Purification and Properties of Rabbit Spermatozoal Acrosomal Neuraminidase

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Treatment of rabbit spermatozoa with 50 mM-MgCl₂ removes the plasma and the outer acrosomal membranes. Subsequent treatment with the detergents Hyamine 2389 and Triton X-100 solubilizes spermatozoal neuraminidase bound to the inner acrosomal membrane. The enzyme was further purified by DEAE-cellulose, Sephadex G-150 and Bio-Gel P-300 column chromatography. The enzyme showed a single major band, with the possibility of some minor contaminants, on disc-gel electrophoresis. It had a specific activity of 0.37 μ mol of sialic acid released/min per mg with purified boar Cowper's-gland mucin as the substrate. The enzyme had marked specificity for 2 $\rightarrow$ 6'-linked sialic acid in glycoproteins. The K_m of spermatozoal neuraminidase was 1.72×10^{-6} M with Cowper's-gland mucin, 1.17×10^{-5} M with fetuin and 8.8×10^{-4} M with sialyl-lactose as substrates. The V_{max} was 0.112 μ mol/min per mg with the Cowper's-gland mucin, 0.071 μ mol/min per mg with fetuin and 0.038 μ mol/min per mg with sialyl-lactose as substrate. The enzyme hydrolysed sheep submaxillary-gland mucin as readily as the Cowper's-gland mucin. The optimum of enzyme activity was at pH 5.0 on the Cowper's-gland mucin and at pH 4.3 on sialyl-lactose. The enzyme activity was unaffected by 20 mM-Na⁺ and -K⁺, but was inhibited by 20 mM-Ca²⁺, -Mg²⁺, -Mn²⁺, -Co²⁺ and -Cu²⁺. The enzyme was unstable in dilute solutions, but could be stored indefinitely freeze-dried at -20°C.

The acrosome, a cap-like structure covering the anterior portion of the mammalian spermatozoon head, was described as a lysosome, as the array of acrosomal enzymes are qualitatively similar to lysosomal enzymes (Allison & Hartree, 1970). Acrosomal enzymes play an important role in penetration of the spermatozoon through the investments of the ovum (Srivastava *et al.*, 1965; McRorie & Williams, 1974). Since the initial observation of neuraminidase-like factor and neuraminidase in spermatozoa (Srivastava *et al.*, 1970), the purification of neuraminidase has been difficult because it is tenaciously bound to acrosomal membranes (Srivastava *et al.*, 1974a). In the present paper we describe the purification and properties of spermatozoal neuraminidase from rabbit spermatozoal acrosomal extracts. As far as is known to us, this is the first report on highly purified mammalian spermatozoal neuraminidase, which has unique substrate specificity and properties, in contrast with bacterial, viral and other mammalian-tissue neuraminidases. This paper also describes a modification of Warren's (1959) method for

measuring free sialic acid. The modified method eliminates chromogen formation due to reactive but uncleaved sialic acid from glycoprotein substrates (Srivastava *et al.*, 1970; Eichberg & Karnovsky, 1966; Brown *et al.*, 1970).

Materials and Methods

Materials

Clostridium perfringens neuraminidase, bovine sialyl-lactose, bovine serum albumin, *p*-dimethylaminobenzaldehyde and *N*-acetylneuraminic acid were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; DEAE-cellulose (DE-52) was from Whatman Biochemicals, Maidstone, Kent, U.K.; Sephadex G-150 was from Pharmacia (Uppsala, Sweden); Bio-Gel P-300 was from Bio-Rad Laboratories, Richmond, CA, U.S.A.; fetuin was from Grand Island Biologicals, Grand Island, NY, U.S.A.; 2-thiobarbituric acid and ammonium pyrrolidine dithiocarbamate were from Eastman Organic Chemicals, Rochester, NY, U.S.A. Hyamine 2389 and Triton X-100 were purchased from Rohm and Haas Co., Philadelphia, PA, U.S.A. All other chemicals were of the highest purity commercially available. Sheep submaxillary-gland mucin and ox

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submaxillary-gland mucin were kindly supplied by Dr. Ward Pigman of New York Medical College, New York, NY, U.S.A.

