NEURAMINIDASE AS A VIRULENCE FACTOR OF *PASTEURELLA MULTOCIDA*

by

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Approved by:

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DEDICATION

To my wife, Uju, and our children, Chuka and Ifeyinwa.

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SECTION I

INTRODUCTION

INTRODUCTION

One major cause of great economic loss to the livestock industry is the bovine respiratory disease complex. It is the most troublesome disease problem of cattle in North America and probably in most other parts of the world (Jensen et al., 1976; Mukkur, 1977; Irwin et al., 1979; Maheswaran and Theis, 1979). The bovine respiratory disease complex consists of at least five clinical disease syndromes: neonatal calf pneumonia, enzootic pneumonia of calves, the shipping fever complex, atypical interstitial pneumonia and pneumonia of adult cattle (Lillie, 1974). A combination of factors are involved in the etiology of the bovine respiratory disease complex (Hamdy and Trapp, 1967). Stress and viral infections in combination with bacterial colonisation of the lower respiratory tract are such factors (Jericho and Langford, 1978). The bacterial species most frequently associated with this complex are Pasteurella multocida and Pasteurella hemolytica while the viruses usually involved as intercurrent infection are Para Influenza-3 and Infectious bovine rhinotracheitis viruses (Gillespie and Timoney, 1981). Pasteurella multocida isolated from the bovine respiratory disease complex in the United States and Canada belong to capsular type A (Carter, 1955). Pasteurella multocida types B and E are involved in haemorrhagic septicaemia (H.S.) - a disease of economic importance common in Asia, Africa,

some parts of South America and Southern Europe (Bain, 1963). Pasteurella infection in man has also been reported (Olsen and Needham, 1952; Wang and Harry et al., 1980).

Despite the public health and economic significance of P. multocida infections, relatively little is known about the virulence factors of this organism (Maheswaran and Thies, 1979).

Although the immunity in pasteurellosis has been shown to be humorally mediated (Bain, 1963; Carter, 1967; Robert et al., 1980), the exact mechanism by which antibodies remove organisms from tissues of the infected host, is poorly understood (Collins, 1973; Woolock and Collins, 1975).

Endotoxin is believed to be a virulence factor because it is responsible for death in H.S. (Rebers et al., 1967). Types A, B, and E of P. multocida are capsulated (Carter, 1958; Peun and Naggy, 1976) and although the capsular material of Streptococcus pyogenes (Barkilius, 1960) and Escherischia Coli (Orskov, 1978) are known to be virulence factors, the capsular material of P. multocida has not been proven to be a virulence factor (Maheswaran and Thies, 1979). When grown in the presence of tryptophan, the enzyme tryptophanase produced by P. multocida catalyses the breakdown of the amino acid into indole, pyruvic acid and ammonia. Indole and a closely-related compound-3-methyl Indole are lipophilic and have been associated with certain diseases (Yokoyama et al., 1975 and Bradely et al., 1978). However,

in vivo production of indole by P. multocida has not been demonstrated and therefore it is not known if the enzyme tryptophenase is a virulence factor or not.

Hyaluronidase, which is a virulence factor of some Gram positive bacteria like Streptococcus and Staphylococcus species, has been demonstrated in P. multocida type B (Carter and Chengappa, 1980) but the investigators could not say whether or not this enzyme plays a role in the pathogenesis of hemorrhagic septicaemia.

The enzyme neuraminidase has been demonstrated in P. multocida (Tsolov and Karadzhov, 1969; Schermann et al., 1970 and Drzniek et al., 1972) but it is not certain that neuraminidase is a virulence factor.

The objective of this study was to attempt to neutralise P. multocida neuraminidase with anti-neuraminidase and determine if the antiserum would protect mice challenged with a homologous P. multocida. It is hoped that this will shed more light into the possible role of neuraminidase as a virulence factor of P. multocida.

SECTION II

LITERATURE REVIEW

LITERATURE REVIEW

Pasteurella multocida was first described in 1880 by Pasteur as the cause of fowl cholera. It was later observed that the fowl cholera bacillus could not be culturally differentiated from the organisms of rabbit septicaemia, Swine plaque and certain pneumionias of cattle. Because of the identity of these organisms and the similarity of the diseases produced by them in various animals, Heuppe (1886) grouped them under one name - bacterium septicaemiae hemorrhagiae. A proposal was later made (Trevisen, 1887) that the several disease-producing agents be recognized as seperate species and grouped under one genus, Pasteurella, named in honor of Pasteur. Rosenbusch and Merchant (1939) suggested that the hemorrhagic septicaemia organisms of the various animals be rather recognized as a single species and urged that the name of the species be Pasteurella multocida - derived from the name "Bacterium bipolare multocidum". Lignieres was the first to apply the name "Pasteurellosis" to the group of diseases caused by Pasteurella multocida.

Pasteurella multocida is a Gram negative, non-spore-forming, facultatively anaerobic, fermentative, coccoid organism. In stained films made from infected tissues, the ends of the short rods are more deeply stained than the central portion giving them a distinct bipolar appearance, hence Pasteur described the organism as "figure of eight

bacillus." The organisms are normally carried as commensals in the oropharynx of many animal species. Under certain predisposing conditions like poor sanitation and ventilation, overcrowding and exposure to cold or viral infection, the organisms multiply extensively and penetrate the physical and immunologic defenses of the respiratory tract. Attempts to produce pasteurellosis in animals not stressed or virus-infected have been unsuccessful (Collier, 1968). Pasteurella multocida is generally regarded as the type species of the genus "Pasteurella" although the species embraces a range of biotypes and serotypes with considerable differences in pathogenicity, host predilection, biochemical activity colonial morphology and antigenic nature. The eighth edition of Bergy's manual lists P. multocida, P. hemolytica, P. pneumotropica and P. ureae as the official species of the genus "Pasteurella."

Cultural and Biochemical Characteristics:

Pasteurella multocida grows best in media containing blood or haematin. It is non hemolytic but most strains produce a brownish discoloration of blood media in regions of confluent growth. Cultures on blood agar have a distinctive smell useful in recognition of the species. The organism does not grow on MacConkey agar and this is one of the characteristics that differentiate it from P. hemolytica. Webster and Burn (1926), Aderson et al., (1930); Hughes, (1930) and Carter and Bigland, (1953)

studied the dissociation pattern of P. multocida on agar. They described three principal colonial variants as mucoid, smooth and rough. The mucoid variant was large and flowing on agar, was capsulated and produced moderate virulence in mice. The smooth (flourescent) type produced medium-sized discrete colonies, was capsulated and highly virulent for mice. The rough (blue) colonies produced small discrete colonies, was non-capsulated and had low virulence for mice. Webster and Burn (1926) observed that many cultures of P. multocida recovered from carrier animals and chronic processes of swine, poultry and rabbits were predominantly the mucoid variant. The smooth type sometimes called the intermediate variants are often seen in avian cultures and laboratory strains that have been maintained for long periods on artificial media (Carter, 1955). There is no clear-cut pattern of growth of P. multocida in liquid media (Elberg and Cheng Lee, 1950). Some variants showed distinct granularity in nutrient broth, some perfectly diffuse growth while others initially grew diffusely and later became granular. In infusion broth, growth is manifested by slight viscid sediment and addition of a few drops of sterile serum greatly increases growth (Gillespie and Timoney, 1981).

Slight biochemical differences are observed among different strains of P. multocida. The biochemical characteristics are listed in Table I (Carter, 1976; Shigidi and Mustafa, 1979; Gillespie and Timoney, 1981). Most canine

strains, unlike other strains, tend to be negative for mannitol and sorbitol (Frederiksen, 1973; Carter, 1976). A few of them that ferment these sugars may represent those strains acquired from the cat or other animals (Carter, 1976). Shigidi and Mustafa (1979) observed H_2S production in 27 out of 43 strains studied.

Table I. Biochemical Characteristics of *Pasteurella multocida*

Characteristics	Sign
Glucose	+ (n.g.)
Mannitol	d
Sucrose	+
Lactose	-
Fructose	+
Galactose	+
Maltose	-
Raffinose	-
Xylose	d
Sorbitol	d
Dulcitol	-
Salicin	-
Indole	+
Nitrate reduction	+ (n.g.)
Urease	-
Simmons Citrate	-
Oxidase cytochrome	+
Gelatin hydrolisis	-
Esculin hydrolisis	-
Hydrogen sulfide	d
Catalase	+
Ornithine decarboxylase	+
Arginine dehydrolase	-
Lysine decarboxylose	-
Tripple Sugar Iron Slant Acid	+
Tripple Sugar Iron Butt Acid	+

d = most strains (16-84%) positive
n.g. = no gas
+ = positive reaction
- = negative reaction

Antigenicity and Serotypes: There are two principal antigenic components of P. multocida (Carter, 1951) - a type-specific polysaccharide associated with the capsule and the somatic antigen. Another antigenic component of P. multocida is endotoxin which is not type specific (Penn and Magy, 1976) but may be a minor protective antigen. The capsular substance has been extracted (Hoffenreich, 1928; Priosky, 1938; Machennan and Rendle, 1957; Bain and Knox, 1961 and Penn and Nagy, 1976) from P. multocida. At least 12 somatic antigens have been described (Namioka and Murata, 1961). Numbers are used to denote different somatic antigens while capsular antigens are written as letters. The antigenic formula of some serotypes may therefore be written as 6:B; 6:E or 5:A. The capsular substance of mucoid variants is made up largely of hyaluronic acid (Carter, 1976) and although this mucoid character is to some extent phenotypic, it may be maintained through many subcultures. The capsulated mucoid variant may be recognised on a culture plate by the action of staphylococcal hyaluronidase (Carter and Rundel, 1975). Smooth variants are also capsulated but the capsular material is a glycoprotein which has been found to be protective against experimental pasteurellosis in mice and H.S. in cattle (Penn and Nagy, 1976; Mukkur, 1978). Rough variants are not capsulated but when passaged in mice or chick embryo, they change into smooth capsulated forms (Carter, 1951).

Most serological procedures for typing P. multocida are based on specificity of the antigenic capsular substance. Various investigators employing different methods classified P. multocida into varying numbers of serotypes. Cornelius, (1929) and Rosenbusch and Merchant (1939) used agglutination and adsorption tests to group P. multocida into 4 types.

Little and Lyon (1943) and Roberts (1947) classified the species into 3 and 8 types respectively. They used passive protection tests. In similar studies, Namioka and Murata (1961) combined capsular and somatic antigen-type results obtained from slide agglutination tests and grouped P. multocida into 15 serotypes. Heddleston et al., (1972) described the gel diffusion precipitin test for serotyping avian strains of P. multocida. They recognised 16 serotypes. Each of these serological classifications was established to correlate a specific serotype with an animal host, specific disease or epizootiological condition. But this relationship could not be established in most cases and none of the classification schemes completely agrees with the other. In a study to compare serotyping systems, Brodgen and Packer (1979) found that cultures of a single serotype often represented more than one serotype in another system and that cultures with one or two serotype antigens in common may differ in other antigens. Six of the 9 Namioka-type strains were inagglutinable in anti serum prepared against Little and Lyon's type strains.

Of the remaining 3 Namioka type strains, 2 reacted with antiserum prepared against Little and Lyon's type I strain. Brogden and Packer attributed this lack of correlation to the antigenic complexity of P. multocida but Carter (1951) stated that the problem arises from the fact that serological methods applied by workers depended on immunological reactions involving specific and nonspecific factors. Consequently strong cross reactions made difficult the unequivocal identification of P. multocida serotypes.

The classification system currently accepted is based on the specificity of the capsular substance of P. multocida. Carter (1955) used the indirect haemagglutination test (I.H.A.) to group P. multocida into four types designated A, B, C, D, based on differences in their capsular antigens. Capsular substance was extracted with saline and adsorbed to human type 'O' red blood cells. Specific antisera were added to treated cells and incubated at room temperature. Marked agglutination was regarded as positive. An additional type E was later identified (Carter, 1961) and type C category was subsequently dropped (Carter, 1963) because of difficulties in recognition. The presence of capsular hyaluronic acid particularly in mucoid type A organisms interfered with their typability by the I.H.A. test. A method was therefore described (Perreau et al., 1962; Carter, 1972) in which cultures were pre-treated with hyaluronidase prior to typing by the I.H.A. technique.

Because the I.H.A. test depends on the presence of capsular substance on the organism, non capsulated, rough types cannot be typed by this method. The Acriflavin test (Allesandrini and Sabatuci, 1973) is employed in identifying these types. Antigenically rough cell types yield clumps, mucoid cells produce slimy precipitate while smooth cells remain suspended in the dye used for this test. Table II lists the common serotypes of P. multocida found in animals.

More recently (Carter, 1976) a proposal was made for 5 biotypes of P. multocida based on the presence or absence of some of the following characteristics: hyaluronidase decapsulation; acriflavin floculation; colonial irridiscence; carbohydrate fermentation; mouse pathogenicity and serum protection tests. The biotypes proposed under this scheme are to be called mucoid; hemorrhagic septicaemia; porcine; canine and feline biotypes. Within the mucoid biotype differences in the somatic antigen among strains may be detected by different serologic procedures (Namioka and Brunner, 1963; Heddleston et al., 1972). Strains in the hemorrhagic septicaemia biotype differ from other strains in the species by possessing two different, serologically recognisable capsular antigens (B and E). On the basis of this, cultures are identified as type B or type E. Both however possess a common somatic antigen (6). Rimler (1978) described the coagglutination test for differentiating the two types. The

Table II. Common Serotypes of Pasteurella multocida in animals. (Gillespie and Timoney, 1981)

Host	Disease	Sero types encountered
Cattle	Hemorrhagic Septicaemis	*6:B; 6:E
Chickens	Fowl cholera	5:A; 8:A
Turkeys	Fowl cholera	9:A
Pigs	Pneumonia	1:A, 3:A, 5:A, 1:D 4:D and 10:D
Sheep	Pneumonia	1:D; 4:D

*Letters denote capsular antigen type

Numbers denote somatic antigen type

test uses specific antibody-coated staphylococci to detect soluble group antigen of serogroup B or E.

Pathogenicity and Immunity

Table II lists some of the diseases caused by different serotypes of P. multocida in various animals. Shipping fever in cattle caused by P. multocida is a broncho pneumonia with moderate amounts of fibrin on lung surface. This is somewhat in contrast to the pneumonia produced by P. hemolytica where amounts of fibrin are much greater and the lesions represent a true fibrinous pneumonia (Scheifer, Ward and Moffat, 1978). Hemorrhagic Septicaemia of cattle, goat and sheep usually takes the pectoral or pneumonic form and occurs mainly in the tropical areas of the world. The nature of the disease in rabbit could be septicaemic and organisms can easily be found in films of blood or spleen pulp after death. Snuffles is the name applied to the milder form of respiratory infection caused by P. multocida in rabbits.

In dogs, P. multocida infection takes the form of cystitis, endocarditis and chronic bronchitis. The canine biotype (Carter, 1976) is responsible for these infections and this biotype differs from the common strains associated with disease in other animals in not being able to ferment mannitol and sorbitol. They are acid agglutinable (Smith,

1958). The feline and canine biotypes are more important in their involvement in wound infections in man than they are as pathogens of cats and dogs. Wound infections resulting from animal bites were among the 316 cases of human pasteurellosis compiled by Hubert and Rosen (1970). The first bacterin used for Pasteurellosis was prepared by Pasteur in 1880 to protect poultry against fowl cholera. This vaccine consisted of living cultures of two grades of virulence given a few days apart. The vaccine is now obsolete but a variety of live vaccines, bacterins and antisera are available for protection against pasteurellosis. Mostly capsulated strains are used for preparation of bacterins and antisera because the capsular substance is the major immunogenic factor of P. multocida.

