Neuraminidase and *N*-Acetylneuraminate Pyruvate-Lyase of *Pasteurella multocida*

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SUMMARY

Neuraminidase was found in nearly all strains of *Pasteurella multocida*, in some strains of *P. haemolytica*, but not in *P. pseudotuberculosis*. The enzyme of *P. multocida* is bound to the bacterial cell. It is inducible by *N*-acetyl-D-mannosamine and by free and bound sialic acid. After solubilization it has a molecular weight of about 250 000, a pH optimum = 6.0, and a Michaelis constant $K_m = 2 \times 10^{-4}$ M when 3'-sialyllactose is used as substrate.

N-acetylneuraminate pyruvate-lyase activity present in *Pasteurella multocida* is higher in the cell-free culture medium than in the bacterium.

It is suggested that the stimulating effect of blood sera on the production of neuraminidase by *Pasteurella multocida* is due to the delayed release of the inducer *N*-acetyl-D-mannosamine.

INTRODUCTION

The enzyme neuraminidase (*N*-acetylneuraminate glycohydrolase, EC. 3.2.1.18) is present in differential bacterial strains, but all belonging to the order of the Pseudomonadales and Eubacteriales (Drzeniek, 1972). In a short communication we have reported the existence of neuraminidase in *Pasteurella multocida* and *P. haemolytica* (Scharmann, Drzeniek & Blobel, 1970). Independently, Tsolov & Karadzhov (1969) and Müller (1971) described the existence of neuraminidase in *P. multocida*. Earlier, Laurell (1959) had found neuraminidase in *P. pseudotuberculosis*.

Neuraminidase of *Pasteurella multocida* exerts a characteristic action on human transferrins: it splits a homogeneous transferrin band from humans, type CC, into five bands when examined by polyacrylamide gel electrophoresis (Scharmann, Brückler & Blobel, 1971). Since this enzyme is bound to the bacterial cell which contains also other enzymatic activities, among them *N*-acetylneuraminate pyruvate-lyase (EC. 4.1.3.3) (Scharmann *et al.* 1970; Müller, 1971), it was necessary to release neuraminidase from the cell and to purify it.

This paper reports differences in the induction of neuraminidase by *N*-acetyl-D-mannosamine and different blood sera, the solubilization, partial purification and characterization of the enzyme, and gives further evidence for *N*-acetylneuraminate pyruvate-lyase activity in *Pasteurella multocida*.

METHODS

Bacteria. One hundred and four strains of *Pasteurella multocida* were investigated; 23 strains of type A, five of type B, one of type C, 14 of type D and four of type E. The remaining strains were not typed. We thank Dr P. Perreau, Maisons-Alfort, France, Dr J. E. Smith, London, and Dr Fujita, Tokyo, for supplying us with these strains. The types corresponded to Carter's classification (Carter, 1967). Five strains of *P. haemolytica*

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(Collection of Animal Pathogenic Micro-organisms, Brno, Czechoslovakia) and six strains of *P. pseudotuberculosis* (Collection of the Institute of Bacteriology, Giessen, Germany) were also tested for neuraminidase activity.

Bacterial count. Viable count determinations were made by plating (Collins & Lyne, 1970). The extinction of the culture was determined at 625 nm in a Bausch & Lomb spectrophotometer (Spectronic 20).

Media. Brain heart infusion (Difco) with 5 % rabbit serum was used in experiments in which the occurrence of neuraminidase activity in *Pasteurella* strains was examined. In order to produce larger amounts of neuraminidase, 1 % (w/v) Casamino acids (Difco) and 10 % (v/v) sheep serum added to the brain heart infusion were used.

