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Increase of Plasma Neuraminidase Activity in Experimental Peritonitis[1] (41381)

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Abstract. A colorimetric assay using 2-(3-methoxyphenyl)-*N*-acetyl-D-neuraminic acid as substrate was used to determine the levels of neuraminidase activity in plasma following experimental peritonitis in rats. Plasma neuraminidase activity was increased 30- to 40-fold 18 hr after induction of peritonitis by ligation and puncture of the cecum. Similar increases were found with endogenous glycoproteins or added fetuin as substrates. From differences in the pH versus activity profile and the stability of the enzyme it appeared that the substantial increase in plasma neuraminidase activity with this form of peritonitis was due, to a large extent, to bacterial neuraminidase, but a contribution by tissue enzymes could not be excluded.

Removal of sialic acid from serum glycoproteins (1) and erythrocytes (2) results in rapid clearance of the desialylated products from the circulation. An increase of neuraminidase in serum caused by bacterial infection or by release of lysosomal enzymes following tissue injury could, therefore, have severe pathological consequences (3). Experimental peritonitis produced by cecal ligation and puncture may be a model for conditions in which large amounts of hydrolases, among them neuraminidase, produced by intestinal bacteria, may enter the systemic circulation.

In spite of the possible physiological significance of serum neuraminidase, very few reports have dealt with its circulating levels (4, 5). This scarcity of data can probably be attributed to the low and unstable activity of mammalian neuraminidase, and to difficulties with the available assay procedures. By employing 2-(3-methoxyphenyl)-*N*-acetyl-D-neuraminic acid as substrate and colorimetric determination of the liberated methoxyphenol with 4-aminoantipyrine and $K_3Fe(CN)_6$ (6) we were able to reliably determine the release of as little as 5 nmole of neuraminic acid in rat serum. The high background absorbance of the commercially available substrate was effectively removed by purification over a silica gel column.

Materials and Methods. *Clostridium perfringens* neuraminidase (Type V) and fetuin (Type III) were purchased from Sigma Chemical Company. *N*-Acetylneuraminic acid (synthetic) used as standard and 4-aminoantipyrine were obtained from Aldrich Chemical Company, and 2-(3-methoxyphenyl)-*N*-acetyl-D-neuraminic acid (MOPNA) from Boehringer-Mannheim Biochemicals. MOPNA (10 mg) was dissolved in 0.1 ml methanol, 0.4 ml chloroform was added, and the sample applied to a column (0.5 cm o.d. × 5 cm) containing 0.6 g activated silicic acid (Unisil, Clarkson Chemical Co.) washed with chloroform. Free methoxyphenol was eluted with 15 ml chloroform, followed by 25 ml of acetone/methanol (9:1, v/v) which eluted the MOPNA. A final elution with 15 ml methanol contained mostly *N*-acetylneuraminic acid and some MOPNA. The purity of the acetone–methanol fraction was checked by the assay for free methoxyphenol (6) and by TLC on silica gel G plates in butanol/propanol/0.1 *N* HCl (1:2:1, v/v) and visualization with resorcinol/HCl (7). The purified MOPNA was dissolved in a small amount of methanol and was stable at −20° for several weeks.

Experimental peritonitis was produced in male Sprague–Dawley rats weighing

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275–325 g by ligation and perforation of the cecum (8). The animals had free access to water but not food, and usually did not survive for more than 48 hr. At 18 hr after cecal ligation, the animals were anesthetized with sodium pentobarbital (30 mg/kg, iv) and a carotid artery was cannulated. Heparin was injected (50 units/kg) and a blood sample was taken via the cannula. Plasma was separated from cells by centrifugation and filtered through Millex GS sterilizing filter units (Millipore Corp.).

In assays with MOPNA, the incubation mixtures contained 100 μl plasma and 150 μg MOPNA dissolved in 100 μl of 0.1 *M* acetate (pH 4.0 to 5.6) or 0.1 *M* phosphate buffer (pH 5.9 to 7.2) and were incubated at 37° for 60 min. Controls were either kept on ice for one hour or consisted of the MOPNA solution incubated with buffer alone, to which the plasma was added at the end of the incubating period. The reactions were stopped by addition of 100 μl PBS (pH 7.0) and 225 μl 1.3 m*M* 4-aminoantipyrine in 1 *M* Tris/HCl buffer (pH 8.5) containing 1.33% ethanol (v/v), followed by the addition of 75 μl of 6 m*M* potassium ferricyanide. After 15 min, the absorbance was read at 500 nm in a Beckman DU-2 spectrophotometer using methoxyphenol (Eastman Kodak) as standard.

