STUDIES ON MUCOPROTEINS

I. THE STRUCTURE OF THE PROSTHETIC GROUP OF OVINE SUBMAXILLARY GLAND MUCOPROTEIN

E. R. B. GRAHAM AND A. GOTTSCHALK

Walter and Eliza Hall Institute of Medical Research, Melbourne, Department of Microbiology, Australian National University, Canberra (Australia)

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SUMMARY

Ovine submaxillary gland mucoprotein (OSM), the most potent of the influenza virus haemagglutinin inhibitors, was found to contain equimolecular amounts of N-acetylneuraminic acid and N-acetylgalactosamine comprising 42 % of the mucoprotein. The prosthetic group was isolated and shown to be the disaccharide 6-α-D-N-acetylneuraminyl-N-acetylgalactosamine by elementary analysis, identification of its components, susceptibility to α-neuraminidase and periodate consumption. The derivative 6-α-D-N-acetylneuraminyl-anhydro-N-acetyl-galactosamine, a chromogen in the direct Ehrlich reaction in the cold, formed from the prosthetic group on gentle alkali treatment, was also prepared. 93 % of the total galactosamine of OSM was oxidized by periodate, and 85 % of the N-acetylneuraminic acid was released by α-neuraminidase concomitant with the loss of biological activity.

INTRODUCTION

The mucoproteins produced by the submaxillary glands of various animal species have been the subject of many investigations (for historical review see GOTTSCHALK[1]). They are the richest and most convenient source of the various types of sialic acid and they were early recognised as potent inhibitors of haemagglutination by influenza viruses[1]. Whereas the sialic acid containing mucoproteins of the serum type, for instance orosomucoid, have a very complex oligo- or polysaccharide as prosthetic group[2], the submaxillary gland mucoproteins contain a relatively simple carbohydrate. For this reason the glandular-type mucoproteins lend themselves more to structural studies of their prosthetic group than do the serum mucoproteins. The main sugar components of the submaxillary gland mucoproteins are N-acetylgalactosamine (NAGal) and sialic acid[3–6]. Bovine submaxillary gland mucoprotein (BSM), as prepared in our laboratory, contains 22.4 % sialic acid (expressed as N-acetylneuraminic acid) and 12.7 % D-galactosamine, *i.e.* about equimolecular quantities of the two components; in addition small amounts of D-glucosamine (2.1 %), D-galactose (0.7 %), L-fucose (0.7 %) and D-mannose (trace) are present[7]. In a previous paper[8] it was shown (a) that the prosthetic group of BSM is a simple disaccharide consisting

of NAGal and sialic acid, (b) that within the disaccharide sialic acid is linked through an α-ketosidic linkage to carbon atom six of NAGal and (c) that the disaccharide is split into its components by α-neuraminidase.

Though BSM inhibits in high dilution haemagglutination with the PR8 strain of influenza virus as indicator, it is practically without effect on the agglutination of red blood cells when tested with other strains of influenza virus as indicators[9]. Ovine submaxillary gland mucoprotein (OSM), on the other hand, is the most potent virus haemagglutinin inhibitor obtained so far[10], and is active against a whole spectrum of indicator viruses. Chemically OSM differs from BSM by the type of sialic acid incorporated in the prosthetic group. Whereas the sialic acid of BSM is a mixture of ON-diacetylneuraminic acid, N-acetyl-O-diacetylneuraminic acid and N-glycolylneuraminic acid, the sialic acid of OSM is nearly exclusively N-acetylneuraminic acid (NANA) with only a very small percentage of N-glycolylneuraminic acid[11]. Also it is easier to prepare from the sheep submaxillary glands a mucoprotein free or practically free from sugars other than NAGal and sialic acid than to isolate such a mucoprotein from the corresponding bovine glands. For these reasons, OSM, a typical glandular mucoprotein, was chosen for a more detailed investigation of its structural features. This paper is concerned with the composition and structure of the prosthetic group.

EXPERIMENTAL

Materials

The crystalline compounds N-acetyl-D-galactosamine, D-galactosamine hydrochloride, N-acetylneuraminic acid and pyrrole-2-carboxylic acid were the preparations used in the previous paper[8].

