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Preparation of Sepharose*-bound α-Ketosides of *N*-Acetylneuraminic Acid and their Interaction with *Vibrio cholerae* Neuraminidase

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The 2-aminoethyl and the 2-aminoethylaminocarbonylmethyl α-ketosides of *N*-acetylneuraminic acid and the 2-aminoethylamide of the 2-hydroxyethyl α-ketoside of *N*-acetylneuraminic acid have been synthesized and coupled to cyanogen bromide activated Sepharose. In the resulting polymers, the *N*-acetylneuraminic acid groups are bound to the matrix by means of α-ketoside (*N*-acetylneuraminic acid-Sepharose) and amide (*N*-acetylneuraminamide-Sepharose) linkages, respectively. Columns of *N*-acetylneuraminic acid-Sepharose equilibrated with buffers of pH 5.5 or 9 adsorbed *Vibrio cholerae* neuraminidase very strongly. The enzyme was effectively desorbed in high yield from the polymer by addition of the benzyl α-ketoside of *N*-acetylneuraminic acid to the eluent. The transformation of the *N*-acetylneuraminic acid-Sepharose to the 7-carbon analogue of *N*-acetylneuraminic acid-Sepharose resulted in a polymer having a slightly weaker affinity for the enzyme. *N*-Acetylneuraminamide-Sepharose did not significantly adsorb the neutraminidase.

Previous studies on the specificity of neuraminidase,[1-3] have revealed that the carboxyl group in the glyconic part of neuraminidase labile α-ketosides of *N*-acetylneuraminic acid, seems essential for the enzymatic cleavage of the α-ketosidic bond. It has also been demonstrated [4-5] that *Vibrio cholerae* neuraminidase is activated by divalent cations such as calcium and manganese, which presumably in conjunction with the carboxyl group in the substrate are essential for the formation of the enzyme-substrate complex.[5] In order to study further the interaction of *N*-acetylneuraminic acid derivatives and neuraminidase, attempts were made to prepare matrix-bound α-ketosides of *N*-acetylneuraminic acid. The enzyme-substrate binding properties have previously been utilized in the purification of *Vibrio cholerae* neuraminidase using erythrocyte stromata.[4]

The present paper reports the coupling of the 2-aminoethylamide of the 2-hydroxyethyl α-ketoside of *N*-acetylneuraminic acid and of the 2-aminoethyl and the 2-aminoethylaminocarbonylmethyl α-ketosides of *N*-acetylneuraminic acid to cyanogen bromide activated Sepharose and some properties of the polymers. In addition, the transformation of matrix-bound *N*-acetylneuraminic acid to its 7-carbon analogue will be described.

The synthesis of the 2-aminoethylaminocarbonylmethyl α-ketoside of *N*-acetylneuraminic acid was conveniently performed by treatment of the previously reported butoxycarbonyl α-ketoside of tetra-*O*-acetyl-*N*-acetylneuraminic acid [2] with anhydrous ethylene diamine. The purification of the nonulosaminic acid derivative, was facilated by the fact that it was not adsorbed by Amberlite IRC 50 (H^+). The 2-aminoethylamide of the 2-hydroxyethyl α-ketoside of *N*-acetylneuraminic acid, having a hydrophilic aglycone, was similarly prepared by treatment of the corresponding methyl ester with ethylene diamine.

The coupling of the 2-aminoethyl [3] and the 2-aminoethylaminocarbonylmethyl α-ketosides of *N*-acetylneuraminic acid and of the 2-aminoethylamide of the 2-hydroxyethyl α-ketoside of *N*-acetylneuraminic acid to Sepharose was per-

* Sepharose is the commercial name for beaded agarose from Pharmacia Fine Chemicals, Sweden.

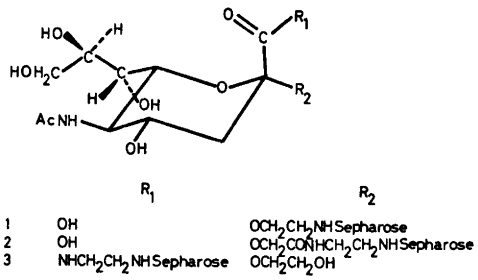

Fig. 1. Sepharose-bound 2-aminoethyl α-ketoside (1) 2-aminoethylaminocarbonylmethyl α-ketoside (2) and 2-aminoethylamide of the 2-hydroxyethyl α-ketoside (3) of *N*-acetylneuraminic acid.

formed as previously described,[6-7] resulting in polymers having the *N*-acetylneuraminic acid groups fixed to the matrix by means of α-ketoside (*N*-acetylneuraminic acid-Sepharose) and amide (*N*-acetylneuraminamide-Sepharose) linkages, respectively (Fig. 1).