As basal medium for the induction tests 1 % (v/v) yeast diffusate was used supplemented with 1 % (w/v) Casamino acids and 0.5 % (w/v) $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$. Yeast diffusate was obtained by dialysing 1 g of yeast extract (Oxoid) against 100 ml of distilled water (Scharmann & Blobel, 1968). *N*-acetyl-D-mannosamine was obtained from Sigma, St. Louis, Missouri, U.S.A., and *N*-acetylneuraminic acid (NANA) from Calbiochem, Los Angeles, California, U.S.A. Bovine sialyllactose together with *N*-acetylneuraminosyl-(α , 2 $\rightarrow$ 3)-D-lactose (alternative name: 3'-sialyllactose) and fetuin were prepared as described previously (Drzeniek, 1970).

Growth of bacteria. Samples of 100 ml of the above-mentioned enriched medium were autoclaved in 1 l Erlenmeyer flasks and inoculated with 5×10^8 bacteria from a culture 6 h old. They were incubated for 66 h at 37 °C in a rotating shaker at 100 rev./min. A final population of 8×10^9 cells/ml was obtained when the medium was enriched with sheep serum. The bacteria were then separated from the culture fluid by centrifugation (27 300 g, 20 min) and washed once with 0.1 M-phosphate buffer, pH 6.5. The cells were suspended in the same buffer (one fifth of the volume of the culture fluid).

Solubilization of neuraminidase. To solubilize the cell-bound neuraminidase from *Pasteurella multocida*, washed bacteria were ultrasonicated at a frequency of 20 kc/s with an instrument of 125 W capacity (Branson Sonifier S 125, Branson Instruments Inc., Danbury, Connecticut, U.S.A.). Treatment periods did not exceed 15 s with intervals between treatments of 1 to 2 min, in order to avoid heat denaturation of the enzyme. In addition, the treated tube was kept at -10 °C. After 20 sonications the sample was centrifuged (31 000 g, 20 min), the supernatant collected, and the sediment resuspended in the same volume of 0.1 M-phosphate buffer, pH 6.5. The sonication procedure was repeated twice to disintegrate all bacteria.

For the NaCl extraction, the phosphate-buffered bacterial suspension was made 0.4 M with respect to sodium chloride. After 15 min of incubation at 37 °C, the cells were centrifuged for 20 min at 31 000 g and the supernatant was collected. The cells were re-extracted twice to obtain a pool of three extracts.

Determination of virulence. White mice (16 to 20 g) were infected with increasing dilutions of a 16 h culture of the appropriate *Pasteurella multocida* strain. The dilutions were made with brain heart infusion broth. In 'high virulence' strains 50 to 500 bacteria were lethal within 48 h, whereas in 'low virulence' strains 5×10^6 to 5×10^9 bacteria were necessary to obtain the same effect.

Enzyme assays. Determination of neuraminidase activity was carried out as described in detail elsewhere (Drzeniek, Seto & Rott, 1966; Drzeniek, 1970). Bacteria or extracts thereof were incubated at 37 °C with 100 µg of bovine sialyllactose (containing about 80 % of 3'-sialyllactose) or with pure 3'-sialyllactose (*N*-acetyl (α , 2 $\rightarrow$ 3)-D-lactose) in 0.1 M-phosphate buffer, pH 6.0, in 0.5 ml final volume. *N*-acetylneuraminic acid liberated from the substrate

Table 1. *Neuraminidase activity in the genus Pasteurella*

Cell-bound neuraminidase activity was determined in washed bacteria in 0.1 M-phosphate buffer, pH 6.0, by measuring the hydrolysis of bovine sialyllactose to free *N*-acetylneuraminic acid.