Although it was over 50 years ago that heat killed formalized bacterins were first used to immunise cattle against pasteurellosis, the value of these biological products is still questionable (Bain, 1955b; Paloty, 1958; Pyke, 1966; Wilkie and Noris, 1976). Nwangota and Thomson, (1980) stated that hemorrhagic septicaemia bacterins have given disappointing results in Africa probably because the serotypes included in the vaccine were not the same as those causing the disease. Field efficacy data on vaccination with bacterins of P. multocida and P. hemolytica in North America, indicate that the immunization programs may not be adequate and that adverse reactions may follow respiratory tract challenge exposure of vaccinated animals

(Friend et al., 1977). The uncertainty about the protective effect of pasteurella bacterins may be attributed to the apparent difficulty in inducing pneumonia with pasteurella species and to the reliance on mouse models for efficacy evaluation (Rushton, 1978; Evans and Wells, 1979) whereas mice are almost insensitive to P. hemolytica and mouse protection tests may be irrelevant to cattle (Collins, 1978). Furthermore, standard experiments in mice assess parenteral immunity in relation to systemic bacterial inoculation (Collins, 1973) whereas the bovine disease prevalent in N. America is pneumonic. Rather than assess systemic immunity, Wilkie et al., (1980) monitored the clinical and pathologic responses of calves immunised with P. hemolytica bacterins and challenged by pumonary exposure. They observed adverse effects on the calves when route of vaccination was subcutaneous. Minor protection with no adverse effects were recorded if vaccination was done intrabronchically. The workers recommended caution in the use of bacterins containing P. hemolytica.

More recently, attempts have been made to use various components of the cells of P. multocida and P. hemolytica for immunisation of animals (Heddleston et al., 1966). Immunogenicity of capsular antigens (Nagy and Penn, 1976; Penn and Nagy, 1976; Mukkur, 1977; Tadayen and Lauermen, 1981), free endotoxins (Rebers and Heddleston, 1974), lipopolysacharide protein complexes (Ganfield et al., 1976, Rebers et al., 1979) and ribosomal fractions (Baba, 1977)

of P. multocida have been studied. The result of these studies indicate that the extracts of these immunogenic factors confer better protection to animals than the intact cell. Potassium thiocyanate extract (KSCN) of P. multocida capsular substance was found (Mukkur, 1977) to be far more effective than formalin treated cells in protecting mice against experimental pasteurellosis. Tadayan and Lauerman, (1981) recorded 55 - 100% protection in mice and hamsters with extracts of P. hemolytica antigen compared to 20 - 60% protection observed with bacterins prepared from the same cell population.

Although, Wells et al., (1979) have shown that cell-mediated immunity plays some role in resistance of lambs to P. hemolytica infection, immunity in pasteurellosis is generally believed to be humorally mediated (Roberts, 1947; Bain, 1963; Carter, 1967; Robert et al., 1980). However, the exact mode of action of antibodies in the removal of organisms from the tissues of the infected host is not clear. Maheswaran and Thies, (1979) demonstrated that heat stable opsonins increased the uptake by bovine neutrolphils of ^{3}[H] thymidine-labelled P. multocida antibodies, thereby suggesting opsonic activity of the antibodies. But Hoffling et al., (1979) showed that rabbit antiserum to P. multocida did not enhance phagocytosis when compared with normal serum in in-vitro tests. They also stated that the antiserum was not bactericidal

although Mukkur (1978) demonstrated bactericidal titres of sera from calves inoculated with KSCN extract of P. multocida. Apart from bactericidal and opsonic activities, antibodies can eliminate invading organisms by neutralization of toxins or enzymes. Pasteurella multocida produces endotoxins and some enzymes. One of the enzymes produced by this organisms is Neuraminidase.

Neuraminidase of Microorganisms

The enzyme neuraminidase (Sialidase; N-acetylneuraminylhydrolase) was first observed in influenza virus Hirst, 1942) where its role in viral adsorption and desorption from erythrocytes was evolved. Hirst first demonstrated that influenza virus becomes attached to red blood cells through sialic acid residues (receptors) embedded in the cell membrane. Such an adsorption is accompanied by agglutination of the cells. On production of neuraminidase by the virus, the enzyme cleaves off the sialic acid residues thereby destroying the receptors and freeing the virus. This was how the name "Receptor Destroying Enzyme" (RDE) was evolved for neuraminidase. McCrea (1946) described RDE of Clostridium perfringes and in the same year, Burnet and Stone observed that culture fluids of Vibrio cholerae rendered human red cells inagglutinable by influenza virus. They described the factor responsible for this as RDE of V. cholerae. It was not until 1949

that Gottoschalk and Lind provided evidence of the chemical activity of RDE and in 1956, it was characterized as a glycosidase (Heimer and Meyer, 1956; Gottoschalk, 1956). The enzyme has been termed "sialidase" (Heimer and Meyer, 1956) or "Neuraminidase" (Gott schalk, 1957) and its action defined as the hydrolytic cleavage of the glycosidic bond joining the keto group of N-acetyl neuraminic acid (sialic acid) to D-galactoseamine (Gott schalk, 1957) in glycoproteins.

Since the first description of neuraminidase in 1946, the enzyme has been described in several other bacteria - Diplococcus pneumonia, (Chu, 1948), Streptococcus species, (Hayano and Tanaka, 1967), Klebsiella aerogenes (Pardoe, 1970), Corynebacterium pyogenes (Muller, 1971); myxoviruses (Wilson and Rafeelson, 1963; Mayron et al., 1961; Seto et al., 1966; Kendal et al., 1969) and protozoa (Watkins and Morgan, 1954). The neuraminidase of P. multocida was first described in 1969 (Tsolov and Karadzhov; 1969) and confirmed in 1970 (Scharmann et al., 1970).

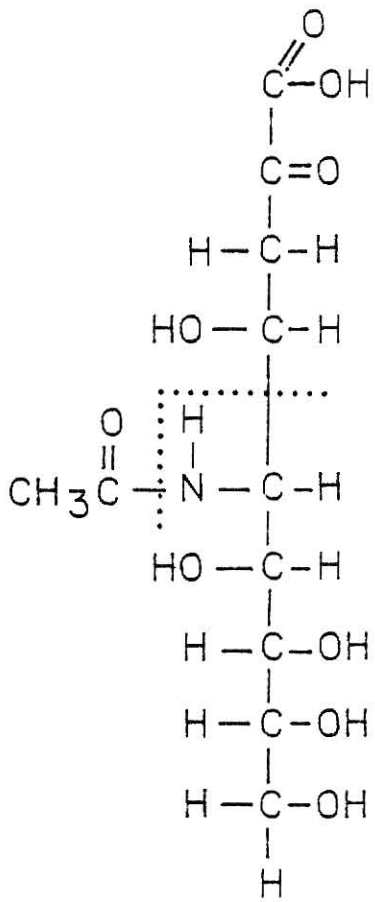
Muller (1974) classified neuraminidase-producing micro-organisms into three groups. Those in group I possess acyl neuraminic acid and their neuraminidase activity is weak. Members of this group include Pseudomonas aeruginosa; Klebsiella aerogenes, Escherichia coli, Proteus vulgaris, and Neisseria meningitidis. Group II micro-organisms produce neuraminidases irregularly. Their enzyme has strong

or weak activity and may play a secondary role in disease pathogenesis. Vibrio fetus and Mycoplasma gallisepticum belong to this group. Micro-organisms in Group III are well established neuraminidase producers. Most of them possess N-acetyl neuraminic acid - aldolase (N-ANA aldolase) activity. Vibrio cholerae, Dipplococcus pneumoniae, Clostridum perfringes, P. hemolytica and P. multocida belong to this group. The neuraminidases of most members of this group show strong activity and have been implicated as virulence factors in several disease processes. The sialidases of all these micro-organisms differ in several properties but one thing common to them all is the removal of sialic acid residues from sialoglycoproteins.

Sialic or neuraminic acids are 9-carbon amino-sugar substances (Fig. I.) which occur as terminal carbohydrate residues in a variety of glycoproteins, mucins, gangliosides and various oligosaccharides. The naturally occuring sialic acids are substituted neuraminic acid derivatives (N-acetyl, N-glycolyl and N,O-diacetyl neuraminic acid).

The function of sialic acids in various tissues and body fluids has been extensively studied principally by observing the effects of selective removal of this residue by digestion with neuraminidases from influenza virus or V. cholerae. From such studies, the role of sialic acid in preserving the conformational structures of

Figure 1. N-acetyl-neuraminic acid.



The dotted lines indicate the substitute amino group.

glycoproteins, as supportive structures and filtration barriers (basement membranes) and as constituents of cellular membranes (red blood cells) have been reported (Spiro, 1969; Spiro, 1970). Sialic acids also contribute in the ability of glycoproteins to participate in enzymatic and hormonal activities, in antigen-antibody interactions, clotting mechanisms, metal and hormone transport (Spiro, 1969). Virtually all serum proteins except albumin contain sialic acid (Haskel et al., 1970) and removal of the sialic acid residue results in such structural alterations that such proteins are rendered non functional. Chemical or enzymatic removal of sialic acid from erythropoetin, human chorionic gonadotropin, follicle stimulating and leutenising hormones completely inactivated these hormones (Haskel et al., 1970). Because neuraminidase removes sialic acid from some vital tissues and fluids, the enzyme plays some role in the pathogenesis of some diseases caused by neuraminidase-producing organisms.

Pathogenic Significance of Microbial Neuraminidase

There are three presuppositions for the pathogenic action of microbial neuraminidases:

Enhancement of microbial survival: This mode of action is particularly important for those organisms that inhabit the respiratory, urogenital and digestive tracts. Neuraminidase, by removing terminal sialic acid from mucins or

salivary glycoproteins, causes the loss in viscosity of such proteins. Consequently, the normal function of those lubricants of entrapping invading micro-organisms, is lost. The enzyme therefore has an adaptive role of enhancing the survival and penetration of epithelial mucous lining by organisms that inhabit mucous tracts. Neuraminidase has in fact been implicated as a virulence factor in Strept. pyogenes, Vibrio cholerae and Corynebacterium diphteriae (Pardoe, 1974). Pasteurella species are respiratory tract inhabitants and neuraminidase has been demonstrated in P. multocida (Tsolov and Karadzhov, 1969; Scharmann et al., 1970; Drzneik et al., 1972) and in P. hemolytica (Frank and Tabatabai, 1981; Scharmann et al., 1970). While Drzneik and his colleagues found no direct correlation between level of neuraminidase activity in different strains of P. multocida and their virulence in mice, Muller and Krasemann (1974) found that P. multocida strains with very high neuraminidase activity were more virulent in mice than those with less enzyme activity. Frank and Tabatabai (1981) found no direct relationship between P. hemolytica neuraminidase levels and pneumonia in sheep but in cattle they claim a relationship exists. Serotypes 1 and 2 are most frequently isolated from cattle and serotype I is more often recovered from cattle with respiratory disease and pneumonic lungs. This serotype was shown to have more neuraminidase activity than serotype 2. There is therefore

some controversy as to whether neuraminidase is a virulence factor in Pasteurella species.

ii. Destruction of functions and structures: The action of neuraminidase on plasma proteins during septicaemia caused by neuraminidase - producing streptococcus species has been demonstrated (Muller, 1974). Immunoelectrophoretic study of serum proteins from septicaemic patients showed altered electrophoretic pattern, shifting towards the cathode as a result of removal of sialic acid that contributes negative charges. Such altered glycoproteins have diminished life span. The neuraminidase of Cl. perfringens, in a similar manner, altered the glycoproteins in infected wounds (Muller, 1970) and the neuraminidase of Bacteroides fragilis caused protein alterations in abscesses caused by this organism (Muller and Werner, 1970). Morell et al., (1971) have shown that oroso mucoid, fetuin, ceruloplasmin, haptoglobulin, alpha-2 macroglobulin, thyroglobulin and lactoferin were promptly removed from circulation by hepatic parenchymal cells after being desialated with Cl. perfringes neuraminidase. Woodruff and Gesner (1969) and Aminoff et al., (1977) also demonstrated similar phenomenon in the survival of erythrocytes and lymphocytes in the circulatory system. Neuraminidase - treated erythrocytes were rapidly removed from circulation by the Kupfer cells of the liver and mononuclear spleen cells.

Histochemical studies of organs of mice dying from intraperitoneal inoculation with purified penumococcal neuraminidase have shown marked decreases in the sialic acid content of the kidney and liver when compared to controls (Kelly and Griff, 1970). Particularly prominent was the loss of all acid mucopolysaccharide from glomeruli of kidney. Toxicity of pneumococcal neuraminidase was, in that study, attributed to the destruction of essential compounds containing sialic acid.

iii. Effects of neuraminidase on cell-surfaces: Removal of sialic acid residues from erythrocyte surface membrane causes increased cell adhesiveness (Gott schalk, 1960; Kemp, 1970; and Caso, 1972). By this mode of action, neuraminidase can cause agglutination (Chu, 1948; Drzniek, 1972; Greenwalk and Steane, 1973), lymphocyte stimulation (Adler et al., 1972; Bentwich et al., 1973; Han, 1972) and thrombocytopaenia (Bosmann, 1972; Choi et al., 1972 and Atherton and Born, 1973). The haemagglutinating activity of Erysipelothrix rheusiopathiae is thought to be related to the high neuraminidase activity of virulent strains. It was postulated (Wellman, 1955) that the high neurominidase activity is in some way involved in the generalized coagulopathy that occurs after swine are inoculated with the organism. Fischer et al., (1971) demonstrated severe thrombosis and a Sanrelli -

Schwartzmann-like syndrome caused by pan agglutinability of red blood cell as an effect of pneumococal neuraminidase action on the cells during an infection in mice. Agglutination of red blood cells occurs when influenza virus attaches to the sialic acid residues on cell membranes. As long as the virus remains attached to the red cells it cannot cause further haemagglutination. When the virus releases neuraminidase, the enzyme destroys the receptors (sialic acid) on the cell surface thereby freeing the virus. The freed virus is fully infective and can now attach to other red blood cells causing more haemagglutination. Viral neuraminidase therefore acts as a sort of spreading factor for the virus.

Isolation and some Properties of Microbial Neuraminidase

Isolation of neuraminidase from various micro organisms has been attempted using different techniques with varying degrees of success. Treatment of influenza virus with ether, alkali, detergents, acetone and lysosomes (Mayron, Barbara and Rafeelson, 1961) released no enzyme activity or destroyed it to varying degrees. Digestion with trypsin at pH 7.0 resulted in release of non sedimentable enzyme activity. Wilson and Rafeelson (1963) and Seto et al., (1966) used α-chymotrypsin and pronase treatment respectively to extract neuraminidase from influenza virus. Automated techniques have also been described

(Kendal and Madely, 1966) involving proteolysis of purified virus concentrates followed by rate density zonal centrifugation.

Extraction of many bacterial neuraminidases is done by growing the organism in liquid medium and recovering enzyme activity in the culture filtrate. Cl. perfringens (Cassidy et al., 1965), V. cholerae (Ada and French, 1961); Streptococcal (Hayano and Tanaka, 1967) and pneumococal (Tanebaum et al., 1970) neuraminidases have been extracted by this procedure. The procedure is most suited for isolation of extracellularly located enzymes. In some bacteria where the enzyme is cell associated, ultrasonication of washed cells has been done (Scharmann et al., 1970; Kelly and Grieff, 1970). The enzyme is recovered from the supernatant of sonicated cell preparation. Pasteurella multocida neuraminidase is strongly cell associated (Scharmann et al., 1970) and has been isolated by ultrasonication. The enzyme isolated by this method, unfortunately precipitated readily at $4^{o}C$ and possessed N-ANA aldolase activity (Drzneik et al., 1972). Drzniek, Scharmann and Balke, (1972) described the NaCl method which is suitable for obtaining P. multocida neuraminidase free from N-ANA aldolase activity.

Neuraminidase is an inducible enzyme. Presence of N-acetyl D-mannose amine or sialic acid greatly improves enzyme yield. It has been suggested (Drzniek et al.,

1972) that the stimulating effect of blood sera on the production of neuraminidase is due to the delayed release of the inducer, N-acetyl D-mannose amine - a breakdown product of sialic acid residues on red blood cell membranes. A study to compare the effects of different sera on the synthesis of neuraminidase by P. multocida, (Drzeneik et al., 1972) showed six to eight-fold higher neuraminidase activity per ml cell culture when medium contained 10% sheep serum as compared to serum-free controls. When bovine, horse, human, rabbit or swine sera were used at the same concentration, lower neuraminidase activity were recorded.