Oxidation of the matrix-bound nonulosaminic acid with periodic acid and reduction of the resulting formyl derivative with sodium borohydride afforded the Sepharose-bound 7-carbon analogues of *N*-acetylneuraminic acid. The polymer beads were not destroyed when treated with periodic acid and sodium borohydride. Further, the beaded form remained unaffected when the Sepharose derivatives were freeze dried or treated with boiling water. On assaying *N*-acetylneuraminic acid-Sepharose at 37 °C with neuraminidase, free *N*-acetylneuraminic acid was released. In contrast the *N*-acetylneuraminamide-Sepharose was resistant to the hydrolytic action of the neuraminidase as well as to 0.05 M hydrochloric acid. By treatment with 0.5 M sulfuric acid at 80 °C, however, the amide linkage could be hydrolyzed and the resulting nonulosaminic acid determined. Under these hydrolyzing conditions, the released acid is probably partly destroyed resulting in an underestimate of the bound *N*-acetylneuraminic acid. Table 1 shows the amounts of *N*-acetylneuraminic acid in different Sepharose conjugates.

All of the *N*-acetylneuraminic acid-Sepharose preparations adsorbed *Vibrio cholerae* neuraminidase very strongly, type V showing a high enzyme binding capacity. Sodium acetate buffer pH 5.5 which according to Mohr and Schramm,[4] is effective in eluting neuraminidase adsorbed to erythrocyte stromata, eluted the enzyme from columns of the Sepharose derivatives only very slowly. The 7-carbon analogue of *N*-acetylneuraminic acid-Sepharose, prepared by periodic acid oxidation and sodium borohydride reduction of the matrix-bound *N*-acetylneuraminic acid, showed a slightly weaker affinity for the enzyme. Attempts to elute the neutraminidase

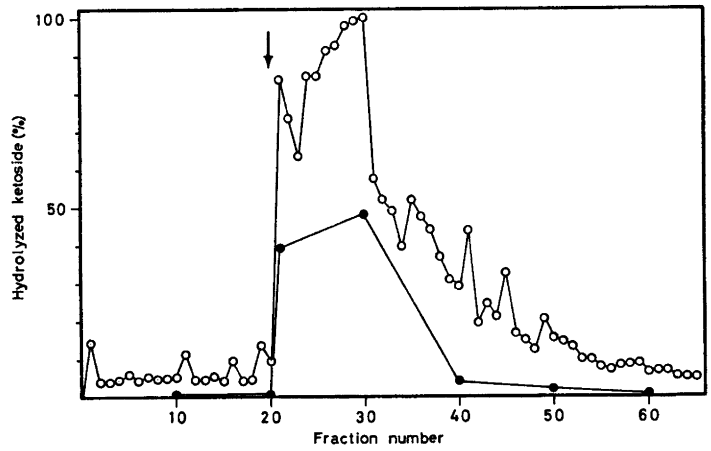

Fig. 2. Activity of *Vibrio cholerae* neuraminidase in the effluent from a column of *N*-acetylneuraminic acid-Sepharose (type V). The enzyme was eluted with 0.1 M sodium acetate buffer pH 5.5, 0.05 M with respect to calcium chloride, containing 200 μg benzyl α-ketoside of *N*-acetylneuraminic acid per ml buffer. The arrow denotes the start of the addition of the benzyl α-ketoside. Incubation was performed for 30 min (●) and 20 h (○). For further details see text.

with sodium borate buffer pH 9 were also unsuccessful. With either of the aforementioned buffers, serum albumin was eluted in a volume corresponding to that of the column. By means of sodium acetate buffer pH 5.5 containing 200 μg benzyl α-ketoside of *N*-acetylneuraminic acid per ml, however, the neuraminidase was desorbed in high yield from columns of *N*-acetylneuraminic acid-Sepharose, demonstrating a specific adsorption of the enzyme to the polymer (Fig. 2).

Vibrio cholerae neuraminidase, when applied to a column of *N*-acetylneuraminamide-Sepharose and eluted with sodium acetate or sodium borate buffer pH 5.5 or 9.0, respectively, was eluted with the total bed volume. In accordance with previous findings,[1-3] the carboxyl group in the glyconic part of the substrate seems to play a dominant role in the formation of the enzyme-substrate complex.

