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STUDIES ON SIALIDASES

by

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A Thesis submitted in fulfilment of the requirements for the degree of Ph.D. of the University of Warwick

August, 1980

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PREFACE

The work described in this thesis was carried out in the Department of Chemistry and Molecular Sciences, University of Warwick, Coventry, England, during the period between October 1977 and August 1980. It is the original work of the author, except where specific acknowledgement is made or implied. This thesis has been submitted at the University of Warwick alone, in fulfilment of the requirements for the degree of Ph.D.

DECLARATION

The work described in this thesis is the original work of the author, except where acknowledgement is made to work and ideas previously published. It was carried out in the Department of Chemistry and Molecular Sciences, University of Warwick, between October, 1977 and August, 1980, and has not been submitted previously for a degree at any institution.

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SYNOPSIS

The subject, "Studies On Sialidases", is introduced in Chapter 1 by a review of the history, biological functions and properties of sialidases.

The development of a rapid and sensitive (detection limit 10^{-6} units) assay system for sialidase, using tritiated fetuin labelled at C-8 of its N-acetylneuraminic acid (NANA) units, is discussed in Chapter 2. Included in this chapter, is the design and application of a radioimmunoassay for sialidases, in which antibodies specific for (NANA) have been obtained. These antibodies were raised in sheep challenged with BSA-colominic acid (the colominic acid was hydrolysed to a chain length of approximately 3 NANA units) conjugate. Further, an attempt has been made to determine the mole to mole ratio of NANA units per sialic acid-containing macromolecule.

The extensive purification of the three sialidases is discussed in Chapter 3, using a double "affinity" column system and hydroxylapatite with which it was possible to separate the two forms of sialidases found in S. griseus and V. cholerae.

The main body of the thesis (Chapter 4), reports on the work undertaken to resolve the controversy pertaining to which amino acids are, or are not, involved in the active centre of sialidases obtained from pathogenic (C. perfringens and V. cholerae) and non-pathogenic (S. griseus) sources. It was found, by chemical modification, that cysteine and lysine amino acid residues do not participate in the catalytic process, whereas on the other hand, arginine, tryptophan and carboxylic amino acid residues have been found to participate in or

near the active centre, either by binding or by actual catalysis, during the catalytic process. Further, the involvement of arginine has been confirmed by differential labelling, isolation of the radiolabelled "active site peptide" and amino acid analysis, using phenyl[^{3}H]glyoxal. The synthesis of the latter, via an inexpensive method, is elaborated upon as well. Preliminary chemical modification studies directed at the carbohydrate residues, covalently attached to these enzymes, reveal the possibility of these moieties participating in the maintenance of the three-dimensional structure of sialidase.

With the aforegoing information, it is concluded that there is no difference between the active centres of sialidases obtained from pathogenic and non-pathogenic bacterial sources. Further, a catalytic mechanism involving the essential amino acid residues in or near the active site of the sialidases is speculated upon.

CHAPTER 1

GENERAL BACKGROUND

1.0 Introduction

One of the two major discoveries[1] made in the first forty years of this century, reawakening research into the chemistry of mucous secretions, concerned a specific constituent of the red blood cell membrane. Hirst (1941) and McClelland and Hare (1941) independently reported that chicken red blood cells were agglutinated by fluids containing infective influenza virus. Exploring this phenomenon, Hirst (1942 a,b) showed that at 4° the influenza virus was adsorbed to the red blood cells and remained so for 18 hr. Further investigation of this viral agglutination of erythrocytes at 37° revealed that the adsorption of the virus to the red blood cells was equally as fast but after 6 hours nearly complete elution of the virus had occurred. Red cells, after such treatment, neither adsorbed fresh virus nor were agglutinated by fresh virus, whereas the eluted virus was functionally intact. It was further established that the virus, after heat treatment at 55° for 30 minutes, lost both infective and eluting powers but retained its haemagglutinating capacity (Hirst 1942 a,b and Briody 1948). Such crippled virus was called indicator virus (Stone, 1949).

