Methods for the Quantitative Estimation of *N*-Acetylneuraminic Acid and their Application to Hydrolysates of Sialomucoids

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The sialic acids (Blix, Gottschalk & Klenk, 1957) are widely distributed components of many sialomucoids (Blix, 1940; Zilliken & Whitehouse, 1958). They are readily obtained from these complexes by mild acid hydrolysis and more specifically by hydrolysis with neuraminidase, an enzyme found in many bacterial filtrates and the myxo group of viruses (Hirst, 1942; Gottschalk, 1958*a*, *b*).

Several techniques have been developed for the determination of the sialic acids, e.g. tryptophan and perchloric acid (Seibert, Pfaff & Seibert, 1948), diphenylamine (Hess, Hahn & Ayala, 1956), the Bial and direct Ehrlich tests (Werner & Odin, 1952), sulphuric acid–acetic acid mixture (Hess, Coburn, Bates & Murphy, 1957) and resorcinol (Svennerholm, 1958). As these methods require heating with strong acids, they cannot distinguish free sialic acids from bound sialic acids, a differentiation necessary to follow the enzymic release of sialic acids from the sialomucoids. This paper describes two procedures, the thiobarbituric acid and the alkali–Ehrlich methods, which have been developed specifically for this purpose. A preliminary account of this work has already appeared (Aminoff, 1959).

A modification of the thiobarbituric acid method for the determination of sialic acid was developed independently by Warren (1959*a*, *b*). The work described here was carried out at about the same time, and there appear to be sufficient differences in the conditions of the reaction and the interpretation of the mechanism to warrant a detailed presentation.

EXPERIMENTAL

Materials and methods

Derivatives of neuraminic acid. *N*-Acetylneuraminic acid was isolated from bovine colostrum and crystallized from methanol. It had the following analytical values: decomposition temperature 171°, $[\alpha]_D^{26} - 36 \pm 1°$, Kjeldahl nitrogen 4·9% and equiv. wt. 299 (for $C_{11}H_{19}O_8N$ mol.wt. 309 and N 4·53%). Both *NO*-diacetylneuraminic acid isomers, obtained from bovine and equine submaxillary mucins (Blix, Lindberg, Odin & Werner, 1956), were kindly made available by Professor G. Blix. A sample of *N*-glycolylneuraminic acid, obtained from pig submaxillary mucin, was kindly provided by Professor E. Klenk. Methoxyneuraminic acid, prepared from bovine submaxillary mucin, was also a gift from Professor Klenk.

Sialomucoids. Through the courtesy of many investigators, samples of the following sialomucoids were made available: bovine salivary mucoid, ox serum mucoid, urine mucoid inhibitor (Dr R. Heimer), crystalline human serum α_1-glycoprotein (Dr K. Schmidt) and colominic acid (Dr W. F. Goebel and Dr S. Roseman).

Bovine colostrum sialomucoids. Skimmed colostrum was exhaustively dialysed against distilled water, diluted with an equal volume of water and made 1 mM with respect to $CaCl_2$. The casein was removed by coagulation with commercial rennin and the supernatant treated with 2 vol. of acetone. The resulting precipitate was extracted with water and the fraction that was obtained between 33 and 50% (v/v) of ethanol contained most of the sialic acid. This material readily dissolved in water to give a clear solution and had 6–7% of sialic acid.

Collocalia *sialomucoids.* 'Edible bird's nest' is predominantly a salivary mucoid with which the *Collocalia* species of swift builds its nest. It is a commercially available oriental delicacy. Insoluble in water, it is readily solubilized by treatment of the finely ground nest with ficin (Lawton, McLoughlin & Morgan, 1956). The solubilized nest was heated in a boiling-water bath for 10 min. to denature extraneous proteins, and the supernatant fluid dialysed to remove low-molecular-weight components and then freeze-dried. The preparations were electrophoretically heterogeneous and contained 10–12% of sialic acid.

Application of Hestrin's (1949) hydroxylamine test for esters and lactones indicated the presence of such groups in colominic acid and Blix's bovine sialic acid, but none in *N*-acetylneuraminic acid or the sialomucoids from bovine colostrum or *Collocalia* salivary mucin.

Determination of N-*acetylneuraminic acid by the thiobarbituric acid method*

Reagents. (*a*) Periodate: 25 mM-periodic acid in 0·125N-H_2SO_4 (pH 1·2). (*b*) Sodium arsenite: 2% solution of sodium arsenite in 0·5N-HCl. (*c*) Thiobarbituric acid: 0·1M solution of 2-thiobarbituric acid (Eastman Organic Chemicals Department, Eastman Kodak Co.) in water, adjusted to pH 9·0 with NaOH. It keeps well for about a month in a dark bottle at 4°. (*d*) Acid butanol: butan-1-ol containing 5% (v/v) of 12N-HCl.