The other sugars were standard preparations exhibiting a single spot on paper-chromatographic examination.

Chromogens I and III were prepared from N-acetyl-D-galactosamine according to KUHN AND KRÜGER[12].

Ovine submaxillary gland mucoprotein (OSM). Fresh ovine submaxillary glands were freed from fat and connective tissue, cut into cherry-size pieces, washed on a Büchner funnel, covered with an equal weight of water and allowed to stand overnight at 0°. After removal of the liquid the cut glands were passed through a coarse meat mincer and extracted 3 times at 1-h intervals with an equal weight of water at 0°. These extracts were discarded to avoid contamination of the final product with blood. The resulting well-washed glandular material was then extracted 4 times with an equal weight of water at 0° for 48 h. These extracts were processed by the method of CURTAIN AND PYE[9] shown by the authors to result in an electrophoretically homogeneous product. As described for the preparation of BSM[8], the addition of two further steps to the procedure was found to improve the product. When 1000 g of cleaned glands were submitted to this procedure, the following yields of lyophilized OSM were obtained: first extract 5 g, second extract 3 g, third extract 2 g and fourth extract 1.5 g.

Sialic acid from OSM was obtained as described for BSM[8].

Indicator viruses were prepared according to standard procedure[13].

Vibrio cholerae neuraminidase (RDE) was prepared in a highly purified state

by Drs. ADA AND FRENCH and kindly presented by them; 7 μg protein were equivalent to 10.000 units.

Ion exchange resins were used in columns of appropriate size. Dowex 50-X4 (50–100 mesh) was prepared and regenerated according to BOAS[14]. Dowex 1-X2 (50–100 mesh) was converted to the formate form by the procedure of SVENNERHOLM[15].

METHODS

The non-amino sugars were determined by the method of GOTTSCHALK AND ADA[5].

Sialic acid and glycolic acid were determined as described previously[8]. All sialic acid values are expressed as N-acetylneuraminic acid (NANA).

Hexosamine determinations were carried out according to RONDLE AND MORGAN[16] 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 OSM, the latter was hydrolysed with 7.4 *N* HCl for 10 h at 100° in a sealed tube. When D-galactosamine hydrochloride was submitted to the same treatment, 88 % of the hexosamine was recovered. All hexosamine values were corrected accordingly.

Periodate oxidation of the disaccharide was carried out as described previously[8]. Periodate oxidation of OSM was performed at pH 5 with excess of potassium and sodium metaperiodate respectively at specified temperatures and times. The excess periodate was reduced by ethylene glycol and the mixture exhaustively dialysed against water.

Standard conditions for RDE treatment of substrates were pH 6.5, 0.005 *M* Ca^{++}, 35° and 20 h.

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

Paper partition chromatography was carried out according to GOTTSCHALK AND ADA[5]. The standard solvent used was *n*-butanol–pyridine–water (6:4:3, v/v). When a compound was chromatographed in more than one solvent, the second solvent was *sec.*-butanol–acetone–acetic acid–water (6:6:3:5, v/v).

Preparation of the prosthetic group of OSM

5 g of lyophilized OSM was dissolved in 500 ml water and the pH adjusted to 8 with saturated barium hydroxide solution. The mixture was quickly heated to 80° in a boiling water bath, 0.95 g $Ba(OH)_2 \cdot 8\ H_2O$ added and the mixture kept for 10 min in a water-bath at 80°. The solution was ice-cooled, acidified to pH 4 with *N* H_2SO_4 and dialysed at 0° against 10 volumes of water for 60 h with occasional stirring. The dialysate, adjusted to pH 4 with *N* H_2SO_4, was applied directly to a Dowex I-formate column (resin bed 20 × 200 mm). The column was washed with water until the positive direct Ehrlich reaction of the effluent, due to the presence of chromogen I, as established chromatographically, disappeared.

The compounds adsorbed to the resin were eluted with formic acid increasing in concentration from 0.1 *N* to 0.4 *N*. The eluate was collected in 12-ml fractions using an automatic fraction collector. The first 564 ml of eluate was orcinol-negative and did not give a direct Ehrlich reaction; it was discarded. The next 108 fractions were orcinol-positive. Each sixth fraction was concentrated *in vacuo* and chromatographed. From the colour reactions of the chromatographed material it was seen that fractions 1–18 consisted almost entirely of NANA and that fractions 54–108

contained mainly substances reacting both with direct Ehrlich reagent in the cold and with orcinol reagent.