Methods

Preparation of acrosomal extracts. Rabbit semen was collected by artificial vagina and pooled. The spermatozoa were washed free from seminal plasma and were extracted first with 0.05M-MgCl₂ and then with 0.2% Hyamine 2389/0.2% Triton X-100 in 50mM-Tris/maleate buffer, pH6.1, as described earlier (Srivastava *et al.*, 1974a). This will be referred to as the sequential method. The MgCl₂ extract was dialysed and freeze-dried. The detergent extract was gradually precipitated with 2vol. of ethanol at -15°C, kept at -15°C for 1h, centrifuged (3000g for 20min), and the precipitate dialysed against 100vol. of glass-distilled water for 24h with one change at 18h at 4°C; the non-diffusible proteins were freeze-dried.

Preparation of Cowper's-gland mucin of the boar. The colourless viscous secretion squeezed from Cowper's gland contains about 25% *N*-acetylneuraminic acid, on a dry-weight basis, bound to the mucoprotein in a 2→6' linkage (Hartree, 1962). Cowper's-gland mucin was further purified as follows: 37g (wet wt.) of mucin from a pair of Cowper's glands was suspended in 100ml of 50mM-Tris/HCl buffer containing 50mM-CaCl₂, 100μg of penicillin G (Calbiochem, La Jolla, CA, U.S.A.)/ml and 100μg of streptomycin sulphate (Calbiochem)/ml, pH8.0, by stirring at 22°C for 1h. Then 10mg of crystallized bovine pancreatic chymotrypsin (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) was added and stirred at 22°C for 20h. The mixture was centrifuged at 10000g for 30min at 2°C. To the supernatant, 50ml of butan-1-ol and 350ml of acetone at -15°C were added; the mixture was then stirred for 10min and stored at -15°C for 4h. After centrifuging (3000g for 20min), the sediment was dissolved in 50mM-Tris/HCl buffer, pH8.0, containing antibiotics (as above), a few drops of octan-2-ol and toluene to prevent frothing and bacterial growth. The solution was stirred at 22°C for 16h to dissolve lumps of mucin formed during the previous precipitation. To 250ml of this solution, adjusted to pH5.0 by adding acetic acid, were added 3.3g of solid zinc acetate, 350ml of glass-distilled water and 150ml of ethanol; the mixture was stirred at 4°C for 20h and centrifuged (3000g for 20min). The zinc acetate/ethanol precipitate was dialysed and freeze-dried as for the barium acetate/ethanol precipitate described below. The zinc acetate precipitate weighed 50mg and was discarded. To the supernatant obtained from zinc acetate/ethanol precipitation was added 10% of 1M-barium acetate, and then a volume of ethanol equal to the combined volumes of the supernatant and barium acetate; the mixture was stirred at 4°C for 4h, centrifuged (3000g for

20min) and the sediment was dissolved in 100ml of glass-distilled water, dialysed next against 2 litres of 10mM-Tris/EDTA, pH6.0, at 4°C for 20h and then against 2 litres of glass-distilled water for 48h, with changes of 2 litres of glass-distilled water at 16h intervals. The non-diffusible glycoprotein was freeze-dried and weighed 2g. A 4mg/ml solution of this glycoprotein, which contained 4μmol of bound *N*-acetylneuraminic acid, and no trypsin or chymotrypsin activity, was used as a substrate, referred to as Cowper's-gland mucin, for spermatozoal neuraminidase. The Cowper's-gland mucin substrate was reasonably pure and had an approximate molecular weight of 200000 (Srivastava *et al.*, 1974b).

Assay of neuraminidase. Unless otherwise indicated, the incubation mixture contained 50mM-sodium acetate buffer, pH5.0, 4μM-Cowper's-gland mucin and 100–200μg of enzyme in a total volume of 1ml. When sialyl-lactose was used as a substrate 50mM-sodium acetate buffer, pH4.3, and 1mM-substrate were used. Incubation was for 1h or more at 37°C. Control tubes contained the same components, but lacked either the substrate or the enzyme or contained heat-denatured enzyme. At the end of incubation samples (0.2ml) were withdrawn and analysed for free *N*-acetylneuraminic acid by a modification of Warren's (1959) method. In some experiments free *N*-acetylneuraminic acid was also measured in 0.5ml samples by the *p*-dimethylaminobenzaldehyde method of Aminoff (1961). One unit of neuraminidase activity is defined as the amount of enzyme that released 1μmol of *N*-acetylneuraminic acid in 1min at 37°C under the conditions of the assay. Specific activity refers to units of enzyme per mg of protein.