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Procedure. A solution of the sample, blank or standard (containing 5–40 μg. of *N*-acetylneuraminic acid) in 0·5 ml. of water is treated with 0·25 ml. of the periodate reagent for 30 min. in a water bath at 37°. The excess of periodate is then reduced with 0·2 ml. of the sodium arsenite. As soon as the yellow colour of the liberated iodine has disappeared (1–2 min.), 2 ml. of the thiobarbituric acid reagent is added and the test sample is covered and heated in a boiling-water bath for 7·5 min. The coloured solutions are then cooled in ice–water and shaken with 5 ml. of the acid butanol. The separation of the two phases is facilitated by a short, rapid centrifuging and the intensities of the colours in the butanol layer are compared at 549 mμ in a spectrophotometer. Despite the apparent discoloration on standing, the extinctions are constant for 2 hr. They decline appreciably in 20 hr.

The absorption spectrum of the colour obtained with *N*-acetylneuraminic acid is identical with that obtained by Warren (1959*b*). The molar extinction at 549 mμ is $70{\cdot}7 \times 10^3$, and the extinction is directly proportional to the concentration of *N*-acetylneuraminic acid.

The oxidation with periodate, resulting in the formation of the chromogen, is more rapid at 37° than at room temperature, and the final colour intensity is dependent on both the pH and the period of oxidation (Fig. 1). The excess of periodate is best removed with acid arsenite; alkaline arsenite, ethylene glycol, glycerol or glucose are not satisfactory as they interfere in the subsequent colour development. The intensity of the colour obtained on heating with thiobarbituric acid is independent of the pH of the reagent over the range 7–10. An alkaline reagent is preferred in order to increase the solubility of the thiobarbituric acid in water, a concentration of 0·1 M being necessary for the maximum colour production.

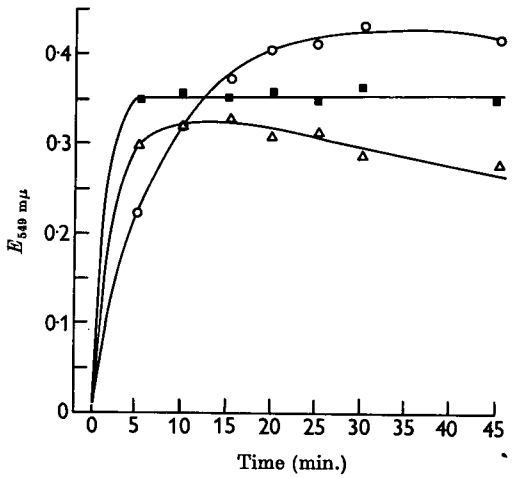


Fig. 1. Effect of the pH of the periodate and the period of oxidation at 37° on the amount of chromogen formed: ○, at pH 1·2; ■, at pH 7·1; △, at pH 8·5.

The coloured complex is both more stable and more soluble in acidified butan-1-ol. The absorption peak of the coloured material is sharper at 549 mμ, and more intense in the butan-1-ol than in the aqueous phase before extraction.

Specificity of the thiobarbituric acid method

Sialomucoids. All the sialomucoids tested give little or no colour. Any interference in the test due to opalescence and discoloration is usually eliminated by the extraction with butan-1-ol.

2-Oxo-3-deoxy sugar acids. Weissbach & Hurwitz (1959) reported that the thiobarbituric acid assay is an excellent method for the estimation of 2-oxo-3-deoxy sugar acids. Under the conditions outlined above, these sugars gave an intense pink colour with the peak also at 549 mμ. Thus a molar extinction of 72×10^3 was obtained with 2-oxo-3-deoxygluconic acid (a gift of Dr E. Racker).

Deoxyribose, 2-aminopyrimidine and sulphadiazine. Waravdekar & Saslaw (1957, 1959) were the first to apply the known reactivity of thiobarbituric acid with aldehydes (Patton & Kurtz, 1951) to the determination of 2-deoxyribose after oxidation with periodate. The periodate oxidation products of both 2-aminopyrimidine and sulphadiazine have also been shown to react (Kohn, 1945; Shepherd, 1948). Under the present conditions, these compounds react to give intense colours with the maximum light-absorption also at 532 mμ. The molar extinction of the colour given by deoxyribose was 124×10^3.

All of these compounds will therefore interfere in the estimation of *N*-acetylneuraminic acid.

Miscellaneous compounds. The following substances were tested and gave no colour: *N*-acetylglucosamine (1 mg.), *N*-acetylmuramic acid (50 μg.), arabinose (1 mg.), ascorbic acid (0·1 mg.), aspartic acid (0·1 mg.), fructose (0·2 mg.), γ-galactonic lactone (0·2 mg.), δ-gluconolactone (0·2 mg.), glucosamine–HCl (1 mg.), glucosaminic acid (1 mg.), histidine–HCl (1 mg.), pyrrole-2-carboxylic acid (100 μg.), ribose 5-phosphate (0·1 mg.), serine (0·1 mg.), streptomycin sulphate (1 mg.), sucrose (0·1 mg.), tartaric acid (0·2 mg.) and threonine (1 mg.).

Under the conditions outlined above, *N*-acetylglucosamine gives no colour. However, when tested in the procedure of Waravdekar & Saslaw (1957) and of Weissbach & Hurwitz (1959), a faint colour was obtained with the maximum absorption of light at 550 mμ. Glucosamine–HCl under the same conditions gave no colour.