However, as early as 1902 Kraus and Ludwig described heat labile bacterial haemagglutinins which were destroyed by heating at 58° .

[1] The other major discovery was the demonstration by Lehn (1930) and Putkonen (1930) that the A, B and O blood group specific substances which are gene controlled and responsible in man for immunological specificity within the A-B-O blood group system, are present not only at the surface of red blood cells in an insoluble form, but are secreted in a water-soluble form in saliva, gastric juice, urine, etc. by about 75% of all persons.

Thomsen (1927) and Friedenrich (1928) also found that, contrary to the high specificity of human erythrocytes in their reaction with iso-antibodies, (iso-agglutination), the red blood cells exposed to bacteria or to their culture fluids were agglutinated by all human serum (panagglutination), known as the Thomsen-Friedenrich phenomenon: yet no explanation was forwarded. Hirst ventured to interpret his findings and the rationale behind his explanation is illustrated in figure 1.0.

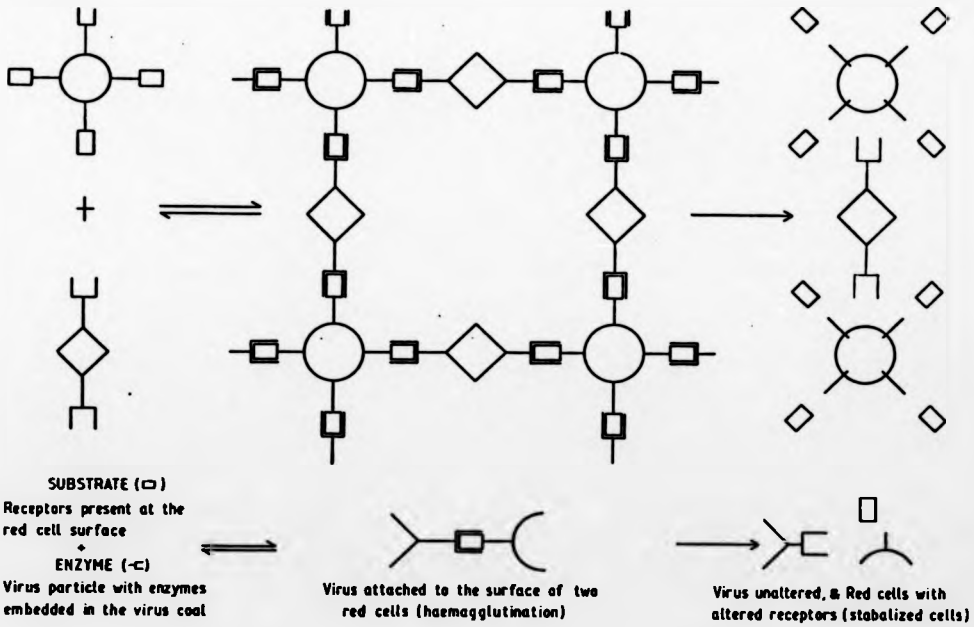


Fig. 1.0 Interpretation of the erythrocyte haemagglutination phenomena (Hirst, 1941).

Evidence in favour of Hirst's theory, that an enzyme present on the surface of the virus was responsible for inactivating the cellular

receptor, was published by Burnet and his co-workers in 1946. They found that pretreatment of erythrocytes with influenza virus rendered them agglutinable by adult human serum. This observation was correlated (Burnet 1951) with the Thomsen - Friedenrich phenomenon of 1928, namely that erythrocytes became panagglutinable after treatment with culture filtrate of bacteria. Burnet (1948) made further important observations that partially purified material from various mucous secretions, a mucoprotein in nature in high dilution, inhibited indicator virus haemagglutination. The same observation was later made by McCrea (1948), Gottschalk and Lind (1949) and Rosenberg et al., (1956), with different glycoproteins.

It was now firmly established that the carbohydrate moeity of erythrocyte surfaces played a role in haemagglutination reactions. Burnet and colleagues also found that these mucoprotein materials irreversibly lost their virus haemagglutinin inhibitory property when treated with living influenza virus or with purified enzymes from Vibrio cholerae or Clostridium perfringens (McCrae, 1947). The Vibrio enzyme was called "receptor destroying enzyme" [RDE] because it also destroyed the virus receptors at the red cell surface, thus protecting them against infection by influenza virus (Burnet, 1951 and Gottschalk, 1957 a,b).