For assays with endogenous glycoproteins or with added fetuin (300 μg/incubation), 200 μl plasma was incubated with 200 μl of 0.1 *M* acetate buffer (pH 5.1) for 2 hr at 37° in 1.5-ml Eppendorf centrifuge tubes. Control samples were kept on ice for 2 hr. At the end of the incubation period, syringe needles were inserted into the lids of the tubes and the tubes heated in a boiling water bath for 3 min. After cooling, 0.6 ml water was added, vortexed, and the precipitated proteins removed by centrifugation in an Eppendorf centrifuge for 10 min at 15,000 *g*. The supernatant and the supernatants of three washes of the precipitates were placed directly onto columns containing 3 ml Dowex 1 × 8 (formate form, 200–400 mesh) washed with 10 ml water, and the acetylneuraminic acid eluted with 9 ml of 1 *N* formic acid (9). After evaporation, the neuraminic acid content was determined by the thiobarbituric acid assay (10). The recovery was checked by addition of 2- to 10-μg amounts of *N*-acetylneuraminic acid to 200 μl rat plasma and found to be 90–95%.

Results and Discussion. The usefulness of MOPNA for the assay of neuraminidase in plasma was first tested by the addition of various amounts of *Cl. perfringens* neuraminidase to 100 μl rat plasma. The assay was found to be linear to 60 nmole methoxyphenol cleaved per 100 μl plasma per hour in the presence of the bacterial neuraminidase and corresponded to the activity determined without the addition of plasma.

The results of the assay for neuraminidase activity in the plasma of control animals and rats with experimental peritonitis are shown in Table I. A striking increase in neuraminidase activity was found in all experimental animals, although the levels were variable in individuals and particularly in the controls, which, however, never exceeded 40 nmole methoxyphenol released per milliliter per hour. These assays were performed in acetate buffer at pH 5.1.

The optimal pH of neuraminidase activity in the experimental animals was 5.1 both in acetate and citrate–phosphate buffer systems (Fig. 1). An additional peak in activity was found at pH 5.9 to 6.3. At pH 7.4 the activity was 50% of the activity at pH 5.9. In cases in which the neuraminidase activity was high enough in the control animals, the pH profile was clearly different from the one in experimental animals. Optimal activity was found at pH 4.6 or lower. The

TABLE I. NEURAMINIDASE ACTIVITY IN RAT PLASMA[a]

	Methoxyphenol released
Control	5.6 ± 6.1 (15)
Experimental peritonitis	204.2 ± 22.1 (16)

[a] Values are expressed as nmole methoxyphenol released/ml plasma/hr at pH 5.1 and are the mean ± SE. Numbers in parentheses are the number of animals studied.

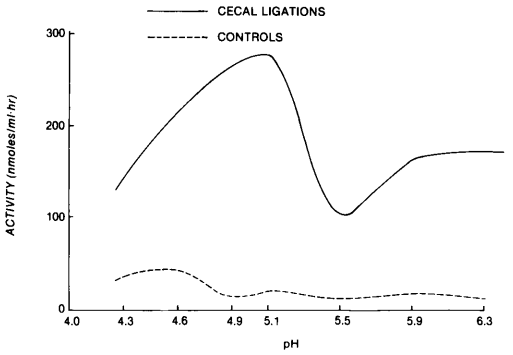

FIG. 1. pH dependence of plasma neuraminidase. The activity was determined as methoxyphenol released from MOPNA as substrate. Sodium acetate buffers were used for pH 4.3 to 5.5 and phosphate buffers for pH 5.9 and higher.

slight increases at pH 5.1 and 5.9 were barely detectable, in agreement with previous findings in human and bovine plasma (11). However, a neuraminidase with a pH optimum of 5.5 had previously been isolated from human and bovine plasma protein fractions (Cohn VI) (11). Soluble cellular neuraminidases with pH optima above pH 5.0 have been described in other rat tissues (12, 13), while the activity in the lower pH range corresponded rather to the lysosomal activities found in leukocytes (14), rat liver (12, 15), and kidney (16).

The large increase in neuraminidase activity at pH 5.1 in the experimental animals coincided with the pH optimum reported for *Cl. perfringens* neuraminidase (17) and suggested a bacterial origin for this enzymatic activity, although the soluble neuraminidases found in mammalian tissues (11–13) also could be responsible.

The enzyme activity was linear with the amounts of plasma added up to 200 μl in the ligated animals and with time up to 2 hr (Fig. 2). The decrease in velocity after 2 hr of incubation (Fig. 2) has also been observed in other tissues and with other substrates (6, 12) and may be partially due to inhibition by free neuraminic acid and the action of serum proteases. Both of these appear to be more effective in the controls. Neuraminidase activity in the plasma of experimental animals was stable for 4 days in the refrigerator but decreased markedly upon freezing and thawing. By contrast, all of the activity in the control plasma was lost during storage for 24 hr at 4°.