Nature of the chromogen

Neither formaldehyde nor formic acid gives a pink colour with thiobarbituric acid under the conditions of the test. The observations of Wilbur, Bernheim & Shapiro (1949) would implicate glyoxylic acid as a possible chromogen. However, glyoxylic acid, obtained by the reduction of oxalic acid with magnesium turnings (Benedict, 1909) or by the oxidation of tartaric acid with periodate (Fleury & Bon-Bernatets, 1936), gave no colour under the conditions of the test.

The coloured complexes of the oxidation products of *N*-acetylneuraminic acid and 2-oxo-3-deoxygluconic acid migrated at the same rate on paper chromatography (with acidified 2-ethoxyethylacetate, *cyclo*hexanone or butan-1-ol as solvent) and on paper electrophoresis at pH 3·45 (citrate–HCl buffer), pH 8·4 (borate buffer) and pH 8·6 (veronal buffer), where the migration was anodic. In contrast, the coloured complex obtained with 2-deoxyribose is neutral and immobile on paper electrophoresis, and on paper chromatography it migrated faster than did the coloured complexes of *N*-acetylneuraminic acid and 2-oxo-3-deoxy sugar acids.

These results imply an identity of the coloured complexes obtained, and hence also of the chromogenic oxidation products obtained with *N*-acetylneuraminic acid and 2-oxo-3-deoxygluconic acid. Unfortunately, this could not be confirmed owing to the apparent lability of the chromogens on paper when tested by chromatography and electrophoresis.

Only 6–16% of the total nitrogen was released as NH_3 as a direct consequence of the oxidation of *N*-acetylneuraminic acid for 20 min. at 37° with 25 mM-periodate in 0·125N-H_2SO_4.

Determination of N*-acetylneuraminic acid by the alkali–Ehrlich method*

This reaction, obtained with the sialic acids but not with sialomucoids, will be referred to as the alkali–Ehrlich reaction to avoid confusion, and to distinguish it from the many colorimetric reactions involving the use of Ehrlich reagent, such as the Elson & Morgan (1933) method for hexosamines, Morgan & Elson (1934) test for *N*-acetylhexosamine, and the direct Ehrlich test (Werner & Odin, 1952), which does not distinguish free from bound sialic acid.

Reagents. (*a*) Borate buffer, pH 8·5: 0·2M-boric acid solution is adjusted to pH 8·5 with NaOH. (*b*) Ehrlich reagent: 1·33% solution of *p*-dimethylaminobenzaldehyde (Eastman Kodak Co.) is prepared in 95% ethanol–analytical-grade conc. HCl (1:1, v/v) solvent. (*c*) 95% Ethanol.

Procedure. To a solution of the sample, blank or standards (containing 10–100 μg. of *N*-acetylneuraminic acid) in 0·5 ml. of water, is added 0·5 ml. of 0·2M-borate at pH 8·5. The solutions are heated at 100° for 45 min., cooled, treated with 3 ml. of ethanol followed by 1 ml. of the Ehrlich reagent and heated at 70° for a further 20 min. (in covered tubes) to develop the colour. Immediately after cooling, the extinctions are determined at 558 mμ.

The absorption spectrum of the violet colour obtained with 100 μg. of *N*-acetylneuraminic acid is shown in Fig. 2. The molar extinction at 558 mμ is $7{\cdot}7 \times 10^3$, and the extinction is directly proportional to the concentration of *N*-acetylneuraminic acid.

The colour obtained is not stable, fading by some 10% at the end of 1 hr. and with the absorption peak shifting to 560 mμ.

Increasing concentrations of alkali result in a more rapid chromogen formation with, however, a concomitant loss in selectivity as sialomucoids also react. Buffered alkaline solutions are more suitable, and, of the buffers tested, borate gives the most intense colours. At pH 8·5 *N*-acetylneuraminic acid gives the maximum colour with the minimum interference from sialomucoids. At this pH, some 40–50 min. heating at 100° is necessary to obtain complete conversion of *N*-acetylneuraminic acid into the chromogen.

A variation in the concentration of *p*-dimethylaminobenzaldehyde from 1·0 to 2·5% in the

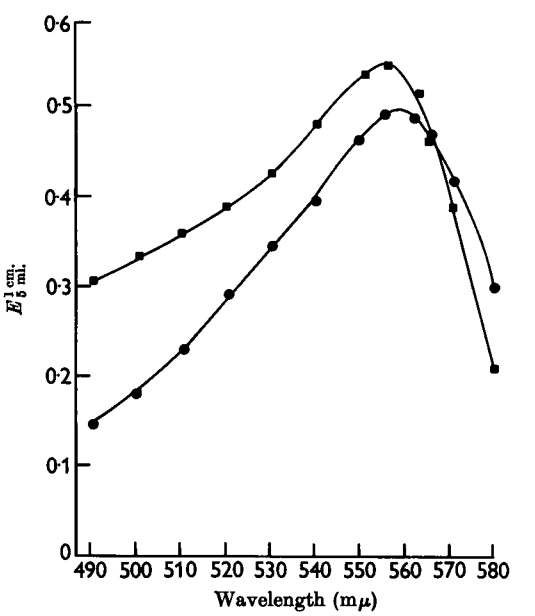


Fig. 2. Absorption spectra of the coloured complexes obtained with (●) *N*-acetylneuraminic acid (100 μg.) and (■) 2-carboxypyrrole (1 μg.) in the alkali–Ehrlich procedure.