Fractions 1–18 were pooled and lyophilized; yield 30 mg: pool I.

Fractions 19–53 were also pooled and lyophilized; yield 130 mg: pool II. When a sample of pool II was chromatographically analysed before and after treatment with α-neuraminidase (RDE), the main component was found to be NANA linked to NAGal; in addition small amounts of free NANA and of NANA bound to chromogens (in the direct Ehrlich reaction in the cold) were present.

Fractions 54–108 were combined and lyophilized; yield 110 mg: pool III.

Pool I was used for the purification and crystallization of NANA according to CORNFORTH, FIRTH AND GOTTSCHALK[17].

Pool II was used for the separation of N-acetylneuraminyl-NAGal (NANA-NAGal). This separation was effected by applying the material of the pool to Whatman 3 MM paper, 2 mg/in. paper, and developing with butanol–pyridine–water. From the thoroughly air-dried paper the area shown on a guiding strip to contain the disaccharide was cut out and the strip eluted with water at 0°. The eluate was applied to a small Dowex I (formate form) column, the column rinsed with water and elution carried out with 0.3 *N* formic acid at 0°. The orcinol-positive eluate was lyophilized and kept over P_2O_5 in vacuo at 0°.

Pool III was used for the isolation of N-acetylneuraminyl-chromogen I (NANA-chromogen I) and of N-acetylneuraminyl-chromogen III (NANA-chromogen III) by paper chromatography as above. The pool contained NANA-chromogen I, NANA-chromogen III and some NANA-NAGal. While NANA-chromogen I separated well on paper from NANA-chromogen III, it did not separate from NANA-NAGal sufficiently for satisfactory elution. For this reason, only fractions 82–108, practically devoid of NANA-NAGal, were applied to paper for isolation of NANA-chromogen I.

When the alkali treated OSM, after dialysis, was submitted to a second alkali treatment as above, the following yields were obtained: 20 mg (pool I), 100 mg (pool II) and 80 mg (pool III).

The combined dialysates resulting from a third and fourth alkali treatment of the mucoprotein material yielded about 240 mg substance which on chemical analysis was found to contain 35 mg hexosamine corresponding to 40 % NANA-NAGal of the total yield. This value compared well with the NANA-NAGal content of the material obtained from the first and the second alkali treatment. Paper chromatography of the material from the third and fourth alkali treatments before and after RDE action showed that it consisted of NANA, NANA-NAGal and NANA-chromogens I and III in approximately the same relative proportion as the material obtained from the first and second treatments.

RESULTS

Sugar analysis of OSM

The analytical values for the component sugars of OSM after appropriate hydrolysis are summarized in Table I.

The sialic acid of OSM is known to be NANA[18]. Determination of the glycolyl content of sialic acid isolated from OSM showed that 0.5 % of the total sialic acid was N-glycolylneuraminic acid. Chromatographic analysis of the isolated hexosamine of

TABLE I

COMPONENT SUGARS OF OSM

Values are expressed in percentage of dried OSM.

Sialic acid	*Hexosamine*	*Galactose*	*Mannose*	*Fucose*
25.4	15.0	0.30	0.15	0.40

OSM showed it to be galactosamine only; in some preparations a trace of glucosamine was seen. In all preparations ninhydrin oxidation of the isolated hexosamine revealed on chromatographic analysis of the oxidized material a single sugar spot coinciding in R_F value and colour shade with D-lyxose.

Identification of the prosthetic group

Elementary analysis and general properties: The material isolated from pool II was snow-white, had strong reducing power and, when dissolved in water, gave a very acid solution. It reacted with both the orcinol and indirect Ehrlich reagents and gave the direct Ehrlich reaction on heating.

For analysis (Microanalytical Laboratory, Australian National University) the material was dried in high vacuum over P_2O_5 at room temperature for several days; on further drying at 55° in high vacuum over P_2O_5 no loss in weight was observed.