Modifications in Warren's (1959) method. After incubation, 0.2ml samples were withdrawn in duplicate from each reaction mixture. One set of samples received 0.1ml of water and the other set of samples received 0.1ml of ammonium pyrrolidine dithiocarbamate (10mM) before the periodate-oxidation step of Warren's (1959) assay. The rest of the procedure was the same as described by Warren (1959). The amount of *N*-acetylneuraminic acid released was determined by subtracting the value in the presence of ammonium pyrrolidine dithiocarbamate from that in its absence.

Other assays. Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as a standard. Acrosin activity was assayed spectrophotometrically with benzoyl L-arginine ethyl ester as the substrate (Srivastava *et al.*, 1974a).

Polyacrylamide-gel electrophoresis. Disc-gel electrophoresis was carried out at pH8.3 by the method of Brewer & Ashworth (1969) on 5, 4 and 3.5% acrylamide gels with Methyl Green as a tracking dye. A

current density of 1.5 mA per gel was applied for 3 h. The gels were stained with 1% Amido Black in 7% (v/v) acetic acid and de-stained with 10% acetic acid.

Results

A solution of Cowper's-gland mucin in acetate buffer reacts in the Warren (1959) method to yield a chromogen, but *N*-acetylneuraminic acid is not released on dialysis as it is in the usual determinations by the Warren method. This macromolecular form of *N*-acetylneuraminic acid was designated as non-diffusible *N*-acetylneuraminic acid (Eichberg & Karnovsky, 1966). Such observations have been previously made for Cowper's-gland mucin (Brown *et al.*, 1970) and for sheep submaxillary-gland mucin (Eichberg & Karnovsky, 1966). The reaction caused by non-diffusible *N*-acetylneuraminic acid can be eliminated by adding 50 mM-NaCl to the reaction mixture. However, the reaction caused by the neuraminidase-like factor, giving rise to non-diffusible *N*-acetylneuraminic acid as described earlier (Srivastava *et al.*, 1970), is not inhibited by NaCl. Similarly the chromogen formed in Warren's (1959) assay by free *N*-acetylneuraminic acid is not inhibited by NaCl. To distinguish between *N*-acetylneuraminic acid released by the true neuraminidase activity by spermatozoal neuraminidase and the non-diffusible form produced by the neuraminidase-like factor when both react in Warren's (1959) assay, advantage was taken of the observations that the addition of ammonium pyrrolidine dithiocarbamate to the reaction mixtures containing Cowper's-gland mucin + Fe^{2+} + ammonium pyrrolidine dithiocarbamate and Cowper's-gland mucin +

ammonium pyrrolidine dithiocarbamate eliminates the non-diffusible *N*-acetylneuraminic acid (Brown *et al.*, 1970). In our modification of Warren's (1959) method, various concentrations of ammonium pyrrolidine dithiocarbamate were added to 0.2 ml samples of reaction products before the periodate oxidation in the Warren (1959) method. The data in Table 1 show that ammonium pyrrolidine dithiocarbamate prevents chromogen formation due to non-diffusible *N*-acetylneuraminic acid and that it is not necessary to add ammonium pyrrolidine dithiocarbamate before the enzyme reaction.

Further, the amount of *N*-acetylneuraminic acid released by spermatozoal neuraminidase or by bacterial neuraminidase from Cowper's-gland mucin when assayed with and without ammonium pyrrolidine dithiocarbamate by Warren's (1959) method and by Aminoff's (1961) method gave identical results within the range of experimental error, $\pm 5\%$.

Therefore, in the experiments described below, the release of free *N*-acetylneuraminic acid from Cowper's-gland mucin by the action of spermatozoal neuraminidase was determined in the presence of ammonium pyrrolidine dithiocarbamate (164 μg) by Warren's (1959) method.

