6-α-D-SIALYL-N-ACETYLGALACTOSAMINE: THE NEURAMINIDASE-SUSCEPTIBLE PROSTHETIC GROUP OF BOVINE SALIVARY MUCOPROTEIN*

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SUMMARY

Very mild alkali treatment of bovine salivary mucoprotein resulted in the release of dialysable bound sialic acid. On treatment with ion-exchange resins a semi-crystalline single compound was isolated from the dialysate. The compound was identified as 6-α-D-sialopyranosyl-N-acetylgalactosamine by elementary analysis, the chemical properties of its components, its susceptibility to α-neuraminidase, its oxygen consumption on periodation and its conversion by alkali to sialyl-substituted chromogens. The yield was 44 %. The near coincidence between total sialic acid of bovine salivary mucoprotein and oxidizable N-acetylgalactosamine suggests the isolated disaccharide to be the main, if not the only, prosthetic group of this mucoprotein.

INTRODUCTION

It is now firmly established that all mucoproteins inhibiting influenza virus haemagglutination contain sialic acid (for nomenclature see BLIX, GOTTSCHALK AND KLENK[1]). In fact, the presence of sialic acid in the molecule is an essential factor endowing a mucoprotein with biological activity, as shown by the loss of activity upon removal of sialic acid by the specific enzyme neuraminidase (for review see GOTTSCHALK[2]). The molecular structure of N-acetylneuraminic acid, a widely distributed species of sialic acid, was first suggested[3] and then proven by synthesis[4] to be that of an aldol condensation product of pyruvic acid and N-acetylhexosamine. The steric configuration of the hexosamine fragment of neuraminic acid was recently elucidated[5,6]. Fig. 1 depicts the configuration of the crystalline β-D-N-acetylneuraminic acid.

BLIX and coworkers[7,8] were the first to isolate in crystalline state sialic acid and

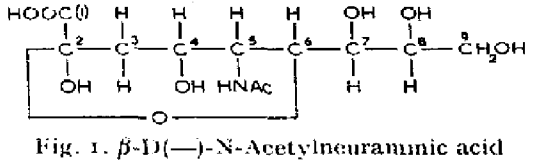

Fig. 1. β-D(—)-N-Acetylneuraminic acid

* This paper is published as part of a tribute from his scientific colleagues to Prof. Dr. E. BERGER, Director of the Virus Laboratory, Children's Hospital, Basle (Switzerland) on the occasion of his 60th birthday.

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D-galactosamine from bovine salivary mucoprotein (BSM) and to recognize them as the main carbohydrate components of this mucoprotein, as distinct from the small amounts of carbohydrate of the serum–mucoprotein type. Further investigations on this virus haemagglutinin inhibitory mucoprotein have shown that dependent on the procedure of its preparation the values for hexosamine and sialic acid vary from 10 to 17 % and from 16 to 28 % respectively. Of the total hexosamine 86 to 92 % is represented by galactosamine, the remainder by glucosamine. All hexosamine residues are N-acetylated. The other sugars present are galactose (0.7 %), fucose (0.7 %) and traces of mannose[9,10]. On mild acid treatment the reducing sialic acid, and sialic acid only, was released from the non-reducing BSM indicating terminal position and engagement in a glycosidic linkage of the acid[11]. When BSM was digested with the receptor-destroying enzyme (RDE) of *Vibrio cholerae*, up to 70 % of the total sialic acid was liberated, thus characterizing the enzyme as glycosidase or ketosidase[10–12]. Subsequently the enzyme was shown to be an O-glycosidase and termed "neuraminidase"[13]. Finally it was recognized as an α-glycosidase[2,14].

When BSM was gently heated with mild alkali, paper partition chromatography revealed the presence in the reaction mixture of a compound split by neuraminidase into sialic acid and a chromogen (in the direct Ehrlich reaction) derived from N-acetylgalactosamine (NAGal) by loss of water[15]. Continued work on the alkaline breakdown products of BSM has resulted in the isolation and purification of an undegraded disaccharide consisting of sialic acid and NAGal. This disaccharide is the main prosthetic group of BSM. The isolation and purification procedures, the properties of the disaccharide, its molecular structure, its susceptibility to neuraminidase and its conversion to sialyl-substituted chromogens in the direct Ehrlich reaction will be described in this paper. These detailed data necessitated reinterpretation of some of the earlier findings[15].

EXPERIMENTAL

Materials

Crystalline 2-acetamido-2-deoxy-D-galactose (N-acetylgalactosamine) was prepared from synthetic galactosamine hydrochloride ($[\alpha]_D^{20}$ + 94°) by treatment with silver acetate and acetic anhydride as described by WHITE[16]: $[\alpha]_{20}^{D}$ + 82° (water); m.p. 163–164°.

Crystalline N-acetylneuraminic acid was prepared according to CORNFORTH, FIRTH AND GOTTSCHALK[1].

The other sugars used were standard preparations exhibiting a single spot on paper chromatography.

Crystalline pyrrole-2-carboxylic acid was obtained by the method of ODDO[17]. Analysis: Found: C 54.07; H 4.73; N 12.92. Calcd. for $C_5H_5O_2N$: C 54.05; H 4.54; N 12.61.