	C	H	N
Found: preparation 1	44.51	6.29	5.68
Preparation 1 after heating at 55°	44.47	6.29	
Preparation 2	44.62	6.34	5.24
Calc. for $C_{19}H_{32}O_{14}N_2$ (512.47)	44.53	6.29	5.47

Identification of the components: 15 mg of the material, dissolved in 2.0 ml 0.1 *N* H_2SO_4, was heated at 80° for 60 min. After cooling the solution was applied to a Dowex 1 (formate form) column and the effluent collected and dried. The column was then eluted with 0.3 *N* formic acid and the eluate lyophilized.

N-acetylgalactosamine: When a sample of the dried effluent was chromatographed in butanol–pyridine–H_2O, the indirect Ehrlich spray showed an intense purple spot at R_F 0.36 coinciding in R_F value and colour shade with NAGal standard (the NAG standard had an R_F value of 0.41) and a weak purple spot at R_F 0.45 (most probably N-acetyltalosamine). On borate-treated paper and with the same solvent purple spots at R_F 0.09 (NAGal) and R_F 0.13 (most probably NATal) were observed (NAG standard R_F 0.19).

When 9.6 mg authentic N-acetyl-D-galactosamine was treated with $Ba(OH)_2$ solution, pH 11.1, at 80° for 10 min and the cations removed, chromatography resulted in spots, reacting with indirect Ehrlich spray, at R_F 0.36 (strong) and R_F 0.45 (weak) as above, the ratio of their colour intensities being 10 : 1. The R_F value of 0.45 is that given in the literature for N-acetyltalosamine[19].

During the gradual release of the prosthetic group from OSM by alkali an even smaller degree of conversion of NAGal to NATal occurred. Our best preparations contained only a few percent of the stereoisomer.

The residual effluent (see above) was heated in 2 *N* HCl at 100° for 5 h and the

solution dried. After oxidation of the dried residue with ninhydrin chromatography revealed only a single reddish spot at R_F 0.42 (lyxose standard R_F 0.42, arabinose standard R_F 0.35), when sprayed with aniline hydrogen phthalate.

N-acetylneuraminic acid: The lyophilized eluate (about 7.5 mg) was dissolved in 4 ml 0.1 *N* NaOH and heated at 100° for 20 min. The solution, adjusted to pH 8, was twice extracted with ether and the extract discarded. The solution was then brought to pH 3 with dilute H_2SO_4 and extracted several times at this pH with ether. The combined ether extracts were washed with water and dried over anhydrous Na_2SO_4. The material extracted with ether was dissolved in ethanol; its absorption spectrum and that of authentic pyrrole-2-carboxylic acid are shown in Fig. 1. From the molecular extinction coefficient of pyrrole-2-carboxylic acid[20] it was calculated that the ether extract contained 0.146 mg pyrrole-2-carboxylic acid, *i.e.* 5.4 % of the NANA present was converted to the pyrrole.

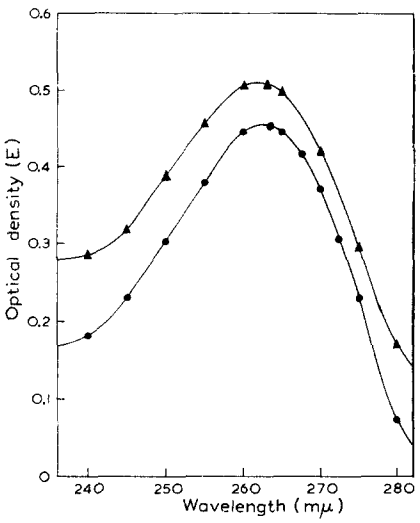

Fig. 1. U.V. absorption spectra in ethanol of authentic pyrrole-2-carboxylic acid and of the ether-soluble material obtained by alkali treatment of the acidic component of the prosthetic group of OSM. ●, authentic pyrrole-2-carboxylic acid; ▲, from prosthetic group.

Ratio of components: 1.68 mg of the material (dried over P_2O_5) was hydrolysed with 0.05 *N* H_2SO_4 at 80° for 60 min and the components of the hydrolysate separated as previously described. Determination of NANA in the lyophilized eluate gave a value of 0.99 mg (theory 1.01 mg). When the dried effluent was compared chromatographically with serial dilutions of NAGal, the amount of N-acetylhexosamine corresponded to the theoretical value of 0.73 mg within the error of the method[5] (± 10 %). The same results were obtained with α-neuraminidase as the hydrolysing catalyst.

