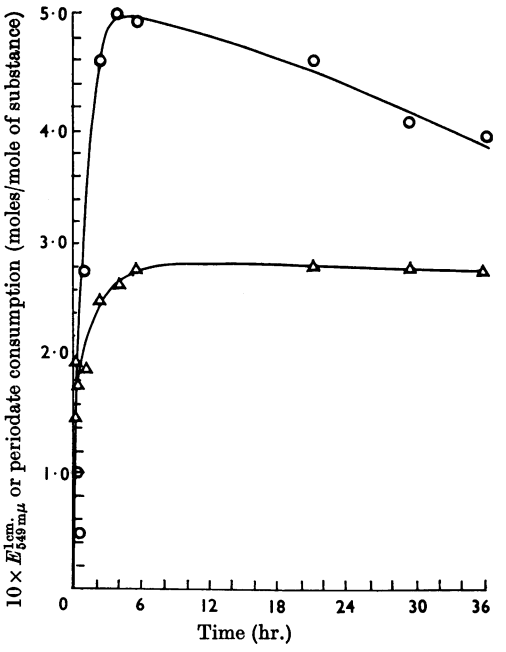


Fig. 2. Periodate consumption (△) and colour formation (○) of *N*-acetylneuraminic acid (0·2mM) in 125mN-H_2SO_4 at room temperature. The initial concentration of $NaIO_4$ was 2·0mM.

methoxyneuraminic acid and C_7-methoxyneuraminic acid (II) will react in the periodate–thiobarbituric acid reaction. That this reaction takes place is demonstrated by a combined estimation of periodate uptake and colour formation of C_7-methoxyneuraminic acid in neutral solution by following the described procedure. The results given in Fig. 1 show that when 1mole of periodate is consumed/mole the extinction reaches a maximum value. Analogous results were obtained with methoxyneuraminic acid under similar conditions. In this case 3moles of periodate were consumed/mole.

However, Warren (1959*b*) and Aminoff (1959, 1961) reported that methoxyneuraminic acid does not react in the periodate–thiobarbituric acid assay. This is due to the fairly acidic conditions of their methods [final concentrations: 3M-phosphoric acid (Warren, 1959*b*); 40mN-sulphuric acid (Aminoff, 1959, 1961)].

Consequently the amino group is protonated and this prevents the oxidation by periodate (McCasland & Smith, 1951). Accordingly, we found no periodate consumption by C_7-methoxyneuraminic acid in acidic solution (3M-phosphoric acid), and no colour formation with thiobarbituric acid was observed. Methoxyneuraminic acid consumed 2moles of periodate/mole in dilute sulphuric acid solution (10 and 125mN) within 5hr. In 3M-phosphoric acid the periodate uptake was 0·5mole/mole in 72hr. For this extremely slow reaction of the hydroxyl groups in this case no explanation is known. In all these latter experiments no colour formation with thiobarbituric acid was obtained. Thus the presented evidence shows that glycosidation of neuraminic acid does not inhibit the periodate–thiobarbituric acid reaction. The non-reactivity of the sialosides in this assay is due to both glycosidation and acylation of the amino group. The latter factor inhibits the rupture of the bond between C-4 and C-5 in the pyranoside ring by periodate. Accordingly, *N*-benzoyl-C_7-methoxyneuraminic acid does not consume periodate. The corresponding C_9 compound consumed 2moles of periodate/mole; in neither experiments was colour formation observed.

Periodate oxidation of N-*acetylneuraminic acid.* It is reasonable to assume that in free sialic acid *N*-acylation also protects the amino group against attack by periodate. Blix *et al.* (1956) and Aminoff (1961) reported only a slight production of ammonia in the periodate oxidation of *N*-acetylneuraminic acid. The periodate oxidation studies of Blix *et al.* (1956) and of Karkas & Chargaff (1964) indicate a rapid uptake of 2moles of periodate/mole; there-

