

Characterization of Neuraminidases Produced by Various Serotypes of *Pasteurella haemolytica*

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Neuraminidases produced by 16 strains of *Pasteurella haemolytica* (serotypes 1 to 16) were characterized by molecular weight, antigenic identity, and substrate specificity. After growth in a chemically defined medium, stage I (lyophilized) culture supernatants were assayed for activity with *N*-acetylneuramin lactose, human α -1-acid glycoprotein, fetuin, colominic acid, and bovine submaxillary mucin. Neuraminidase produced by *P. haemolytica* serotype A1 (Ph A1) was purified by a combination of salt fractionation, ion-exchange chromatography on DEAE-Sephacel, and gel filtration on Sephadex G-200. Purified Ph A1 neuraminidase was used to immunize rabbits, and the resultant antiserum reduced the activity of Ph A1 neuraminidase by 46%. This antiserum also reduced the activity of neuraminidase produced by the other serotypes by between 15 and 66%. Molecular weight estimates of the neuraminidases produced by the various serotypes were obtained by gel filtration chromatography on Sephadex G-200. Fifteen of the 16 serotypes examined produced a neuraminidase with a molecular weight of approximately 150,000 to 200,000. One serotype (serotype 11) produced no material with neuraminidase activity. In addition, all 15 high-molecular-weight neuraminidases showed similar substrate specificities. That is, they were all most active against *N*-acetylneuramin lactose and least active against bovine submaxillary mucin. On the basis of these results, it appears that the high-molecular-weight neuraminidases produced by the different *P. haemolytica* serotypes are quite similar.

Pasteurella haemolytica serotype A1 (Ph A1) is arguably the most important pathogen involved in acute fibrinohemorrhagic pneumonias that develop in market-stressed stocker-feeder calves after shipment (10). Because of the tremendous pathology that occurs in the lungs of cattle and goats suffering pneumonia caused by Ph A1 (15), we have attempted studies to examine some of the bacterial exoproducts that could conceivably account for this damage. Most recently, our studies have examined the neuraminidase produced by *P. haemolytica* (17). Neuraminidase production by *Pasteurella* species was first reported by Scharmann et al. in 1970 (16). These investigators showed that 102 of 104 strains of *Pasteurella multocida* and 3 of 5 strains of *P. haemolytica* produced the enzyme. The next examinations of the *P. haemolytica* neuraminidase, performed by Frank and Tabatabai (6, 18), demonstrated that type strains 2, 3, 8, 10, and 11 did not produce a neuraminidase while type strains 1, 4, 5, 6, 7, 9, and 12 did. At the time of those reports (i.e., 1981), there were only 12 known serotypes of *P. haemolytica* (6). In their second study, these authors examined the relationship between serotype and neuraminidase production by bovine and ovine field isolates (18). Their work suggested that neuraminidase production was serotype specific. They also demonstrated that antiserum produced against intact *P. haemolytica* was capable of neutralizing neuraminidase activity, indicating an association of this extracellular enzyme with the bacterial cell wall (18).

Neuraminidase has been implicated as a virulence factor in bacteria such as *Streptococcus agalactiae* (12), *Streptococcus pneumoniae* (9), *Corynebacterium diphtheriae* (13), and *Vibrio cholerae* (14). One of the possible mechanisms for this activity was proposed by Gottschalk (7). He showed that

the removal of sialic acid from salivary glycoproteins inhibited the protective capability of these secretions against potentially pathogenic microorganisms. In this situation, exogenous neuraminidase (produced by the invading bacterium) would act to help the organism survive in vivo defense mechanisms.

The majority of acute bovine fibrinohemorrhagic pneumonias are produced by Ph A1 strains. All Ph A1 strains thus far examined appear capable of elaborating very high levels of neuraminidase (6, 17, 18). The only *P. haemolytica* neuraminidase that has been characterized to date is the serotype 1 enzyme (17). We therefore thought it would be of importance to further examine the characteristics of the neuraminidases produced by the other serotypes (serotypes 2 through 16). We were specifically interested in determining whether the serotype 1 enzyme differs in any way from the other non-serotype 1 enzymes. In this study, we examined the molecular weight, substrate specificity, and antigenic structure of this enzyme produced by non-serotype 1 *P. haemolytica* strains.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Sixteen strains of *P. haemolytica* (serotypes 2 through 16 were kindly supplied by G. H. Frank) were employed in this study. Serotypes 3, 4, 10, and 15 were biotype T strains, while all other strains employed in this study were biotype A. Ph A1 was isolated from a confirmed case of acute bovine respiratory disease. Cultures were stored at -70°C in reconstituted double-strength powdered milk on filter paper. For routine use, frozen cultures were thawed and incubated for 24 h at 37°C on nutrient agar (Difco, Detroit, Mich.) plus 5% bovine erythrocytes. The frozen stock cultures were transferred only once before use in experiments. Colonies from