Susceptibility to α-neuraminidase: When a sample of the material was treated with α-neuraminidase (RDE) and together with the control and reference substances chromatographed, complete cleavage of the prosthetic into NANA and NAGal was observed. As with acid hydrolysis of the material, the enzymic hydrolysis revealed a small amount of NATal.

Periodate oxidation: 3.6 mg of the material was oxidized at pH 4.0 with 0.01 *M* KIO_4 at 7°. The rate of periodate consumption is shown in Fig. 2.

Conversion by alkali of the prosthetic group to NANA-chromogen I and the enzymic cleavage of NANA-chromogen I: 5 mg of the material was treated with 1.0 ml 0.2 *N* Na_2CO_3 solution at 80° for 10 min. After removal of the cations with Dowex 50 (H^+) the resulting material was chromatographed before and after treatment with neuraminidase. Whereas the original material did not react with direct Ehrlich reagent in the cold, after alkali treatment a strong direct Ehrlich-reacting spot (in the cold) at R_F 0.20 was seen which on RDE treatment moved to R_F 0.61 (chromogen I standard R_F 0.61) concomitant with the appearance of an orcinol-reacting spot at R_F 0.15 (NANA standard R_F 0.15).

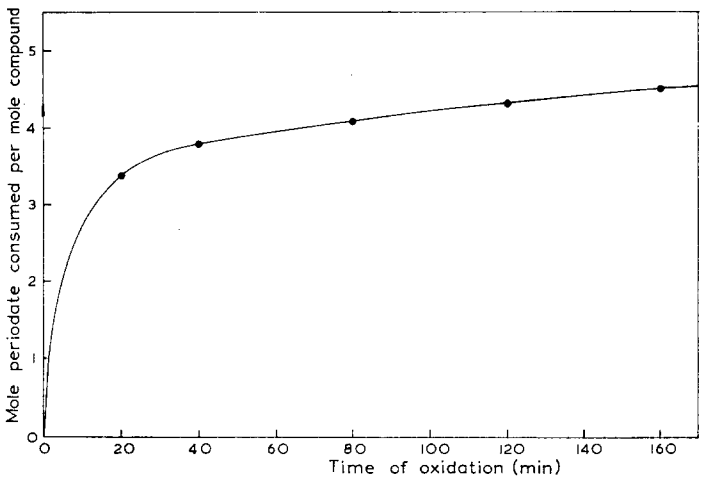

Fig. 2. Consumption of periodate by N-acetylneuraminyl-N-acetylgalactosamine. Conditions: 0.01 *M* KIO_4, pH 4, temperature 7°.

Identification of N-acetylneuraminyl-chromogen I formed by alkali treatment of OSM

The material obtained from pool III by selective elution from paper and purification on Dowex I (formate form) reacted even in high dilution immediately in the cold with Ehrlich reagent. It was found to be susceptible to α-neuraminidase and to behave chromatographically before and after enzymic treatment in the same manner as NANA-chromogen I produced by alkali treatment of the prosthetic group (see above).

The snow-white, non-crystalline material was dried over P_2O_5 in high vacuum at room temperature for several days; no weight was lost on further drying at 55°. Analysis:

Found: C, 46.47; H, 6.36; N, 5.32; ash, 0.84 %.

Calc. for $C_{19}H_{30}O_{13}N_2$ (494.45): C, 46.15; H, 6.12; N, 5.67 %.

1.33 mg of the dried material was hydrolysed with 0.05 *N* H_2SO_4 at 80° for 60 min and NANA after adsorption to and elution from Dowex 1-formate determined quantitatively. Found: 0.80 mg (theory 0.83 mg).

NANA-chromogen III, isolated from pool III as above and chromatographed, revealed a single orcinol- and direct Ehrlich-reacting spot at R_F 0.28; the RDE

treated material gave rise to an orcinol-positive spot at R_F 0.15 (coincident with NANA standard) and a direct Ehrlich-positive spot at R_F 0.78 (coincident with chromogen III). The yield of NANA-chromogen III was insufficient for further analysis.