Mayron et al., (1961) demonstrated the specificity of the glycosidase action of neuraminidase. They failed to detect the liberation of free amino acids, sugars or peptides from N-acetyl neuraminyl-lactose (N-AN Lac) by influenza virus neuraminidase. N-acetyl neuraminic acid was the only detectable component of the substrate released. Substrates for neuraminidases include N-AN Lac, mucins, sialomucoids orosomucoid and fetuin. Some of these are better substrates than others for specific enzymes. The pH optimum of a given enzyme may vary from one substrate to another and different enzymes may vary in their pH optima for the same substrate. Molecular weight of neuraminidases range from 10,000 - 250,000 daltons. Some enzymes require calcium or other divalent metal ions for activity while

others do not. Table III summarizes some properties of some bacterial neuraminidases. Apart from Ethylene diamine tetra acetic acid (EDTA), cyanide, glutathione, phosphotungstic acid and sialic acid also exert inhibitory effects on neuraminidase. Other chemical inhibitors include N-substituted oxamic acids (Edmond et al., 1966) 3-4 dihydroisoquinolines (Brammer et al., 1968) Thiopyruvic acids and 2-(0-aminophenyl) benzimidazole analogs (Haskel et al., 1969).

Stability of neuraminidase is critically affected by temperature and pH. Heating at 70° for 30 minutes at pH 6.5 almost completely inactivated influenza virus neuraminidase (Mayron et al., 1961). V. cholerae neuraminidase is stable for 6 months when freeze dried and stored under vacuum at 0°C. Cassidy et al., (1969) improved the stability of Cl. perfringes neuraminidase by storing the dilute enzyme preparation in presence of albumin.

Purification and Assay for Neuraminidase

The process of specific adsorption of neuraminidase to and elution from red blood cells was first introduced by Burnet and Stone in 1947 and was one important step in purification of crude neuraminidase of influenza virus. Ada, French and Lind, (1961) combined this procedure with fractional precipitation and chromatography to purify V. cholerae neuraminidase. Ion exchange chromatography on Diethylaminoethylcellulose (DEAE) has been employed to

Table III. Properties of some bacterial Neuraminidases. (Muller, 1974; Ada, French and Lind, 1961)

Neuraminidase from	Molecular weight (daltons)	Calcium activation	EDTA inhibition	pH optimum	Substrate	Michaelis Constant
1. Vibro cholera	10,000	+	+	5.6	SL	1.2×10^{-3}
2. Pasteurella multocida	250,000		-	6.0	SL	2.0×10^{-4}
3. Clostridium perfringens	56,000	-	-	5.5	OM	5.0×10^{-5}
4. Diplococcus pneumoniae	88,000	-		6.5	F	2.4×10^{-4}
5. Group K strept		+	+	5.5	BSM	6.0×10^{-5}
6. Group A strept		+		5.5-7	OM	3.5×10^{-4}
7. Group B strept		+		5.5-7	SL	3.5×10^{-5}
8. Group C strept		+		5.5-7	BSM	4.9×10^{-5}
9. Group G strept		+		5.5-7	BSM	2.5×10^{-4}
10. Strept sanguis (viridans)	250,000	-		5.5-7	BSM	5.4×10^{-5}
11. Corynebacterium diphteriae	34,000-65,000	+	+	5.5-6.0	BSM	1.7×10^{-4}
12. E.rheusiopathiae	210,000	+		6.0	SL	9.0×10^{-4}

SL = Sialyl Lactose

OM = Orosomucoid

F = Fetuin

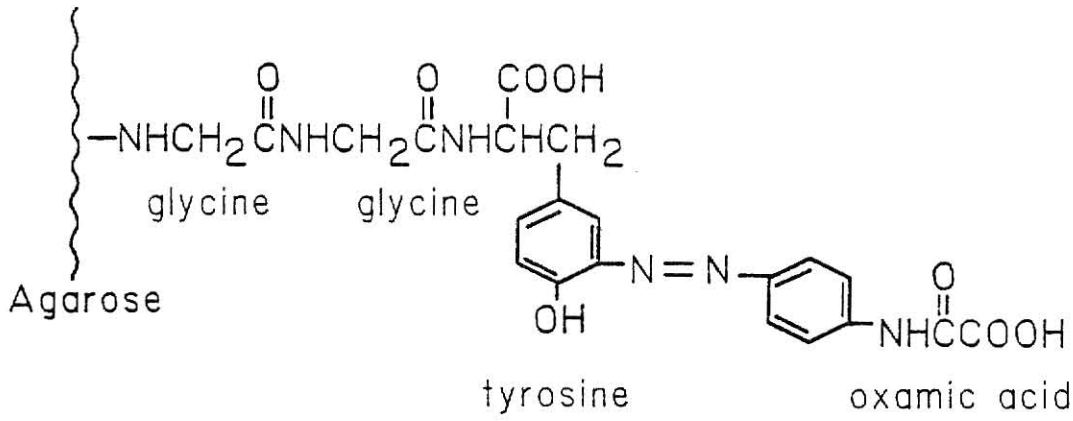
BSM= Bovine Submaxillary mucin

purify Cl. perfringens neuraminidase (Cassidy et al., 1965), Group K Streptococcus sialidase (Hayano and Tanaka, 1967) pneumococcal neuraminidase (Tanebaum et al., 1969; Stahl and O'Toole, 1972). Other methods that have been described include gel filtration for purification of P. multocida neuraminidase (Drzeniek et al., 1972), Cl. perfringens neuraminidase (Cassidy et al., 1965) and Diplococcus pneumonia neuraminidase (Hughes and Jeanloz, 1964). Cuatrecasas and Iliano, (1971) described the affinity chromotography procedure for purifying neuraminidases of V. cholerae, Cl. perfringens and influenza virus.

This method is a functional purification approach that, exploits one of the most unique properties of enzymatic active sites - that of "recognition." A selective adsorbent is made by covalently attaching, through azo linkage, a neuraminidase inhibitor, N-(p-aminophenyl) oxamic acid, to agarose beads which possess a three amino acid spacer arm (Fig. 2). The enzyme activity present in crude enzyme preparations is extrated by the column containing this adsorbent and elution is achieved by modifying the pH and ionic strength of the buffer. This column may also be used to concentrate neuraminidases or remove inactivated forms of the enzyme in already purified enzyme preparations.

Assay for neuraminidase activity involves allowing the enzyme act on a suitable substrate. The released

Figure 2. The adsorbent used in selective purification of neuraminidases by affinity chromatography.



The diazonium derivative of N-(p-aminophenyl) oxamic acid was coupled to agarose-gly-gly-tyr.

sialic acid is then measured. Sialic acid has been detected and measured by procedures like the Orcinol (Klenk and Langerbeins, 1941), tryptophan - per chloric acid (Siebert and Pfaff, 1948), direct Ehrlich (Werner and Odin, 1952), Resorcinol (Svennholm, 1957) and the diphenylamine method (Saifer and Gerstenfeld, 1957). These procedures are not only relatively insensitive but in general have a low specificity and cannot be used for the measurement of sialic acid in unpurified biological materials (Warren, 1959). More over, because these procedures involve heating with strong acids, they cannot distinguish free from bound sialic acid - a differentiation necessary to follow the enzyme release of sialic acid from substrates. (Aminoff, 1961). Warren, (1959) and Aminoff (1961) described a colorimetric method - the Thiobarbituric Acid Assay (TBA) for the measurement of sialic acid. This method is based upon the oxidative cleavage of the 9-carbon N-acetyl neuraminic acid (N-ANA) to yield β-formylpyruvic acid. The subsequent reaction of this product with thiobarbituric acid forms a chromophore which is extracted by organic solvents. The color production varies linearly with the concentration of N-ANA over the range of 0.01 to 0.06 μ moles (Warren, 1959). The TBA differs from the earlier assay procedures in that it measures only the free sialic acid and can therefore be used to measure the sialic acid released from substrates by neuraminidase.

Although the TBA has been satisfactoriy used as a standard method, Ziegler and Hutchinson (1972) stated that the multistep procedures involved in the assay induce variations associated with biphasic extractions and chemical transfers. Comb and Roseman (1960) and Brunetti et al., (1963) had earlier described a coupled enzyme assay for determination of sialic acid and in 1972, Ziegler and Hutchinson described a modified coupled enzyme system for measuring viral neuraminidase activity. This procedure measures the rate of release of N-ANA by neuraminidase and dispenses with the need for multistep colorimetric determinations. It involves the use of three enzymes - N-ANA aldolase, Lactic acid dehydrogenase (LDH) and reduced Nicotinamide adenine dinucleotide (NADH). Neuraminidase releases N-ANA from a substrate and the N-ANA is cleaved to N-acetyl-D-mannoseamine and pyruvic acid by N-ANA aldolase. The pyruvate is then reduced to lactic acid by LDH as NADH is converted to the oxidised form NAD. The ultimate determination of the coupled reaction is provided by the rate of change of adsorbance at 340 nM as NADH is oxidised.

Antigenicity and Neutralization of Neuraminidase

Immunological studies have shown that neuraminidases from different organisms or even different strains of the same organism can be serologically distinguished from one

another. Rafeelson et al., (1963) showed that guniea pig antiserum against Asian strain of influenza virus specifically neutralised the activity of the enzyme produced by this strain but was without effect on the activity of the enzyme produced by PR-8 strain of the same virus. Schulman et al., (1968) and Kendal and Madely (1969) in similar studies showed that many influenza virus strains possess neuraminidases which differ from each other in both their antigenic identity and kinetic characteristics. Serological cross reactions may however occur between neuraminidases of different organisms. Hayano and his co-workers described cross-reactions between Streptococcus sanguis and Streptococcus uberis neuraminidases and a serologically homologous enzyme in the group C, G and L streptococcus. Groups A, B and E streptococcus produce a serologically distinct, group-specific neuraminidase. Warren and Spearing (1963) reported cross reaction between the neuraminidases of Cl. perfringens and C. diphtheriae. Scharmann and Blobel (1972) showed absence of serological cross reactivity between neuraminidase of P. multocida on the one hand and those of V. cholerae, D. pneumoniae, Group K strept., C. diptheriae and Cl. perfringens on the other. A weak cross inhibition was found between neuraminidases of several strains of P. multocida and P. hemolytica.

SECTION III
(Part I)

PURIFICATION OF *PASTEURELLA MULTOCIDA* NEURAMINIDASE BY AFFINITY CHROMOTOGRAPHY AND GEL FILTRATION

ABSTRACT

Neuraminidase was extracted from an 18 hr culture of P. multocida grown on Dextrose starch Agar base enriched with 2.3 μM of N-acetyl-D-mannosamine. The crude enzyme extract was purified by Affinity chromatography and gel filtration. With affinity chromatography, 50-60% of enzyme activity in the extract was recovered. Enzyme activity was determined by assaying the purified enzyme according to the Warren's Thiobarbituric acid assay procedure.

One unit of activity was defined as that amount of enzyme that will cause the release of one micromole of sialic acid per minute at 37° and pH 6.5 from bovine sialo mucoid. The relatively low recovery rate of enzyme activity achieved with affinity chromatography purification was believed to be due to insufficient knowledge of certain kinetic properties of P. multocida neuraminidase and more work was recommended in this area.

INTRODUCTION

The bovine respiratory disease complex is one of the most serious disease problems of cattle industry in the United States and most other parts of the world (Mukkur, 1977; Irwin et al., 1979). A combination of factors are involved in the etiology of the disease complex. Bacterial colonisation of upper respiratory tract along with concurrent viral infection and/or environmental stress may trigger the condition. Pasteurella multocida is one of the bacteria species usually associated with the disease complex. The organism causes disease in a variety of domestic animals and man. Despite the economic importance of pasteurellosis, there is lack of complete understanding of the virulence factors of P. multocida (Maheswaran and Thies, 1979). Pasteurella multocida produces the enzyme neuraminidase (Tsolov and Karadzhov, 1969; Schormann et al., 1970 and Drzeniek et al., 1972). That neuraminidase could be a virulence factor of P. multocida is suggested by the role of this enzyme in the pathogenesis of some other diseases (Wellman, 1955; Kelly and Grieff, 1970; Muller, 1974; Pardoe, 1974; Frank and Tabatabai, 1981).

The objective of this study was to extract and purify P. multocida neuraminidase as a step in the investigation of the possible role of this enzyme as a virulence factor of P. multocida.

MATERIALS AND METHODS

Bacteria:

Twenty seven bovine isolates of P. multocida, collected from the clinical Microbiology laboratory, of Kansas State University, were screened for cell bound neuraminidase using a slight modification of a described method (Frank and Tabatabai, 1981). The isolate that had the highest enzyme activity was selected for this study.

Screening of isolates:

Pasteurella multocida cultures were grown overnight at 37^{o} on blood agar base (Difco laboratories, Detroit, Mich.) supplemented with 5% bovine blood. A suspension of bacteria approximating an absorbance of 1.83 at 549 nm was made in 5 ml of 0.1 M Sodium Acetate buffer pH 6.0. Five hundered microliters of this suspension was added to 0.4 ml of bovine colostral sialomucoid prepared according to the method of Aminoff (1961). Five hundred microliters of the acetate buffer was added and the mixture incubated for 30 mins in 37^{o} waterbath. The released sialic acid was measured by a slight modification of the Thiobarbituric acid assay (Warren, 1959). Enzymatic reaction in the incubating mixture was stopped by addition of 1 milliliter of 5% phosphotungstic acid (PTA) in 2.5 N HCl.

Following centrifugation at 8000 xg for 10 mins, 1 millilitre of the supernatant was assayed for free sialic acid. Two hundred micro liters of 0.2 M sodium metaperiodate in 9 M phosphoric acid was added to the supernatant and the mixture incubated at room temperature for 20 mins. The reaction was stopped by addition of 1 milliliter of 0.755 M Sodium arsenite in 0.5 M Sodium sulfate and tubes were shaken to liberate iodine. To develop the chromogen, 3 ml of 0.6% Thiobarbituric acid (4.5 gm 2 x crystallised Thiobarbituric acid m 750 ml of 0.5 M sodium sulfate) was added to the tubes which were then placed in boiling water bath for 15 mins. After cooling in ice bath for 5 mins, the pink chromogen was extracted by adding 4.6 ml of cyclohexanone (Fischer Scientific Comp., Fairlwan N. J.) followed by vortexing and centrifugation at 8000 xg for 10 mins. The absorbance of the upper cyclohexanone phase was read in a 240 Gilford Spectrophotometer at a wavelength of 549 nm against a similar phase from a control mixture containing buffer instead of cell suspension.

Enzyme Extraction: This was done by two methods.

(a) Ultrasonication: The method described by Scharmann et al., (1970) was followed. Pasteurella multocida was grown for 18 hrs on 5% bovine blood agar base medium. Cells were harvested, washed twice in 0.1 m sodium phosphate buffer pH 6.5 and re-suspended in the same buffer.

The cells were sonicated at 70 watts for forty five times. Each sonic treatment lasted 15 secs and treatments were applied at 1 min intervals. Sonicated cell suspension was made 1 M with respect to NaCl and incubated at 37° for 20 mins. After centrifugation at 27,000 x g for 20 mins, the supernatant was adjusted to 85% saturation with solid $(NH_4)SO_4$ and kept in the refrigerator overnight. The foamy substance that rose to the surface was skimmed off and assayed for enzyme activity by the Thiobarbituric acid method (Warren, 1959).

(b) NaCl extraction: This was done according to a described method (Drzeniek, Schormann and Balke, 1972). Pasteurella multocida was grown for 24 hrs on blood agar base supplemented with 5% bovine blood. A special medium was later used instead of the blood agar medium for reasons to be discussed later. This medium was made from Dextrose Starch agar base (Difco Laboratories, Detroit, Mich.) Supplemented with 2.3 µM N-acetyl-D-mannoseamine (Sigma Chemical Comp., St. Louis, MO) per liter of medium. The bacterial growth was washed off the plates with 0.1 M sodium phosphate buffer pH 6.5. Cell suspension was maintained at 37°C by carrying out this procedure in a water bath. The suspension was made 0.4 Molar with respect to NaCl and incubated on a rotating shaker at 37° for 15 mins followed by centrifugation at 27,000 x g for 20 mins. The supernatant was saved while cells were again resuspended

in the buffer and reextracted two more times as described. The supernatants were pooled as crude enzyme extract.

Enzyme purification: Two methods were used.