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TABLE 1. Release of sialic acid from various substrates by neuraminidase^a from 16 serotypes of *P. haemolytica*

Substrate ^b	Sialic acid released by serotype ^c :						
	1	2	3	4	5	6	7
<i>N</i> -Acetylneuramin lactose	0.233 ± 0.01	0.142 ± 0.002	0.042 ± 0.005	0.025 ± 0.004	0.145 ± 0.001	0.179 ± 0.008	0.149 ± 0.004
Fetuin	0.071 ± 0.004	0.026 ± 0.005	0.027 ± 0.002	0.023 ± 0.003	0.019 ± 0.003	0.035 ± 0.001	0.024 ± 0.002
α-1-Acid glycoprotein	0.081 ± 0.003	0.019 ± 0.003	0.038 ± 0.001	0.029 ± 0.001	0.022 ± 0.001	0.016 ± 0.008	0.017 ± 0.001
Colominic acid	0.047 ± 0.006	0.006 ± 0.004	0.022 ± 0.003	0.014 ± 0.005	0.006 ± 0.001	0.010 ± 0.001	0.010 ± 0.003
BSM	0.016 ± 0.001	0.004 ± 0.001	0.012 ± 0.003	0.009 ± 0.001	0.007 ± 0.001	0.006 ± 0.001	<0.002

^a Stage I neuraminidase was prepared as described previously (17).

^b The substrate concentration was 1 mg/ml.

^c Serotypes 3, 4, 10, and 15 are biotype T; all others are biotype A. All values are expressed in micromoles of sialic acid released per minute per milligram of protein and represent an average of two determinations.

^d Activity less than 0.002 μmol of sialic acid released per min per mg of protein was considered negative.

the blood agar were used to initiate cultures in defined medium. The medium and growth conditions used were the same as those described previously (17) except that each culture was grown in 250 ml of chemically defined RPMI 1640 medium buffered with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.2; GIBCO, Grand Island, N.Y.).

Purification of the extracellular neuraminidase produced by Ph A1 and molecular weight estimates of the neuraminidases produced by the various *P. haemolytica* serotypes. The extracellular neuraminidase of Ph A1 was purified by the procedure of Straus et al. (17), including lyophilization (stage I), 40 to 60% ammonium sulfate fractionation (stage II), ion-exchange chromatography on DEAE-Sephacel (stage III), gel filtration chromatography on Sephadex G-200 (stage IV), and rechromatography on Sephadex G-200 (stage V). Gel filtration chromatography on a Sephadex G-200 column (2.5 by 90 cm; Pharmacia Fine Chemicals) was performed to determine the molecular weights of the neuraminidases produced by serotypes 1 through 16. The following elution volumes were determined by recording the volume at which these proteins displayed a maximal A_{280} : blue dextran, 2,000,000; β-amylase, 200,000; alcohol dehydrogenase, 150,000; bovine serum albumin (BSA), 67,000; ovalbumin, 43,000; chymotrypsinogen A, 25,000; RNase, 13,700. Partition coefficients were then determined for the eluted peaks of each standard protein in addition to the peaks of neuraminidase activity. The molecular weight of each neuraminidase was estimated by interpolation from a graph of partition coefficient versus \log_{10} molecular weight (17), or if a neuraminidase eluted from the gel filtration column at the same elution volume as that of a standard protein, that molecular weight was recorded for that enzyme preparation.

Assays. The amount of neuraminidase activity present in concentrated culture supernatant fluids or purified preparations was quantitated by measuring the amount of sialic acid released from five substrates as described previously (17). The five substrates employed, with known α-ketosidic linkages, included *N*-acetylneuramin lactose, colominic acid, fetuin, α-1-acid glycoprotein, and bovine submaxillary mucin (BSM; Sigma Chemical Co.). Protein determinations were performed in duplicate by the method of Lowry et al. (11), in which BSA (Sigma) was used as the standard.