Purification of spermatozoal neuraminidase

(1) *Extraction of the enzyme from the acrosomes.* As previously reported, spermatozoal neuraminidase is a membrane-bound enzyme, and is therefore extracted by the subsequent detergent treatment of spermatozoa previously treated with MgCl_2 (Srivastava *et al.*, 1974a). The crude detergent extracts show high neuraminidase-like-factor activity and low spermato-

Table 1. Effect of ammonium pyrrolidine dithiocarbamate on Warren's (1959) assay of non-diffusible and free *N*-acetylneuraminic acid from Cowper's-gland mucin

Samples of non-diffusible *N*-acetylneuraminic acid formed in Cowper's-gland mucin solution by neuraminidase-like factor, *N*-acetylneuraminic acid released by neuraminidases and synthetic *N*-acetylneuraminic acid were assayed without and with ammonium pyrrolidine dithiocarbamate, which was added before the periodate-oxidation step.

Sample (0.2 ml)	Ammonium pyrrolidine dithiocarbamate (μg)	Warren's chromogen (μmol)
Non-diffusible <i>N</i> -acetylneuraminic acid in Cowper's-gland mucin solution	0	0.013
	2	0.013
	10	0.003
	20	0.000
Non-diffusible <i>N</i> -acetylneuraminic acid catalysed by neuraminidase-like factor	0	0.038
	180	0.000
<i>N</i> -Acetylneuraminic acid released by spermatozoal neuraminidase	0	0.058
	180	0.056
<i>N</i> -Acetylneuraminic acid released by <i>Cl. perfringens</i> neuraminidase	0	0.048
	180	0.046
Free synthetic <i>N</i> -acetylneuraminic acid	0	0.025
	180	0.026
	0	0.048
	180	0.047

zoal neuraminidase activity. These observations confirm the previous report on neuraminidase-like factor (Srivastava *et al.*, 1970). Therefore, for the purpose of further purification, all those extracts which showed neuraminidase-like factor or spermatozoal neuraminidase activities were pooled, as it was previously reported that acrosomal extracts containing the former yielded the latter on further purification (Srivastava *et al.*, 1970). All further purification was carried out at 4°C.

(2) *DEAE-cellulose chromatography*. The enzyme was extracted from the freeze-dried acrosomal extract by 0.1M-potassium phosphate buffer, pH 6.5, and dialysed against several changes of 0.02M-potassium phosphate buffer, pH 6.5, for 3 h. The dialysed enzyme solution was then added to a column (2 cm × 15 cm) of DEAE-cellulose (DE-52) which was previously equilibrated with the same buffer. After the sample was adsorbed, the column was eluted with 300 ml of the same buffer until the effluent contained no protein. The column was eluted with 200 ml of 0.05M-potassium phosphate buffer containing 0.05M-, 0.2M- and then 0.5M-NaCl (200 ml of each). The peaks of protein from each elution were pooled, freeze-dried and dialysed against 0.10M-sodium acetate buffer, pH 5.0, and assayed for activity. The neuraminidase activity was eluted with the 0.2M-NaCl buffer.

(3) *Sephadex G-150 chromatography*. The enzyme obtained from the DEAE-cellulose column was concentrated to 3.0 ml by Aquacide (Calbiochem) and dialysed against several changes of 0.05M-sodium acetate buffer, pH 5.0, and applied to a column (2.2 cm × 35 cm) of Sephadex G-150 (medium grade), which was pre-equilibrated with the same buffer for 3 days. The column was then developed with 0.05M-sodium acetate buffer, pH 5.0, 1.0 ml fractions being collected. A protein peak devoid of neuraminidase activity was eluted in the void volume, and another peak that contained most of the activity was obtained between tubes 65 and 75. These fractions were then pooled, concentrated by Aquacide to 3.0 ml and dialysed against the sodium acetate buffer, pH 5.0. The dialysed enzyme was then rechromatographed on the same Sephadex G-150

column to remove possible contaminants. The enzyme was eluted in the same position and in nearly a symmetrical peak. At this stage the enzyme was purified about 29-fold over the crude extract.