(a) Affinity Chromatography: This was done according to a described method (Cuatrecases and Iliano, 1971). The crude enzyme extract was thoroughly dialysed against 0.05 M sodium phosphate buffer pH 6.5 using Amicon DC-2 HF dialyser (Amicon Corp., Lexington, Mass.). A column 1.5 cm. in diameter and 6.5 cm long was packed with N-(P-aminophenyl) oxamic acid-agarose (Sigma) gels and thoroughly equilibrated with the phosphate buffer. The dialysed extract was passed through the column at a flow rate of 2.2 mls/min. The optical density of the effluent at 280 nm wavelength was monitored on a 240 Gilford Spectrophotometer (Gilford Instrument Lab., Inc., Oberlin, Ohio). The column was then washed with 0.05 M sodium phosphate buffer pH 6.5 until absorbance at 280 nm approximated zero. Elution of the enzyme adsorbed to the column was achieved with 0.1 M sodium bicarbonate buffer pH 9.1. To rapidly lower the pH to which the purified enzyme was exposed, the effluent fractions were collected in tubes containing 0.5 ml of 0.5 M sodium phosphate buffer pH 5.0. The effluent fractions were assayed for neuraminidase activity according to the Warren's Thiobarbituric acid method. Fractions with enzyme activity were pooled, rechromatographed, concentrated, and stored frozen at -60^{o}C in 2 ml aliquots.

If the effluent collected while the crude enzyme was being passed through the column retained some enzyme activity, it was rechromatographed as described above. Modifications of this standard procedure were made in an attempt to elute the enzyme in a more concentrated form. Instead of the recommended 0.1 M sodium bicarbonate buffer pH 9.1, the enzyme was eluted with 0.4 M sodium bicarbonate buffer pH 8.9; 0.4 M NaCl in 0.1 M $NaHCO_3$ buffer pH 9.1 and 1.4 M NaCl in 0.1 M $NaHCO_3$ buffer pH 9.1. Furthermore, various sizes of columns were used in place of the regular 1.5 cms x 6.5 cms columns. These columns measured 2.5 cm (diameter) x 5 cm (long); 2.5 cm x 30 cm.; 0.5 cm x 20 cm and 0.5 cm x 30 cm .

(b) Gel filtration: A column 1.6 cm in diameter and 85 cm long was packed with Biogel-A 0.5 M (Biorad Laboratories, Richmond, Calif.) and equilibrated with 0.05 M sodium acetate buffer pH 5.5. Three hundred milliliters of crude enzyme extract was thoroughly dialysed against the acetate buffer and concentrated to 15 mls with the Fleaker™ concentrator (Spectrum Medical Industries, L.A.). Four milliliters of the concentrated enzyme was passed through the column at the flow rate of 2 mls/min. Effluent was collected in 2.2 ml fractions, concentrated, and stored frozen at -60°.

Enzyme Assay

The Thiobarbituric acid assay (Warren, 1959) was the method used. The standard reaction mixture in a tube contained the following substances: 0.4 ml of bovine colostral sialomucoid prepared according to the method of Aminoff (1961); 0.5 ml of 0.1 M sodium phosphate buffer pH 6.5; 0.1 ml of the purified enzyme. The mixture was incubated for 15 mins in 37^{o}C water bath. The enzyme reaction was stopped with 1 ml of 5% phosphotungstic acid. Tubes were centrifuged and 0.2 ml of the supernatant assayed for free sialic acid as described earlier except that 4.3 ml of cyclohexanone was used instead of 4.6 ml of the reagent as used in the modified assay.

One unit of enzyme was defined as that amount of enzyme that causes the release of one micromole of sialic acid per minute from bovine sialomucoid at 37^{o} and pH 6.5.

Protein determination

This was done according to the Biorad (Biorad Laboratories, Richmond, Calif.) standard protein assay procedure using bovine gammaglobulin as standard. The protein content of the enzyme preparation was determined from a standard curve.

Gel Diffusion Test

Enzyme extracts from cultures grown on Blood Agar plates (N-1) and Dextrose Starch Agar plates (N-2) were checked for presence of bovine antigens. A 1% solution of Agarose (Becton, Dickinson Corp, MI) was cast on 4 ml gel bond film (Marine Colloids Div. FMC Corp. Rockland). One central and six peripheral wells, 1.5 mm in diameter and 7.5 mm apart were made on the agarose gel. A 1:10 dilution of bovine serum was placed in two of the peripheral wells as controls. N-1 and N-2 were placed in the peripheral wells as in Fig. 5. The center well contained anti-bovine serum. The test was incubated overnight in a humified chamber at room temperature. Precipitation bands were observed and recorded.

Immunoelectrophoresis

The enzyme preparation used was purified by affinity chromatography. The antineuraminidase and normal serum were raised in rabbits as described in section IV of this thesis. One gram of Seakem (M.E.) Agarose (Marine Colloids Div. F.M.C. Corp., Rockland) was dissolved in 100 mls of 0.05 M sodium barbital buffer pH 8.6 and the gel cast on 4 mil gel bond film. Two parallell troughs, each 6 cm long and 3 mm wide were made 3 cm apart and a well, 3 mm in diameter was made in between the troughs as in Fig. 6.

The well was filled with the enzyme and electrophoresis was done for 45 mins. The buffer used was 0.05 M sodium barbital buffer pH 8.6 and 60 volts of current was applied. Following electrophoresis, the antineuraminidase and normal serum were applied to the troughs and the test incubated overnight at room temperature. Any precipitin lines observed were recorded. Electrophoresis was repeated under three different experimental conditions in an attempt to achieve maximum separation of the enzyme protein:

i. 0.05 M sodium phophate buffer pH 6.5 was used and electrophoresis carried out for 45 mins.

ii. 0.1 M sodium phosphate buffer pH 6.5 was used and electrophoresis carried out for 2 hrs.

iii. 0.05 M sodium barbital buffer pH 8.6 was used and electrophoresis carried out for 2 hrs.

RESULTS

Screening of isolates

Eighteen out of the 27 isolates screened produced neuraminidase to varying degrees. Culture #50859 produced the enzyme that possessed the highest activity and this isolate was selected for the study. This isolate was identified as Carter's serogroup A, strain KSU-LM-50859. Table IV summarizes the results of the test.

Enzyme Extraction

The extracts prepared by ultrasonication did not have any neuraminidase activity while those made by the NaCl method yielded enzyme activity. The first of the three NaCl extracts pooled together, always had the highest enzyme activity.

Enzyme Purification

The chromatograms of typical purification by affinity chromatography and gel filtration are presented in Figs. 3 and 4 respectively. With the affinity chromatography method, most of the purified enzyme was usually recovered in the first three or four fractions following the application of the eluant (0.1 M sodium bicarbonate buffer pH 9.1). Subsequent fractions still retained some enzyme activity. The activity slowly trailed off until it was virtually zero.

Eluting the enzyme with the various solutions listed in the materials and methods, and the use of different size of columns, did not change this pattern. Rechromatography of the purified enzyme did not result in further concentration of the enzyme. With gel-filtration, some enzyme activity was also lost in the effluent and the purified fraction did not emerge in a concentrated form.

Protein determination

The protein content of different batches of enzyme preparations varied. Values determined were rather low even after concentrating the purified enzyme. One batch of enzyme purified by affinity chromotography had protein content of 0.5 mg/ml while that purified by gel filtration contained 0.26 mg protein per ml of purified enzyme.

Gel diffusion tests

Precipitation lines observed are shown in Fig. 5. Enzyme extracts prepared from cultures grown on bovine blood agar plates contained bovine antigens while the cultures grown on Dextrose starch agar plates yielded enzyme extracts that did not contain bovine antigen.

Immunoelectrophoresis

When the enzyme preparation was electrophoresed for

45 mins using either 0.05 M sodium barbital buffer pH 8.6 or 0.05 M sodium phosphate buffer pH 6.5, there was no migration. Two bands were observed at the point of enzyme application (Fig. 6). When electrophoresis was carried out for 2 hrs using 0.1 M sodium phosphate buffer pH 6.5, the enzyme was again separated into two bands but there was no migration either. A slight migration of the two bands towards the anode was observed when 0.05 sodium barbitol buffer pH 8.6 was used and elctrophoresis carried out for 2 hrs (Fig. 7).

DISCUSSION

In this study, ultrasonicated cell extracts had no enzyme activity. A low yield of 10% of original enzyme activity has however been reported (Schormann et al., 1970) with this method. Neuraminidase had been easily extracted from culture supernatants of V. cholerae (Ada, French and Lind, 1961), Cl. perfringens (Cassidy et al., 1965); Group K streptococcus (Hayano and Tanaka, 1967). The enzyme was in these cases extracellularly located in the organisms. Pasteurella multocida neuraminidase is known to be cell associated (Scharmann et al., 1970, Drzeniek et al., 1972) and culture fluid had less than 5% of total recoverable enzyme activity (Scharmann et al., 1970). The NaCl method used in this study was a fast and reasonably efficient

procedure for extracting P. multocida neuraminidase. Further studies to be reported in Section VI of this thesis indicated that our NaCl extracts did not possess N-ANA aldolase activity in agreement with the finding of Drzeniek, Scharmann and Balke, (1972). The presence of bovine antigen in affinity chromatography-purified extracts, prepared from cultures grown on bovine blood agar plates, indicated that N-(para aminophenyl) oxamic acid chelated the bovine antigen. It is therefore recommended that if affinity chromatography is to be used for purifying P. multocida neuraminidase, the cultures should not be grown on media containing bovine blood. The two protein bands observed in immunoelectrophoresis study indicated that the enzyme preparation was not homogeneous. There was probably a contaminating protein from the cell wall components of the organism. Another possible explanation is based on the tendency of P. multocida neuraminidase to aggregate at neutral pH conditions. During affinity chromatography purification, the enzyme was eluted with 0.1 M $NaHCO_3$ buffer pH 9.1 and collected in 0.5 M Na_2PO_4 buffer pH 5.0. The ultimate pH to which the purified enzyme was exposed was therefore around the neutral range. Aggregation probably occured. The two bands may therefore represent aggregate forms of the same enzyme. One other possibility is that the second protein band represents an isoenzyme form of P. multocida neuraminidase. Chromatography indications

of multiple forms of neuraminidase exist in descriptions of purification of this enzyme in Corynebacterium diphtheriae (Mariyania and Barksdale, 1967); Streptococcus strains (Hayano and Tanaka, 1967) Cl. perfringens (Cassidy et al., 1965) and Diplococcus pneumoniae (Tanebaum et al., 1969). Existence of multiple forms of P. multocida neuraminidase has not been described. The results of this study do not prove this possibility.

With the affinity chromatography purification method, 50 - 60% of original enzyme activity in the crude extract was recovered. This recovery rate compares favourably with the results of some workers who used other purification schemes. Seto et al., 1969 and Stahl O'Toole (1971) achieved 50-90% recovery rate using DEAE sephadex chromatography on viral and pneumococcal neuraminidases respectively. Rechromatography of purified enzyme did not result to further concentration of the enzyme and this was in agreement with the finding of Cuatrecasas and Iliano (1971). Despite the use of various solutions and different sizes of columns, the enzyme was not eluted in a concentrated form. The chromatograms indicated protein peaks that rose fairly sharply but trailed off rather gradually. This pattern of column emergence was not similar to what was obtained by Cuatreasas and Iliano (1971) in purifying Vibrio cholerae, influenza virus and Cl. perfringens neuraminidases by affinity chromatography. The "trailing off"

of enzyme activity and the relatively low enzyme yield may be explained by the fact that in affinity chromatography, procedural conditions tend to be individualised to specific enzymes (Cuatrecasas and Iliano, 1971). Although the principle of the method remains the same in all cases, special consideration should be given to the role of ions, metals, pH and temperature for each enzyme under consideration. Pasteurella multocida neuraminidase has not hitherto been purified by affinity chromatography and more work is needed to refine the procedure to suit purification of this and other enzymes.

Fig. 3. Five hundred and fifty milliliters of crude enzyme extract was exhaustively dialysed against 0.05 M Na_2PO_4 buffer pH 6.5. A column 1.5 cms x 6.5 cms was thoroughly equilibrated with the same buffer and the dialysed extract was passed through the column at a flow rate of 2.2 mls /min. The optical density of the effluent at 280 nm was monitored on a 240-Gilford Spectrophotometer. The column was washed with 0.05 M Na_2PO_4 buffer pH 6.5 and the enzyme eluted with 0.1 M $NaHCO_3$ buffer pH 9.1. Aliquots of the purified fractions were assayed for neuraminidase activity according the Warren's Thiobarbituric acid assay. The absorbance at 280 nm and 549 nm were plotted against the fraction numbers.

Symbols: —●— Absorbance at 280 nm

—0— Absorbance at 549 nm

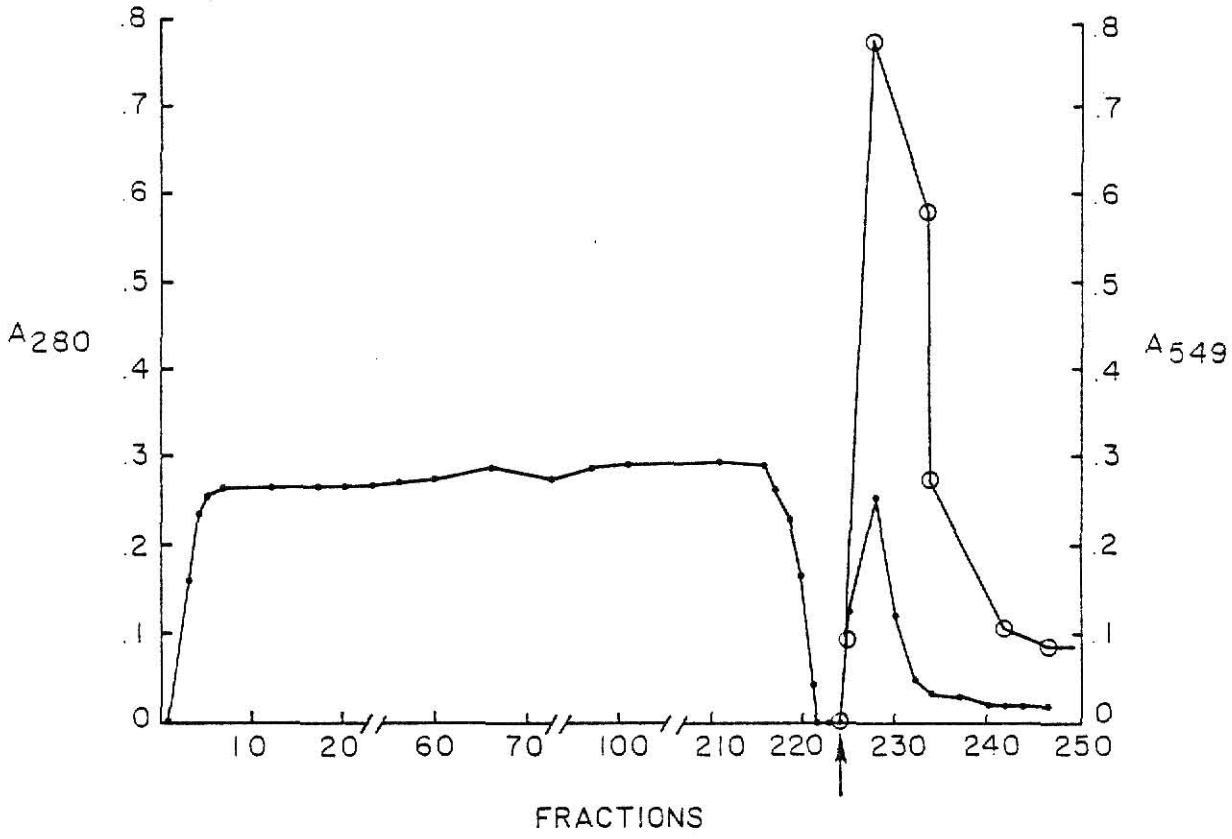


Fig. 3. Affinity Chromatography of P. multocida neuraminidase on Agarose-oxamic acid (Sigma) column.

Fig. 4. Three hundred milliliters of crude enzyme extract, thoroughly dialysed against 0.05 M sodium acetate buffer pH 5.5, was concentrated to 15 mls. A column 1.6 x 85 cms, packed with Biogel-A 0.5 M, was equilibrated with the acetate buffer and 4 ml of the concentrated enzyme was passed through the column at a flow rate of 2 ml/min. The absorbance of the effluent at 280 nm was monitored in a 240-Gilford Spectrophotometer. Elution of the enzyme was done with the same acetate buffer and purified fractions collected were assayed for neuraminidase activity according to Warren's Thiobarbituric acid assay. The absorbance at 280 nm and 549 nm were plotted against the fraction numbers.