Chromogens I and III were prepared from N-acetylgalactosamine according to KUHN AND KRÜGER[18].

Bovine salivary mucoprotein (BSM). Fresh bovine salivary glands were washed, freed from fat and connective tissue, put through a coarse meat-mincer and extracted with an equal volume of water once at 2° for 9 h and then four times at 2° for 24 h. The first extract was discarded. The other extracts were processed according to

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CURTAIN AND PYE[10], two further steps being added. Immediately before fractionation with methanol the solution was passed through a Seitz filter pad (Horman-Ekwip grade D3), and subsequent to the final dialysis the solution was centrifuged at 3000 rev./min for 30 min at 0°, the supernatant being then used directly or stored at —20°.

Sialic acid from BSM was obtained by heating the mucoprotein with 0.1 *N* H_2SO_4 for 1 h at 80°, followed by neutralization of the mixture to pH 6.5 with $Ba(OH)_2$, dialysis and removal of cations from the concentrated dialysate by means of Dowex 50 (see below). The effluent from the column was treated according to CORNFORTH, FIRTH AND GOTTSCHALK[4].

Influenza B virus (Lee Strain) was purified by procedure 3 as described by GOTTSCHALK AND PERRY[20].

Receptor-destroying enzyme (RDE) of *Vibrio cholerae* was prepared according to ADA AND FRENCH[21] and kindly supplied by them.

Ion exchange resins were used in columns of appropriate size. Dowex 50 – x4 (50–100 mesh) was prepared and regenerated according to BOAS[22]. Dowex 1 – x2 (50–100 mesh) was converted to the formate form by the procedure of SVENNERHOLM[23].

Crystalline N-acetyl-D-glucosamine-anilide was kindly donated by Professor F. WEYGAND, Munich.

Methods

Sialic acid was determined by BIAL's orcinol method in the modification by SVENNERHOLM[24] using N-acetylneuraminic acid (NANA) as standard. All sialic acid values quoted are expressed as NANA. For the determination of sialic acid bound in the disaccharides a solution of the latter was heated at pH 1–2 for 45 min at 80° and the sialic acid adsorbed to Dowex 1 - formate using 0.3 *N* formic acid for subsequent elution. Authentic NANA treated in the same way was used as standard.

Hexosamine determinations were carried out according to RONDLE AND MORGAN[25] on the vacuum-dried acid hydrolysate of the test material. Unless otherwise stated all hexosamine values refer to the free base. In order to liberate hexosamine from BSM, the latter was hydrolysed with 2 *N* HCl for 18 h at 100°. It was found that hydrolysis for more than 18 h did not increase the hexosamine values: 75, 85 and 92 % respectively of the maximum value being obtained after 3, 6 and 9 h hydrolysis. A correction factor of 1.05 was applied to the hexosamine value obtained to allow for destruction of N-acetylgalactosamine and/or galactosamine, based on the recovery of hexosamine after treatment of N-acetylgalactosamine with 2 *N* HCl at 100° for 15 h. In the case of the disaccharide the hexosamine determination was preceded by treatment with *N* HCl for 4 h at 100°. As standard in this determination N-acetyl-galactosamine similarly treated was used. On several occasions the hexosamine present in the hydrolysate was isolated according to BOAS[22] prior to the determination; this precaution had no significant effect on the result.

Glycolic acid was determined according to KLENK AND UHLENBRUCK[26].

Periodate oxidations were carried out with a threefold excess of periodate in the dark; 0.01 *M* KIO_4 solutions were used. The disaccharide was oxidized at pH 4.5 and 7° for specified times. The excess periodate was reduced to iodate at neutral pH using an aqueous "quenching" solution, and the excess of standard sodium arsenite was back-titrated with standard iodine as described by JACKSON[27]. BSM was oxidized at pH 4.5 under specified conditions, the excess periodate reduced by ethylene glycol,

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and the mixture dialysed exhaustively against water. In a standard experiment excess of periodate was ascertained by back-titration at the end of the oxidation period.

Dry matter: an aliquot of the mucoprotein solution was dried at 92° and weighed.

Paper partition chromatography was carried out as described by GOTTSCHALK[8,38]. Unless specified otherwise the solvent used was *n*-butanol–pyridine–water (6:4:3, v/v).

Preparation of 6-α-D-sialyl-N-acetylgalactosamine and sialyl-substituted chromogens

Three hundred ml of 0.6% BSM solution was adjusted to pH 8 with saturated aqueous $Ba(OH)_2$ solution and quickly brought to 80° in a boiling water bath. After addition of solid $Ba(OH)_2 \cdot 8H_2O$ to a final concentration of 0.01 *N*, the mixture was transferred to an 80° water bath and kept there for 15 min, the pH being readjusted to 10.5 at short intervals (because of liberation of acetic acid from O-acetyl groups). After cooling in ice the mixture was neutralized to pH 6.5 with *N* H_2SO_4 and dialysed against ten volumes of distilled water for 60 h at 0–2° with occasional stirring. The dialysate was then concentrated *in vacuo* at 36° to a volume of about 30 ml and the concentrate applied to a Dowex 50 (H^+ form) column. The column was rinsed with water from a reservoir and the acidic portion of the effluent collected. A small aliquot of this effluent and of the original BSM solution were analysed for sialic acid. The sialic acid values of 300 ml of the BSM solution and of the total effluent were found to be 383 mg and 34.5 mg respectively, *i.e.* during the above alkaline treatment of BSM 9% of the total sialic acid was rendered dialysable (not allowing for loss due to equilibrium across the membrane).