Species	Type	Number of strains tested	Neuraminidase	
			Detected	Not detected
<i>Pasteurella multocida</i>	A	23	23	0
	B	5	5	0
	C	1	1	0
	D	14	14	0
	E	4	4	0
	Untyped	57	55	2
<i>P. haemolytica</i>	Untyped	5	3	2
<i>P. pseudotuberculosis</i>	Untyped	6	0	6

after 15 min of incubation was measured according to Aminoff's thiobarbiturate method (Aminoff, 1961). The examined sample was properly diluted to obtain a straight line dependence between the amount of liberated *N*-acetylneuraminic acid and enzyme concentration: 15 µg of *N*-acetylneuraminic acid liberated in 0.5 ml of the test volume was the upper limit in our test procedure. The dependence of enzymatic cleavage of the substrate upon time and enzyme concentration has been described by us (Scharmann *et al.* 1970). In gel filtration experiments neuraminidase activity of the diluted fractions was determined using fetuin as substrate. The separated fractions were incubated for 15 min at 37 °C in 0.1 M-acetate buffer, pH 6.0, with 1.5 mg fetuin in a final volume of 0.5 ml. Free sialic acid was measured according to Aminoff's method and expressed as *N*-acetylneuraminic acid.

N-acetylneuraminate pyruvate-lyase (EC. 4.1.3.3) activity was determined by measuring the decrease of *N*-acetylneuraminic acid. Ten µg of *N*-acetylneuraminic acid in 0.3 ml of 0.05 M-acetate buffer, pH 5.5, and 0.2 ml of the enzyme solution were incubated at 37 °C for 15 min. The reaction was stopped by the addition of sodium periodate, as generally used in the Aminoff procedure, and the remaining *N*-acetylneuraminic acid was measured.

Chromatography. Separation of *N*-acetylneuraminic acid and of *N*-acetyl-D-mannosamine was performed by descending chromatography on Schleicher & Schüll [(3345), Dassel, Federal Republic of Germany] paper, 2043b, in butylacetate:acetic acid:water (3:2:1). Paper chromatograms were stained after 16 h using the direct Ehrlich reaction for the visualization of *N*-acetylneuraminic acid (Gottschalk, 1960) or the Morgan-Elson reaction for detection of *N*-acetyl-D-mannosamine (Horton, 1969).

RESULTS

Evidence of neuraminidase in Pasteurella. Preliminary experiments with *Pasteurella multocida* showed that this species contains neuraminidase (Scharmann *et al.* 1970). Using a medium consisting of brain heart infusion and 5 % rabbit serum, we were able to determine a maximum of neuraminidase activity of *P. multocida* strain A-43 after 60 to 70 h growth at 37 °C. Between 90 % and 95 % of the enzyme activity was bound to the cells; only 5 to 10 % of the neuraminidase activity could be found in the cell-free supernatant. Therefore, 103 different *P. multocida* strains were examined for their content of cell-bound neuraminidase under the conditions for maximal enzyme activity given above. The highest neuraminidase activity was observed in the serological groups A and D as well as in some untyped strains. The strains of the serological group B showed only slight enzyme activity. Additionally, a

Table 2. *Effect of different sera on the synthesis of neuraminidase by Pasteurella multocida*

Pasteurella multocida, strain A-43, was grown in brain heart infusion (Difco) supplemented with 1 % Casamino acids (Difco) in the absence and presence of 10 % of human and of different animal sera. After 66 h of incubation at 37 °C, the amount of viable cells was counted by plating. Neuraminidase activity of the cells was determined as described in Methods. The amount of *N*-acetylneuraminic acid (NANA) liberated within 15 min in a test volume of 0.5 ml by the appropriate dilution of the bacterial cells was converted into the amount of NANA which in our test procedure would be liberated by 1 ml of the cells.

Medium	<i>Pasteurella multocida</i> cells/ml	Neuraminidase activity: μg NANA/ml of cells
No serum	8×10^8	240
Bovine serum	1×10^9	340
Horse serum	1.2×10^9	400
Human serum	1.5×10^9	500
Rabbit serum	2×10^9	640
Swine serum	2.5×10^9	920
Sheep serum	8×10^9	2750

few strains of *P. haemolytica* and *P. pseudotuberculosis* were tested for neuraminidase activity under the same conditions in order to find out if these *Pasteurella* strains also contained neuraminidase (Table 1). Only three out of five *P. haemolytica* strains were positive for neuraminidase. No neuraminidase activity was found in *P. pseudotuberculosis*.