Symbols: —●— Absorbance at 280 nm

—0— Absorbance at 549 nm

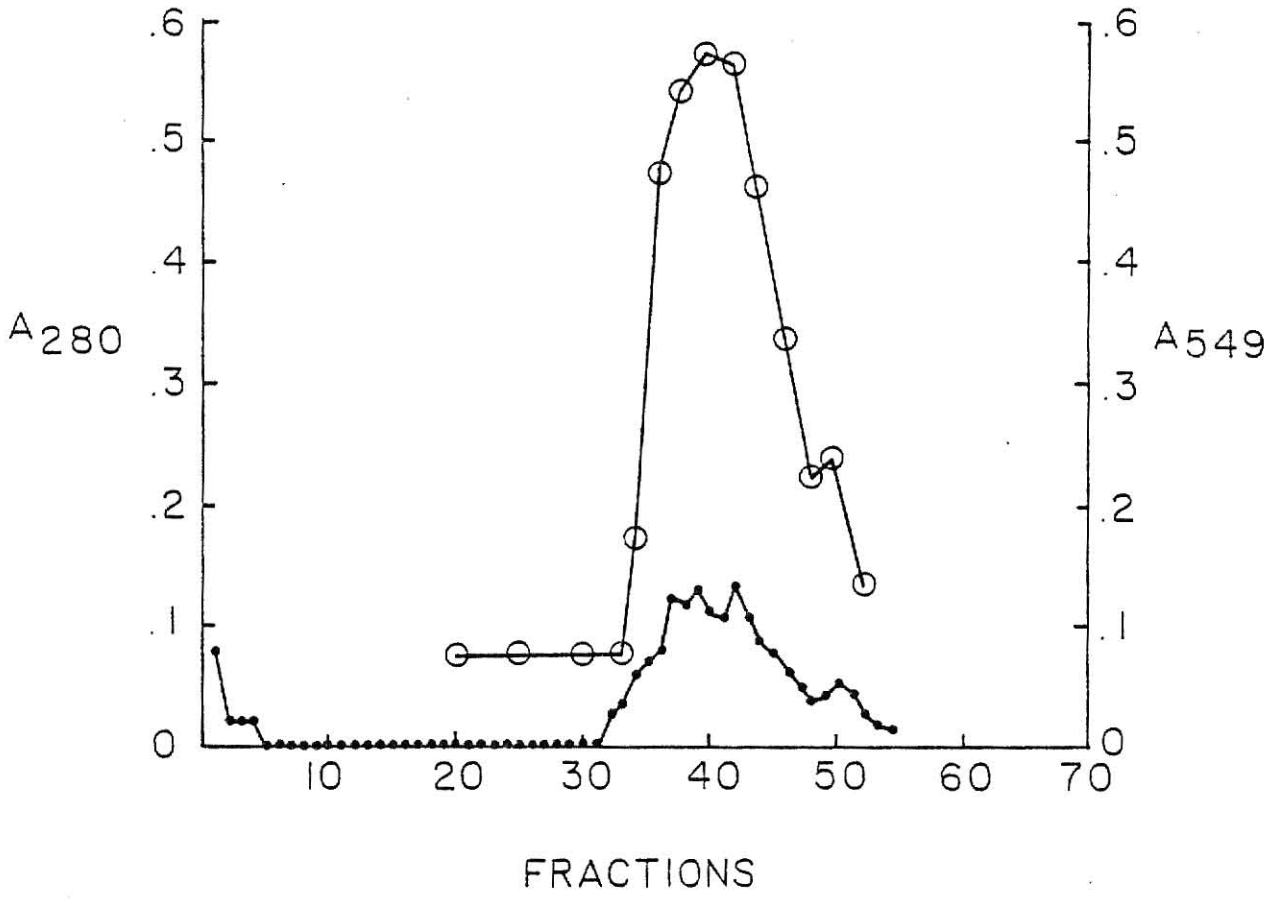


Fig. 4. Gel filtration of P. multocida neuraminidase on Biogel-A 0.5 M column.

Fig. 5. A 1% agarose solution was made and cast on a 4 mil. gel bond film. One central and six peripheral wells were made. Antibovine serum was placed in the center well. A 1:10 dilution of bovine serum was placed in two of the peripheral wells as controls. The remaining peripheral wells contained the two neuraminidase extracts. The test was incubated overnight at room temperature and precipitin bands were observed.

B S = bovine serum

N-1 = Neuraminidase extracted from cultures grown on bovine blood agar plates.

N-2 = Neuraminidase extracted from cultures grown on Dextrose starch agar supplemented with 2.3 μM N-acetyl D-mannose amine.

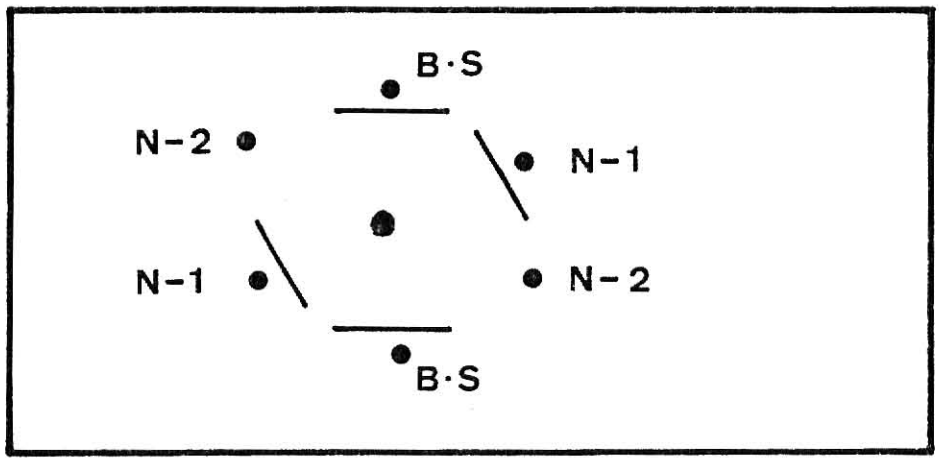


Fig. 5. Gel diffusion test on affinity chromatography - purified neuraminidase extracted from P. multocida cultures grown on bovine blood agar plates and Dextrose starch agar supplemented with 2.3 µM of N-acetyl-D-mannosamine.

Fig. 6. Electrophoresis was carried out for 45 mins using either 0.05 M sodium barbital buffer pH 8.6 or 0.05 M sodium phosphate buffer pH 6.5 or electrophoresis was carried out for 2 hrs using 0.1 M sodium phosphate buffer pH 6.5.

Fig. 7. Electrophoresis was carried out for 2 hrs using 0.05 M sodium barbital buffer pH 8.6. Current applied in all cases was 60 volts.

Fig. 6.

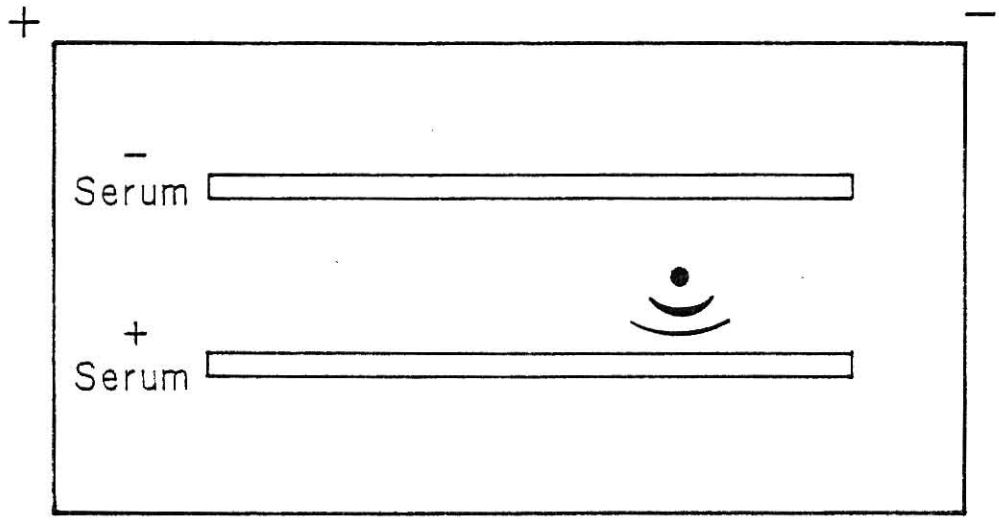
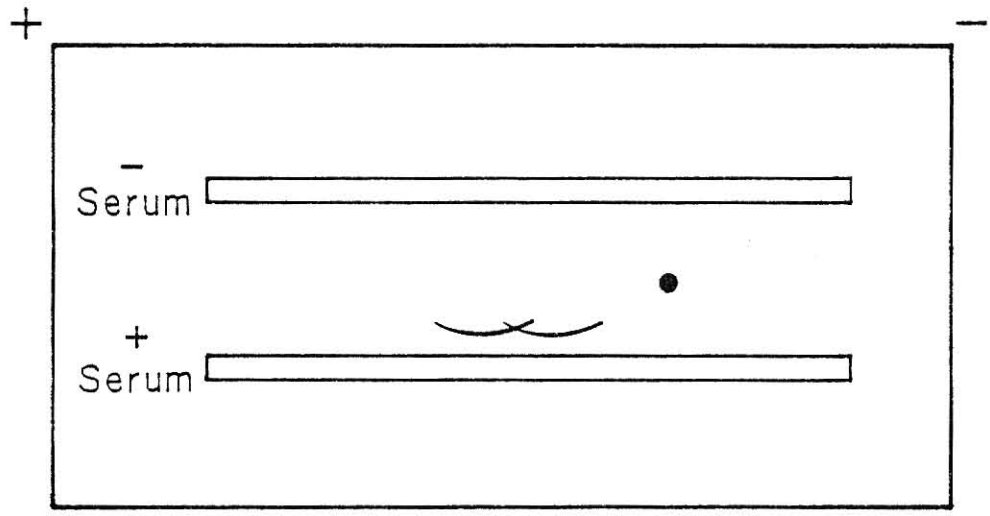


Fig. 7.



Figs. 6 and 7. Immunoelectrophoresis of Pasteurella multocida neuraminidase purified by affinity chromatography.

Table IV. Screening test of Pasteurella multocida isolates for cell-bound neuraminidase.

Culture #	Turbidity (A549)	Enzyme Assay (A549)	μ moles N-ANA released per ml cell suspension
50910	1.808	0.110	0.0396
50914	1.799	0.025	0.009
*50859	1.800	0.221	0.0796
50915	1.803	0.108	0.0389
50860	1.805	0.082	0.0295
50141	1.807	0.113	0.0407
50909	1.800	0.141	0.0508
50806	1.820	0.003	0.0011
50907	1.821	0.145	0.0522
50917	1.823	0.188	0.0677
50939	1.830	0.018	0.0065
50872	1.797	0.135	0.0486
50879	1.799	0.108	0.0388
50906	1.798	0.146	0.0526
1062	1.820	0.103	0.0371
50880	1.800	0.118	0.0425
50946	1.880	0.122	0.0439
50942	1.800	0.087	0.0313
50140	1.830	0.024	0.0086
50912	1.801	0.001	0.0004
50934	1.831	0.021	0.0076
50859	1.802	0.166	0.0597
50807	1.808	0.186	0.0669
50818	1.832	0.185	0.0666
50931	1.826	0.108	0.0389
50941	1.805	0.077	0.0277
50139	1.830	0.140	0.0504

*Selected for study.

Details of all calculations involved in this thesis may be found in the Appendix section.

SECTION IV

(Part II)

NEUTRALISATION OF *PASTEURELLA MULTOCIDA* NEURAMINIDASE ACTIVITY BY ANTISERUM

Abstract

Antibody to Pasteurella multocida neuraminidase was raised in rabbits by repeated inoculation with a mixture of equal volumes of Complete Fruend's adjuvant and purified enzyme. When the enzyme was treated with normal rabbit serum, the enzyme activity determined by Warren's Thiobarbituric acid assay was 2.08 units/mg. Following treatment with the antiserum, the enzyme was partially neutralized and the activity determined was 0.335 unit/mg. This residual activity was probably due, in part, to the action of neuraminidase on serum glycoproteins releasing sialic acid which contributed to the optical density at 549 nm determined by enzyme assay.

The antiserum did not cause agglutination of a P. multocida cell suspension in a slide agglutination test. This was believed to be due to the fact that the antiserum was not specific for the intact bacterial cell since it was prepared against the enzyme extract.

Introduction:

The enzyme neuraminidase has been implicated as a virulence factor in several species of bacteria (Wellman, 1955; Kelly and Grieff, 1970; Muller, 1971; and Pardoe, 1974). The role of the enzyme in the pathogenesis of some diseases has also been described (Morell et al; 1971; Muller, 1974; and Aminoff et al, 1977). Pasteurella multocida produces neuraminidase (Tsolov and Karadzhov, 1969; Scharmann et al., 1970; Drzniek, Scharmann and Balke, 1972) but it is not certain whether the enzyme is a virulence factor of this organism. Immunity in pasteurella infections has been demonstrated by passive transfer studies to be humorally mediated (Roberts, 1947; Bain 1963; Carter, 1967 and Robert et al., 1980) but the mode of action of the antibodies is not completely understood (Collins, 1973; Woolock and Collins, 1975). Hoffling et al (1979) demonstrated that rabbit antiserum to P. multocida was not bactericidal and did not enhance phagocytosis when compared with normal serum in in-vitro tests. Neutralization of enzymes or toxins is one other way immune antibodies can combat microbial infections (Tizzard, 1977). Antiserum specific for influenza virus neuraminidase cannot neutralize the virus itself but has neutralized the neuraminidase activity of a homologous virus strain (Rafeelson et al., 1963) and also reduced the yield and release of virus from infected cells (Kilbourne et al., 1968). Lee and

Howe (1965) and Hayano, Tanaka and Okuyama (1969) in similar studies, produced antiserum to streptococcal and pneumococcal neuraminidases. The antiserum in each case neutralized the enzyme activity of the respective organism. The objective of this study was to neutralize P. multocida neuraminidase with antiserum to the enzyme.

MATERIALS AND METHODS

Antiserum Production:

Four pasteurella free New Zealand white rabbits were identified as #98, #100, #101 and #102. Nasal swabs were taken from each rabbit and culture yielded staphylococcus, streptococcus and micrococcus species. The rabbits were bled, serum harvested and frozen at -60^{o}C as normal serum.

Animal Inoculation:

Four milliliters of P. multocida neuraminidase purified by affinity chromatography was mixed thoroughly with an equal volume of complete Fruend's adjuvant (Difco Laboratories, Detroit, Mich.) Rabbits #98, #100 and #102 were inoculated with 2.4 ml of this mixture containing 0.78 mg of enzyme. One milliliter of the mixture was given intramuscularly at two sites and the rest injected subcutaneously

at 12 different sites. Rabbit #101 was not inoculated. Two weeks later, the inoculated rabbits received intramuscular injection of 1 ml of affinity chromatography - purified enzyme containing 0.95 mg/ml protein. On day 28, post inoculation, these three rabbits were test-bled and on day 30 each of them was given 3 mls of a mixture of equal volumes (4 mls) of incomplete Fruend's adjuvant and affinity chromatography - purified enzyme containing 0.95 mg/ml protein. One milliliter of the mixture was given intramuscularly and the rest subcutaneously as described earlier. The final bleeding of the rabbits was done on day 55 post inoculation.

Immunodiffusion Test:

One gram of Seakem (M.E.) Agarose (Marine Colloids Div., F.M.C. Corp., Rockland) was dissolved in 100 mls of distilled water and the gel cast on a 4 mil. gel bond film (Marine Colloids Div. F.M.C. Corp., Rockland). One central and six peripheral wells were cut on the agar as described in Section III of this thesis. In one set of wells, the positive serum collected from rabbits #98, 100 and 102 were placed on the peripheral wells while the center well contained neuraminidase purified by affinity chromatography (Figure 8A). The pre-inoculation serum was used as control in another set of wells (Figure 8B). The test was incubated overnight in a humidified chamber at room temperature. Precipitin bands were observed and recorded as in Figure 8A.