Analysis of these results revealed that loss of biological activity of the red cell receptors and of the inhibitory mucoids on digestion with active influenza virus or with receptor destroying enzyme was due to the activity of two similar enzymes.

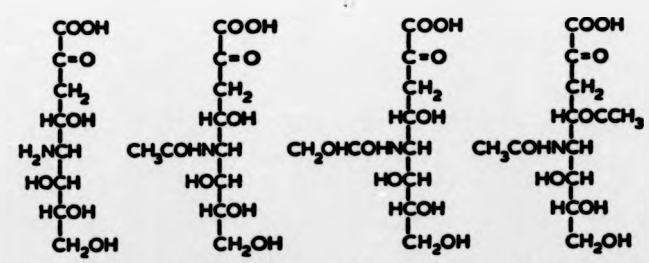
Information regarding the chemical structure of the substrate-product of the viral enzyme and the receptor destroying enzyme was provided by Gottschalk and Lind (1949). They presented evidence that, concomitant with the loss of biological activity of the potent inhibitor ovomucin, upon treatment with living virus and receptor destroying enzyme, a low molecular weight compound was released. It was characterized as an N-acylhexosamine residue (Gottschalk, 1951). Later, from the laboratories of Blix, Klenk and Gottschalk, it was disclosed that all virus haemagglutinin inhibitors are glycoproteins containing sialic acid and that sialic acid is the constituent split off from the virus particle and the receptor destroying enzyme in the inactivation reaction (Faillard & Klenk, 1955). The same was shown for the receptor substance at the red cell surface (Gottschalk, 1960). The soluble virus haemagglutinin inhibitors turned out to be chemical analogues of the specific influenza virus receptor substance at the erythrocyte surface. As in the case of the blood-group substances, the specificity characterizing the agglutinogen-agglutinin reaction and its inhibition was shown to reside in the carbohydrate group of the agglutinogen and the inhibitor respectively (Gottschalk, 1972).

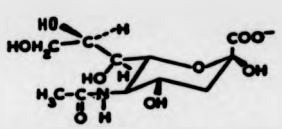
Sialic acid now became the centre of attraction. Its structure elucidation and its ever-present distribution in nature are so intimately interwoven with the subject of this thesis that a very brief account of its history seems appropriate.

At the time of sialic acid being discovered by Blix (1936) in bovine submaxillary gland mucoprotein, the interest in mucous secretions was

so low that it went unnoticed and was only named sialic acid as late as 1952 (Blix, et al.,). A substance with similar properties but without reducing power was isolated by Klenk (1941) when he treated certain types of human brain glycolipids with 5% methanolic hydrochloric acid at 105° . Klenk (1942) termed the product "methoxy neuraminic acid" to be recognized later as a methylglycoside.

In the nomenclature of Blix et al., (1957), sialic acid is the group name for acylated derivatives of a nine carbon sugar with an amino group in the molecule called neuraminic acid [Fig. 1.1] Neuraminic acid [Fig. 1.1(i)] never occurs in nature unsubstituted; usually it is found in either its N-acetylated form [Fig. 1.1(ii)] as the N-glycolyl derivative [Fig.1.1(iii)] or as various disubstituted derivatives [eg. N,O diacetyl] N-acetylneuraminic acid [Fig. 1.1(iv)] and probably all other sialic acids occur in the pyranose form [Fig. 1.1(v).





N-acetylneuraminic acid in pyranose form.

(V)

Fig.1.1 Structures of some sialic acids.