Identification of N-acetylneuraminic acid formed by alkali treatment of OSM

Crystalline NANA as obtained from pool I was dried over P_2O_5 in high vacuum and analysed (Microanalytical Laboratory, C.S.I.R.O., Melbourne).

Found: C, 42.6; H, 6.4; N, 4.6%.

Calculated for $C_{11}H_{19}O_9N$: C, 42.7; H, 6.2; N, 4.5 %.

Effect of periodate oxidation on the hexosamine recovery from OSM

10 ml 0.01 *M* KIO_4 or 3 ml 0.06 *M* $NaIO_4$ was added to 1.0 ml of a solution containing about 5 mg OSM. After oxidation and dialysis the hexosamine contents of the oxidized OSM and its control were determined. In one experiment the hexosamine recovery from oxidized OSM was compared with that from OSM treated with RDE (70,000 units) prior to oxidation. This was done to see what effect the unmasking of the hexosamine by removal of the terminal sialic acid may have on the oxidizability of the hexosamine. The results of the periodate experiments are shown in Table II. An experiment with BSM is included to compare the oxidizability of the hexosamine in the two mucoproteins.

TABLE II

EFFECT OF PERIODATE OXIDATION OF OSM ON THE HEXOSAMINE RECOVERY

Oxidizing agent	*Conditions of oxidation*		*Hexosamine recovery in percentage of control*
	Hours	*°C*	
KIO_4	3	10	55.6
KIO_4	24	10	15.2
KIO_4	24	20	8.7
$NaIO_4$	3	20	20.1
$NaIO_4$	6	20	16.9
$NaIO_4$	24	15	6.7
$NaIO_4$ after RDE treatment of OSM	24	15	6.1
$NaIO_4$ on BSM	24	20	29.2

Yield of N-acetylneuraminyl-NAGal and conversion products

In the experiment detailed under "*Preparation*", of the total NANA present, namely 1.16 g, 260 mg (= 22.5 %) was rendered dialysable by the first alkali treatment. Calculated as NANA, in pools I, II and III 178 mg was recovered in the form of the prosthetic group and compounds derived from it. On the second treatment, of 170 mg NANA made dialysable 130 mg NANA was recovered in the form of the prosthetic group and conversion products. Corresponding figures for NANA from the combined third and fourth treatments were 203 and 141 mg respectively. In these four treatments 55 % of the total NANA was detached from OSM in dialysable form; 71 % of this NANA was recovered in the prosthetic group and conversion products thereof. Further alkali treatments still yielded appreciable amounts of the prosthetic

group thus indicating that the residual 45 % of NANA in the mucoprotein was also bound to NAGal.

Biological activities of OSM and BSM and their reduction by neuraminidase

The influenza virus haemagglutinin inhibitory titres of OSM and BSM with the PR8 and Lee strains as indicators and the reduction of these titres by neuraminidase (RDE) are shown in Table III.

TABLE III

INHIBITORY TITRES PER MILLIGRAM MUCOPROTEIN AND THEIR REDUCTION BY NEURAMINIDASE (RDE)

Conditions of test: Serial dilutions of mucoprotein in 0.25 ml volume mixed with 4 agglutinating doses of indicator virus in 0.025 ml and incubated at 4° for 60 min; then 0.025 ml of 5% fowl red blood cells added and pattern read after further 30 min at 4°.
Inhibitory titre is defined as the dilution factor at which 0.25 ml of the test solution prevents 3 out of 4 haemagglutinating doses of a particular indicator virus from aggregating fowl red blood cells.

Indicator	*OSM*	*OSM–RDE*	*BSM*	*BSM–RDE*
PR8 – i	$1.18 \cdot 10^6$	$6.86 \cdot 10^3$	$4.8 \cdot 10^4$	$1.42 \cdot 10^3$
Lee – i	$1.30 \cdot 10^5$	$2.44 \cdot 10^3$	$4.96 \cdot 10^2$	$3.48 \cdot 10$

Quantitation of sialic acid release from OSM and BSM by neuraminidase

Concomitant with the reduction in inhibitory titres of OSM and BSM on neuraminidase treatment sialic acid was released.