Determination of Equivalence Point:

This was done by a gel-diffusion test run as follows:

Two prallel troughs each measuring 6 cms long and 1 mm wide were cut 2.5 cm. apart in an agar gel prepared as described earlier. Seven wells, 2 mm in diameter and 4 mm apart were made in-between the troughs (Figure 9). The antiserum of rabbit #102 was diluted 1:2 with 0.1 m sodium phosphate buffer pH 6.5. Twofold dilutions of affinity chromatography - purified enzyme were made up to 1:64. Into each of 7 numbered tubes was placed 0.1 ml of each of the various enzyme dilutions to which was added equal volume of the 1:2 dilution of antiserum. After thorough mixing, the tubes were incubated at room temperature for 5 mins. The seven wells were filled with aliquots of the incubated mixtures from respective tubes. Undiluted antigen was placed in one of the troughs while the other trough was filled with the 1:2 antiserum dilution. The test was incubated overnight in a humidified chamber at room temperature. Precipitin bands were observed and recorded (Fig. 9).

Enzyme Neutralization:

This was done according to a described method (Hayano and Tanaka, 1968) with minor modifications. Two microliter quantities of purified enzyme, positive serum, negative

serum or 0.1 m sodium phosphate buffer pH 6.5 was added to 12 numbered tubes according to the protocol in table V. The contents of the tubes were properly mixed and all tubes were incubated in 37^{o} waterbath for 30 mins. Four hundred microliters of bovine colostral sialomucoid (BSM) prepared according to the method of Aminoff (1961) was placed in 6 fresh tubes. The substrate was diluted with 0.5 ml of 0.1 M sodium phosphate buffer pH 6.5. Instead of the substrate, 0.9 ml of the buffer was added to another 6 tubes. One milliliter of each reaction mixture in tubes 1 - 6 incubated earlier was added to the first set of 6 tubes containing BSM. One milliliter of the incubated reaction mixture in each of tubes 7 - 12 was similarly added to the remaining 6 tubes that contained buffer. All tubes were thoroughly mixed, and incubated for 15 mins in a 37^{o} waterbath. The enzymatic reaction in the tubes was stopped by addition of 1 ml of 5% phosphotungstic acid. The tubes were centrifuged at 8000 x g for 10 mins and 0.5 ml of the supernatants were assayed for released sialic acid according to Warren's Thiobarbituric acid assay. The test was run in triplicate and the average results calculated as recorded in table V.

Slide Agglutination Test:

One drop of anti-neuraminidase serum was mixed with a small suspension of P. multocida cells on a glass slide. The mixture was examined for agglutination.

RESULTS

The inoculated rabbits produced antibody to neuraminidase as indicated by the precipitin bands in figure 8A. There were no bands in the control test run with normal serum. The equivalence point determination indicated that a 1:2 dilution of positive serum neutralized the activity in a 1:2 dilution of the enzyme preparation. The absolute value of the antiserum titer was not determined. The neutralization test indicated that neuraminidase activity was effectively, though not completely, neutralized by antiserum. The antiserum however did not cause slide agglutination of P. multocida cell suspensions.

DISCUSSION

The evaluation of the extent of neutralization of enzyme activity by antiserum was complicated by the action of the enzyme on the sialoglycoproteins contained in the serum samples. Virtually all serum glycoproteins, with the exception of albumin, contain sialic acid (Haskel et al., 1971) and evidence has been presented (Morell et al., 1971) to show that neuraminidase desialates serum glycoproteins. The sialic acid released from the serum samples contributed to absorbance read during the enzyme assay

following the neutralization test. This was the reason 6 of the 12 test samples were run without substrate. The only substrate the enzyme could act on was the serum glycoproteins. That way it was determined that the sialic acid released from the glycoproteins of the positive and normal sera contributed to an absorbance at A549 of 0.012 and 0.073 respectively (table V). After taking this into consideration, there was still some amount of residual, unneutralized enzyme activity. While it was possible that the antiserum did not have titer high enough to neutralize all the activity, the residual activity could have been due to an isoenzyme of P. multocida neuraminidase. The possibility of this enzyme existing in two different forms was suggested in Section III of this thesis. It was thought that the antiserum was specific for only one of the isoenzymes whose activity was neutralized and that the other isoenzyme was responsible for the unneutralized enzyme activity. The test did indicate, however, that the antiserum neutralized some of the enzyme activity as demonstrated by comparing the enzyme activity calculated after treating the enzyme with positive serum and normal serum (table V). The in-vitro neutralization of enzyme activity by antiserum suggested that neuraminidase could be a virulence factor of P. multocida.

The inability of the antiserum to agglutinate P. multocida cell suspension could be explained by the fact that the antiserum was not raised against the intact cell.

Although neuraminidase of P. multocida is known to be cell associated (Scharmann et al., 1970), the enzyme may not be so superficially located to be able to react with anti neuraminidase to cause agglutination.

Figure 8. Immunodiffusion test with rabbit antibody to *P. multocida* neuraminidase.

The agar was cast and wells made as described earlier. The serum used was collected 28 days post inoculation.

A = post inoculation serum

B = pre inoculation serum

Both centre wells contain neuraminidase purified by affinity chromatography.

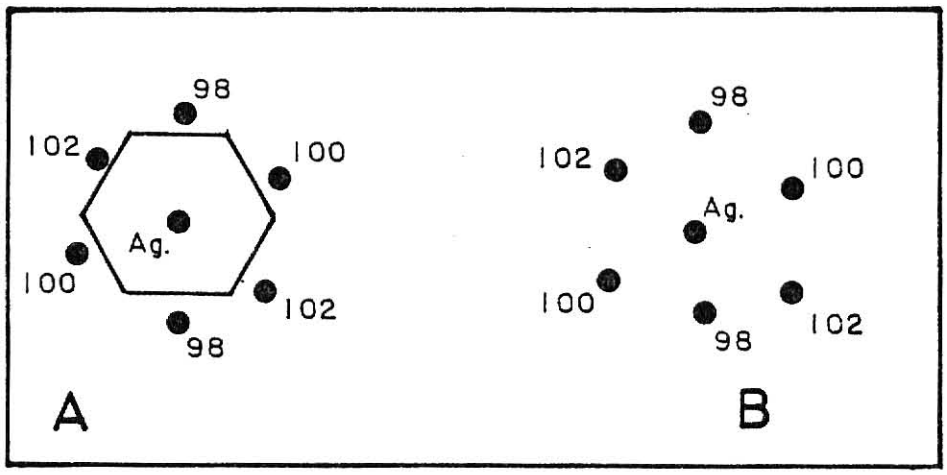


Figure 8: Immunodiffusion test with rabbit antibody to P. multocida neuraminidase.

Figure 9. Determination of equivalence point.

Mixture of equal volumes of various twofold dilutions of the enzyme and 1:2 dilution of positive serum were incubated at room temperature for 5 mins. Aliquots of various mixtures were placed in respective wells. One of the troughs contained undiluted enzyme while the other contained the 1:2 dilution of positive serum. Test was incubated overnight at room temperature in a humidified chamber.

1. 0.1 ml 1:1 Ag + 0.1 ml 1:2 dil. positive serum
*2. 0.1 ml 1:2 Ag + 0.1 ml 1:2 dil. positive serum
3. 0.1 ml 1:4 Ag + 0.1 ml 1:2 dil. positive serum
4. 0.1 ml 1:8 Ag + 0.1 ml 1:2 dil. positive serum
5. 0.1 ml 1:16 Ag + 0.1 ml 1:2 dil. positive serum
6. 0.1 ml 1:32 Ag + 0.1 ml 1:2 dil. positive serum
7. 0.1 ml 1:64 Ag + 0.1 ml 1:2 dil. positive serum

* Equivalence point.

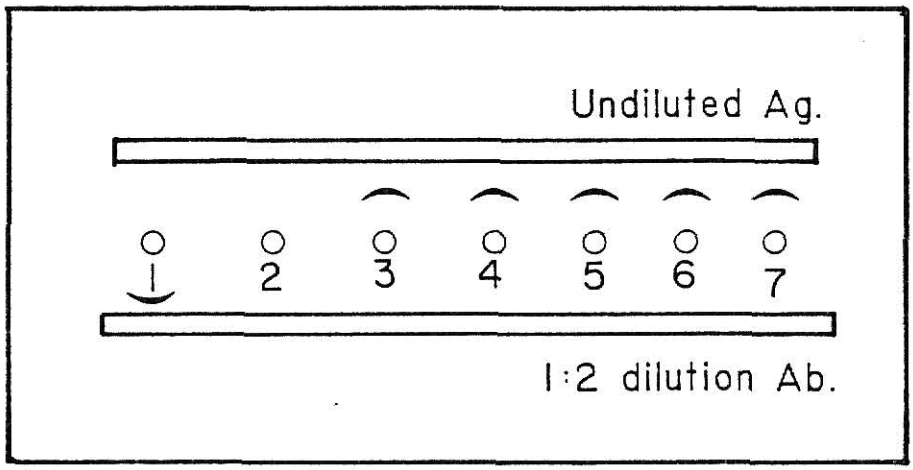


Figure 9: Determination of equivalence point

Table V. Neutralization of Pasteurella multocida neuraminidase by antiserum

Tube #	Enzyme	+Serum	-Serum	Buffer	BSM	A549			Average A549	Enzyme Activity in unit/mg
1	+	+	-	-	+	0.058	0.074	0.071	0.067	0.335
2	+	-	+	-	+	0.386	0.428	0.435	0.416	2.08
3	+	-	-	+	+	0.349	0.350	0.348	0.349	1.97
4	-	+	-	-	+	0.024	0.024	0.019	0.022	0.11
5	-	-	+	-	+	0.019	0.009	0.008	0.012	0.06
6	-	-	-	+	+	0.002	0.004	0.003	0.003	0.015
7	+	+	-	-	-	0.011	0.011	0.014	0.012	0.06
8	+	-	+	-	-	0.061	0.080	0.076	0.073	0.365
9	+	-	-	+	-	0.000	0.001	0.001	0.001	0.005
10	-	+	-	-	-	0.000	0.000	0.000	0.000	-
11	-	-	+	-	-	0.000	0.000	0.000	0.000	-
12	-	-	-	+	-	0.000	0.000	0.000	0.000	-

+ sign = substance added to tube
- sign = substance not added to tube.

SECTION V

(Part III)

MOUSE PROTECTION STUDIES WITH RABBIT ANTISERUM TO *PASTEURELLA MULTOCIDA* NEURAMINIDASE

ABSTRACT

A group of 60 mice were passively immunized by intraperitoneal injection of 0.5 ml of rabbit antiserum to P. multocida neuraminidase. For control three other groups each comprising of 48 mice received different pretreatments. One group received intraperitoneal injection of 0.5 ml of sterile physiological saline while the other received 0.5 ml of normal rabbit serum intraperitoneally. The third group did not receive any pretreatment. Two days post treatment, cages of mice in each group were challenged by intraperitoneal injection of serial 10-fold dilutions of an 18 hr broth culture of the same Pasteurella multocida strain from which the neuraminidase was extracted.

Thirteen days after challenge, the experiment was terminated and the LD_{50} calculated for each group. Results indicated that the antineuraminidase serum protected mice against challenge with homologous P. multocida.

INTRODUCTION

The enzyme neuraminidase has been implicated as a virulence factor in several species of bacteria (Kelly and Greiff, 1971; Pardoe, 1974; Ray, 1977; Smith, 1977 and Milligan et al., 1978). Pasteurella multocida produces

neuraminidase (Tsolov and Karadzhov, 1969; Scharmann, 1970; and Drzniek et al., 1972), but it is not certain that the enzyme is a virulence factor in this organism. Muller and Kraseman (1974) reported that P. multocida strains producing high levels of neuraminidase activity were more virulent in mice than those producing low levels of activity and concluded that neuraminidase production by P. multocida was associated with virulence. Frank and Tabatabai (1981) established a correlation between pasteurella-caused pneumonia in cattle and high neuraminidase activity of Pasteurella hemolytica type 1. Drzniek, Scharmann and Balke (1972), on the other hand, found no correlation between neuraminidase activity of different strains of P. multocida and their virulence in mice. The investigators concluded that neuraminidase was probably not a virulence factor of P. multocida.

Antiserum to P. multocida neuraminidase neutralized the activity of the enzyme in in-vitro test as reported in Section IV of this thesis. If neuraminidase is indeed a virulence factor of P. multocida, antiserum prepared against the enzyme should protect mice challenged with homologous P. multocida.

The objective of this study, therefore, was to determine if mice passively immunized with rabbit antiserum to P. multocida neuraminidase were more resistant to infection than untreated, placebo or normal serum-injected mice.

MATERIALS AND METHODS

Animals:

Two hundred and four mice weighing, on the average, 29.8 grams were used for this study. The mice were arranged in 4 groups, A, B, C and D. Each of the groups A, B and C had 8 cages and each cage contained 6 mice. There were 10 cages in group D and each cage also contained 6 mice.

Pre-treatment:

Group A mice did not receive any pre-treatment. Groups B and C mice received intraperitoneal injection of 0.5 ml of sterile physiological saline and normal (pre-immune) rabbit serum respectively. All mice in group D were injected intraperitoneally with 0.5 ml of rabbit antiserum to *P. multocida* neuraminidase.

Bacterial Count:

Serial ten-fold dilutions of an 18 hr broth culture of *P. multocida* were made in sterile physiological saline. The dilutions ranged from 10^{-2} to 10^{-11}. Three blood agar plates were each inoculated with 0.5 ml of the bacterial suspension from each of the dilutions. Following overnight incubation of the plates at 37^{o}, the colonies of bacteria

were counted and average counts of the 3 plates were recorded for each dilution (Table VI).

Challenge Experiment:

On the second day after pre-treatment, individual cages of mice within each group were challenged intra-peritoneally with 0.5 ml of one of the serial 10-fold dilutions of an 18 hr broth culture of homologous P. multocida. In groups A, B and C, the dilutions ranged from 10^{-4} through 10^{-11} while in Group D, the dilutions ranged from 10^{-2} through 10^{-11}. After 13 days post challenge, all the surviving mice were euthanized and the experiment terminated.

Determination of LD_{50}:

The challenge dose was expressed in colony forming units (c.fu) and the LD_{50} calculated by the method of Karber (1931).

RESULTS

It was determined from the bacterial count that the original broth culture of P. multocida contained 9×10^6 c.fu. The LD_{50} for group A mice was a dilution of $10^{-6.17}$ of the original broth culture. This dilution contained

6.08 c.f.u. For group B mice, a dilution of $10^{-6.83}$ containing 1.33 c.f.u was determined to be the LD_{50}. The LD_{50} for group C mice was a dilution of 10^{-7} containing 0.9 c.f.u while for the passively immunized group D mice, the LD_{50} was a dilution of $10^{-2.5}$ containing 28460.5 c.f.u. A summary of the results is presented in tables VII - X.

DISCUSSION

The fact that a far greater number of organisms were needed to kill 50% of the protected mice than was needed for the unprotected groups of mice suggested that the anti-neuraminidase did protect mice against challenge with homologous P. multocida. Although animal protection tests are subject to some experimental variations because of differences between animals in their susceptibility to infections among other factors (Tizzard, 1977), the degree of resistance to infection demonstrated by the immunized mice in this study, indicated that the antiserum to neuraminidase was protective in mice. It was therefore concluded that neuraminidase is indeed a virulence factor of P. multocida.

The importance of antibodies in immunity to pasteurellosis was suggested by Collins (1973) who reported the successful transfer of immunity against P. multocida infection in mice by passive transfer of serum antibodies.