N-acetylneuraminic acid was first prepared from animal sources by Klenk and Faillard (1954). In 1955 (a,b), Gottschalk proposed that NANA was formed from pyruvate by an aldol condensation with N-acetyl - hexosamine. Comb and Roseman (1958) proved, by enzymic procedure (aldolase from Clostridium perfringens), that N-acetyl - mannosamine is the amino sugar incorporated into N-acetylneuraminic acid (NANA). The isolation of various types of sialic acids was achieved by Blix and co-workers (Blix et al., 1956 and Blix & Lindberg, 1960), a difficult and tedious task when the instability and the elucidation of the nature, number and position of their substituent acyl groups are considered.

Categorizing the enzyme product required the enzyme to be classed as well. Heimer and Meyer (1956) proposed that the name "sialidase" be used when the product of enzymic hydrolysis is sialic acid and that "receptor destroying enzyme" [RDE] be used when the destruction of biological activity is the result [as influenza haemagglutination could be inactivated by enzymes other than sialidase (Muller, 1974a)]. Gottschalk, (1957 a,b) suggested that the enzyme be termed "neuraminidase", and defined it as the specific α-glycosidase cleaving the ketosidic linkage joining the potential keto group of a terminal N-acylated neuraminic acid to an adjacent sugar residue in a disaccharide, trisaccharide or polysaccharide (Gottschalk, 1958). The term neuraminidase has gained fairly wide acceptance. However, a few investigators prefer the term "sialidase" as the more correct term because neuraminic acid, the unstable parent compound, is now known not to be the product of enzymic action and has not been found as such in

nature. The product generated is the free N, or N, O- substituted neuraminic acid, namely sialic acid. It is for this reason we chose the term "sialidase" as opposed to "neuraminidase". The systematic name and function as listed by the International Union of Biochemistry (1978), is Acylneuraminyl hydrolase, EC 3.2.1.18, and it catalyses the hydrolysis of 2,3-, 2,6- and 2,8- glycosidic linkages joining terminal non-reducing N- or O-acylneuraminyl residues to galactose, N-acetylhexosamine, or N- or O-acylated neuraminyl residues in oligosaccharides, glycoproteins, glycolipids or colominic acid [Fig. 1.2]

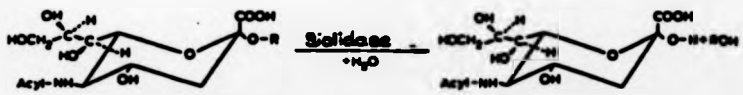


Fig. 1.2 Action of sialidase on the glycosides of N-acylneuraminic acids. Acyl = acetyl or glycolyl in naturally occurring substances; R = monosaccharides, oligosaccharides, glycoproteins, glycolipids, aliphatic or aromatic alcohols. For restrictions see under enzyme specificity. Assignment of the anomeric configuration to sialidase susceptible bonds of N-acylneuraminic acid according to Yu & Ledeen (1969)

1.1 Occurrence Of Sialidase

Sialidase is widely distributed in microorganisms and animal tissues. Viruses and bacteria as well as avian and mammalian tissues are known

to possess sialidase activity.

1.1.1 Viral Sialidases

Viral sialidases appear to be restricted to myxoviruses [Table 1.0 from Fenner, 1968] and are an integral part of this group of viruses. The term "myxoviruses" was given to a group of viruses which has a "high affinity for mucins" (Andrews et al., 1955), indicating the presence of sialidase on their surfaces. Further evidence in favour of the enzymes' superficial arrangement is that they are inhibited by antibodies directed against the envelope components of the virus (Maeno & Kilbourne, 1970). The myxoviruses are divided into ortho, para and, possibly, a third subgroup, meta myxoviruses (Melnick, 1972).

Table 1.0

The Sub-Divisions Of Myxovirus

Myxovirus	Source	Comments
Ortho	Influenza Type A. of: Man, swine, horse duck, fowl	Share type specific nucleoprotein antigen. They all possess sialidase
	Influenza Type B.	Distinctive nucleoprotein antigen recovered from man. They all possess sialidase
	Influenza Type C.	
Para	Mumps	All possess sialidase
	Newcastle Disease	
	Influenza 1, 2, 3 & 4	
	Sendai 5	
	Measels, Distemper, Rinderpest	Do not possess sialidase

The enzyme is described, along with the other major viral surface antigen, haemagglutinin, as being associated with spike-like projections on the outer surface of the virion (Laver & Valentine, 1969). The sialidase spikes are thought to be embedded in the hydrophobic portion of the surface membrane (Kendal et al., 1968), between haemagglutinin spikes (Seto, 1964) [Fig. 1.3].