Ehrlich reagent has no effect on the intensity of the colour obtained. The concentration of 1·33% was used for convenience, as the same reagent is used in the estimation of hexosamines (Elson & Morgan, 1933). Less acid in the Ehrlich reagent results in slower colour development; stronger acid gives rise to a more-labile coloured complex.

Specificity of the alkali–Ehrlich method

Sialomucoids. Most of the sialomucoids tested give a negligible amount of colour.

2-Carboxypyrrole. An immediate and intense purple colour is obtained on adding the Ehrlich reagent to 2-carboxypyrrole in the cold. In the procedure for estimation of *N*-acetylneuraminic acid, 2-carboxypyrrole gives a pinker colour and the two absorption spectra are quite distinct, although both show peaks of absorption at 554–560 mμ (Fig. 2). The intensity of colour given by 2-carboxypyrrole at 555 mμ is equivalent to a molar extinction of 309×10^3.

Hexosamines and derivatives. Glucosamine–HCl (1 mg.), glucosaminic acid (1 mg.) and *N*-acetylmuramic acid (50 μg.) give no colour at all. *N*-Acetylglucosamine gives but a trace of colour, equivalent to 0·014 mole of *N*-acetylneuraminic acid.

Miscellaneous. The following substances in 1 mg. amounts give no detectable colour: L-glutamine, D-glutamic acid, L-histidine, 2-oxo-3-deoxygluconic acid (23 μg.), proline and tryptophan. L-Hydroxyproline gives a very faint pink colour with a peak of absorption at 560 mμ, and 1 mole gives an intensity equivalent to 0·01 mole of *N*-acetylneuraminic acid. Indole gives a strong pink colour with cold Ehrlich reagent, which intensifies on heating at 70°. Under the conditions of the test, it gives a double-peaked absorption curve with maxima at 540 and 560 mμ, and an intensity equivalent to a molar extinction of 146 and 159×10^3 respectively.

Neither fructose (1 mg.) nor lysine–HCl (0·1 mg.) alone or in admixture in this proportion gives any colour in this test, in contrast with the faint but distinct colours that they give in the hexosamine and *N*-acetylhexosamine tests (Immers & Vasseur, 1950, 1952; Aminoff, Morgan & Watkins, 1952).

Nature of the chromogen

Heating with the borate buffer at pH 8·5 converts the *N*-acetylneuraminic acid into a derivative with marked absorption in the ultraviolet. Even though the u.v. absorption of the heated product continues to rise fairly uniformly throughout the 105 min. investigated, the rate of colour formation resulting from the addition of the Ehrlich reagent indicates a diphasic reaction with the point of inflexion after approx. 25 min. heating with the borate (Fig. 3). The u.v.-absorption spectra of the solutions of *N*-acetylneuraminic acid for various periods of heating are shown in Fig. 4. They appear similar to that given by 2-carboxypyrrole in 0·1 M-borate, pH 8·5, with the maximum absorption at 257 mμ and a molecular extinction of 11 500.

The chromogens obtained on heating *N*-acetylneuraminic acid with borate buffer were examined by paper electrophoresis and partition chromatography. Ehrlich reagent (as modified by Partridge, 1948) was used to spray the strips and, as has been previously observed by Berggård & Odin (1958), butanol–acetic acid–water (4:1:5) gives the best resolution. With this solvent system, at least four visible spots were obtained with R_F 0·14, 0·41, 0·67 and 0·87. The chromogen with R_F 0·41 predominates in the material heated for 45 min. and decreases with the longer heating periods. It would thus appear to be the principal chromogen involved in the alkali–Ehrlich estimation.

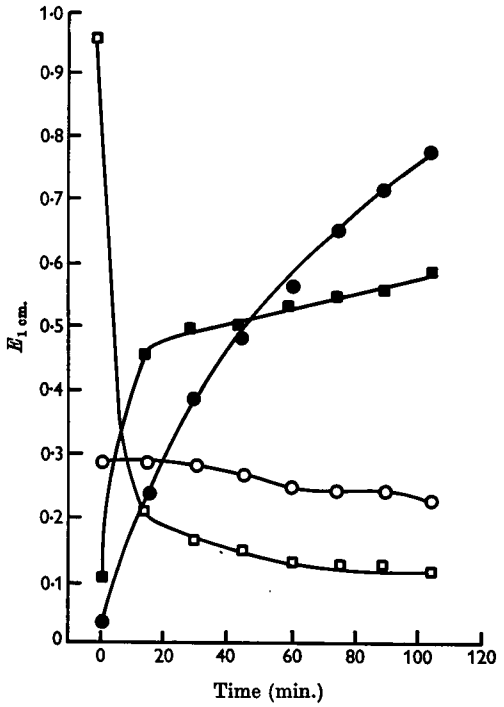


Fig. 3. Effect of the period of heating of *N*-acetylneuraminic acid in borate buffer, pH 8·5 (0·1 M), on: ●, *E* at 260 mμ; ■, subsequent colour development (at 558 mμ) on the addition of the Ehrlich reagent as in the alkali–Ehrlich procedure. ○, □, Stability of the sialic acid under these conditions as determined by the resorcinol and thiobarbituric acid procedures respectively.