Previous studies (Bains, 1955; Dhanda, 1958) suggested that immunity to pasteurella infections was capsular type - specific but more recent studies (Heddleston and Rebers, 1972) suggested that it is somatic serotype - specific. It would therefore be desirable to obtain an antigen preparation that could be used as an immunogen to impart immunity not only to the homologous but also to the heterologous serotypes of P. multocida. Neuraminidase could be such an antigen. Although homologous P. multocida was used for the challenge experiment in this study, Scharmann and Blobel (1971) reported that neuraminidase from P. multocida types B and D were inhibited by neuraminidase antisera from all the other serotypes. There is therfore the possibility that anti-neuraminidase of a particular strain of P. multocida could offer passive protection against heterologous serotypes. More work is needed to investigate this possibility. The need for a better immunogen to protect animals against pasteurellosis is stressed by the finding that the value of the bacterins currently in use is still questionable despite the fact that they have been used to immunize cattle for over 50 years (Paloty, 1958; Pyke, 1966; Wilkie and Norris, 1976). More recently, attempts have been made to use various extracts of different serotypes of Pasteurella multocida for immunization of animals. Purified lipopolysaccharides extract (Rebers, et al., 1979) and potassium thiocyanate extract (Mukkur, 1979) of P. multocida

have been studied. The results indicate that these extracts induce, by far, better protection in mice than bacterins made from the intact cells. Protection of mice against pasteurella infection by passive immunization with anti-neuraminidase has not hitherto been reported. It is believed that neuraminidase, like the other extracts already studied, would induce good protection against pasteurellosis. This needs to be investigated in the continued search for a better immunogen for protecting an imals against pasteurella infections.

Table VI. Bacterial count of ten-fold dilutions of an 18 hr broth culture of Pasteurella multocida.

Dilutions:	Colony forming units/0.5 ml			
	Plate 1	Plate 2	Plate 3	Average
10^{-2}	T.N.C.	T.N.C.	T.N.C.	-
10^{-3}	T.N.C.	T.N.C.	T.N.C.	-
10^{-4}	T.N.C.	T.N.C.	T.N.C.	-
10^{-5}	92	89	91	90
10^{-6}	10	13	11	11
10^{-7}	4	5	3	4
10^{-8}	N.G.	N.G.	N.G.	-
10^{-9}	N.G.	N.G.	N.G.	-
10^{-10}	N.G.	N.G.	N.G.	-
10^{-11}	N.G.	N.G.	N.G.	-

N.G. = No growth.

T.N.C. = Too numerous to count.

Table VII. Summary of results of mouse protection studies with antiserum to Pasteurella multocida neuraminidase.

	GROUP A (No pretreatment)							
	OBSERVED				ACCUMULATED			
Dilution	Dead	Alive	Ratio Dead / Total	%	Dead	Alive	Ratio Dead / Total	%
10^{-2}	N.D.*							
10^{-3}	N.D.							
10^{-4}	6	0	6/6	100	16	0	16/16	100
10^{-5}	5	1	5/6	83.3	10	1	10/11	90.9
10^{-6}	4	2	4/6	66.7	5	3	5/8	62.5
10^{-7}	1	5	1/6	16.7	1	8	1/9	11.1
10^{-8}	0	6	0/6	0	0	14	0/14	0
10^{-9}	0	6	0/6	0	0	20	0/20	0
10^{-10}	0	6	0/6	0	0	26	0/26	0
10^{-11}	0	6	0/6	0	0	32	0/32	0

LD_{50} dil = $10^{-6.17}$ containing 6.08 c.f.u.

* Not done.

Table VIII. Group B (Saline pretreatment)

Dilution	OBSERVED				ACCUMULATED			
	Dead	Alive	Ratio $\frac{\text{Dead}}{\text{Total}}$	%	Dead	Alive	Ratio $\frac{\text{Dead}}{\text{Total}}$	%
10^{-2}	N.D.							
10^{-3}	N.D.							
10^{-4}	6	0	6/6	100	20	0	20/20	100
10^{-5}	5	1	5/6	83.3	14	1	14/15	93.3
10^{-6}	5	1	5/6	83.3	9	2	9/11	81.8
10^{-7}	4	2	4/6	66.7	4	4	4/8	50
10^{-8}	0	6	0/6	0	0	10	0/10	0
10^{-9}	0	6	0/6	0	0	16	0/16	0
10^{-10}	0	6	0/6	0	0	22	0/22	0
10^{-11}	0	6	0/6	0	0	28	0/28	0

LD_{50} dilution = $10^{-6.83}$ containing 1.33 c.f.u.

Table IX. Group C (Negative serum pretreatment)

Dilution	OBSERVED				ACCUMULATED			
	Dead	Alive	Ratio $\frac{\text{Dead}}{\text{Total}}$	%	Dead	Alive	Ratio $\frac{\text{Dead}}{\text{Total}}$	%
10^{-2}	N.D.							
10^{-3}	N.D.							
10^{-4}	6	0	6/6	100	21	0	21/21	100
10^{-5}	6	0	6/6	100	15	0	15/15	100
10^{-6}	6	0	6/6	100	9	0	9/9	100
10^{-7}	2	4	2/6	33.3	3	4	3/7	42.9
10^{-8}	1	5	1/6	16.7	1	9	1/10	10
10^{-9}	0	6	0/6	0	0	15	0/15	0
10^{-10}	0	6	0/6	0	0	21	0/21	0
10^{-11}	0	6	0/6	0	0	27	0/27	0

LD_{50} dilution = 10^{-7} containing 0.9 c.f.u.

Table X. Group D (positive serum pretreatment)

Dilution	OBSERVED				ACCUMULATED			
	Dead	Alive	Ratio $\frac{\text{Dead}}{\text{Total}}$	%	Dead	Alive	Ratio $\frac{\text{Dead}}{\text{Total}}$	%
10^{-2}	3	3	3/6	50	6	3	6/9	66.7
10^{-3}	2	4	2/6	33.3	3	7	3/10	30
10^{-4}	1	5	1/6	16.7	1	12	1/13	7.7
10^{-5}	0	6	0/6	0	0	18	0/18	0
10^{-6}	0	6	0/6	0	0	24	0/24	0
10^{-7}	0	6	0/6	0	0	30	0/30	0
10^{-8}	0	6	0/6	0	0	36	0/36	0
10^{-9}	0	6	0/6	0	0	42	0/42	0
10^{-10}	0	6	0/6	0	0	48	0/48	0
10^{-11}	0	6	0/6	0	0	54	0/54	0

LD_{50} dilution = $10^{-2.5}$ containing 28460.5 c.f.u.

SECTION VI

(Part IV)

STUDIES ON SOME KINETIC PROPERTIES OF *PASTEURELLA MULTOCIDA* NEURAMINIDASE

ABSTRACT

Using bovine colostral sialomucoid as substrate, the enzyme activity of Pasteurella multocida neuraminidase at various pH levels ranging from 5.0 - 7.0, were determined. The maximum enzyme activity was observed at a pH of 6.5 and it was concluded that the pH optimum for P. multocida neuraminidase was 6.5 under these experimental conditions.

An optimal incubation time of 15 mins was established for the above enzyme-substrate interaction by incubating P. multocida neuraminidase with bovine colostral sialomucoid for different periods of time ranging from 10 - 30 mins. Pasteurella multocida neuraminidase extracted by the Nacl method and purified by affinity chromatography was incubated with a known amount of N-acetyl neuraminic acid (N-ANA). For control, the same amount of N-ANA was incubated with buffer instead of enzyme. The amount of N-ANA left at the end of incubation was the same in both cases and it was concluded that P. multocida neuraminidase extracted by the Nacl procedure did not possess N-ANA aldolase activity. The effect of N-ANA aldolase activity on the estimation of neuraminidase activity of micro organisms was briefly discussed.

Introduction:

The term neuraminidase has been used (Muller, 1974) to describe a group of enzymes differing from one another in many properties but with a common ability to hydrolize the α-0-ketosidic bond between sialic acid and carbohydrates in sialoglycoproteins. The pathologic significance of microbial neuraminidase has been attributed (Kelly and Grieff, 1970; Fischer et al., 1971; Greenwalt and Steane, 1973) to this ability of the enzyme to remove sialic acid from vital tissues and body fluids. Important differences in other properties of different neuraminidases have been reported (Muller, 1974) as listed in Table III. Whereas the properties of Vibrio Cholera, (Ada, French and Lind, 1961) Clostridium perfringens (Cassidy et al., 1965) and influenza virus (Mayron et al., 1961) neuraminidases have been thoroughly studied, there are only scanty reports on the properties of P. multocida neuraminidase. Scharmann, Drzniek and Blobel, (1970) and Drzniek et al., (1972) reported a pH optimum of 6.0 using 0.1 M sodium phosphate buffer and bovine sialyl lactose (BSL) (Sigma Chem. Comp. St. Louis, MO) as substrate. There are no reports of this study repeated with other substrates. Drzniek et al., (1972) reported that P. multocida neuraminidase extracted by the Nacl method did not possess N-acetyl neuraminic acid (N-ANA) aldolase activity. This report has not been confirmed by another independent study. In enzyme-substrate

interactions, the enzyme activity is known (Scharmann, et al., 1970) to be directly dependent on the time of incubation and enzyme concentration. Drzniek, Scharmann and Balke (1972) and Scharmann et al., 1970 incubated P. multocida neuraminidase and BSL for 15 mins. It was considered useful to establish the optimal incubation time for the enzyme and substrate being used for this study.

The objectives of this study were:

i. To determine the pH optimum for P. multocida neuraminidase activity using bovine colostral sialomucoid as substrate.

ii. To determine the optimal incubation time for the above enzyme-substrate interaction.

iii. To find out if P. multocida neuraminidase extracted by the Nacl method possessed N-ANA aldolase activity.

MATERIALS AND METHODS

pH Optimum:

This was done according to a method described by Drzniek et al., (1972). Five milliliters of P. multocida neuraminidase purified by affinity chromatography and 10 ml quantities of bovine colostral sialomucoid prepared according to the method of Aminoff (1961) were thoroughly

dialized against each of these buffer solutions:

0.1 M sodium acetate buffer pH 5.0,

0.1 M sodium acetate buffer pH 5.5,

0.1 M sodium phosphate buffer pH 6.0,

0.1 M sodium phosphate buffer pH 6.5, and

0.1 M sodium phosphate buffer pH 7.0.

Four hundred microliters of the substrate dialyzed against each of the buffer solutions was placed in each of 5 test tubes to which was added 0.5 ml of the respective buffer. One microliter of the enzyme dialyzed against each buffer solution was added to the respective tubes and the tubes were incubated in 37° waterbath for 15 mins. The enzymatic reaction in the tubes was stopped by the addition of 1 ml of 5% phosphotungstic acid (P.T.A.) in 2.5 N Hcl. Following centrifugation at 8000 x g for 10 mins, 0.2 ml of the supernatant of each tube was assayed for enzyme activity according to the Warren's Thiobarbituric acid assay (T.B.A.) as described earlier. The test was run in triplicate and the average result calculated (Table XI).

Time Course Study:

Four hundred microliters of bovine colostral sialomucoid prepared according to the method of Aminoff (1961) was placed in each of five test tubes and 0.5 ml of 0.1 M sodium phosphate buffer was added to the tubes. The mixture was placed in 37^{o} water bath and allowed to equilibrate for 5 mins. One microliter of a 1:2 dilution of P. multocida neuraminidase, purified by affinity chromatography, was then added to each of the tubes. The enzymatic reaction in the first tube was stopped after 10 mins of incubation in the 37^{o} water bath, by addition of 1 ml of 5% P.T.A. in 2.5 N Hcl. The reaction in subsequent tubes was similarly stopped at 5 mins. intervals. Following centrifugation at 8000 x g for 10 mins, 0.2 ml of the supernatant of each tube was assayed for enzyme activity according to the Warren's T.B.A. assay. The experiment was conducted in triplicate and the average result was calculated. The enzyme activity was expressed in terms of micromoles of sialic acid released per mg protein (Table XII).

N-Acetyl Neuraminidase Acid Aldolase Activity:

This was done by a slight modification of a described method (Drzniek et al., 1972). A 0.2 % solution of N-acetyl

neuraminic acid (Sigma Chem. Comp. St. Louis, MO) was diluted 1:8 and 0.4 ml of this dilution was added to each of six test tubes. One microliter of 0.1 M sodium phosphate buffer pH 6.5 was added to 3 of the 6 tubes. To each of the remaining 3 tubes was added the same volume of P. multocida neuraminidase extracted by the Nacl method and purified by affinity chromatography as described in Section III. All tubes were incubated in a 37^{o} water bath for 30 mins. The enzymatic reaction in the tubes was stopped by addition of 1 ml of 5% P.T.A. in 2.5 N Hcl. The tubes were centrifuged at 8000 x g for 15 mins. Two microliters of the supernatant of each tube was assayed for any sialic acid left after incubation. The Warren's T.B.A. procedure described earlier was used for the assay. The result was presented in table XIII.

RESULTS

pH Optimum:

The results were presented in table XI. Units of enzyme activity were expressed as percentages of the highest activity recorded at the pH of 6.5. A plot of enzyme activity versus the pH values was made (Figure 10). From the plot, it was determined that the pH optimum was 6.5.

Time Course Study:

The results were presented in table XII. A plot of micromoles sialic acid released per mg. protein, versus time was made (Figure 11). From the linear part of the curve, the optimal incubation time of 15 mins was determined.

N-acetyl Neuraminic Acid-aldolase Activity:

Micromoles of sialic acid left after incubation was calculated as presented in table XIII. The values were the same in both the tests incubated with the enzyme and those incubated with buffer. Since there was no decrease in the amount of N-ANA in tubes after incubation with the enzyme, it was concluded that P. multocida neuraminidase extracted by the Nacl method did not possess N-ANA aldolase activity.

DISCUSSION

Muller (1974) stated that the pH optimum for neuraminidases may vary within two pH units depending on the substrate. It was therefore not surprising that the pH optimum of 6.5 established in this study for P. multocida

neuraminidase was different from that of 6.0 reported by Scharmann et al., (1970) and Drzniek et al., (1972). These investigators used BSL as substrate while bovine colostral sialomucoid was used in this study.

The optimal incubation time of 15 mins determined in this study agreed with what was reported by Scharmann et al. (1970) and Drzniek et al. (1972). Incubation times of 5 mins (Cassidy et al., 1965) and 30 mins (Hayano and Tanaka, 1969) have also been reported for Clostridium perfringens and streptococcal neuraminidases respectively. Establishing the optimal incubation time for a particular enzyme-substrate interaction is necessary for following the course of such reactions.

The enzyme, N-ANA aldolase first described in 1956 by Heimer and Meyer, has been demonstrated (Muller, 1974) in majority of micro organisms producing neuraminidase at high levels with the exception of Erysipelothrix rheusiopathiae. N-ANA-aldolase breaks down N-acetyl-neuraminic acid (sialic acid) into N-acetyl D-mannose amine and pyruvic acid. In organisms producing both neuraminidase and N-ANA aldolase, the sialic acid released by the action of neuraminidase on an appropriate substrate is further degraded by N-ANA-aldolase with the result that free sialic acid measured by colorimetric methods, like the T.B.A. procedure, does not truly reflect the enzyme activity. Drzniek, Scharmann and Balke, (1972) stated that while P. multocida

neuraminidase extracted by ultrasonication had N-ANA aldolase, extraction by the Nacl method yielded neuraminidase that was free of N-ANA aldolase activity. This study confirms that Nacl extracts of P. multocida neuraminidase further purified by affinity chromatography did not possess N-ANA-aldolase. The attempt to extract P. multocida neuraminidase by ultrasonication was unsuccessful as reported in Section III of this thesis. It was therefore not possible to confirm that such extracts do possess N-ANA aldolase activity. However, since the Nacl method was very efficient and reliable, the procedure is recommended for the isolation of P. multocida neuraminidase free of N-ANA aldolase activity.

Figure 10. The purified enzyme and substrate were dialyzed against various buffer solutions with varying pH values ranging from 5 - 7. One microliter of the enzyme dialyzed against each buffer solution was assayed for enzyme activity and the activity was expressed as a percentage of the highest enzyme activity observed at pH 6.5.