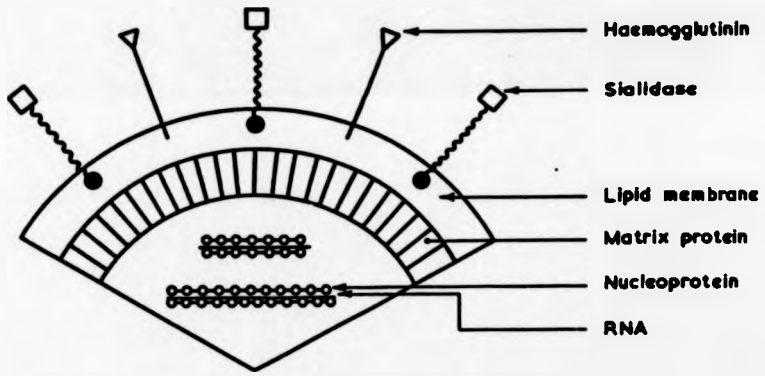


Fig.1.3 Schematic diagram of Influenza A Virus (Laver & Valentine, 1969 and Wrigley et al: 1973)

In the case of paramyxoviruses (Leprat & Aymard, 1979) both haemagglutinin and sialidase activities reside on the same glycoprotein whereas, in the orthomyxoviruses, these activites reside on independent glycoproteins.

Generally, sialidase represents 5% to 10% of the total viral protein. The synthesis of sialidase is controlled by the viral genome and the viral sialidase is not acquired from a cellular supply of the host

(Lipkind & Tsvetkova, 1967 and Ueda et al., 1978).

Because of the phenomenon of antigenic variation, sialidases from most viral strains will differ antigenically, physiochemically and kinetically. However, designation of the antigenic kinship of the sialidase of a particular strain of virus is now conventionally indicated in the viral nomenclature (Kendal & Madelay, 1969 and Wildy, 1971).

1.1.2 Bacterial Sialidases

Eubacteriales and Pseudomonadales are the two major orders of bacteria producing sialidases (Drzeniek, 1972). It has also been found in the orders Mycoplasmatales (Sethi & Muller, 1972 and Roberts, 1967), Actinomycetales (Myhill & Cook, 1972 and Kunimoto et al., 1974) and Arthrobacter of the Coryneform group (Flashner et al., 1977 and Uchida et al., 1977, 1979) (Table 1.1).

Table 1.1

The Physical Association Of Sialidase And Its Source

Bacteria producing cell bound sialidase	References
Corynebacterium diphteria	Moriyama & Barksdale, 1967
Klebsiella aerogenes	Pardee, 1970
Pasteurella multocida	Schauwecker et al., 1970
Bacteria producing extracellular sialidase	
Arthrobacter sialophilus	Flashner et al., 1977
Arthrobacter ureafaciens	Uchida et al., 1977
Clostridium perfringens	McCrea, 1947 and Rahm et al., 1957
Diplococcus pneumoniae	Chu, 1948 and Lee & Howe, 1966
Streptococci	Hayano & Tanaka, 1969
Bacteria producing both cell bound and extracellular sialidase	
Actinomyces viscosus	Costello et al., 1979
Actinomyces naeslundii	

The bacterial sialidases are either cell bound or exuded into their growth media.

Because bacterial sialidases were regarded almost exclusively as model substances of viral sialidases, interest flourished and the discovery of bacterial sialidase entered a logarithmic phase during 1970 - 1974 (Muller, 1974).