(4) *Bio-Gel P-300 chromatography*. The rechromatographed enzyme obtained from the Sephadex G-150 column was concentrated by Aquacide to 3.0 ml, dialysed against several changes of sodium acetate buffer, pH 5.0, and then applied to a column (2.2 cm × 35 cm) of Bio-Gel P-300 which was pre-equilibrated with the same buffer for 3 days. The column was then developed with 0.05M-sodium acetate buffer, pH 5.0. The enzyme was eluted in a symmetrical peak, and all the fractions across the peak had a constant specific activity of about 0.37 $\mu\text{mol/min}$ per mg. The fractions with the constant specific activity were pooled, dialysed and freeze-dried.

Results of a typical purification process are summarized in Table 2. The final purification from the crude extract was 46-fold with a yield of 41%. The purified enzyme preparation had no acrosin activity.

Disc-gel electrophoresis

The purity of the enzyme was determined by disc-gel electrophoresis at various gel concentrations and pH values. Purified spermatozoal neuraminidase did not migrate into gels at pH 4.3. At pH 8.3, the enzyme in 5%, 4% and 3½% acrylamide gels showed a major component with some minor components (Plate 1).

Location of the enzyme

Extraction of the acrosomes with 0.05M-MgCl₂ in Tris/maleate buffer, pH 6.1, which was shown to extract only the outer acrosomal-membrane proteins (Srivastava *et al.*, 1974a), did not release any neuraminidase activity. On the other hand, all the neuraminidase activity was extracted when the acrosomes were treated subsequently with Hyamine and Triton X-100. Such treatment was reported to solubilize only the inner-membrane proteins

Table 2. *Purification of spermatozoal neuraminidase*

The enzyme was assayed by the standard procedure as described in the text. Cowper's-gland mucin concentration was 4 μM . Specific activity is defined as μmol of *N*-acetylneuraminic acid formed/min per mg of protein under the conditions given in the text.

Fraction	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Recovery (%)
1. Acrosomal crude extract	112	0.90	0.008	—
2. DEAE-cellulose chromatography	24	0.48	0.02	53
3. Sephadex G-150 chromatography	2	0.46	0.23	51
4. Bio-Gel P-300 chromatography	1	0.37	0.37	41

EXPLANATION OF PLATE I

Analytical gel electrophoresis of purified spermatozoal neuraminidase

The electrophoresis was performed at pH 8.3 on gels of different acrylamide concentrations: (a) 5%, (b) 4% and (c) 3.5%. Purified enzyme (60 μ g) from the Bio-Gel P-300 column was applied on gels (a) and (b) and 30 μ g on gel (c). The gels were stained with 1% Amido Black and de-stained with 10% (v/v) acetic acid.

(Srivastava *et al.*, 1974b). Thus the enzyme appears bound to the inner membrane of the spermatozoal acrosome.

Kinetic studies

The rate of hydrolysis of substrate was directly proportional to enzyme concentration between 0.1 and 0.3 mg of enzyme protein. Under these conditions and at 37°C, the rate of hydrolysis of Cowper's-gland mucin was linear with time for 1 h, after which time the rate decreased. This also applies when either fetuin or sialyl-lactose was used as a substrate. The rate of hydrolysis was linear with time for 2 h when the incubation was carried out at 22°C. Addition of another 4 μ M-Cowper's-gland mucin substrate to the enzyme mixture after 1½ h of incubation did not increase the rate of hydrolysis. This shows that the substrate was not the limiting factor and that the enzyme is labile at 37°C. Considerable loss of activity was observed when the enzyme was preincubated at 37°C before the assay; approx. 22% of the activity was lost on preincubation of the enzyme for 1 h at 37°C, and 70% after 2 h. When the preincubation was carried at 22°C, only 10% of the neuraminidase activity was lost after 2 h. Efforts to stabilize the enzyme at 37°C with phospholipids, dithiothreitol, bovine serum albumin or increased buffer concentration or pH (6.5) were without success. The enzyme was stable for at least 2 months when stored at higher enzyme concentration and at -20°C in 50 mM-sodium acetate buffer, pH 5.0, or in 50 mM-potassium phosphate buffer at pH 6.5. However, activity was rapidly lost when the enzyme was stored in dilute solution. The enzyme was also stable in the freeze-dried state for several months.