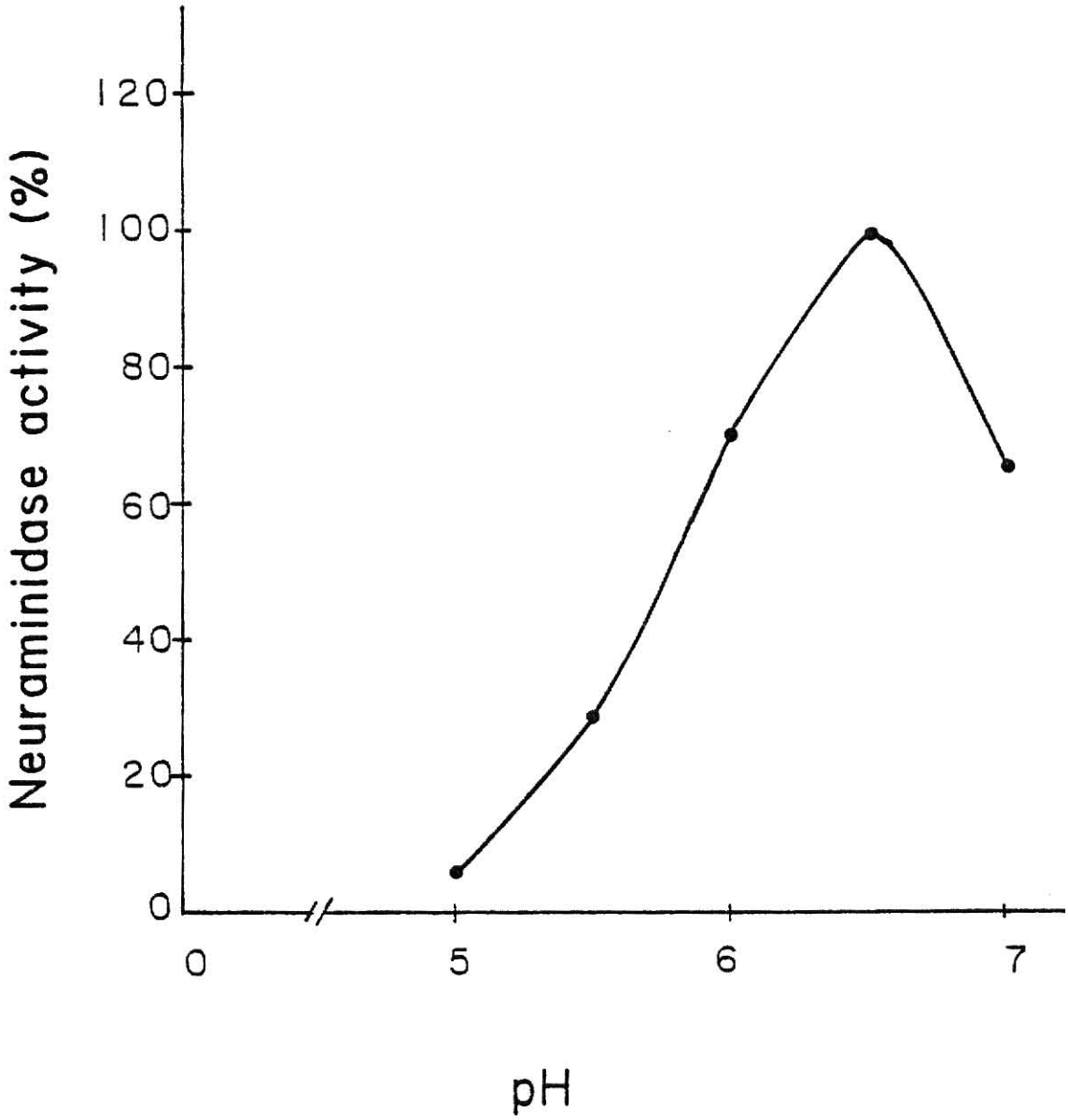


Figure 10. pH optimum study.

Figure 11. One microliter of a 1:2 dilution of purified neuraminidase was incubated for various periods of time ranging from 10 - 30 mins. The enzyme activity for each period of incubation was calculated and expressed in terms of micromoles of sialic acid released per mg protein.

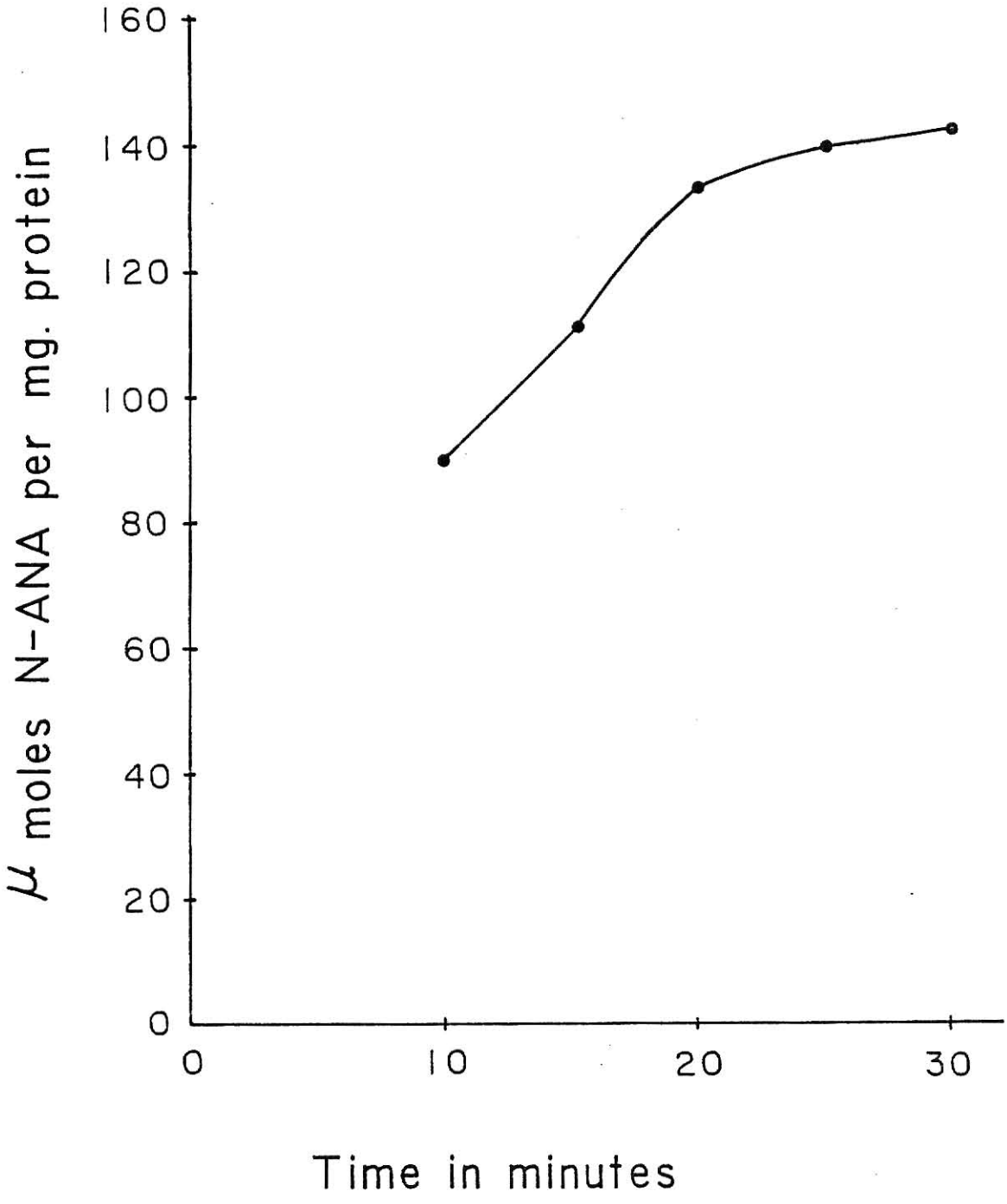


Figure 11. Time course study.

Table XI. pH Optimum Study

Buffer Solution	A549			Average	Enzyme Activity unit/mg	Unit as % of highest activity
0.1 M Sod. Acetate buffer pH 5.0	0.023	0.021	0.025	0.023	0.28	5.37
0.1 M Sod. Acetate buffer pH 5.5	0.127	0.109	0.115	0.117	1.46	28.02
0.1 M Sod. Phosphate buffer pH 6.0	0.298	0.302	0.300	0.300	3.75	71.9
0.1 M Sod. Phosphate buffer pH 6.5	0.417	0.418	0.416	0.417	5.21	100
0.1 M Sod. Phosphate buffer pH 7.0	0.256	0.301	0.278	0.347	3.47	66.6

Table XII. Time Course Study.

Time in minutes	A549			Average	μ Moles N-ANA / mg protein
10	0.231	0.240	0.246	0.239	81.6
15	0.275	0.296	0.297	0.289	108.4
20	0.342	0.346	0.365	0.356	133.5
25	0.368	0.367	0.380	0.372	139.5
30	0.382	0.384	0.382	0.381	142.8

Table XIII. N-acetyl neuraminic acid-aldolase activity.

Reaction Mixture	A549			Average	μ Moles N-ANA
N-ANA + Enzyme	0.655	0.652	0.654	0.6536	0.4902
N-ANA + Buffer	0.653	0.660	0.649	0.6536	0.4902

SECTION VII

APPENDIX

DETAILS OF CALCULATIONS AND PREPARATION OF SOME REAGENTS

A. DETAILS OF CALCULATIONS

1. Screening test for cell bound neuraminidase

Reaction mixture consisted of:

0.4 ml substrate

0.5 ml buffer

0.5 ml cell suspension

1.0 ml phosphotungstic acid.

2.4 ml of total reaction mixture.

Out of this, 1 ml was assayed, therefore a multiplication factor of 2.4 was applied in the calculation.

μ Moles N-ANA released = A549 x 0.075

Applying the multiplication factor, μ Moles N-ANA released

= A549 x 0.075 x 2.4

But this was released by 0.5 ml cell suspension. μ Moles N-ANA/ml cell suspension = A549 x .075 x 2.4 x 2.

For culture #50859, A549 = 0.221

μ Moles N-ANA/ml of cell suspension

= 0.221 x 0.075 x 2.4 x 2 = 0.0796.

2. Enzyme neutralization test.

One unit of enzyme activity was defined as that amount of enzyme that will cause the release of 1 micromole of sialic acid from bovine colostral sialomucoid in 1 min at 37° and pH 6.5 (Section III)

μ Moles sialic acid = A549 x 0.075

$$\text{unit/mg} = \frac{\text{Micromoles N-ANA liberated} \times 4}{15 \times \text{mg enzyme in initial reaction mixture}}$$

Protein content of enzyme = 0.04 mg/ml

But 0.1 ml enzyme was used in the initial reaction mixture,

mg enzyme in the mixture = .004. When A549 = 0.067.

$$\text{Enzyme Activity} = \frac{0.067 \times 0.075 \times 4}{15 \times .004}$$

$$= 0.335 \text{ unit/mg}$$

3. Mouse protection studies

According to Spearman-Karber method.

$$\text{Log } LD_{50} = \text{log of dil. with 100\% response} - \frac{\text{sum of \% mortality at each dil.}}{100} - 0.5 \times \text{log of dilution factor}$$

For Group A mice, for example

Dilution with 100% response = 10^{-4}

$$\text{Log } LD_{50} = -4 - \left(\frac{100 + 83.3 + 66.7 + 16.7}{100} - 0.5\right) \times (1)$$

$$= (-4 - 2.17)\ (1)$$

$$= -6.17$$

Hence LD_{50} was the dilution, $10^{-6.17}$.

The colony forming units (c.f.u.) was calculated as follows:

Original broth had 9×10^6 c.f.u. $10^{-6.17}$ dil. would have

$(9 \times 10^6)(10^{-6.17}) = 6.08$ c.f.u.

4. pH Optimum:

μ moles N-ANA released = A549 x 0.075.

But because 0.2 ml was assayed out of a total reaction mixture of 2 ml, a multiplication factor of 10 was applied.

μ Moles N-ANA = A549 x 0.075 x 10.

Enzyme contained 0.04 mg/ml, but 0.1 ml of enzyme was used. So, protein content of reaction mixture = 0.004 mg

Enzyme activity in units/mg $= \frac{A549 \times 0.075 \times 10}{15 \times 0.004}$ where

15 = incubation time in mins.

When A549 = 0.023

Enzyme activity $= \frac{0.023 \times 0.075 \times 10}{15 \times 0.004}$

$= 0.28$ units/mg

Highest activity observed at pH 6.5 was 5.21 units/mg.

Activity expressed as % of highest activity $= \frac{0.28}{5.21} \times 100$

$= 5.37\%$

5. Time course study:

μ Moles N-ANA released = A549 x 0.075

Apply multiplication factor of 10 for the same reason as given above.

μ Moles N-ANA = A549 x 0.075 x 10.

Enzyme had protein content of 0.04 mg/ml. One microliter of a 1:2 dilution of enzyme was used. Therefore, protein content of reaction mixture was 0.002 mg.

Hence $\frac{\mu \text{ Moles N-ANA}}{\text{mg protein}} = \frac{A549 \times 0.075 \times 10}{0.002}$

When A549 = 0.239.

$\frac{\mu \text{ Moles N-ANA}}{\text{mg protein}} = \frac{0.239 \times 0.075 \times 10}{0.002}$

$= 89.6$

6. N-ANA aldolase activity:

μ Moles N-ANA = A549 x 0.075

Apply multiplication factor of 10

μ Moles N-ANA = A549 x 0.075 x 10.

If A549 = 0.6536, μ moles N-ANA

$= 0.6536 \times 0.075 \times 10$

$= 0.4902$

B. REAGENTS PREPARATION

1. 0.2 M sodium meta periodate in 9M H_3PO_4.

Mol. wt. of sod. meta periodate = 213.9. Prepared 250 ml solution by dissolving 10.69 gm sodium meta periodate in 151.8 ml of H_3PO_4 and making up to a volume of 250 ml with 98.2 ml of distilled H_20.

2. 0.755 M sodium arsenite ($NaAsO_2$) in 0.5 M sodium sulfate with 0.1 N H2504.

Mol. wt. of $NaAsO_2$ = 129.91.

Prepared by dissolving 25 gm of $NaAsO_2$ in 250 ml of 0.5 M

sodium sulfate (F.W. = 142.04) and adding 5 ml of 5 N H_2SO_4. Slight heating was used to effect solution.

3. 5% phosphotungstic acid in 2.5 N Hcl.

Prepared by dissolving 25 gm of phosphotungstic acid in 500 ml of 2.5 N Hcl.

4. 0.6% 2 x crystallized Thiobarbituric acid in 0.5 M Na_2SO_4.

Prepared as follows:

a) Dissolved 25 gm of 2-Thiobarbituric acid in 600 ml distilled H_2O. Steaming was necessary to effect solution.

b) Allowed solution to cool and obtained precipitated crystals by filtration.

c) Re-crystallized the precipitate as above and dried it in a desicator.

d) Weighed out 4.5 gm of the 2 x crystallized 2-Thiobarbituric acid in 750 ml of 0.5 M Na_2SO_4. Solution was effected by steaming; filtered when cool and the supernatant used for enzyme assays.

SECTION VIII

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NEURAMINIDASE AS A VIRULENCE FACTOR OF *PASTEURELLA MULTOCIDA*

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Abstract

Neuraminidase of Pasteurella multocida strain KSU-LM-50859 was extracted from cell suspensions with 0.4 M NaCl. The crude extract was purified by affinity chromatography on Agarose-oxamic acid column and gel filtration on Biogel-A 0.5 M. The affinity chromatography procedure yielded 50-60% of total enzyme activity - a result that was comparable to enzyme activity recovery rates obtained for other enzymes by some investigators. Pasteurella multocida neuraminidase has not hitherto been purified by affinity chromatography. More work is needed to refine the procedure for the purification of this and other enzymes. Immunoelectrophoresis of the affinity chromatography purified enzyme yielded two bands indicating that the enzyme preparation was probably not homogeneous. The bands were believed to represent aggregate forms of the same enzyme. In vitro neutralization of the activity of P. multocida neuraminidase was achieved with rabbit antiserum to the enzyme. Neutralization, however, was incomplete probably because the antibody titer was not high enough to neutralize all the enzyme activity.

Mice passively immunized with rabbit antiserum to P. multocida neuraminidase were, by far, more resistant to challenge infection with homoloous P. multocida than control mice which were either untreated or injected with

physiological saline or normal serum. The conclusion was therefore drawn that neuraminidase was indeed a virulence factor of P. multocida.

A pH optimum of 6.5 was determined for P. multocida neuraminidase using bovine colostral sialomucoid as substrate. A time course study indicated that the optimal incubation time for the enzyme - substrate interaction was 15 mins. Pasteurella multocida neuraminidase extracted by the NaCl method did not possess N-acetyl neuraminic acid aldolase activity in agreement with the report of Drzniek et al. (1972).