As time passed, researchers (Gottschalk & Bhargava, 1971; Muller, 1970 and Vertiev et al., 1978) began to associate pathogenicity with sialidase; the genus Bacteriodes (Muller & Werner, 1970) and Vibrio (Muller, 1973) were cited. This relationship between sialidase and pathogenicity is now known not to be the case because, in the genus Bacillus (Fraser, 1978) and Neisseria (Zhuravleva, 1978), it has been shown that pathogenic Clostridium botulinum did not produce sialidase as opposed to the pathogenic Clostridium perfringens and a non-pathogenic strain of Neisseria was a sialidase producer as opposed to the pathogenic Neisseria meningitidis which is a sialidase producer.

It is now suggested that sialidase production may be of value in (i) taxonomic studies (as in the case of viruses) and, (ii) in studies of pathogenicity and virulence (on the assumption that increased virulence is related to increased sialidase activity) (Fraser, 1978).

Recent years have seen an increase in the number of non-pathogenic sialidase producing bacteria of the genus Clostridium (Fraser, 1978),

Neisseria (Zhuravleva, 1978), Streptomyces (Kunimoto et al., 1974) and especially Arthrobacter (Flashner, et al., 1977 and Uchida et al.,1977).

Of the higher protistan, protozoal sialidase is found in the culture media of the Mastigophora and Sporozoa orders (Watkins, 1953 and Crampen et al., 1979).

1.1.3 Mammalian Sialidases

Mammalian sialidases, unlike the viral or bacterial sialidases, are an integral component of all organs containing sialyl-compounds. The period 1962 to 1973 has seen the association of sialidase with virtually every major organ of the mammalian body (Carubelli et al., 1962, Zvetkova, 1965 and Tulsiani et al., 1973). With the emergence of more sophisticated methods for isolation, preparation and characterization, the mammalian sialidases, at a cellular and sub-cellular level, are associated primarily with lysosomes (Jibril & McCay, 1965 and Venerando et al., 1978) and plasma membranes (Schengrund et al., 1972 & 1979) of the various organs. It has been found in the blood serum (Schauer et al., 1976), brain cytosol (Venerando et al., 1978) and cultured epitheliod cell lines (Tallman et al., 1977 and Thomas et al., 1979) as well.

Schengrund and coworkers (1979) have stated that the sialidase of spleen plasma membrane are similar to those associated with the plasma

membranes of cells from other organs. It is now clear that mammalian sialidase can be found as a soluble enzyme, as a cell surface component of individual organs or in both forms in a single organ (Tulsiani & Carubelli,1970 and Venerando et al., 1978). High sialidase activity, being associated with organs such as the intestines, brain and liver which have a high turn-over of sialic acid containing compounds, is governed by the age and physiological condition of the organ (Dickson & Messer, 1978).

1.2 Biological Function Of Sialidases

1.2.1 Viral sialidases

Over the years, many roles have been proposed for the function of viral sialidase namely, viral binding to the host cell surface by enzyme-substrate interaction, viral penetration into the host cell, dissemination of newly formed viruses from the host cell and destruction of substances which protect the cell surface from virus binding. As each theory was put to the test, conflicting lines of evidence arose. Few of the important findings are elaborated upon below.

Confirmation of Hirst's theory (1965) which is that the binding of myxoviruses to cell surfaces is mediated by enzyme-substrate interaction, came from an elegant experiment performed by incorporating

fetuin (containing N-acetylneuraminic acid) into liposomes: these artificial membranes bound ortho and paramyxoviruses but not adeno or herpesviruses. Antibodies directed against fetuin also prevented the binding of these viruses (Tiffany & Blough, 1971). The inconsistencies pertaining to the latter lie in the fact that indicator virus binds red blood cells (Stone, 1949) and that purified viral sialidase does not adsorb red blood cells (Laver, 1964 and Drzeniek et al., 1966).

This does not mean that there is no enzyme-substrate interaction between sidalidase of the myxovirus and the cell surface containing N-acetylneuraminic acid. This enzyme was shown to split off N-acetylneuraminic acid from substances with which it came into contact (Drzeniek, 1970).