The present results demonstrate that *N*-acetylneuraminic acid-Sepharose and its derivatives, being water insoluble substrates of *Vibrio cholerae* neuraminidase, should be useful for enzyme-substrate binding studies and for the purification of neuraminidases from other sources, as well as for studies on virus and bacteria.

EXPERIMENTAL

Material and methods. The same general methods were used as previously reported.[1-3] Neuraminidase from *Vibrio cholerae* (glycoprotein *N*-acetylneuraminyl-hydrolase, EC 3.2.1.18) was purchased from Behring-Werke, Marburg; 1 ml containing 500 units (producer's specification). The 2-aminoethyl α-ketoside and the benzyl α-ketoside of *N*-acetylneuraminic acid were prepared as previously described.[3,1,8]

Syntheses. 2-Benzyloxyethyl α-ketoside of tetra-O-acetyl-N-acetylneuraminic acid. Peracetylated *N*-acetylneuraminic acid (1 g) was transformed to its 2-chloro derivative as described by Meindl and Tuppy [8] and condensed with 2-benzyloxyethanol (5 g) in the presence of silver carbonate (0.5 g) and pulverized Drierite (2 g). After reacting overnight, the mixture was treated as previously described for the preparation of the butoxycarbonylmethyl α-ketoside of *N*-acetylneuraminic acid.[2] The product obtained from the chloroform layer was crystallized from water. The yield was 280 mg (24 %) m.p. 165–169 °C (decomp.), $[\alpha]_D^{25}$ −12° (*c* 1.0; methanol) Found: C 55.06; H 6.13; N 2.24. $C_{28}H_{37}NO_{14}$ requires C 54.99; H 6.10; N 2.29).

2-Hydroxyethyl α-ketoside of methyl tetra-O-acetyl-N-acetylneuraminate. The peracetylated benzyloxyethyl α-ketoside of *N*-acetylneuraminic acid was transformed to its methyl ester with diazomethane. A solution of the ester (200 mg) in ethanol–water (1:1, 20 ml) was added to palladium oxide (100 mg) in ethanol–water and hydrogenated overnight at room temperature. Removal of catalyst and evaporation of solvent resulted in a chromatographically pure product which was dissolved in water and freeze-dried. The yield was 150 mg (88 %). $[\alpha]_D^{25}$ −20° (*c* 1.0; chloroform). (Found: C 48.50; H 5.95; N 2.55. $C_{21}H_{32}NO_{14}$ requires C 48.27; H 6.17; N 2.68).

2-Aminoethylamide of 2-hydroxyethyl α-ketoside of N-acetylneuraminic acid. The 2-hydroxyethyl α-ketoside of the methyl tetra-*O*-acetyl-*N*-acetylneuraminate (20 mg) was dissolved in anhydrous ethylene diamine (0.2 ml). After being left overnight at room temperature, the reaction mixture was concentrated to dryness and the residue dried for several hours over diphosphorus pentoxide in vacuum at room temperature, dissolved in methanol and precipitated with ethyl ether. The resulting chromatographically homogeneous product (10 mg) was dissolved in 0.1 M sodium carbonate buffer pH 10.2 (2 ml). This solutions was used in the coupling procedure.

2-Aminoethylaminocarbonylmethyl α-ketoside of N-acetylneuraminic acid. A solution of crystalline butoxycarbonyl α-ketoside of tetra-*O*-acetyl-*N*-acetylneuraminic acid [2] (100 mg) in anhydrous ethylene diamine (0.5 ml) was left overnight at room temperature. After evaporation of solvent, the residue was taken up in water and the solution put on a 1 × 5 cm Amberlite IRC 50 (H^+) column. Elution with water resulted in a chromatographically pure α-ketoside. After freeze-drying, the yield was 55 mg (76 %). $[\alpha]_D^{25}$ −19 °(*c* 1.0; water) (Found: C 41.99; H 6.74; N 9.68. $C_{15}H_{27}N_3O_{10}.H_2O$ requires C 42.15; H 6.84; N 9.83).

Periodate oxidation. The matrix-bound *N*-acetylneuraminic acid (0.4 ml) was treated with a solution of periodic acid (20 mg) in water (1 ml) for 30 min at room temperature. The resulting 6-formylhexulosonic acid derivative was filtered off and washed with water, 0.1 M hydrochloric acid, and water.