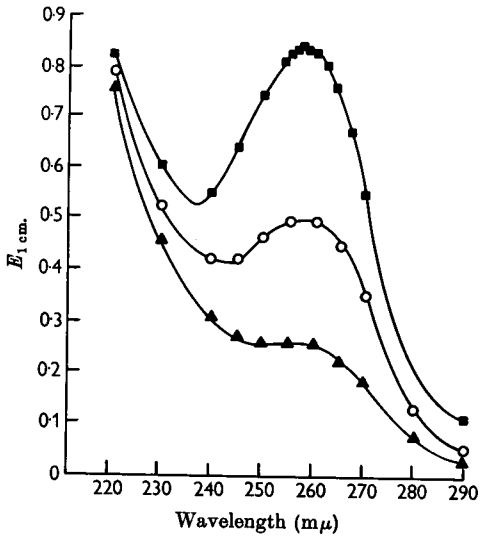


Fig. 4. Absorption spectra of *N*-acetylneuraminic acid (100 μg.) heated at 100° for various periods in 0·1 M-borate buffer, pH 8·5: ▲, 15 min.; ○, 42 min.; ■, 105 min.

Table 1. *Comparison of the reactivity of various derivatives of neuraminic acid in the thiobarbituric acid and alkali–Ehrlich determinations*

Conditions of the determinations are described in the text. Derivatives of neuraminic acid were used without prior drying over P_2O_5.

Substance	$10^{-3}\epsilon$ Thiobarbituric method (549 mμ)	$10^{-3}\epsilon$ Alkali–Ehrlich method (558 mμ)
N-Acetylneuraminic acid	70·7	7·7
N-Glycolylneuraminic acid	44·4	7·9
NO-Diacetylneuraminic acids:		
(*a*) Bovine	0	6·1
(*b*) Equine	65·4	6·1
Methoxyneuraminic acid	0	0

Applications of the two methods of determining N-acetylneuraminic acid

Comparison of derivatives of neuraminic acid. The results obtained with the various derivatives of neuraminic acid are expressed in terms of molecular extinction and summarized in Table 1. The reactivity of bovine sialic acid in the thiobarbituric acid test can be greatly increased on removal of the *O*-acetyl groups by cold alkali or mild acid hydrolysis (1 hr. with 0·01 N-HCl at 84°). The reactivity of the bovine sialic acid in the alkali–Ehrlich procedure can be attributed to the rapid hydrolysis of the *O*-acetyl group under the alkaline conditions of the test.

Stability of N-acetylneuraminic acid to acid and alkali. Table 2 summarizes the results obtained on treatment of *N*-acetylneuraminic acid with acid. Confirming the observations of Svennerholm (1958), *N*-acetylneuraminic acid appears to be fairly stable when heated in 0·1 N-HCl at 84° as determined by the resorcinol method. However, when assayed by the thiobarbituric acid and alkali–Ehrlich methods, there are distinct indications of degradation, the moiety essential for a positive colour with thiobarbituric acid being the more labile. With 0·01 N-HCl at 100° the degradation is more enhanced. Neither the original solution of *N*-acetylneuraminic acid nor the resulting acid-treated material reacts with hydroxylamine, and the possibility of lactone formation on heating with the HCl is thereby eliminated.

The thiobarbituric acid-reacting moiety of *N*-acetylneuraminic acid is also extremely unstable in alkali, as seen in Fig. 3. Again this marked lability of *N*-acetylneuraminic acid to alkali is not as readily detected by the resorcinol method.

Rate of release of N-acetylneuraminic acid from sialomucoids on acid hydrolysis. Three sialomucoids were chosen for this study: solubilized *Collocalia* mucoid, the sialomucoids from bovine colostrum and colominic acid, presumably a polymer of sialic acid (Barry, 1958).

Table 2. *Stability of N-acetylneuraminic acid to acid as determined by the resorcinol, alkali–Ehrlich and thiobarbituric acid methods*

In each case the colour produced is expressed as a percentage of that given by the untreated substance.

Period of heating (min.)	0·1 N-HCl at 84°: Resorcinol (580 mμ)	Alkali–Ehrlich (558 mμ)	Thiobarbituric acid (549 mμ)	0·01 N-HCl at 100°: Resorcinol (580 mμ)	Alkali–Ehrlich (558 mμ)	Thiobarbituric acid (549 mμ)
5	99	99	100	105	99	100
30	95	92	98	98	100	80
60	96	80	83	98	91	65
120	88	81	77	82	84	44
240	80	71	61	58	65	26

The rate of release of sialic acid from the sialomucoid of *Collocalia* by acid hydrolysis is almost the same when determined by the thiobarbituric acid or alkali–Ehrlich procedure (Fig. 5). A similar behaviour was also observed with bovine colostrum. However, with colominic acid, there is an apparently greater release of *N*-acetylneuraminic acid as measured by the alkali–Ehrlich method (Fig. 5). This suggests that the sialic acid, as released from colominic acid by mild acid hydrolysis, is still substituted in such a way that it does not give the full colour with the thiobarbituric acid assay. This substituent is presumably alkali-labile and thereby does not interfere in the alkali–Ehrlich determination. The positive hydroxylamine test given by colominic acid and the isolation of *NO*-diacetylneuraminic acid (the bovine isomer) from the hydrolysis products of the endotoxins of *Escherichia coli* (DeWitt & Rowe, 1959) suggest that this substituent is the *O*-acetyl group.