5.0 ml mucoprotein solution, containing 0.88 mg of the protein, were digested with 5000 units RDE under standard conditions for specified time, followed by exhaustive dialysis at 0°. Sialic acid was determined in the mucoprotein solution after dialysis and in appropriate controls. From OSM 76 % and 85 % of the total NANA were released after 7 h and 24 h respectively. The corresponding values for BSM were 68 % (7 h) and 76 % (24 h).

DISCUSSION

The dialysable material obtained from OSM by mild alkali treatment was separated into three distinct fractions: pool I (11 %), pool II (48 %) and pool III (41 %). The main constituent of each pool was isolated and analysed.

The compound from pool II consists of NANA, identified by conversion to pyrrole-2-carboxylic acid, and N-acetylgalactosamine, identified chromatographically and by oxidation to lyxose, in equimolecular proportion as shown by quantitation of the constituents. The elementary analysis conforms to $C_{19}H_{32}O_{14}N_2$ in agreement with the above composition. Cleavage by α-neuraminidase of the reducing saccharide into NANA and NAGal indicates α-ketosidic linkage of NANA to NAGal. The presence of this linkage with a deoxy and a carboxyl group vicinal to the ketosidic carbon atom accounts for the ease of hydrolysis by very dilute acid. The consumption by one mole of the compound (in the aldehydo form) of 4 moles IO_4^-, 2 moles per component, excludes substitution at carbon atoms 4 and 5 of NAGal. Conversion by mild alkali of the compound to NANA-chromogen I (a chromogen reacting in the

cold with Ehrlich reagent) without elimination of chromogen I excludes substitution at C_3 and C_4 of NAGal. Therefore, 6-α-D-N-acetylneuraminyl-NAGal is the only structure compatible with the evidence presented (see Fig. 3); a more detailed discussion of this evidence has been given in the previous paper[8].

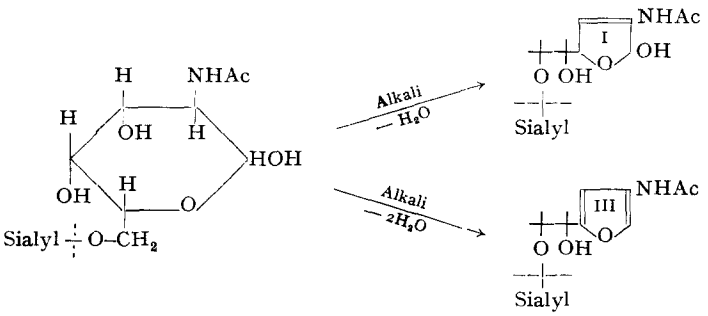

Fig. 3. Conversion by alkali of 6-α-D-N-acetylneuraminyl-N-acetylgalactosamine to NANA-chromogen I and NANA-chromogen III. Sialyl = N-acetylneuraminyl; ––– bond susceptible to α-neuraminidase.

The statement that in the compound, isolated from pool II, the N-acetylhexosamine moiety is NAGal requires a qualification. As observed previously[8], small amounts of N-acetyltalosamine were released by dilute acid and by neuraminidase from the compound. The identification of NATal is based on (a) its colour reaction with the indirect Ehrlich reagent only, (b) its chromatographic identity with the substance formed in small quantity from authentic NAGal under the alkaline conditions of the release of the prosthetic group, (c) its oxidation to lyxose and (d) its identical R_F value with that reported in the literature. Its formation by epimerization of NAGal in alkaline medium is paralleled by the epimerization of N-acetylglucosamine to N-acetylmannosamine under similar conditions[21].

The high recovery from OSM of NANA in the form of NANA-NAGal and its conversion products on repeated mild alkali treatment taken together with the 1:1 ratio of NANA to NAGal in OSM and with the oxidizability of 93 % NAGal present in OSM provides strong evidence that 93 % of the total NANA is linked to carbon atom six of NAGal and that the disaccharide NANA-NAGal is the main prosthetic group of OSM. This means that in OSM as in BSM[8] and in the serum-type urine inhibitory mucoprotein[22] a considerable number of prosthetic groups are distributed along the protein chain. In the urinary inhibitor approximately 120 individual prosthetic groups are attached to the protein core[23]. In OSM (11.3% *N*) about one out of eight amino acids carries a prosthetic group.