The effluent was put on a Dowex 1-formate column which was washed with water until the direct Ehrlich reaction of the effluent (free chromogen I) became negative. Gradient elution with formic acid (0.05 to 0.4 *N*) was effected, the orcinol-positive eluate collected in eighteen 100 ml fractions, and a concentrated sample of each fraction was chromatographed. The first ten fractions, giving an orcinol reaction and indirect Ehrlich reaction and direct Ehrlich reaction on heating only and running on the chromatogram as a single compound, not coincident with free NANA, were pooled and lyophilized. This pool is referred to as fraction I. The lyophilized material was snow-white and weighed 25 mg.

The residual fractions were also pooled and lyophilized and are referred to as fraction II. The yield was about 22 mg. The chromatogram of this fraction revealed three individual spots reacting both with direct Ehrlich reagent in the cold and with orcinol reagent. In addition the orcinol reagent showed a weak spot corresponding to that given by fraction I.

The whole procedure may be repeated many times with the same BSM solution without much decrease in yield of fractions I and II. As it is difficult to avoid infection of the solution over a longer period, it is not advisable to repeat the procedure more than 4 or 5 times.

RESULTS

A. *Identification of fraction I as 6-α-D-sialopyranosyl-N-acetylgalactosamine*

1. *Elementary analysis and general properties.* The snow-white material was crystalline in appearance. Analysis of fraction I (by Microanalytical Laboratory, C.S.I.R.O.): Found: C, 44.57; H, 6.06; N, 5.32. Calculated for $C_{19}H_{32}O_{14}N_2$: C, 44.52; H, 6.29; N, 5.47. It dissolved readily in water yielding in 1% concentration

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a solution of pH about 2. Even when kept *in vacuo* at 0°, the compound slowly degraded from the reducing end.

The material strongly reduced Benedict's reagent. The rate of reduction coincided with that of N-acetylgalactosamine (NAGal). It was noted that the rate of reduction of NAGal is much higher than that of N-acetylglucosamine (NAG). Thus with equimolar amounts of NAGal and the material the reduction of Benedict's reagent was already very marked after 20 and 30 sec respectively, whereas under the same conditions NAG required more than 2 min for the same colour change.

2. *Identification of sialic acid.* Twenty mg of the material was heated in 2 ml of 10 % (w/v) $Ba(OH)_2 \cdot 8H_2O$ at 100° for 8 h, the mixture acidified at 0° with N H_2SO_4 to pH 3 and extracted with ether. When the ether extract was chromatographed on paper, spraying with direct Ehrlich reagent revealed two purple spots at R_F 0.58 and 0.78 coinciding respectively in R_F value, colour shade and stability with authentic pyrrole-2-carboxylic acid and with the compound formed from NAGal upon similar alkaline treatment. The same two compounds were previously obtained by alkaline treatment of BSM[28]. When sialic acid was released from the material by hydrolysis (pH 2, 80°, 45 min) and absorbed to and eluted from Dowex 1-formate, alkaline treatment of the eluate, as above, resulted in ether-extractable material which, on chromatography, displayed only the spot coinciding with pyrrole-2-carboxylic acid. When the Dowex 1 eluate was tested in the orcinol reaction, the iso-amyl alcohol extract had an absorption maximum at 575 mμ identical with that of authentic NANA similarly treated.

BSM is known to contain N-acetyl-O-diacetylneuraminic acid and N-glycolylneuraminic acid, the latter possibly also being O-acetylated[29]. All O-acetyl groups will be removed from BSM by the alkali treatment used in the detachment of the prosthetic group. On analysis of the isolated sialic acid from the BSM preparation, a glycolic acid content of 3.4 % was found, *i.e.* 14.5 % of the total sialic acid is N-glycolylneuraminic acid. The effect on the elementary analysis of replacement of N-acetyl by N-glycolyl in 14.5 % of the sialic acid residues falls within the limits of the error. When the isolated sialic acid was chromatographed in *n*-butanol–*n*-propanol–0.1 N HCl (1:2:1, v/v) and sprayed with resorcinol–trichloracetic acid[30], a purple spot at R_F 0.44 (coinciding with NANA standard) and a very faint spot at R_F 0.33 (N-glycolylneuraminic acid) were observed.

3. *Characterization of the amino sugar.* 2.5 mg of the material dissolved in 0.8 ml water (final pH 2) was heated at 80° for 40 min, the solution concentrated and chromatographed on paper. Indirect Ehrlich spray disclosed purple spots at R_F 0.12, coinciding with NANA standard, and at R_F 0.34, coinciding with NAGal standard; no spot corresponded to NAG standard at R_F 0.38. There was, however, a weak purple spot at R_F 0.44 due most probably to N-acetyl-talosamine known to have this R_F value in the solvent system used[31].