Seto, Kendal, Dowdle and their co-workers (1967, 1970, 1974 respectively), observed that sialidase-specific antibodies do not inhibit the adsorbtion onto or penetration of myxoviruses into permissive cells, but affect the release of viruses from the cell by binding newly formed viruses to each other and to the membrane of the cell surface. Thus it was postulated that the biological function of sialidases was to release mature virus from the cell surface. Such eluted viruses could then attack fresh cells and increase the efficiency of viral propagation by circumventing localized "overkill". However, Becht et al., (1971) observed that monovalent antibodies which inhibited sialidase of ortho myxoviruses did not inhibit the release of virus particles from infected cells. Also, high concentrations of a viral sialidase inhibitor, 2-deoxy-2, 3

dehydro-N-acetylneuraminic acid, (dehydro NANA), added to Newcastle disease virus had no effect on the multiplication of the viruses (Drzeniek, 1972).

When the environment of the influenza virus is considered, one is inclined to believe that sialidase is an evolutionary tool required to penetrate the mobile mucous of the respiratory tract, thereby not only giving the virus access to the underlying cells but also protecting it against confinement in a coating of host mucin (Gottschalk & Bhargava, 1971). The latest theory as proposed by Rosenberg and Schengrund (1976) is that newly synthesized sialidase molecules, for which the viral genome codes are incorporated into the surface membrane of the host cell and occupy a position similar to the mammalian sialidase. By removing adjoining sialic acid residues from the surface of the host cell membranes, the enzyme serves to alter the physiochemical properties of the plasma membrane so as to permit budding and envelopment of the formed and exiting virus. This hypothesis still remains to be tested.

To resolve the present uncertainty about the function of viral sialidase, it will be necessary to opt for a biochemical and genetic approach; the former studying the kinetics, substrate specifications and structure-function relationship of the pure solubilized enzyme and the latter, verifying Rosenberg and Schengrund's theory and further elaborating on the genetics of antigenic variation related to sialidase.

1.2.2 Bacterial Sialidases

The function(s) of bacterial sialidase, although subject to speculation, appears slightly more clear-cut than that of viral sialidases. Bacterial sialidases are known to alter the characteristics of important sialic acid-containing macromolecules in mammalian cell surfaces, matrices and circulatory material (see Table 1.2).
Because of the latter, two possible roles for bacterial sialidases can be considered; the first having its emphasis on the inducible (Pardoe, 1970; Nees *et al.*, 1975 and Hoffler, *et al.*, 1978) nature of the enzyme and the other on cell-cell interaction.

In the primary areas of bacterial infection such as the respiratory and intestinal tracts, the invading bacteria will encounter an adequate supply of inducers and substrates for the rapid synthesis of sialidase, the initial effect of which is to remove the sialic acid and, in turn, is followed by other enzymes (Ashwell & Morell, 1974) which subsequently remove other sugars which can also serve as a ready source of energy for the parasites. Following from the initial attack and rapid generation of sialidase, the protective properties of the mucous layer are now drastically altered and its much reduced viscosity can no longer trap and impede the bacterial onslaught upon the underlying cells.

The function of bacterial sialidase at a subcutaneous level is elaborated upon by Costello *et al.*, (1979), who have shown that sialidase is essential for the adhesion of a human strain of

Table 1.2

Experimental Removal Of Sialic Acid From Biologically Important Sialic Acid-Containing Substances By Microbial Sialidases