Effect of different sera on the synthesis of neuraminidase by Pasteurella multocida. *Pasteurella multocida* strain A-43 was found to produce the highest neuraminidase activity but also to require a rich medium for its cultivation. In order to evaluate the optimal growth conditions, we chose a medium consisting of brain heart infusion and 1 % (w/v) Casamino acids supplemented with 5 to 20 % (v/v) different sera. In these experiments a six- to eightfold higher neuraminidase activity per ml cell culture was measured when the medium contained 10 % (v/v) sheep serum, as compared to the serum-free control. This increase of neuraminidase activity in the presence of sheep serum was paralleled by the increase of cell number per ml of cell culture, measured as extinction. If other sera at 10 % (v/v) concentration were used, we received, compared to sheep serum, lower neuraminidase activities and cell numbers (Table 2). The relation of neuraminidase activity to cell number, however, was always constant. The enzyme present in 3×10^7 cells cleaved approximately 10 μg NANA from 100 μg of bovine sialyllactose within 15 min at 37 °C in 0.1 M-phosphate buffer, pH 6.0.

Induction of neuraminidase. Scharmann *et al.* (1970) described the inducibility of neuraminidase in *Pasteurella multocida* by *N*-acetyl-D-mannosamine, fetuin, *N*-acetylneuraminic acid, and sialyllactose. The highest neuraminidase activities per cell were obtained by using *N*-acetyl-D-mannosamine for the induction of the enzyme. Fig. 1 shows differences in the time-dependent appearance of neuraminidase activity of *P. multocida* grown in basal medium (1), after addition of *N*-acetyl-D-mannosamine to the basal medium (2), and in a medium containing brain heart infusion, Casamino acids, and sheep serum (3). Neuraminidase activity of bacteria grown in basal medium reached its maximum already after 6 to 10 h, independent of whether the medium contained *N*-acetyl-D-mannosamine or not. In the presence of *N*-acetyl-D-mannosamine, neuraminidase activity was (with the same number of cells) ten times higher than in the absence of this substance. The maximum of neuraminidase activity and maximal cell numbers were reached at the same time. Due to the fact that after 22 h of incubation the cells started to flocculate, no activities occurring after that time were measured.

Fig. 1. Production of neuraminidase by *Pasteurella multocida*. 1 ml of a 6 h culture of *P. multocida*, strain A-43, containing 4×10^8 cells was inoculated into 100 ml of three different culture media and incubated at 37 °C. Neuraminidase activity was expressed as μg *N*-acetylneuraminic acid (NANA) liberated by 1 ml of cell culture (see Table 2). The number of cells per ml of cell culture was determined by plating. (1) Basal medium: 1 % yeast diffusate + 1 % Casamino acids + 0.5 % $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$. (2) Basal medium (1) containing 2-mM *N*-acetyl-D-mannosamine. (3) Brain heart infusion + 1 % Casamino acids + 10 % sheep serum.

Fig. 2. Induction of neuraminidase in *Pasteurella multocida*. *N*-acetyl-D-mannosamine (2 mM) was present in a culture of *P. multocida* in basal medium (containing initially 4×10^8 cells in 100 ml) at the time of inoculation or after 16 and 39 h of incubation at 37 °C (arrows). Neuraminidase activity was measured and expressed in μg *N*-acetylneuraminic acid per ml of cell culture. ●—●, Neuraminidase activity of *P. multocida* grown in basal medium; ○—○, neuraminidase activity after addition of *N*-acetyl-D-mannosamine to the culture.