The origin of NANA-chromogen I, the main component of pool III, is obvious from the convertibility by mild alkali of NANA-NAGal to NANA-chromogen I. NANA-chromogen I is identified as 6-α-D-N-acetylneuraminyl-anhydro-NAGal by elementary analysis, susceptibility to α-neuraminidase and by its origin. NANA-chromogen III, a minor component of pool III, is derived from the same source, as established previously[8].

The compound comprising pool I is NANA, identified by elementary analysis of the crystalline material. It may arise by two reaction mechanisms: (a) NANA (2 → 6) NAGal may be transformed under the impact of alkali to the ketoside of a

β-hydroxy-carbonyl compound; the glycosidic linkage of this type of compound is known to be alkali-sensitive[24]. (b) Since 7 % of the total hexosamine is unoxidizable, this fraction may be present in OSM in the form of NANA (2 → 3) NAGal which on alkali treatment would decompose by elimination to chromogen I, actually found in the dialysate of alkali treated OSM (see "*Preparation*"), and NANA.

The presence in our OSM preparation of very small amounts of sugars characteristic of the serum-type mucoprotein would suggest them to be the carbohydrate moiety of a contaminant rather than intrinsic components of OSM. If so, the contamination of glandular mucoproteins by small amounts of serum-type mucoproteins is assessed more sensitively by carbohydrate analysis than by electrophoresis.

The high biological activity of OSM is demonstrated by the observation that $0.85 \cdot 10^{-6}$ mg will inhibit 4 haemagglutinating doses of indicator virus (influenza strain PR8) compared with $0.21 \cdot 10^{-4}$ mg of BSM. The biological activity is strikingly reduced by α-neuraminidase, in OSM to 0.6 %, in BSM to 3.0 % of original activity. Similar observations have been reported by others[9,10]. The fact that neuraminidase removes from OSM and BSM 85 % and 76 % respectively of the total sialic acid present, and sialic acid only[25], strongly suggests the terminal sialic acid in inhibitory mucoproteins to be the main anchoring group for the influenza virus particle.

The relationship between chemical structure and inhibitory activity of OSM and of BSM will be treated separately[26].

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HYDROLYSIS OF LECITHINS BY VENOM PHOSPHOLIPASE A

I. STRUCTURE OF THE ENZYMICALLY FORMED LYSOLECITHINS

G. V. MARINETTI, J. ERBLAND, K. TEMPLE AND ELMER STOTZ
Department of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, N.Y. (U.S.A.)

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SUMMARY

Rat-liver [^{32}P]lecithin was hydrolyzed by snake venom phospholipase A to give quantitative yield of [^{32}P]lysolecithins. The lysolecithins were oxidized in buffered aqueous solutions in the pH range 2.8 to 7.0, with bromine or permanganate to yield a keto-lysolecithin derivative. The extent and rate of oxidation is pH dependent. Bromine oxidation is more rapid at pH 6.8 than at pH 5.5 or 2.8. Permanganate oxidation is rapid at pH 5.5 but very slow at pH 6.8. During the oxidation partial concomitant hydrolysis occurs.

The keto-lysolecithin can be separated and measured by paper chromatography and thus permits the determination of the α-lysolecithin isomer. The amount of this isomer was found to vary between 54–94 % depending on the pH at which the bromine oxidation is carried out. Definitive evidence for the formation of the lysolecithinic acid has not yet been obtained.

Passage of lysolecithin through silicic acid causes ester migration from the beta to the alpha position of glycerol. This is reflected by an increase in the keto-lysolecithin upon bromine oxidation.

A novel reaction occurs when lysolecithin is treated with bromine in chloroform or methanol. Brominolysis rather than oxidation takes place with the formation of lysophosphatidic acids and choline.

A discussion of these findings is presented.

INTRODUCTION

In a previous paper[1] it was shown that permanganate oxidation of enzymically produced lysolecithins yielded two products, one of which was a ketone derivative. Acid hydrolysis of the oxidized lysolecithins gave rise to at least four water soluble phosphate compounds. These data provided evidence that the lysolecithins were a