after a slow consumption of a third mole/mole occurred. The formation of β-formylpyruvic acid would require at least 5 moles of periodate/mole. To study the relation between periodate consumption and chromogen formation with *N*-acetylneuraminic acid a number of experiments were performed. By following the described procedure *N*-acetylneuraminic acid (0·2 mM) was oxidized with periodate (2·0 mM) under similar conditions to those mentioned by the various authors (Weissbach & Hurwitz, 1959; Aminoff, 1961; Warren, 1959*b*). The amount of periodate consumed/mole of substance in 125 mN-sulphuric acid at room temperature was 2·8 and 3·1 moles; in 125 mN-sulphuric acid at 37° 3·3 and 3·4 moles; at pH 7 at room temperature 3·3 moles; in 3 M-phosphoric acid at room temperature 3·0 and 3·0 moles. The first of these experiments is shown in Fig. 2 as an illustrative example.

The extinction reached a maximum at the time when approx. 3 moles of periodate were consumed/mole. In all experiments the periodate uptake ceased at the given value. Apparently 1 mole of *N*-acetylneuraminic acid consumes 3 moles of periodate and not the number required for the formation of β-formylpyruvic acid. Moreover, this latter substance itself reduces periodate on account of the activated methylene group. Indeed, in a similar experiment with 3-deoxy-L-*erythro*-hexulosonic acid (Fig. 3) the periodate consumption rapidly exceeded the calculated amount for the formation of β-formylpyruvic acid (2 moles/mole).

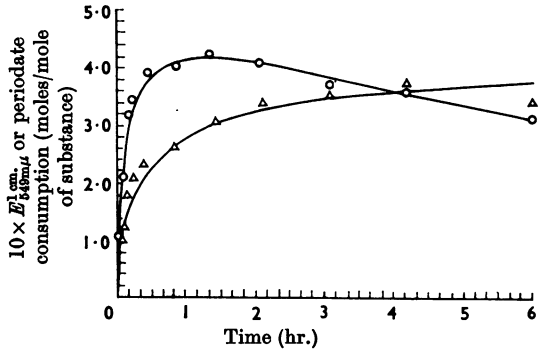
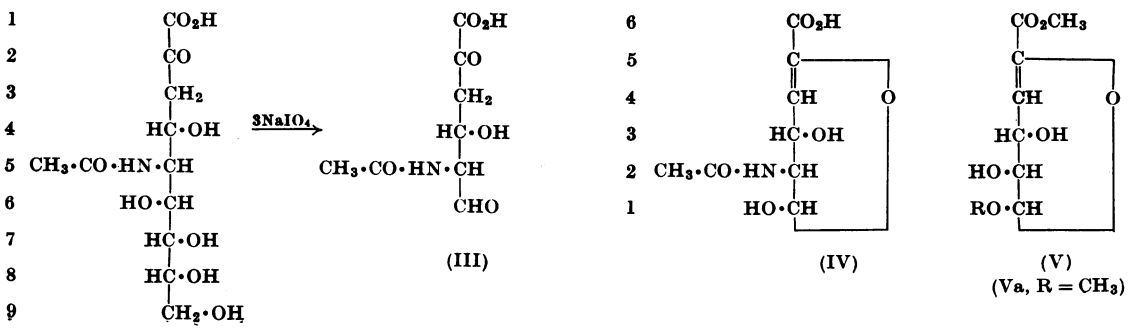


Fig. 3. Periodate consumption (△) and colour formation (○) of 3-deoxy-L-*erythro*-hexulosonic acid (0·2 mM) in unbuffered solution at room temperature. The initial concentration of $NaIO_4$ was 2 mM.