Table 3. *Cell-bound and solubilized neuraminidase*

Pasteurella multocida, strain A-43, was grown for 66 h in an enriched medium to a density of about 8×10^9 cells/ml and then separated from the culture filtrate and washed. The washed cells were ultrasonicated or extracted with 0.4 M-NaCl. Neuraminidase activity was expressed in μg NANA per 1 ml of cell culture or the appropriate amount derived thereof. The protein content of the sample was determined with Folin-Ciocalteu's phenol reagent as described by Kabat & Meyer (1964) using bovine serum albumin as standard.

Preparation	Neuraminidase activity (μg NANA/ml)	Protein (mg/ml)	Specific enzymatic activity $\frac{\mu\text{g NANA released}}{\text{mg protein}}$
Cell-free culture filtrate	240	18.2	13
Washed cells	2625	0.6	4370
Supernatant of ultra-sonicated cells	1350	0.4	3380
NaCl extract	220	0.03	7350

In cells grown in a rich medium consisting of brain heart infusion, Casamino acids and sheep serum, the maximal neuraminidase activity was observed after 50 to 70 h; at a point when the cell density was already decreased. After approximately 72 h the cells began to clot and flocculate. The velocity of cell division in this medium was identical to that in basal medium.

A comparison of the highest level of neuraminidase activity per bacterium in different culture media showed that in basal medium 3×10^8 cells were necessary to produce that amount of neuraminidase which could cleave 10 μg *N*-acetylneuraminic acid from 100 μg bovine sialyllactose within 15 min. In the presence of *N*-acetyl-D-mannosamine only 2×10^7 cells were needed for the same neuraminidase activity. In the medium containing sheep serum 3×10^7 cells were necessary.

The data in Fig. 1 show that neuraminidase activity induced by *N*-acetyl-D-mannosamine appeared during the logarithmic growth phase of the cells. Therefore, we investigated the influence of *N*-acetyl-D-mannosamine on the induction of neuraminidase at different times after inoculation and also when cell flocculation was observed. As demonstrated in Fig. 2, the addition of *N*-acetyl-D-mannosamine always resulted in a considerable increase of neuraminidase activity, even if added 39 h after inoculation.

We also tried to induce this enzyme by addition of *N*-acetyl-D-mannosamine to those *Pasteurella multocida* strains which did not show neuraminidase activity. No neuraminidase activity could be detected in these cases.

Virulence. *Pasteurella multocida* strains showed great differences in their virulence in mice. Our experiments indicated also great differences in the neuraminidase activity of different strains. Therefore, we endeavoured to answer the question of a possible correlation existing between neuraminidase activity and virulence of *P. multocida* in mice. A comparison of bacterial virulence in mice with the measured neuraminidase activity of different *P. multocida* strains failed to result in a correlation of these parameters. The fact that neuraminidase is probably not a virulence factor of *P. multocida* is supported further by tests in which a neuraminidase-free strain (no. 34) showed the same virulence in mice as the neuraminidase-rich strain A-43.

Solubilization of neuraminidase from *Pasteurella multocida* cells. For the characterization of *Pasteurella multocida* neuraminidase it was necessary to detach the enzyme from bacterial cells. Therefore, cells harvested after 66 h of inoculation were treated in different ways and

Fig. 3. pH-Optimum of the neuraminidase of *Pasteurella multocida*. Neuraminidase of *P. multocida* partially purified by the ammonium sulphate procedure was incubated at 37 °C for 15 min with 100 µg 3'-sialyllactose in 0.1 M-acetate buffer (pH 3.5 to 5.5) or in 0.1 M-phosphate buffer (pH 6.0 to 7.5). Liberated *N*-acetylneuraminic acid was determined according to Aminoff's (1961) procedure. Neuraminidase activity was expressed in per cent of the maximal value found at pH 6.0.