Neutralization of heterologous *P. haemolytica* neuraminidases with antiserum to Ph A1 neuraminidase. New Zealand White rabbits were injected subcutaneously with 1 ml (200 μg) of the stage V Ph A1 neuraminidase which was emulsified with an equal volume of Freund's complete adjuvant (Difco). Two weeks after the first subcutaneous injection, the rabbits were injected again with the same preparation in

Freund's incomplete adjuvant. One week later, a blood sample was obtained from these rabbits by cardiac puncture. Rabbit serum was separated from clotted blood by a method described previously (3). The presence of neutralizing antibody to all high-molecular-weight neuraminidases of the 16 serotypes (except for serotype 11) was determined by using the neuraminidase assay (17). The various stage I preparations (100 μl) were incubated with either 0.1 ml of preimmune serum or 0.1 ml of immune serum in the presence of 0.1 mg of fetuin, 10 mM CaCl₂, and 33.3 mM sodium acetate (pH 6.5) in a final volume of 0.4 ml. Each set of assays included a substrate blank, and enzyme reactions were initiated by the addition of the enzyme to the remaining components. The enzyme and serum preparations were incubated together for 90 min at 4°C before the assay was begun, to allow the antibody to react with the enzyme. After incubation, the percent reduction in neuraminidase activity was determined by performing the neuraminidase assay (17) for 60 min at 37°C and comparing the difference in activities between the preimmune and immune sera. Statistical evaluations were performed by employing Student's *t* test in a pairwise comparison.

RESULTS

Substrate specificity of neuraminidases produced by strains of different *P. haemolytica* serotypes. The substrate specificity of stage V neuraminidase produced by Ph A1 has already been described (17). This enzyme was shown to be active against all substrates utilized in these studies but was only weakly active against BSM. This was shown to be the case for a stage I neuraminidase preparation from a different Ph A1 strain (Table 1). Ph A1 stage I neuraminidase was most active against *N*-acetylneuramin lactose but was 15-fold less active against BSM. Indeed, this same pattern could be seen for all other serotypes that produced this enzyme. With only rare exceptions, *P. haemolytica* neuraminidases were most active against *N*-acetylneuramin lactose and least active against BSM. Reactivity against fetuin, α-1-acid glycoprotein, and colominic acid usually fell between these two extremes. Serotype 11 *P. haemolytica* produced no material with neuraminidase activity against any of the five substrates examined. Serotypes 7, 10, and 14 produced enzymes that had no detectable activity against BSM. All serotypes produced a neuraminidase that had comparable specific activities except for Ph A1. Ph A1 neuraminidase had the highest specific activity against all substrates, usually averaging twice the specific activities of the neuraminidase produced by the other serotypes. However, the biotype T strains (serotypes 3, 4, 10, and 15) consistently demonstrated less

TABLE 1—Continued

Sialic acid released by serotype ^c :								
8	9	10	11	12	13	14	15	16
0.122 ± 0.006	0.143 ± 0.007	0.017 ± 0.001	<0.002 ^d	0.174 ± 0.001	0.179 ± 0.002	0.130 ± 0.003	0.025 ± 0.003	0.142 ± 0.001
0.025 ± 0.002	0.044 ± 0.001	0.016 ± 0.001	<0.002	0.039 ± 0.008	0.067 ± 0.003	0.015 ± 0.005	0.021 ± 0.001	0.029 ± 0.001
0.024 ± 0.006	0.029 ± 0.005	0.015 ± 0.003	<0.002	0.014 ± 0.002	0.022 ± 0.001	0.014 ± 0.004	0.018 ± 0.002	0.007 ± 0.001
0.003 ± 0.001	0.009 ± 0.001	0.012 ± 0.001	<0.002	0.007 ± 0.001	0.025 ± 0.003	0.022 ± 0.001	0.030 ± 0.003	0.010 ± 0.001
0.004 ± 0.001	0.002 ± 0.001	<0.002	<0.002	0.003 ± 0.001	0.010 ± 0.002	<0.002	0.007 ± 0.001	0.003 ± 0.001

neuraminidase activity against *N*-acetylneuramin lactose than did the biotype A strains. The differences in specific activity between the various serotypes were not significantly different as evaluated by Student's *t* test.