Effect of pH

The spermatozoal neuraminidase showed different pH optima depending on the substrate used. The effect of pH on the rate of hydrolysis is illustrated in Fig. 1. With acetate buffer, the optima is at pH 5.0 with Cowper's-gland mucin and at pH 4.3 with sialyl-lactose as substrate.

Metal ion requirements

The activity of the purified enzyme was not changed on addition of 20 mM-Na⁺ and K⁺; the activity was inhibited by 40% by the same concentration of Ca²⁺ and Mg²⁺, by 60% by Co²⁺, by 80% by Mn²⁺ and by 95% by Cu²⁺ as compared with the enzyme alone. All cations used were their chloride salts.

Spermatozoal neuraminidase does not seem to require thiol groups, and the addition of 10 mM-N-ethylmaleimide or 1 mM-iodoacetamide did not inhibit the neuraminidase activity.

Characterization of reaction products

The free *N*-acetylneuraminic acid released by

Fig. 1. Effect of pH on the activity of spermatozoal neuraminidase

The purified enzyme (50 μ g) was incubated with 0.4 mg of Cowper's-gland mucin (●), or 0.4 mg of sialyl-lactose (■), containing 50 mM-sodium acetate buffer, pH range 3.6-5.2, or 50 mM-Tris/maleate buffer, pH range 5.4-8.0, for 1 h at 37°C.

spermatozoal neuraminidase gave a spot identical with pure synthetic *N*-acetylneuraminic acid as determined by paper chromatography with *n*-butyl acetate/acetic acid/water (3:2:1, by vol.) as solvent (Spiro, 1960).

Substrate specificity

The rates of hydrolysis of three substrates, Cowper's-gland mucin (mol.wt. 200000), fetuin (44500) and bovine sialyl-lactose (633), were tested with the purified spermatozoal neuraminidase and bacterial neuraminidase. When identical quantities of the two enzymes were used, there was a significant difference in the rate and extent of hydrolysis of the substrates tested, as shown in Table 3. Whereas bacterial neuraminidase hydrolyses sialyl-lactose to nearly the same extent as Cowper's-gland mucin, spermatozoal neuraminidase hydrolyses sialyl-lactose at about one-quarter of the rate for Cowper's-gland mucin. In this respect, it appears that spermatozoal neuraminidase preferentially

Table 3. Comparison of the specificity of spermatozoal and bacterial neuraminidase

The reaction mixture contained 50mM-sodium acetate buffer, pH 5.0 for Cowper's-gland mucin and fetuin, or pH 4.3 for sialyl-lactose, 0.4mg of each substrate and 0.1 mg of either the spermatozoal neuraminidase obtained after Sephadex G-150 step or the bacterial enzyme. The reaction was carried at 37°C for 1h. Results are the average of three experiments. AcNeu, *N*-acetylneuraminic acid.

Substrate	AcNeu released (μmol) by	
	Spermatozoal neuraminidase	Bacterial neuraminidase
Cowper's-gland mucin	1.11	1.19
Fetuin	0.48	0.73
Sialyl-lactose	0.30	1.05

Fig. 2. Determination of K_m of spermatozoal neuraminidase with Cowper's-gland mucin as the substrate. The reaction mixture contained 50mM-sodium acetate buffer, pH 5.0, 0.2mg of spermatozoal neuraminidase and the indicated Cowper's-gland mucin concentrations in a total volume of 1ml. The incubation was for 1h at 37°C; ●, in the presence of protamine; ▲, in absence of protamine (insert). K_m was calculated on the basis of *N*-acetylneuraminic acid (AcNeu) released in the presence of protamine.

hydrolyses 2→6-linked substrates, whereas the bacterial enzyme hydrolyses both the 2→3 and 2→6 types of linkage equally well. The spermatozoal neuraminidase could not completely hydrolyse the 2→3-linked sialyl-lactose even after prolonged

incubation for 24h. The spermatozoal enzyme also hydrolysed sheep submaxillary-gland mucin, a substrate similar to Cowper's-gland mucin (Boursnell *et al.*, 1970), just as well as Cowper's-gland mucin. The close structural analogies between Cowper's-gland and sheep submaxillary-gland mucin suggest that the carbohydrate consists of 6- α -D-sialyl-*N*-acetylgalactosamine residue linked to hydroxyl groups of serine and threonine (Boursnell *et al.*, 1970). However, the extent of hydrolysis of bovine submaxillary mucin was approximately one-third that of Cowper's-gland or sheep submaxillary-gland mucin.