10 mg of the material was treated with N HCl at 100° for 4 h, and the filtered solution applied to Dowex 50 (H^+ form). The eluate, concentrated and chromatographed, showed a single spot at R_F 0.17 (D-galactosamine–HCl standard R_F 0.17, D-glucosamine–HCl standard R_F 0.20) when sprayed with ninhydrin or the ELSON-MORGAN reagents[32]. When the eluate was analysed for hexosamine, the resulting coloured solution exhibited maximum absorption at 530 mμ as do similarly treated 2-deoxy-2-aminohexoses. Oxidation of the eluted material with ninhydrin followed

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by chromatography resulted in a single spot coinciding in R_F value and colour shade (aniline hydrogen phthalate spray) with authentic lyxose (lyxose standard R_F 0.42, arabinose standard R_F 0.35).

4. *Ratio of components in the material.* The sialic acid content of 2.2 mg of the material was found to be 1.30 mg (as NANA). Since 14.5 % of the total sialic acid is N-glycolyl-neuraminic acid, and since this species in the orcinol reaction has a 30 % higher molar absorbancy than NANA[33], the recovery of sialic acid was 95 % of the theory.

The hexosamine content of 1.8 mg of the material was found to be 0.50 mg, *i.e.* 80 % of the theory.

Therefore the ratio hexosamine/sialic acid = 0.84.

5. *Enzymic hydrolysis of the compound.* 8.7 mg of the material was dissolved in 0.8 ml water, the pH adjusted to 6.5 and 0.2 ml taken as control. The residual was digested with 40,000 units RDE (27 μg dry weight) at 35° for 6 h. Control and assay were treated with Dowex 50 (H^+ form), concentrated to half their original volumes and chromatographed. The results are shown in Fig. 2 under A and AE. After removal

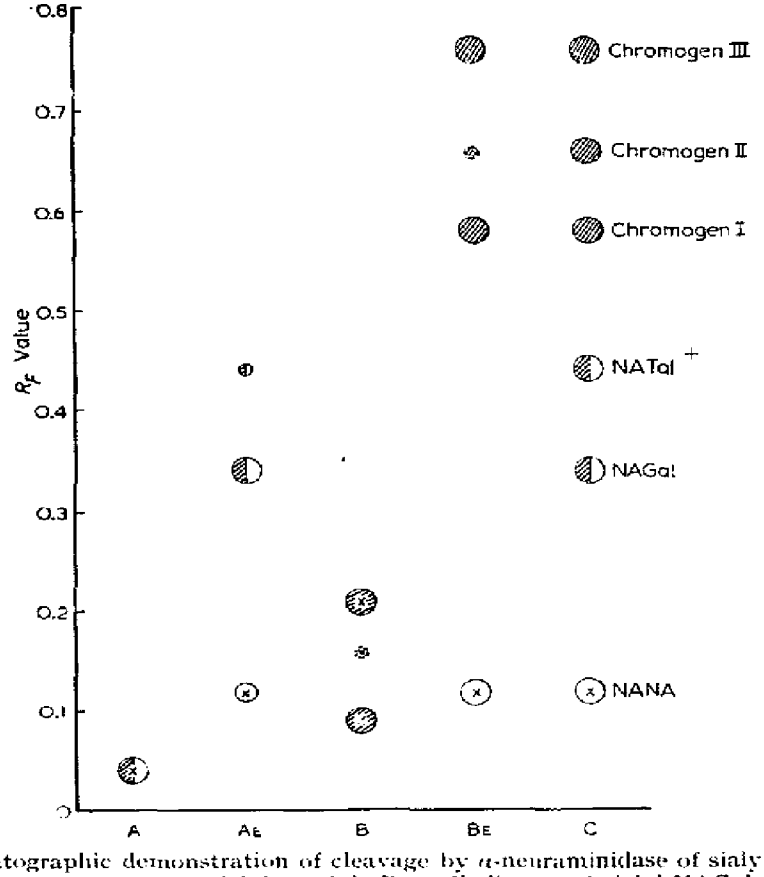

Fig. 2. Chromatographic demonstration of cleavage by α-neuraminidase of sialyl-NAGal before and after alkali treatment. A = sialyl-NAGal; B = alkali-treated sialyl-NAGal; AE and BE = A and B after treatment with neuraminidase; C = reference substances. Spots shown: ◐ gives reddish-purple colour with indirect Ehrlich reagents; ● gives a reddish-purple colour with Ehrlich spray in the cold; × gives a reddish colour with orcinol spray. ○ not regularly observed. Solvent: *n*-butanol–pyridine–water, 6:4:3 v/v. + R_F value according to CRUMPTON[31].

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of the anions by Dowex I the rechromatographed assay gave only the spots corresponding to N-acetylhexosamines.

6. *Periodate oxidation of the material.* 4.2 mg of the material and 2.1 mg of NAGal respectively were oxidized at pH 4.5 with excess KIO_4 at 7° and the IO^-_4 consumption determined at various intervals. The results are shown in Fig. 3.