Molecular weight estimation of neuraminidases produced by all *P. haemolytica* serotypes. Stage I neuraminidases from all *P. haemolytica* serotypes were chromatographed on Sephadex G-200 to purify the enzyme to obtain a molecular weight estimate of each. All 15 serotypes produced high-molecular-weight neuraminidases of approximately the same molecular weight. These enzymes were in the 150,000 to 200,000 molecular weight range. Serotypes 2, 3, 4, 5, 7, 8, 9, 13, 14, and 16 produced high-molecular-weight neuraminidases with a molecular weight of approximately 200,000. Figure 1 illustrates the elution profile of stage I *P. haemolytica* serotype 7 on Sephadex G-200. The *P. haemolytica* serotype 7 neuraminidase eluted from Sephadex G-200 at the same

point as β -amylase, indicating a molecular weight of approximately 200,000. Serotypes 1, 6, 10, 12, and 15 produced high-molecular-weight neuraminidase with a molecular weight of approximately 150,000. Figure 2 illustrates the elution profile of stage I Ph A1 on Sephadex G-200. The Ph A1 neuraminidase eluted from Sephadex G-200 at the same point as does alcohol dehydrogenase, indicating a molecular weight of approximately 150,000.

Neutralization of neuraminidases produced by heterologous *P. haemolytica* serotypes with anti-Ph A1 neuraminidase. In the presence of immune serum, a 45.3% reduction in activity of the homologous stage I Ph A1 enzyme preparation was observed (Table 2). This was shown to be statistically significant at a *P* value of 0.0042. The stage I heterologous neuraminidase preparations obtained from strains of the other serotypes were also incubated with either preimmune or immune serum to stage V Ph A1 neuraminidase and then