Table 4. Degradation of *N*-acetylneuraminic acid by *Pasteurella multocida*

Pasteurella multocida, strain A-43, was grown for 66 h to a density of about 1×10^{10} cells/ml and then centrifuged at 31000 *g* for 20 min. The bacteria were washed three times with 0.1 M-phosphate buffer, pH 6.0, and resuspended in this buffer to the original density. One part of the washed bacteria was ultrasonicated, another part was extracted with 0.4 M-NaCl. A 0.2 ml portion of the prepared sample was incubated with 100 µg *N*-acetylneuraminic acid dissolved in 0.3 ml of 0.05 M-acetate buffer, pH 5.5. After 15 min at 37 °C *N*-acetylneuraminic acid still present in the sample was determined. The observed disappearance of *N*-acetylneuraminic acid was expressed in per cent of the amount present at time zero in the sample.

Time after inoculation	NANA-degradation (%) after incubation at 37 °C for 15 min with			
	Cell-free culture medium (%)	Washed cells (%)	Ultrasonicated bacterial supernatant (%)	NaCl extract (%)
24	81	37	45	0
48	60	27	27	0
72	53	24	15	2

Fig. 4. Effect of substrate concentration upon the liberation of *N*-acetylneuraminic acid from 3'-sialyllactose by neuraminidase of *Pasteurella multocida*. Increasing concentrations of the substrate 3'-sialyllactose were incubated for 15 min at 37 °C in 0.1 M-phosphate buffer, pH 6.0, and the liberated *N*-acetylneuraminic acid was determined. The rate of hydrolysis of 3'-sialyllactose (left ordinate) was plotted against the substrate concentration (lower abscissa): Δ — Δ . The reciprocal values were calculated according to Lineweaver & Burk (1934) and plotted ($\circ$ — $\circ$) on the right ordinate and upper abscissa as already described in a previous paper (Drzeniek *et al.* 1966).

centrifuged at 31000 *g* for 20 min. The neuraminidase activity of the supernatant was measured. After ultrasonication, up to 60 % of the total neuraminidase was found in the supernatant. This amount could be increased by 10 % by means of subsequent extraction with 0.4 M-NaCl. NaCl extraction without ultrasonication showed that this method could release approximately 10 % of the cell-bound neuraminidase.

Unfortunately, neuraminidase preparations obtained by ultrasonication precipitated readily when left standing at 4 °C, and their specific activity was smaller than that of untreated cells (Table 3). They also contained the enzyme *N*-acetylneuraminate pyruvate-lyase (Table 4). Therefore, we used NaCl extraction for the preparation of solubilized neuraminidase. The NaCl extracts were mixed with solid ammonium sulphate at 4 °C (510 g $(\text{NH}_4)_2\text{SO}_4$ to 1 l of extract). The precipitate was centrifuged at 2200 *g* for 20 min at 4 °C, suspended in 10 ml of water, and dialysed against H_2O . The neuraminidase yield of the ammonium sulphate purification step was 60 to 80 %; the specific activity increased by a factor of 4 to 10 in different preparations.

Thermal stability. Neuraminidase preparations partially purified by the ammonium sulphate procedure containing 0.33 mg protein/ml were incubated without substrate in 0.1 M-phosphate buffer, pH 6.0, for 15 min to 24 h at 37 °C and thereafter tested in the standard assay. No decrease of activity was noted up to 60 min. Twenty-four h later the neuraminidase activity was still 93 %.

pH-Optimum. Fig. 3 shows the liberation of *N*-acetylneuraminic acid from 3'-sialyllactose at different pH values. The maximum of neuraminidase activity was measured at pH 6.0.

Fig. 5. Gel filtration of neuraminidase of *Pasteurella multocida* on Sephadex G 150. Neuraminidase preparations obtained by extraction of *P. multocida* with 0.4 M-NaCl were subjected to gel filtration on a Sephadex G 150 column (1.6 × 123 cm). For the elution, a 0.05 M-phosphate buffer, pH 6.0, containing 0.1 M-KCl was used. Fractions (2 ml) were collected. Ordinate: neuraminidase activity, expressed in $\mu\text{g}/0.5 \text{ ml}/15 \text{ min}$ of *N*-acetylneuraminic acid (NANA), liberated from fetuin as substrate by properly diluted fractions (see Methods). Abscissa: fraction number of the eluate from the Sephadex column.