Two further points emerge from these studies and are illustrated in Fig. 6. First, the sialic acid of *Collocalia* mucoid, in contrast with that of colostrum or colominic acid, appears to be rapidly degraded by the acid when assayed by the thiobarbituric acid method. Secondly, this rapid degradation is not reflected in the resorcinol and alkali–Ehrlich procedures (see also Table 2), which would suggest that the thiobarbituric acid assay involves a more 'labile' moiety of the sialic acid molecule.

Release of sialic acid from sialomucoids by the sialidase of Vibrio cholera. A partially purified specimen of sialidase prepared from *Vibrio cholera* was used for these studies. The rate of release of sialic acid from the sialomucoids of *Collocalia* salivary mucin and bovine colostrum is shown in Fig. 7. The course of hydrolysis was followed by the thiobarbituric acid and the alkali–Ehrlich procedures. There is a rapid release of sialic acid, with little or no subsequent loss of free sialic acid, indicating the absence of a *N*-acetylneuraminic acid aldolase (Comb & Roseman, 1958) in this preparation of the sialidase.

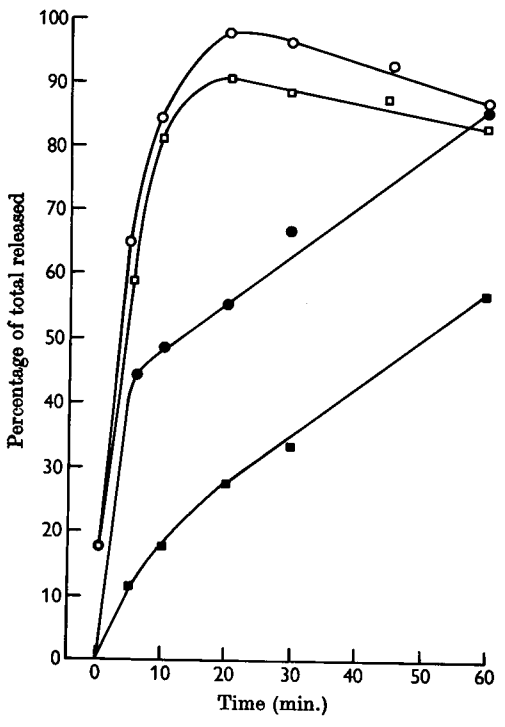


Fig. 5. Rate of release of *N*-acetylneuraminic acid by hydrolysis with 0·1 N-HCl at 84° from *Collocalia* mucoid as determined by alkali–Ehrlich (○) and thiobarbituric, acid (□) procedures; and from colominic acid by alkali–Ehrlich (●) and thiobarbituric acid (■) procedures.

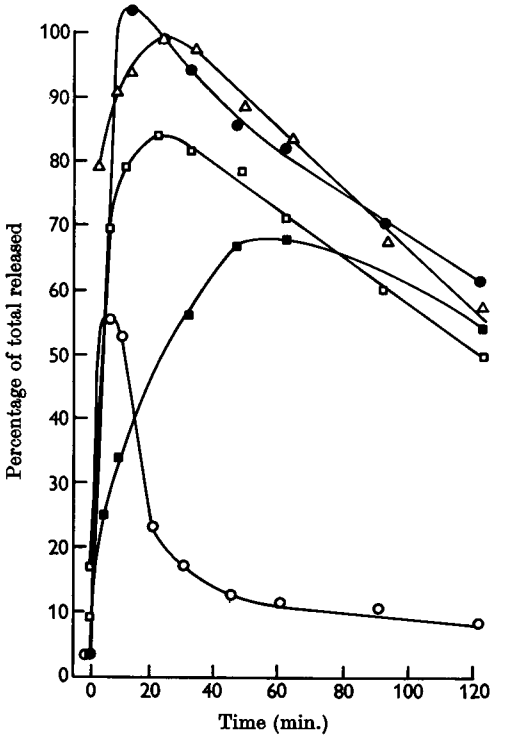


Fig. 6. Rate of release of *N*-acetylneuraminic acid by hydrolysis with 0·01 N-HCl at 100° as determined by the thiobarbituric acid procedure. ●, Bovine colostrum; ■, colominic acid; ○, *Collocalia* sialomucoids; △, □, sialic acid content of the *Collocalia* mucoid simultaneously determined by the resorcinol and alkali–Ehrlich procedures.