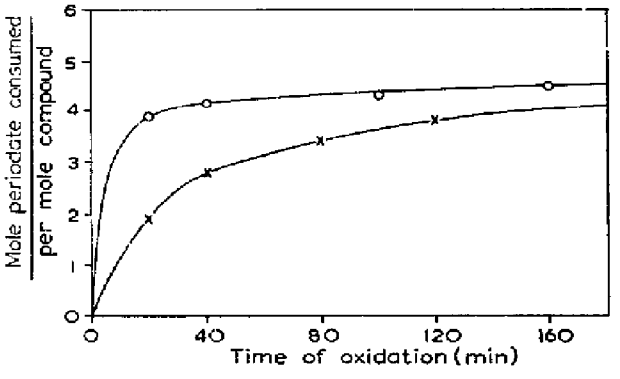

Fig. 3. Consumption of periodate by sialyl-NAGal and by NAGal. Conditions: 0.01 *M* KIO_4, 7°. O = sialyl-NAGal, X = NAGal.

7. *Conversion of the material by alkali to sialyl-substituted chromogens and their enzymic cleavage.* 2 mg of the material was dissolved in 1.0 ml of 0.1 *N* Na_2CO_3 solution and heated for 5 min at 80°. After removal of the cations half of the solution (S) was treated with RDE (10,000 units) at pH 6.5 and 35° for 4 h, the cations again removed and the resulting mixture, together with the other half of S, chromatographed. The results are seen in Fig. 2 under B and BE. The spot at R_F 0.66, which was not seen in all experiments, coincided with a chromogen sometimes observed when NAGal was heated in dilute alkali; it was designated as chromogen II by KUHN AND KRÜGER[18].

In order to correlate the free chromogens I, II and III to their respective bound chromogens, in a similar experiment the zones corresponding to the bound chromogens (R_F 0.09 and 0.21) were eluted from the chromatogram. The eluates, after concentration, were appropriately treated with RDE and then rechromatographed. The resulting spot of free chromogen I corresponded to the bound chromogen at R_F 0.09 and that of free chromogen III to the bound chromogen at R_F 0.21. The same results were obtained when the bound chromogens were treated with influenza B virus (Lee strain).

When fraction II was chromatographed before and after RDE treatment, the same picture with regard to bound and free chromogens was obtained as that described above for fraction I after alkali treatment, and after alkali treatment followed by digestion with RDE.

B. *Hexosamine analysis of BSM before and after periodate oxidation*

Analysis of BSM for hexosamine and sialic acid gave mean values of 14.8 ± 0.51 and 22.4 ± 0.16 respectively. The ratio of galactosamine to glucosamine was found to be 6:1, as assessed chromatographically.

For the periodate experiments freshly prepared BSM solutions, not previously

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frozen, were used. In aliquots of this material the dry weight, sialic acid content and hexosamine content were determined. About 5 mg BSM (1.0 ml of solution) was oxidized with periodate and subsequently analysed for hexosamine. In two experiments dried BSM, obtained by lyophilizing the above BSM solution, was homogenized with water and the reconstituted BSM solution centrifuged at 2000 rev./min for 10 min to remove insoluble material. Lyophilized BSM is less soluble and almost devoid of inhibitory capacity (Lee indicator) indicating partial denaturation. The results including the conditions of oxidation are shown in Table I and Fig. 4.

TABLE I

EFFECT OF PERIODATE OXIDATION OF BSM ON THE HEXOSAMINE RECOVERY

Condition of oxidation		Total hexosamine of oxidized BSM / Total hexosamine of control	Galactosamine oxidized*
°C	Hours	%	%
12	7	53.0	54.9
12	12	52.2	55.9
20	7	50.7	57.6
20	16	45.2	64.9
20	24	38.9	71.3
20	24	37.8	72.6
20	60	29.2	82.6
20	12	27.6**	84.5
20	17	25.1**	87.4

* The glucosamine in BSM is not oxidized (see text).
** In this experiment partly denatured BSM was used (see text).

Chromatographic analysis of hexosamines isolated from BSM before and after periodate oxidation revealed a considerable decrease in galactosamine concentration after oxidation, but no appreciable difference in glucosamine concentration.

By submitting 3.2 mg of aniline-N-acetyl-D-glucosaminide (anilide) to periodate oxidation followed by reduction of excess periodate, acid hydrolysis and the Rondle-Morgan procedure, it was ascertained that the oxidation products of N-acetyl-hexosaminides do not produce a colour with Ehrlich's reagent.

Analyses of oxidized BSM for sialic acid and hexosamine, before and after dialysis, did not show significant variations, indicating no loss of these substances during dialysis.