Fig. 6. Determination of the molecular weight of *Pasteurella multocida* neuraminidase. The elution volume of neuraminidase (5) derived from Fig. 5 and of 4 molecular weight standards (1 to 4) on the same Sephadex G 150 column was used for the calculation of the molecular weight of *P. multocida* neuraminidase. The procedure was described in detail elsewhere (Balke & Drzeniek, 1969). V_0 = void volume; V_e = elution volume. Molecular weight standards: (1) trypsin inhibitor; (2) ovalbumin; (3) bovine serum albumin (monomer); (4) bovine serum albumin (dimer).

Fig. 7. Conversion of *N*-acetylneuraminic acid to *N*-acetyl-D-mannosamine by a cell-free extract of *Pasteurella multocida*. Bacteria (4×10^{10}) were suspended in 4 ml of an 0.1 M-phosphate buffer, pH 6.0, ultrasonicated and centrifuged for 20 min at 31 000 g. A 0.2 ml sample of the supernatant was incubated for 18 h at 37 °C with 1 mg of *N*-acetylneuraminic acid dissolved in 0.3 ml of 0.05 M-acetate buffer, pH 5.5 (= sample) and subsequently subjected to descending paper chromatography in the following system: paper, Schleicher-Schüll 2043 b; solvent, butyl acetate/acetic acid/water = 3/2/1; time, 16 h. The paper chromatogram was cut into two pieces. *N*-acetylneuraminic acid (NANA) was visualized by the direct Ehrlich reaction as described by Gottschalk (1960). *N*-acetyl-D-mannosamine (ManNAc) was detected by the Morgan-Elson reaction as given by Horton (1969). The control was treated in the same way as the sample, but the *P. multocida* extract was omitted.

Dependence of NANA liberation on substrate concentration. Fig. 4 shows the liberation of *N*-acetylneuraminic acid from 3'-sialyllactose in dependence of the substrate concentration. Evaluation of these tests according to Lineweaver & Burk (1934) results in a Michaelis constant of $K_m = 2 \times 10^{-4}$ M.

Test for determination of molecular weight of Pasteurella multocida neuraminidase. To determine the molecular weight of the neuraminidase isolated by NaCl extraction from *P. multocida*, a gel filtration of the preparations was performed on Sephadex G 150 as described earlier (Balke & Drzeniek, 1969). These experiments show (Fig. 5) that the neuraminidase of *P. multocida* moves as a single band, no lower molecular weight material being detected. The elution volume (V_e) of neuraminidase was 43.8 ml; the void volume (V_0) was determined at 40.1 ml of the eluate. From these values and molecular weight standards the molecular weight can be calculated by extrapolation to be about 250 000 daltons (Fig. 6).

N-acetylneuraminate pyruvate-lyase activity in Pasteurella multocida. Incubation of *P. multocida* in a medium containing 2 mM of *N*-acetylneuraminic acid for 7 h at pH 6.0 and 37 °C showed less than 0.02 mM of the substance to be present after this time in the test system. It was thought that the disappearance of *N*-acetylneuraminic acid was caused by the enzyme *N*-acetylneuraminate pyruvate-lyase. In order to show unequivocally that the described decrease of *N*-acetylneuraminic acid was caused by *N*-acetylneuraminate pyruvate-lyase, the appearance of *N*-acetyl-D-mannosamine was shown by paper chromatography when the supernatant of ultrasonicated bacteria was used as source of the enzyme (Fig. 7).

From Table 4 it is obvious that higher activities of *N*-acetylneuraminate pyruvate-lyase are present in the cell-free supernatant than in washed bacteria, independent of the time of growth of the bacteria. Ultrasonication of bacteria (as used for the liberation of neuraminidase) also liberated *N*-acetylneuraminate pyruvate-lyase activity into the cell-free supernatant. However, the treatment of bacteria with 0.4 M-NaCl solutions did not have a releasing effect on *N*-acetylneuraminate pyruvate-lyase.