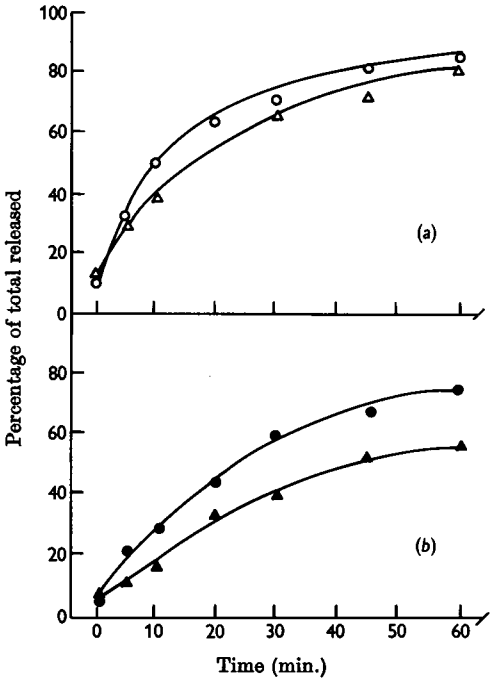


Fig. 7. Rate of release of *N*-acetylneuraminic acid by hydrolysis at 37° with the *Vibrio cholera* sialidase. (*a*) *Collocalia*, (*b*) bovine colostrum sialomucoids, as determined by (○, ●) the thiobarbituric acid and (△, ▲) the alkali–Ehrlich procedures.

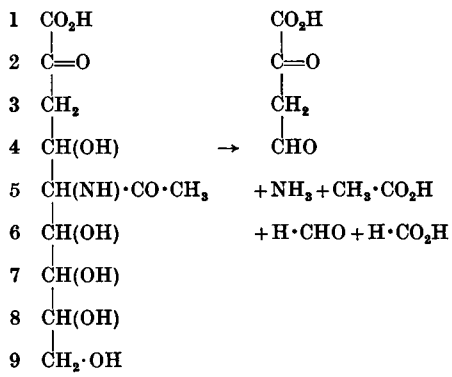
Both methoxyneuraminic acid and colominic acid were unaffected by the sialidase of *Vibrio cholera*, as determined by either the thiobarbituric acid or alkali–Ehrlich procedure.

DISCUSSION

Two alternative methods have been described for the determination of free sialic acid. Together with the classical methods for the assay of total sialic acid (direct Ehrlich test, Bial, resorcinol etc.), it is now possible to determine the total, bound and free, sialic acid in a mixture, and to follow the hydrolysis and degradation of sialic acid from sialomucoids.

The thiobarbituric acid method as outlined here is slightly more sensitive than is the method described by Warren (1959*b*), and, moreover, experience with the two methods has indicated a greater freedom from interference in complex biological systems (S. Roseman, personal communication). A study of the conditions of assay has indicated that the high acidity of the periodate reagent as recommended by Warren (1959*b*) is not essential to obtain a colour. Indeed, a more selective choice of conditions of oxidation can be more informative. For instance (*a*) in the assay here presented, the thiobarbituric acid method can distinguish *N*-acetylneuraminic acid from the bovine *NO*-diacetylneuraminic acid, and (*b*) oxidation at pH 1·2 and 7 will distinguish between acid-labile compounds and free sialic acid. Thus the cytidine monophosphate derivative of sialic acid (Comb, Shimizu & Roseman, 1959) reacts quantitatively when oxidized at pH 1·2, and gives but a trace of colour when oxidized at pH 7, whereas sialic acid still gives an intense colour (Fig. 1).

The evidence presented above indicates that the coloured complex given by *N*-acetylneuraminic acid in the thiobarbituric acid procedure is identical with that obtained with 2-oxo-3-deoxy sugar acids. According to Weissbach & Hurwitz (1959) and Srinivasan & Sprinson (1959), β-formyl pyruvate is the periodate oxidation product of 2-oxo-3-deoxy sugar acids responsible for giving a colour with the thiobarbituric acid reagent. One should therefore postulate an oxidative cleavage of the *N*-acetylneuraminic acid by periodate between C-4 and C-5.



It is difficult, however, to reconcile this interpretation of the mechanism of the reaction with the behaviour of certain derivatives of neuraminic acid in the thiobarbituric acid procedure. The studies of Blix *et al.* (1956) and Blix (1958) on the structure of the sialic acids suggest that the *O*-acetyl group in the bovine sialic acid is located at C-7, and in the equine isomer at C-4. Contrary to expectation, the equine isomer gave substantial colour whereas the bovine isomer gave no colour at all. Preliminary *O*-deacetylation of the bovine sialic acid by treatment with alkali at room temperature, or by mild acid hydrolysis, results in complete reactivity in the thiobarbituric acid procedure.

The absorption spectrum of the colour obtained by Gottschalk & Lind (1949), under conditions somewhat similar to those used here in the alkali–Ehrlich test, would seem to suggest that the

chromogen obtained might be related to that given by *N*-acetylglucosamine in the Morgan & Elson (1934) test. The subsequent identification of *N*-acetylhexosamine in the alkaline-degradation products of *N*-acetylneuraminic acid by Kuhn & Brossmer (1956) and Zilliken & Glick (1956) would appear to substantiate this possibility. However, the evidence presented here indicates that we are dealing with a different chromogen. The absorption spectrum in both the ultraviolet and visible range for *N*-acetylneuraminic acid and *N*-acetylglucosamine (Aminoff *et al.* 1952) are quite distinct. Moreover, *N*-acetylglucosamine hardly reacts in the alkali–Ehrlich test and, conversely, *N*-acetylneuraminic acid gives no colour in the test for *N*-acetylhexosamines.