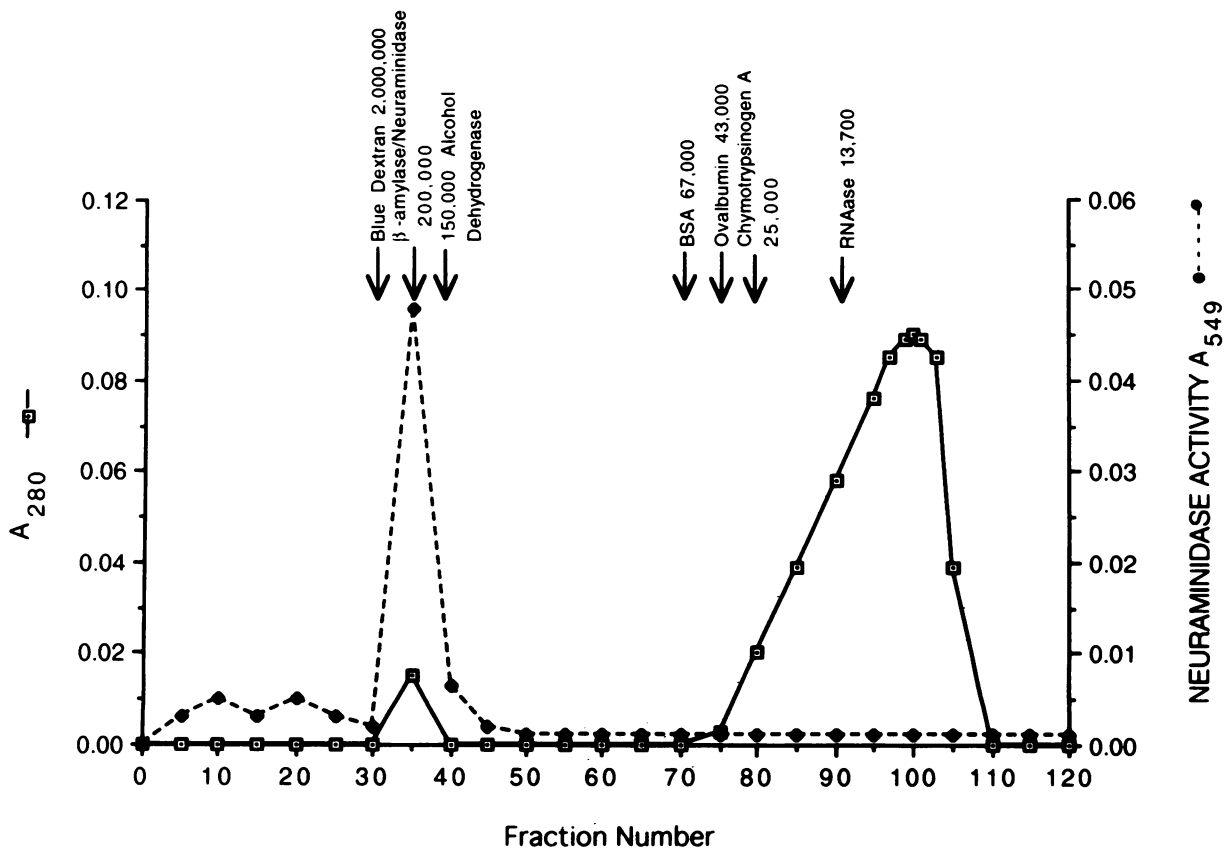


FIG. 1. Sephadex G-200 elution profile of stage I neuraminidase from *P. haemolytica* serotype 7. Stage I enzyme was applied to the column and eluted with 10 mM sodium citrate buffer (pH 6.5) at 4°C. Every fifth tube was assayed for neuraminidase activity by the procedure of Aminoff (1). Calibration of the column with blue dextran and proteins of known molecular weights was performed with the same buffer.