DISCUSSION

As in the case of *Corynebacterium diphtheriae* (Moriyama & Barksdale, 1967) and *Klebsiella aerogenes* (Pardoe, 1970), the neuraminidase of *Pasteurella multocida* is bound to the bacterial cell. It can be liberated from *P. multocida* by ultrasonication or NaCl extraction. After liberation from the bacterial cell the enzyme displays a high tendency to aggregate at neutral pH values. The molecular weight of *P. multocida* neuraminidase found by means of gel chromatography is in the range of 250 000. This value is much higher than all molecular weights of bacterial neuraminidases reported up till now which were found to be in the range of 60 000 to 90 000 (Drzeniek, 1972). Only the neuraminidase of *Streptococcus viridans* shows approximately the same molecular weight as *P. multocida* neuraminidase (E. Balke, W. Scharmann & R. Drzeniek, in preparation). The reason for this high molecular weight of some bacterial neuraminidases is unknown.

The solubilized enzyme has the same pH-optimum and kinetic characteristics as the cell-bound enzyme. The determined pH-optimum of pH 6.0 is in the range found for other bacterial neuraminidases (Gottschalk & Drzeniek, 1972). The Michaelis constant with $K_m = 2 \times 10^{-4}$ M for *N*-acetylneuraminosyl (α , 2 $\rightarrow$ 3)-D-lactose as substrate is the lowest reported.

Pasteurella multocida neuraminidase is an inducible enzyme. The highest enzyme activity was obtained by the addition of *N*-acetyl-D-mannosamine to the growth medium. This finding is in agreement with earlier reports on the inducibility of neuraminidase of different bacteria. Thus, neuraminidase of *Vibrio cholerae* (Ada & French, 1959; French & Ada, 1959), *Diplococcus pneumoniae* (Kelly, Greiff & Farmer, 1966), and *Klebsiella aerogenes* (Pardoe, 1970) was induced by substances containing sialic acid. *Vibrio cholerae* neuraminidase was induced by *N*-acetyl-D-mannosamine (Ada & French, 1959).

Our recent experiments reveal that prolonged incubation of *Pasteurella multocida* in a medium containing blood sera of various animals results in a delayed increase of neuraminidase activity when compared to bacteria grown in the presence of *N*-acetyl-D-mannosamine. *N*-acetyl-D-mannosamine acts very rapidly, the maximum of neuraminidase activity being reached after 6 h, as compared to 50 to 60 h with a medium containing sheep serum. This delayed appearance of neuraminidase is not due to differences in the amount of bacteria since the inoculum and the bacterial growth curves were identical in these experiments. Furthermore, *N*-acetyl-D-mannosamine promptly induces neuraminidase activity, not only during the logarithmic growth of the bacteria, but also in a later period. Therefore, it has to be assumed that the delayed inducing effect of animal sera on neuraminidase activity in *P. multocida* is caused by a slow release of the low molecular weight inducer.

We reported earlier that *N*-acetylneuraminic acid also induces neuraminidase activity, though to a lower degree when compared on a molecular basis with *N*-acetyl-D-mannosamine (Scharmann *et al.* 1970). The fast disappearance of *N*-acetylneuraminic acid observed in cultures of *Pasteurella multocida* and the presence of *N*-acetylneuraminate pyruvate-lyase in these cells suggest that *N*-acetyl-D-mannosamine is the neuraminidase inducer. The immediate action of *N*-acetyl-D-mannosamine at all stages of bacterial growth and its higher

inducing effect are in agreement with this statement. The specific action of *N*-acetyl-D-mannosamine is also supported by the lack of neuraminidase induction by *N*-acetyl-D-glucosamine.

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