Sodium borohydride reduction. Sepharose-bound α-ketoside of *N*-acetylneuraminic acid (0.2 ml) after periodate oxidation was treated with sodium borohydride (20 mg) in water (1 ml). After 60 min at room temperature, the resulting 7-carbon analogue of the *N*-acetylneuraminic acid derivative was filtered off and washed as above.

Preparations of columns. Activation of Sepharose (2 ml) with cyanogen bromide was performed as previously described.[6-7] The pH of the reaction mixture was held at 11 by means of 0.1 or 2 M sodium hydroxide. After 30 to 60 min at room temperature, crushed ice was added to the reaction mixture. The acti-

Table 1. Amounts of *N*-acetylneuraminic acid, released from different Sepharose conjugates by treatment with neuraminidase or acid.

α-Ketoside of *N*-acetylneuraminic acid	Type of Sepharose	Cyanogen bromide used for activation (mg/ml wet gel)	Released *N*-acetylneuraminic acid (μmol/ml wet gel) with neuraminidase	with 0.05 M hydrochloric acid	Type
2-aminoethyl	2 B	300	0.3	0.6	I
2-aminoethyl	2 B	50	0.04	0.1	II
2-aminoethylamino-carbonylmethyl	2 B	50	0.04	0.1	III
2-aminoethylamino-carbonylmethyl	4 B	50	0.7	1.6	IV
2-aminoethylamino-carbonylmethyl	4 B	300	1.3	3.2	V
2-aminoethylamide of 2-hydroxyethyl	4 B	300	–	0.2[a]	VI

[a] Hydrolysis with 0.5 M sulfuric acid. See text.

vated Sepharose was rapidly filtered off and washed cold 0.1 M sodium carbonate buffer pH 10.2 (20 ml). The *N*-acetylneuraminic acid derivative having a free primary amino group (10 mg), dissolved in the same buffer as above (2 ml), was added to the polymer and the mixture was left overnight at 5 °C. The mixture was filtered, the filtrate being saved for further couplings, and the polymer was washed with 0.1 M sodium hydroxide, 0.1 M hydrochloric acid and distilled water. Columns of the Sepharose derivatives were equilibrated with the appropriate buffer and the column experiments were performed at *ca.* 0 °C.

The enzyme labile fraction of the bound *N*-acetylneuraminic acid was determined by incubation of the Sepharose derivative (0.2 ml) with neuraminidase (100 μl) for 24 h at 37 °C in 0.05 M Tris-maleate buffer pH 6.40, (0.005 M with respect to calcium chloride) in a total volume of 0.60 ml. Acid hydrolysis of α-ketosidically bound *N*-acetylneuraminic acid was performed by heating the Sepharose derivative (0.2 ml) in 0.1 M hydrochloric acid (0.2 ml) for 60 min at 60 °C. Samples of 200 μl were analyzed according to Warren.[9] The amount of *N*-acetylneuraminic acid, bound as amide was estimated by treatment of the polymer (0.2 ml) with 1 M sulfuric acid (0.2 ml) at 80 °C for 60 min and samples of 200 μl were treated as above. The results from the couplings are shown in Table 1.

Column experiments. The neuraminidase (0.5 ml) was applied to 5 × 5 mm Sepharose columns which, after 15 min, were washed with 0.1 M sodium acetate buffer pH 5.5 and sodium borate buffer pH 9, both being 0.01 M with respect to calcium chloride. Fractions (2 ml) were collected and a flow rate of about 0.2–0.3 ml/min was used. To each fraction was added a solution of benzyl α-ketoside of *N*-acetylneuraminic acid (400 μg) in buffer (50 μl). After incubation for 30 min and 20 h at 37 °C, aliquots of 200 μl were removed from each fraction and released *N*-acetylneuraminic acid analyzed as above. When using sodium borate buffer pH 9, the effluent was adjusted to pH 7 with 0.1 M ice-cold hydrochloric acid prior to the incubation with benzyl α-ketoside of *N*-acetylneuraminic acid.

Desorption of neuraminidase from the columns was performed with 0.1 M sodium acetate buffer pH 5.5, 0.01 M with respect to calcium chloride, containing 200 μg of the benzyl α-ketoside of *N*-acetylneuraminic acid per ml buffer. The effluent was directly incubated for 30 min and 20 h at 37 °C and aliquots (200 μl) from the 2 ml fractions were analyzed as above. In all experiments the temperature of the Sepharose columns was kept at *ca.* 0 °C.

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