The relative rates of hydrolysis of Cowper's-gland mucin, fetuin and sialyl-lactose as a function of substrate concentrations were determined by plotting $1/v$ against $1/[S]$ (Dixon & Webb, 1964) in order to estimate K_m and V_{max} . Fig. 2 shows the data for Cowper's-gland mucin. This substrate appeared to be inhibitory at higher concentrations. With fetuin and sialyl-lactose, normal linear kinetics were observed. The apparent K_m of spermatozoal neuraminidase was $1.72 \times 10^{-6} \text{M}$ for Cowper's-gland mucin in the presence of protamine, $1.17 \times 10^{-5} \text{M}$ for fetuin and $8.8 \times 10^{-4} \text{M}$ for sialyl-lactose. The V_{max} values were $0.112 \mu\text{mol/min per mg}$ on Cowper's-gland mucin, $0.071 \mu\text{mol/min per mg}$ on fetuin and $0.038 \mu\text{mol/min per mg}$ on sialyl-lactose.

Higher concentrations of Cowper's-gland mucin (above $6 \mu\text{M}$) were inhibitory, whereas higher concentrations of fetuin or sialyl-lactose did not inhibit the reaction. This might be due to the fact that Cowper's-gland mucin is a higher-molecular-weight glycoprotein with polyanionic properties. Inhibition by Cowper's-gland mucin was reversed by the addition of protamine (Fig. 3, insert).

Discussion

This is the first report of highly purified spermatozoal neuraminidase. The following evidence supports the conclusion that it is indeed of spermatozoal acrosomal origin and its presence in acrosomal extracts is not due to any extraneous contamination. The entire extraction procedure was carried out in the presence of antibiotics. The initial extract of washed spermatozoa with MgCl_2 , removing the plasma and outer acrosomal membranes, is devoid of neuraminidase-like factor (Srivastava *et al.*, 1970) or spermatozoal neuraminidase activity. The subsequent detergent extraction, removing the inner acrosomal membrane, yields these two activities. Any contaminating neuraminidase of non-spermatozoal origin should have been removed during washing of spermatozoa and certainly by the MgCl_2 treatment. When the pooled MgCl_2 extracts were subjected to the same steps of purification as those of detergent extracts, none of the fractions

showed any spermatozoal neuraminidase activity. Spermatozoal neuraminidase obtained by purification of the detergent extracts cannot be due to any artifact during purification. The substrate specificity and properties of spermatozoal neuraminidase strongly argue against any contamination. Unlike the membrane-bound neuraminidase of rat liver, which preferentially hydrolyses the 2→3' linkage in sialyl-lactose and has no activity towards glycoprotein substrates, fetuin and others (Kishore *et al.*, 1975), spermatozoal neuraminidase preferentially hydrolyses the glycoprotein Cowper's-gland mucin, in which the linkage of sialic acid is 2→6' (Table 3). The bacterial neuraminidase from *Clostridium perfringens* attacks the 2→3' link of sialyl-lactose at approximately twice the rate of attack on the 2→6' link (Cassidy *et al.*, 1965). The bacterial neuraminidase cleaves all the bound sialic acid from Cowper's-gland mucin, however. Partially purified rat liver neuraminidases hydrolyse sialyl-lactose more efficiently than sheep submaxillary-gland mucin (Tulsiani & Carubelli, 1970), which is a substrate resembling Cowper's-gland mucin (Boursnell *et al.*, 1970). Human leucocyte neuraminidase hydrolyses sialyl-lactose in preference to sheep submaxillary-gland mucin, but it does not hydrolyse fetuin (Yeh *et al.*, 1971). The partial hydrolysis of fetuin relative to that of Cowper's-gland mucin by spermatozoal neuraminidase may be due to the fact that fetuin is reported to contain 2→6'-linked *N*-acetylneuraminic acid in addition to 2→3' links (Spiro & Bhoyroo, 1974). Slight hydrolysis of sialyl-lactose by spermatozoal neuraminidase may also be due to the fact that the commercially available sialyl-lactose contains some 2→6' links in addition to predominantly 2→3' links. The purified neuraminidase from rat heart does not hydrolyse neuraminyl-lactose, but has a $V_{\max}$ of 1.43 $\mu\text{mol/h}$ per mg with fetuin (Tallman & Brady, 1972). Fetuin is the preferred substrate for this enzyme. However, the $V_{\max}$ of purified rat heart neuraminidase is considerably lower than that of purified spermatozoal neuraminidase with fetuin, even though fetuin is the less preferred substrate for spermatozoal neuraminidase. The specific activity of purified spermatozoal neuraminidase is considerably higher than that of partially purified rat liver neuraminidase (Tulsiani & Carubelli, 1970). On the other hand, the specific activity of purified spermatozoal neuraminidase is several hundredfold lower than that of purified *Clostridium* neuraminidase (Cassidy *et al.*, 1965).