C. *Yield of sialyl-NAGal and of sialyl-substituted chromogens*

In the experiment detailed under "Preparation", of the total sialic acid (383 mg as NANA) of the BSM treated 9% was rendered dialysable, *i.e.* 34.5 mg. In fraction I, consisting of 25 mg analysed sialyl-NAGal, an equivalent of 15.1 mg = 43.8% of the dialysable sialic acid was recovered. In fraction II, comprising sialyl-chromogen I, sialyl-chromogen III, sialyl-NAGal and sialyl-chromogen II in order of decreasing concentration, 22 mg of material was collected. Assuming an average molecular weight of 494 (sialyl-chromogen I) for the constituents of this fraction, a further 13.8 mg = 40% of the dialysable sialic acid was recovered. Total recovery of dialysable sialic acid as sialyl-NAGal and its conversion products amounted to 84%. It is noteworthy that sialyl-NAGal is a weaker acid than the sialyl-chromogens and that the

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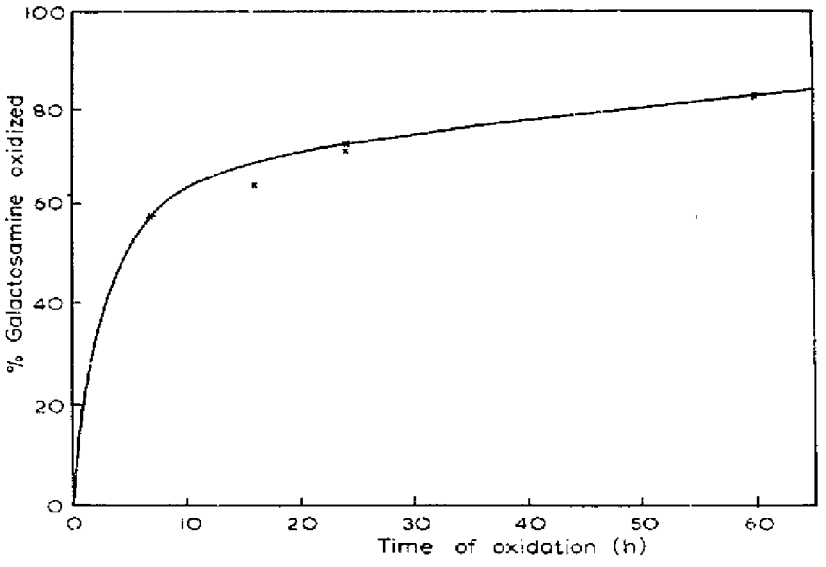

Fig. 4. Progress with time of periodate oxidation of galactosamine of BSM. The galactosamine oxidized is expressed in % of total galactosamine. Conditions of oxidation: 0.01 *M* KIO_4, 20°.

disaccharide is not an inhibitor of haemagglutination by heat-inactivated virus (PR8 and Lee strains).

D. *Yield of N-acetylhexosamine from NANA*

A solution containing 28 mg NANA and having a final $Ba(OH)_2$ concentration of 0.01 *N* was heated at 80° for 15 min, cooled and neutralized with dilute H_2SO_4. After removing and washing the precipitate, the pooled supernatants were concentrated *in vacuo* to a small volume. The N-acetylhexosamine content of the concentrate, as assessed by the semi-quantitative chromatographic method[9], was found to be 0.15 mg, expressed as NAG. Since N-acetylmannosamine is known to be the N-acetylhexosamine portion of sialic acid and to give in the indirect Ehrlich reaction only 55 % of the colour intensity of NAG[5], the recovery indicates that, under the alkaline conditions of BSM treatment, not more than 1.5 % of the sialic acid could be transformed to N-acetylhexosamine or chromogen derived from it.

DISCUSSION

The data presented leave little doubt that the compound isolated from BSM by gentle alkali treatment, purified and collected in fraction I consists of sialic acid (85.5 % N-acetylneuraminic acid and 14.5 % N-glycolylneuraminic acid) and N-acetylgalactosamine in the ratio 1:1. This is evidenced by the elementary analysis of the semi-crystalline compound and by the following results. (1) The specific enzyme neuraminidase splits the compound into two components; the one is chromatographically indistinguishable from authentic NANA, the other is chromatographically identical with NAGal. (2) The acidic component is split off from the compound at pH 2.0 at 80° in 45 min; it is absorbed to and eluted from Dowex I (formate form) under the same conditions as NANA prepared from BSM and it forms with Bial's

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orcinol reagent a reddish-purple colour exhibiting an absorption maximum at the same wavelength (575 mμ) as NANA. (3) On prolonged alkali treatment the acidic component is converted to pyrrole-2-carboxylic acid as is NANA. (4) On heating at 100° with *N* HCl, known to decompose sialic acid, the neutral component is recovered as hexosamine-HCl as shown by its adsorption to Dowex 50 (H^+form) and by the adsorption maximum at 530 mμ of the coloured substance formed when the eluate from the Dowex 50 column is submitted to the Elson-Morgan reaction. Its identity as galactosamine is indicated by the chromatographic coincidence of the neutral component, released enzymically or on mild acid hydrolysis (pH 2), with authentic NAGal in a solvent separating NAGal, N-acetylglucosamine and N-acetyltalosamine. Apparently a very small contribution to the neutral component is made by N-acetyltalosamine. Further evidence for the *cis*-position of the OH-groups at C-3 and C-4 of the hexosamine component is its chromatographic coincidence with authentic D-galactosamine and its oxidation with ninhydrin to lyxose.