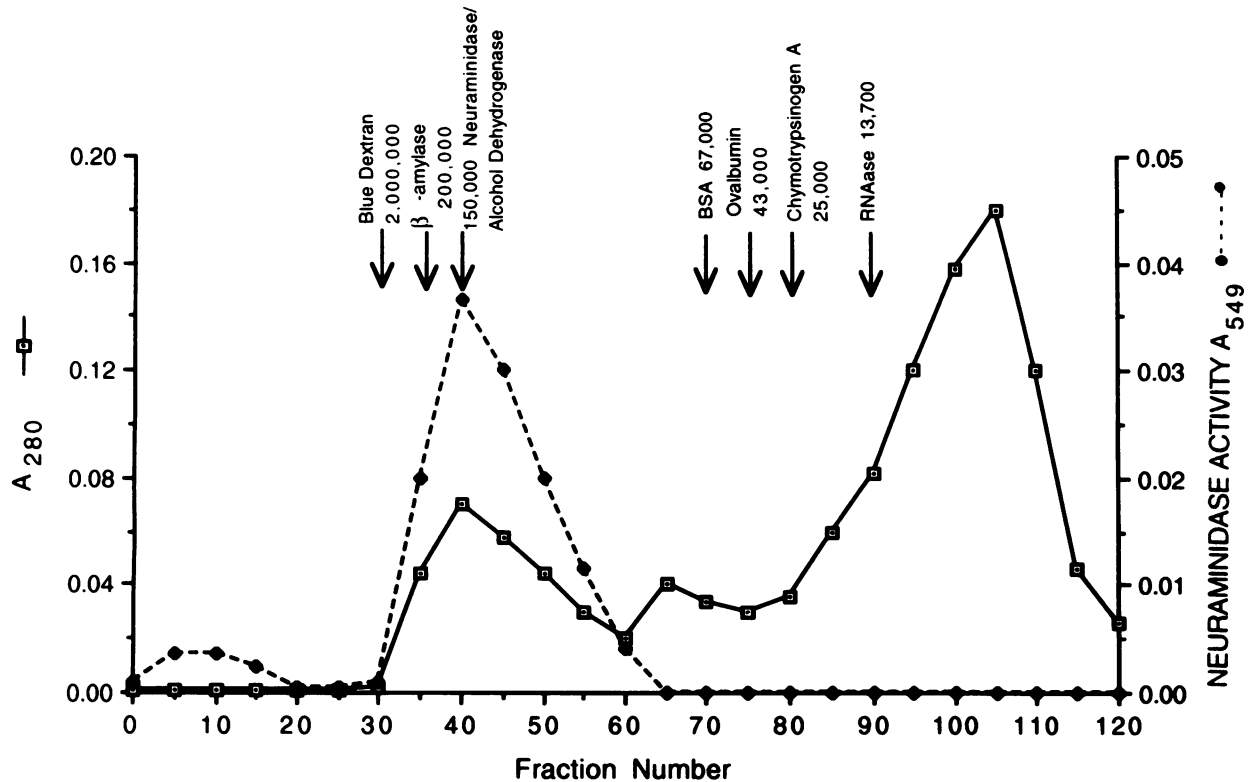


FIG. 2. Sephadex G-200 elution profile of stage I neuraminidase from Ph A1. Stage I enzyme was applied to the column and eluted with 10 mM sodium citrate buffer (pH 6.5) at 4°C. Every fifth tube was assayed for neuraminidase activity by the procedure of Aminoff (1). Calibration of the column with blue dextran and proteins of known molecular weights was performed with the same buffer.

assayed for activity. In the presence of immune serum, the percent reduction in neuraminidase activity of stage I preparations obtained from heterologous serotypes (Table 2) ranged from 14.5% (serotype 15) to 65.7% (serotype 16). It is interesting to note that the two strains (serotypes 10 and 15) whose neuraminidases demonstrated the least neutralization by anti-Ph A1 neuraminidase antibody (16.4 and 14.5%, respectively) were biotype T strains. The percent reductions in neuraminidase activity between the preimmune and immune serum incubations were relatively similar among the strains of the different serotypes as evidenced by the observation that there was no statistically significant difference among them.

DISCUSSION

The purpose of this study was to determine whether the neuraminidases produced by *P. haemolytica* serotypes were similar with regard to molecular weight, substrate specificity, and antigenic identity. We have recently characterized the production of this enzyme by a Ph A1 strain isolated from a case of bovine pneumonia (17). In that study, we used a method for producing the enzyme that had been developed for serotype 1 strains. We were concerned that we may have influenced our results by using an assay that was optimal for serotype 1 neuraminidase but not for neuraminidases produced by other *P. haemolytica* serotypes. Therefore, we attempted to examine the relationship between the Ph A1 neuraminidase and the neuraminidases produced by the other serotypes.

The first characteristic examined was substrate specificity.

TABLE 2. Antibody neutralization by Ph A1 antineuraminidase of neuraminidases produced by heterologous serotypes of *P. haemolytica*

Serotype	Postincubation activity ^a		% Reduction in activity ^b
	Preimmune serum	Immune serum	
1	9.732 ± 0.64	5.321 ± 0.14 ^c	45.3
2	5.792 ± 0.13	2.155 ± 0.13	62.7
3	4.243 ± 0.06	1.886 ± 0.13	55.5
4	3.637 ± 0.26	2.357 ± 0.06	35.1
5	7.274 ± 0.13	2.896 ± 0.06	60.1
6	6.937 ± 0.06	3.839 ± 0.06	44.6
7	7.812 ± 0.67	3.738 ± 0.03	52.1
8	8.924 ± 0.16	4.310 ± 0.40	51.7
9	7.072 ± 0.63	4.445 ± 0.53	37.1
10	3.468 ± 0.03	2.896 ± 0.06	16.4
11	ND ^d	ND	ND
12	12.124 ± 0.07	6.398 ± 0.24	47.2
13	10.642 ± 0.94	8.419 ± 0.07	20.9
14	6.567 ± 0.34	3.637 ± 0.60	44.6
15	4.647 ± 0.06	3.973 ± 0.09	14.5
16	11.113 ± 0.67	3.806 ± 0.37	65.7

^a Stage I neuraminidase activities after incubation with serum. Either preimmune or immune serum was incubated with stage I enzyme from the various *P. haemolytica* serotypes, and activity against fetuin was assayed. The activities (micromoles of sialic acid released per minute per milliliter) represent a mean of two determinations.

^b The percent reduction in neuraminidase activity was obtained by comparing neuraminidase activities after a 90-min preincubation on ice and a 60-min incubation at 37°C in the presence of preimmune and immune sera and dividing the difference by the preimmune value.

^c Statistically different from preimmune serum postincubation activity against Ph A1 neuraminidase ($P = 0.0042$) as determined by Student's *t* test in a pairwise comparison.

^d ND, no neuraminidase activity detected.