The optimum activity of spermatozoal neuraminidase with Cowper's-gland mucin as a substrate is at pH 5.0, and with sialyl-lactose at pH 4.3. It is not uncommon for an enzyme to have different pH optima with different substrates. *Clostridium* neuraminidase has an optimum at pH 4.5 with sialyl-

lactose (Cassidy *et al.*, 1965); rat liver neuraminidase has optima at pH 5.0 and at pH 5.8 (Tulsiani & Carubelli, 1970); membrane-bound rat liver neuraminidase has the optimum activity at pH 4.2 (Kishore *et al.*, 1975). The K_m values of spermatozoal neuraminidase are also significantly different from those of bacterial neuraminidase (Cassidy *et al.*, 1965) and of rat liver neuraminidases (Kishore *et al.*, 1975; Tulsiani & Carubelli, 1970). Spermatozoal neuraminidase behaves like soluble rat liver neuraminidase in that it is inhibited by Cu^{2+} and is unaffected by Na^+ and K^+ (Kishore *et al.*, 1975). On the other hand, Ca^{2+} and Mg^{2+} have no effect on soluble or lysosomal-bound rat liver neuraminidase (Tulsiani & Carubelli, 1970), but these ions inhibit spermatozoal neuraminidase. Golgi neuraminidase of rat liver is stimulated by Ca^{2+} or Zn^{2+} (Kishore *et al.*, 1975). *Clostridium* neuraminidase does not require Ca^{2+} or bivalent metal ions, but *Vibrio* neuraminidase activity is dependent on Ca^{2+} in the reaction mixture (Cassidy *et al.*, 1965). Thus spermatozoal neuraminidase has distinctive metal-ion effects compared with other neuraminidases.

The modification in Warren's (1959) assay enables one to determine whether the chromogen formation is due to free or reactive non-diffusible *N*-acetylneuraminic acid. This modification should be of special value in assaying enzymes in the crude state. The mechanism by which ammonium pyrrolidine dicarbamate prevents the chromogen formation by non-diffusible *N*-acetylneuraminic acid is not known. However, in several hundred assays, the modification always gave reliable results for distinguishing between neuraminidase-like factor and spermatozoal neuraminidase activities. Alternatively, one can use Aminoff's (1961) *p*-dimethylaminobenzaldehyde method of assay for free *N*-acetylneuraminic acid, since non-diffusible *N*-acetylneuraminic acid is not reactive in this assay provided that sufficient free *N*-acetylneuraminic acid is released.

The role of spermatozoal neuraminidase in fertilization of the ovum is not known. It has been demonstrated that pretreatment of rabbit ova with spermatozoal neuraminidase renders them incapable of fertilization (Gould *et al.*, 1971a) and resistant to digestion with trypsin (Gould *et al.*, 1971b). The zona pellucida of the ovum is a mucoprotein containing sialic acid (Soupart & Noyes, 1964).

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