To ascertain that the observed activities were truly due to release of neuraminic acid, some samples were incubated without addition allowing the use of endogenous plasma glycoproteins as substrates. Fetuin was added as an additional substrate in other plasma samples. Liberated sialic acid was determined according to Aminoff (10) after removal of plasma proteins by heat denaturation. This procedure was shown to yield effective recovery of free *N*-acetylneuraminic acid added exogenously to plasma samples, and was much faster and more efficient than the dialysis of the incubation mixture previously used for removal of free sialic acid from plasma proteins (5). Although the activities observed with fetuin and endogenous substrates were not quite as high as those with MOPNA, there was still a substantial increase compared to the control animals, in which neuraminidase activity toward the natural substrates was barely detectable (Table II). In their studies of *Vibrio cholerae* and hamster cell neuraminidase, Santer *et al.* (6) also reported that much longer incubation times than with MOPNA were required to release the same amount of *N*-acetylneuraminic acid from natural substrates.

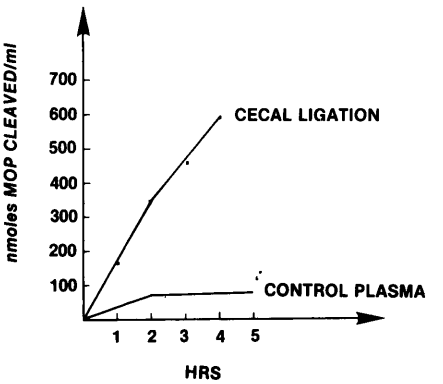

FIG. 2. Time course of the neuraminidase assay using MOPNA as substrate. Incubations contained 100 μl plasma in acetate buffer, pH 5.1, as described under Methods. Data points for control plasma are within the curve. MOP: methoxyphenol.

TABLE II. COMPARISON OF NEURAMINIDASE ACTIVITIES USING MOPNA AND GLYCOPROTEINS AS SUBSTRATES[a]

	MOPNA[b]	Fetuin	Endogenous glycoproteins
Control	5.3	1.0 (20)	—
Experimental peritonitis	101	36 (36)	32 (32)
	161	64 (40)	40 (25)
	130	51 (39)	40 (31)

[a] Values are expressed as nmole neuraminic acid released/ml plasma/hr and numbers in parentheses represent percentage of activity with MOPNA. Values for fetuin represent the contribution of both fetuin and endogenous glycoproteins as substrates.

[b] 2-(3-Methoxyphenyl)-*N*-acetyl-D-neuraminic acid.

The effect of increasing substrate concentrations on enzymatic activity is presented in a Lineweaver–Burk plot in Fig. 3. The curve was obtained by computer fitting of the data using the method by Bliss and James (18) as described by Hanson *et al.* (19). The K_m from these data was 0.5 m*M* for MOPNA as substrate, which is comparable to the K_m reported for mammalian (12, 14, 20) as well as *Cl. perfringens* neuraminidases with other substrates (21).

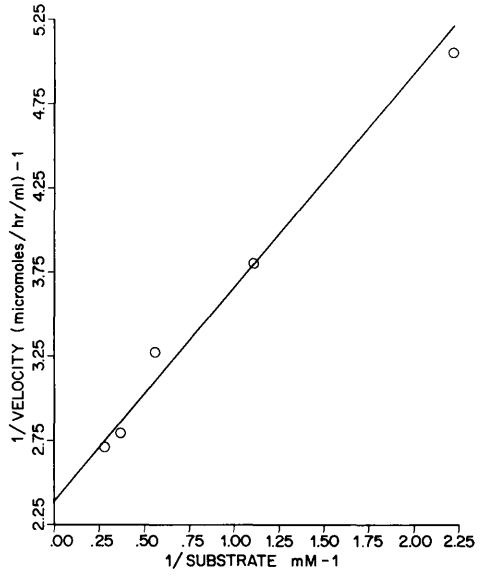

FIG. 3. Lineweaver–Burk plot of neuraminidase activity in the plasma of experimental animals using MOPNA as substrate, incubated at pH 5.1 for 1 hr. The line represents the theoretical curve obtained by computer fitting (18,19) and ○ represents experimental data.

In the only other report of elevated levels of plasma neuraminidase, which occurred in patients with gas gangrene (5), the K_m was found to be 4 m*M*.

The similarity of pH optima, K_m, and stability of the plasma neuraminidase found in experimental peritonitis to the neuraminidase of *Cl. perfringens* suggests that the observed neuraminidase activity may be of bacterial origin. Neuraminidase secreted by *Cl. perfringens* in the peritoneum may enter the systemic circulation via the lymphatics (3). However, contributions by injured cells and tissues containing soluble neuraminidases of similar characteristics (11, 12, 14) cannot be excluded.

The increase in neuraminidase levels in plasma in this condition and in patients with gas gangrene (5) poses interesting questions regarding the effects on endogenous plasma proteins and erythrocytes, and their survival in the circulation.

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