With compounds of known α -ketosidic linkages, neuraminidase produced by Ph A1 was found to be active on all substrates examined; however, activity was very low against BSM (Table 1). These results are similar to those obtained in a previous study (17), in which neuraminidase produced by a Ph A1 strain was highly active against *N*-acetylneuramin lactose, human α -1-acid glycoprotein, and fetuin but was only slightly active against BSM. Similar substrate specificities were observed for all neuraminidases produced by the various serotypes. This type of substrate specificity by a bacterial neuraminidase has been observed before. For example, neuraminidases produced by a variety of other bacteria, including *V. cholerae* (4), *S. pneumoniae* (8), *Arthrobacter* species (9), and *Clostridium perfringens* (4), have been shown to cleave sialic acid from more than one substrate. This is in contrast to the neuraminidase produced by *S. agalactiae* which is active against only BSM (2). In any event, Ph A1 neuraminidase released more sialic acid from the five substrates than did the other serotypes. This seems to be the only correlation that we can draw between serotype and neuraminidase production. We are currently examining a large number of *P. haemolytica* field isolates to determine whether there is a correlation between enzyme production and serotype.

Another interesting observation was that *P. haemolytica* serotype 11 did not produce a neuraminidase. The reason for this remains unclear. However, Frank and Tabatabai (6) also reported that this *P. haemolytica* serotype did not produce a neuraminidase. These authors (6) reported that type strain *P. haemolytica* serotypes 2, 3, 4, and 10 also failed to produce detectable neuraminidase activity. In this study, we demonstrate that all serotypes of *P. haemolytica* except for serotype 11 produce this enzyme. One possible explanation for why we were able to show neuraminidase production by serotypes 2, 3, 4, and 10 while Frank and Tabatabai (6) were not is that we were measuring extracellular enzyme production by these organisms while Frank and Tabatabai measured mostly cell-bound enzyme.

We next examined the molecular weights of the neuraminidases produced by the various *P. haemolytica* serotypes. The molecular weight for Ph A1 neuraminidase was previously reported to be 150,000 (17), determined by gel filtration on Sephadex G-200. This was confirmed in this study (Fig. 2), and it was also shown that serotypes 6, 10, 12, and 15 produced a neuraminidase with a similar molecular weight on Sephadex G-200. Serotypes 2, 3, 4, 5, 7, 8, 9, 13, 14, and 16 all produced a neuraminidase with a molecular weight of approximately 200,000 on Sephadex G-200 (Fig. 1). These molecular weights are higher than those previously determined for the neuraminidases of a variety of other bacteria, including the neuraminidases of the group B streptococci (molecular weight, 120,000 to 180,000) (2), *Arthrobacter* species (molecular weight, 87,000) (5), *V. cholerae* (molecular weight, 68,000) (4), *C. diphtheriae* (molecular weight, 65,000) (13), and *S. pneumoniae* (molecular weight, 88,000) (4).

Purified neuraminidase (stage V from Ph A1) was used in the immunization of New Zealand White rabbits to produce antibody specific for the enzyme produced by this strain. The presence of antibody to this enzyme was detected by the use of the standard neuraminidase assay and was based on the reduction of thiobarbituric acid-reactive material after a 90-min incubation at 4°C of the enzyme-antibody mixture (Table 2). The results of this study indicate that neuraminidases produced by strains of different serotypes of *P. haemolytica* are quite similar antigenically, at least regarding

the presence of similar antigenic sites making up the enzymatically active portion of the molecule.

Two interesting, unexplained observations were made regarding biotype T strains. Biotype T strains apparently produce a neuraminidase that has less activity against *N*-acetylneuramin lactose than do their biotype A counterparts. In addition, the two neuraminidases that were least neutralized by anti-Ph A1 neuraminidase antibody were biotype T strains (serotypes 10 and 15). The significance of these findings remains unclear.

In this report, we show that the neuraminidases produced by the various serotypes of *P. haemolytica* are neutralized by specific anti-Ph A1 neuraminidase antibody and are similar in molecular weight and substrate specificity. Thus, investigators can study these enzymes by using the characterization scheme developed previously (17) for Ph A1 neuraminidase, without concern that the assay techniques would be inadequate for neuraminidases produced by other *P. haemolytica* serotypes. The contribution, or lack thereof, of Ph A1 neuraminidase production to the virulence of this organism remains unknown. At the present time, there is no evidence that neuraminidase-negative *P. haemolytica* strains are less virulent than neuraminidase producers. We are currently examining this possibility.

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