In the isolated compound sialic acid is linked α-ketosidically to N-acetylgalactosamine to form a reducing disaccharide, as proved by: (1) α-neuraminidase splits the compound into its components; (2) the rates of reduction of Benedict's reagent by the compound and by NAGal are almost the same; (3) the same chromogens are obtained from free NAGal treated with mild alkali and from the compound treated with mild alkali followed by neuraminidase action; (4) the ease of hydrolysis.

Concerning the carbon atom of NAGal involved in the glycosidic linkage with sialic acid the evidence presented is compatible only with substitution at C-6. The compound, on mild alkali treatment, readily forms a chromogen (for the Ehrlich reaction) with the sialic acid still attached to it in a neuraminidase susceptible linkage. N-Acetylglucosamine substituted at C-4 does not produce a chromogen upon alkali treatment. A 3-substituted N-acetylglucosamine forms a chromogen, but in doing so eliminates the substituent (β-elimination). Furthermore the consumption within

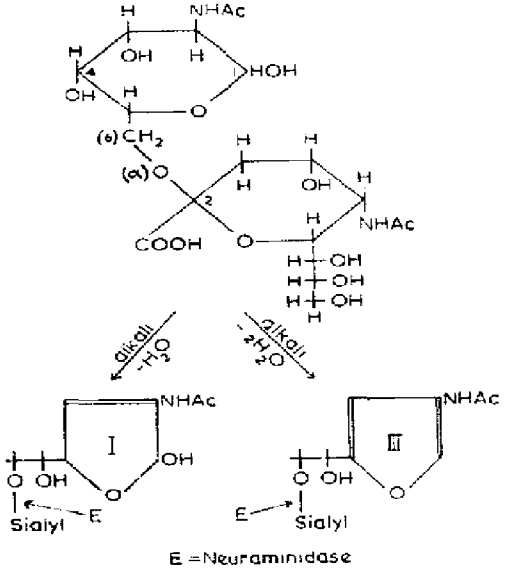

Fig. 5. Conversion by alkali of 6-α-D-sialyl-NAGal to sialyl-substituted chromogens I and III.

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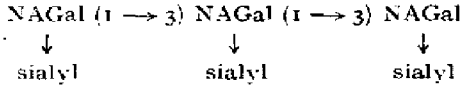
40 min of 4 moles periodate per mole compound clearly indicates that 2 moles periodate are consumed by the NAGal moiety of the compound, since ketosidically bound NANA has been shown to consume 2 moles periodate per mole[34]. The consumption of 2 moles periodate by the NAGal residue in its aldehyde form excludes substitution at C-4 and C-5. As substitution at C-3 is excluded for the reason given above, substitution at C-6 is the only structure left which will provide three vicinal OH-groups (at C-3, C-4 and C-5) as required for the consumption of 2 moles periodate.

On the evidence presented the compound isolated in fraction I is defined as 6-α-D-sialopyranosyl-N-acetylgalactosamine. The conversion by mild alkali of this disaccharide to the sialyl-substituted chromogens I and III and their identity with the sialyl-substituted chromogens collected in fraction II leaves no doubt on the origin of the main constituents of fraction II (see Fig. 5). The formation of these 6-substituted chromogens is in analogy to the result of alkali treatment of 6-β-D-galactosyl-N-acetylglucosamine[35].

The question then arises whether sialyl-NAGal as such is the prosthetic group or rather the repeating unit of an oligo- or polysaccharide. Obviously structures like sialyl–NAGal–sialyl–NAGal or sialyl–sialyl–NAGal–NAGal are incompatible with the experimental data. However, a branched structure in which sialyl (2 → 6) NAGal residues are joined through 1 →3 linkages between the NAGal residues*



would not contradict the results discussed so far, since by alkali treatment such an oligosaccharide would, by the mechanism of β-elimination, break down from the reducing end to sialyl-substituted chromogens and sialyl-NAGal. The results of a detailed study of the effect of periodate oxidation of BSM on the recovery of intact hexosamine are incompatible with this type of structure. Obviously in such a structure only the terminal NAG unit is oxidizable by periodate; therefore for a chain with 2, 3, or 4 repeating units the recovery of galactosamine after oxidation would be 50, 66 and 75 % respectively, with the ratio sialyl-NAGal/sialyl-chromogens decreasing with increasing chain length. Against that only 12.6 % galactosamine is recovered after 17 h oxidation of partly denatured BSM and 17.4 % galactosamine after prolonged oxidation of native BSM, *i.e.* up to 87.4 % of the total galactosamine is oxidized. Loss of hexosamine due to overoxidation would have to be preceded by cleavage of the linkage involving its potential reducing group. This event can be excluded from the non-dialysability of any sialic acid of oxidized BSM.

In BSM sialic acid and galactosamine are in equimolar proportion, and most likely all sialic acid is bound to NAGal as indicated by the recovery of sialyl-NAGal and its anhydro derivatives in 84 % yield (referred to that portion of total sialic acid rendered dialysable by alkali) and by the similar recovery of these sialyl compounds on repeated alkali treatment of BSM. The unoxidized fraction of NAGal (12.6 %) may be unoxidizable either because of lack of α-glycol groupings or because of inaccessibility to the oxidizing agent. The observation that after rapid oxidation of the first 60–65 % galactosamine the oxidation rate considerably decreases (see Fig. 4)

* This structure was suggested to us by Dr. J. W. CORNFORTH, London, in the early stages of the work.