Although the evidence reported here, and in the literature, strongly suggests that pyrrole-2-carboxylic acid is one of the end products of the alkaline degradation of sialic acid, the nature of the immediate chromogens obtained in the alkali–Ehrlich method of determining *N*-acetylneuraminic acid is still unresolved. More than one chromogen is formed, and the principal chromogen obtained after 45 min. heating in the borate buffer has R_F 0·41 in butan-1-ol–acetic acid–water (4:1:5).

The thiobarbituric acid and the alkali–Ehrlich procedures for the determination of sialic acid are based on different principles, and appear to involve different moieties of the sialic acid molecule. Of the two, the thiobarbituric acid method is superior, not only for its greater sensitivity, but also qualitatively for its greater facility in detecting possible modifications of the sialic acid molecule.

However, following the kinetics of mild acid hydrolysis of sialomucoids by both methods can, as has been illustrated, reveal certain aspects of the structure of the complex mucoids. The results obtained, moreover, emphasize the danger of using the thiobarbituric acid procedure alone to determine the sialic acid content of mucoids, as has been suggested by Warren (1959*b*).

SUMMARY

1. Two colorimetric procedures, the thiobarbituric acid and alkali–Ehrlich, have been developed for the determination of free sialic acid in the presence of bound sialic acid.

2. These methods apparently involve reactions with different moieties of the molecule.

3. Complementing each other and the existing methods of determining total sialic acid in sialomucoids, they serve as valuable tools in the study of the degradation and release of sialic acids from sialomucoids.

4. The structural implications derived from the application of these tests are briefly discussed.

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Studies on Carbohydrate-Metabolizing Enzymes

7. YEAST ISOAMYLASE*

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Although the starch- and glycogen-metabolizing enzymes of higher plants and animals have been intensively studied [for reviews, see Manners (1953) and Whelan (1958)], the related yeast enzymes have attracted comparatively little attention. For example, the presence of phosphorylase in yeast extracts was reported by Schaffner & Specht (1938) and Cori, Colowick & Cori (1938), but the enzyme does not appear to have been highly purified, whereas both potato and muscle phosphorylase have been crystallized. Further, the properties of yeast branching enzyme have only recently been reported (Gunja, Manners & Khin Maung, 1960), although potato Q-enzyme was first investigated 16 years ago.

This paper is concerned with a yeast 'debranching' enzyme, hereafter referred to as isoamylase, which hydrolyses the outer α-(1 $\rightarrow$ 6)-glucosidic inter-chain linkages in amylopectin and glycogen. Nishimura (1931) noted that yeast extracts contained an enzyme, 'amylosynthease', which could liquefy starch and accelerate the action of normal amylases, although it differed in thermolability from α- and β-amylases. Further studies by Nishimura & Minagawa (1931) and Minagawa (1932) showed that enzyme action on glutinous rice starch, which was optimum at pH 6·2 and 20°, caused an increase in iodine-staining power. Amylosynthease was therefore regarded as a starch-synthesizing enzyme. Later, Meyer & Bernfeld (1942) observed that yeast extracts attacked both starch and its β-dextrin. A small increase (7–15%) in the β-amylolysis limit of the latter was noted. The impure enzyme preparation also hydrolysed maltose and isomaltose. The action of amylosynthease on rice starch was re-examined by Maruo & Kobayashi (1951). The product had a lower molecular weight, higher β-amylolysis limit, stained bluish-purple with iodine, and showed a tendency to retrograde from solution. These results were considered to be caused by extensive debranching of the starch, and the new name isoamylase was proposed for the enzyme. This implies a starch-degrading function. This activity is similar to that of R-enzyme on amylopectin (Hobson, Whelan & Peat, 1951).

In view of our interest in the structure and metabolism of glycogen (Manners, 1957), the action of yeast isoamylase on this and related polysaccharides was investigated. It should be noted that R-enzyme has no action on normal glycogen (Peat, Whelan, Hobson & Thomas, 1954; Fleming & Manners, 1958). As stated in the preliminary account of this work (Manners & Khin Maung, 1955) we first encountered isoamylase as an impurity in yeast-phosphorylase preparations.

* Part 6: Liddle, Manners & Wright (1961).

METHODS AND MATERIALS

Analytical methods. The methods used for paper chromatography, the determination of reducing sugars and the analysis of polysaccharides (by α- and β-amylolysis and periodate oxidation) have been described (Gunja *et al.* 1960).

Determination of isoamylase activity. The action of a debranching enzyme causes an increase in both the β-amylolysis limit, and iodine-staining power of glycogen but no appreciable increase in reducing power. The changes may be used as a measure of activity although since neither property is directly related to the number of α-(1$\rightarrow$6)-glucosidic linkages hydrolysed, such values are relative rather than absolute.

Digests containing approximately equal weights of glycogen and isoamylase preparation (usually 1 or 2 mg./ml. in a total volume of 25 ml.) were incubated at pH 5·9