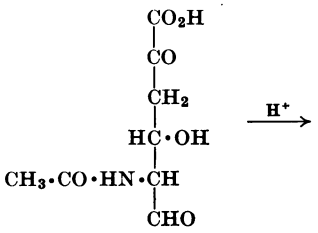
Nature of the oxidation product. Since the periodate uptake ceased at about 3 moles/mole with *N*-acetylneuraminic acid it can be concluded that β-formylpyruvic acid is not a product of the oxidation of this substance. This chromogen must be formed from the resulting moiety during the subsequent condensation with thiobarbituric acid in acidic solution at 100°. From the periodate consumption of 3 moles/mole it follows that the resulting moiety (pre-chromogen) is a 4-deoxyhexos-5-uluronic acid (III). Analogues of the tautomeric form of III (IV) are known. Heim & Neukom (1962) described the synthesis of methyl (methyl β-L-*threo*-hexos-4-enepyranosid)uronate (Va). Indeed this compound, prepared according to the prescription of Heim & Neukom (1962), reacted readily with thiobarbituric acid in acidic solution without pretreatment with periodate. The coloured substance had a spectrum identical with that obtained with *N*-acetylneuraminic acid in the periodate–thiobarbituric acid assay. This agrees with the reports of Albersheim, Neukom & Deuel (1960*a*,*b*) that the unsaturated oligouronides [which contain (V) as the non-reducing end] obtained from pectins after enzymic degradation or by heating in a borate buffer reacted directly with thiobarbituric acid to give the same red pigment. The identity of the pigments obtained from compound (Va) by direct condensation with thiobarbituric acid and from *N*-acetylneuraminic acid in the periodate–thiobarbituric acid reaction was also suggested by the results of paper chromatography. The pigment derived from compound (Va) is a methyl ester and was therefore compared with the pigment obtained

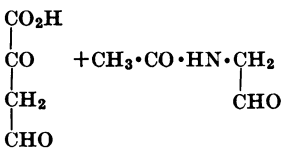


from *N*-acetylneuraminic acid esterified with diazomethane. To compare the pigments derived from the acids, compound (Va) was saponified with 2N-sodium hydroxide at room temperature for 1hr. before the condensation with thiobarbituric acid was performed. The corresponding pigments migrated at the same rate with butan-1-ol–acetic acid–water (4:1:1, by vol.) and tetrahydrofuran as solvents.

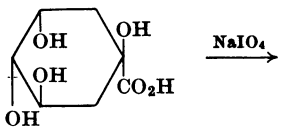
DISCUSSION

The periodate–thiobarbituric acid reaction with the sialic acids follows a different path from that followed with the 3-deoxyaldulosonic acid. Acylation of the amino group affords an effective protection against periodate attack even under strongly acidic conditions. The oxidation of the sialic acids results in a pre-chromogen from which the chromogen β-formylpyruvic acid is formed by an aldol splitting between C-4 and C-5 (numbering of neuraminic acid):





This pre-chromogen is an unstable substance, as is indicated by the decrease of the extinction curve in Fig. 2. The proposed path explains the fact that sialosides do not react in the periodate–thiobarbituric acid assay: the substitution at C-2 prevents the oxidation at C-6 by stabilizing the pyranoside ring. The observations of Aminoff (1961), that equine sialic acid (*N*-acetyl-4-*O*-acetylneuraminic acid) does react in this assay and bovine sialic acid (*N*-acetyl-7-*O*-acetylneuraminic acid) does not, are easily explained by (and lend support to) the pre-chromogen theory. Blockage of the hydroxyl group at C-7 prevents periodate attack of the bond between C-6 and C-7, rupture of which is necessary for pre-chromogen formation. The acetyl group at C-4 of equine sialic acid does not inhibit this reaction. The acetyl group is apparently hydrolysed during the reaction with thiobarbituric acid before the aldol splitting. The lower extinction found with equine sialic acid in comparison with *N*-acetylneuraminic acid may indicate that hydrolysis and aldol splitting were not complete. An analogous aldol splitting may explain the finding of Mesnard & Devaux (1964) that quinic acid reacts in the periodate–thiobarbituric acid assay, yielding a pigment with $\lambda_{max.}$ at 549mμ. The product of the oxidation of quinic acid with periodate is the dialdehyde of citric acid:



$$OHC\cdot CH_2\cdot C(OH)(CO_2H)\cdot CH_2\cdot CHO$$

The formation of β-formylpyruvic acid from this pre-chromogen may follow the same mechanism as with the sialic acids:

$$OHC\cdot CH_2\cdot C(OH)(CO_2H)\cdot CH_2CHO \xrightarrow{H^+}$$

$$OHC\cdot CH_2\cdot CO\cdot CO_2H + CH_3\cdot CHO$$

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