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taken together with the finding[11] that α-neuraminidase action on BSM ceases after removal of 64 % of the total sialic acid would suggest a minor fraction of the prosthetic groups to be inaccessible to periodate ions.

The isolated disaccharide is the simplest substrate for neuraminidase prepared so far. As with most carbohydrases acting on oligosaccharides, the specificity of neuraminidase is directed mainly towards the glycosyl (neuraminyl) moiety and only to a lesser degree towards the partner in the glycosidic linkage[2]. Thus sialyl-NAGal, the sialyl-chromogens and sialyl-lactose are readily cleaved by α-neuraminidase.

Though the presence of some sialyl-N-acetyltalosamine in fraction I is merely suggested on chromatographic evidence, its formation from sialyl-N-acetylgalactosamine by epimerization under alkaline conditions would be in agreement with the ease of epimerization of N-acetylglucosamine under similar conditions[6].

NOTE ADDED IN PROOF

For the preparation of the disaccharide the use of Zeokarb 215 (40–100 mesh) seems to be preferable to Dowex 50.

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REFERENCES

1 G. BLIX, A. GOTTSCHALK AND E. KLENK, *Nature*, 179 (1957) 1088.
2 A. GOTTSCHALK, *Advances in Enzymology*, 20 (1958) 135.
3 A. GOTTSCHALK, *Nature*, 176 (1955) 881.
4 J. W. CORNFORTH, M. E. FIRTH AND A. GOTTSCHALK, *Biochem. J.*, 68 (1958) 57.
5 D. G. COMB AND S. ROSEMAN, *J. Am. Chem. Soc.*, 80 (1958) 497.
6 R. KUHN AND R. BROSSMER, *Ann.*, 616 (1958) 221.
7 G. BLIX, *Z. physiol. Chem.*, 240 (1936) 43.
8 G. BLIX, L. SVENNERHOLM AND I. WERNER, *Acta Chem. Scand.*, 6 (1952) 358.
9 A. GOTTSCHALK AND G. L. ADA, *Biochem. J.*, 62 (1956) 681.
10 R. HEIMER AND K. MEYER, *Proc. Natl. Acad. Sci. U.S.*, 42 (1956) 728.
11 A. GOTTSCHALK, *Biochim. Biophys. Acta*, 20 (1956) 560.
12 H. FAILLARD, *Z. physiol. Chem.*, 307 (1957) 62.
13 A. GOTTSCHALK, *Biochim. Biophys. Acta*, 23 (1957) 645.
14 R. KUHN AND R. BROSSMER, *Angew. Chem.*, 70 (1958) 25.
15 A. GOTTSCHALK, *Biochim. Biophys. Acta*, 24 (1957) 649.
16 T. WHITE, *J. Chem. Soc.*, (1940) 428.
17 B. ODDO, *Gazz. chim. ital.*, 39 (1909) 649.
18 R. KUHN AND G. KRÜGER, *Chem. Ber.*, 90 (1957) 264.
19 C. C. CURTAIN AND J. PYE, *Australian J. Expll. Biol. Med. Sci.*, 33 (1955) 315.
20 A. GOTTSCHALK AND B. T. PERRY, *Brit. J. Expll. Pathol.*, 32 (1951) 408.
21 G. L. ADA AND E. L. FRENCH, *Australian J. Sci.*, 13 (1950) 82.
22 N. F. BOAS, *J. Biol. Chem.*, 204 (1953) 553.
23 L. SVENNERHOLM, *Acta Soc. Med. Upsaliensis*, 61 (1956) 75.
24 L. SVENNERHOLM, *Arkiv Kemi*, 10 (1957) 577.
25 C. J. M. RONDLE AND W. T. J. MORGAN, *Biochem. J.*, 61 (1955) 586.
26 E. KLENK AND G. UHLENBRUCK, *Z. physiol. Chem.*, 307 (1957) 266.
27 E. L. JACKSON, in R. ADAMS, *Organic Reactions*, J. Wiley and Sons, New York, Vol. 2, 1944, p. 341.
28 A. GOTTSCHALK, *Biochem. J.*, 61 (1955) 298.
29 G. BLIX, *Trans. 4th Intern. Congr. Biochem., Vienna*, 1958, Symposium 1.
30 L. SVENNERHOLM, *Nature*, 181 (1958) 1154.
31 M. J. CRUMPTON, *Nature*, 180 (1957) 605.
32 S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238.
33 L. SVENNERHOLM, *Acta Chem. Scand.*, 12 (1958) 547.
34 E. KLENK, H. FAILLARD, F. WEYGAND AND H. H. SCHÖNE, *Z. physiol. Chem.*, 304 (1956) 35.
35 R. KUHN, H. H. BAER AND A. GAUHE, *Chem. Ber.*, 88 (1955) 1713.