Substrate	Effect on substrate	References
Blood group antigens	Loss of M antigen	Yokoyama & Trams, 1962
Blood platelets	Spontaneous aggregation	Hovig, 1965
Erythrocytes	Increased sensitivity to lysis	Perone et al., 1964
Erythrocytes	Unmasking of ABH blood groups	Saber et al., 1965
Erythrocytes	Increased ingestion by macrophages	Lee, 1968
Monocytes	Increased phagocytosis	Weiss et al., 1966
Lymphocytes	Decreased homing	Gesner et al., 1969
Transfused lymphocytes	Decrease in lymphnodes and spleen, increase in liver	Woodruff & Gesner, 1969
Human lymphocytes	Activation release of growth factor	Novogradsky et al., 1980
Human lymphocytes	Increased lysis susceptibility	Grathaus et al., 1971
Human lymphocytes	Activation, release of inhibitor factor	Greineder et al., 1979
Human lymphocytes	Enhance expression of cellular IgG; decrease of IgM	Itoh & Kumagai, 1980
Lymphoid cells	Increased susceptibility to cytolysis by complement	Roy et al., 1971
Chicken fibroblasts	Stimulation of division and sugar uptake	Veheri et al., 1972
Hamster kidney fibroblast	Stimulated ricin binding	Rosen, S.W. & Hughes, R.C. 1977
F S H	Loss of biological activity	Finne 1978
HCG	Loss of biological activity	van Hall et al., 1971
Serum alkaline phosphatase	Differential release of sialic acid from isoenzymes	Robinson & Pierce, 1964
γ-Glutamyl transpeptidase	Increase in acid stability	Szewczuk & Connell, 1964
Dopamin β-hydroxylase	Decreases stability to proteolytic degradation	Aquino et al., 1980
Erythropoietin	Loss of activity in vivo	Schooley & Mahlmann, 1971
Synaptic structure	Abolishes synaptic transmission	Tauc & Hinzen, 1974
Nervous system cells in culture	Activation of octapyrophosphatase	Stefanovic et al., 1975
Lung tumour cells	Reduced metastasis	Gasic & Gasic, 1962
Mouse sarcoma cells	Increased immunogenocity	Currie & Bagshawe, 1969
Leukemia L1210 tumour cells	Increased immunogenocity	Bekesi et al., 1971
Membrane glycoproteins	Increased binding to liver cell membranes	Pricer & Ashwell, 1971
Macrophages	Increased bacterial adhesion	Allen & Cook, 1970
Mouse islets of Langerhans	Decreased insulin secretion	Maier & Pfeiffer, 1971
Post coital intravenous injection in mice	Suppression of pregnancy	Gasic & Gasic, 1970
Injection into mice	Delay of skin graft rejection	Medoff & McKenna, 1973
Mouse trophoblasts	Increases transplant immunity in recipient mice	Taylor, Hancock & Gowland, 1979
Embryomesoderm cells	Altered shape	Wakely & England, 1978
Glomerular basement membrane	Loss of endothelium and epithelium	Kanwar & Farquhar, M.G., 1980
Macrophage glycolipids	Loss of biological activity	Lui et al., 1980

Actinomycetes to red blood cells. They observed that bacterial haemagglutination incurred two steps (i) the action of sialidase is to expose the galactosyl groups on the red blood cell surface and (ii) the binding of the newly exposed groups by lectin-like molecules on the Actinomycetes cells. Such a mechanism may enable bacterial surfaces to adhere to host cells in the presence of asialo-membrane proteins containing the newly exposed galactose termini. This provides support for the idea that interactions of the lectin- carbohydrate type, represents a significant mechanism for the attachment of microorganisms to mammalian cell surfaces (Ofek et al., 1978).

An example where both proposed roles of bacterial sialidases come into play, is the action of V. cholerae where it is believed that the V. cholerae sialidase augments the action of cholera toxin by (i) degrading the glycoprotein mucosa, thus facilitating the choleragen reaching its receptor rapidly and (ii) removing the sialic acid residues from the gangliosides, thereby priming the ganglioside, G_{GM1}, the receptor for choleragen (Stark et al., 1974).

1.2.3 Mammalian Sialidases

A molecule as negatively charged as sialic acid will, to some degree, control the configuration of the site upon which it is borne in addition to any other interaction which it may have with neighbouring glycoproteins on the same membrane. Therefore the effect of sialidase can be expected not only to uncover previously protected asialo-

heterosaccharides, but also to affect membrane structure in general.

In the endeavour to determine the function of mammalian sialidase, virtually every known serum sialoglycoprotein has been subjected to bacterial sialidase *in vitro* and the results have implicated sialidase with the biological effects listed in Table 1.2. For a possible *in vivo* interpretation from the table, two significant events occur. After the action of sialidase there follows either a cell-to-glycoprotein interaction or a cell-to-cell interaction, both of which are synonomous with all the listed biological events.

The cell-to-glycoprotein interaction is normally associated with the catabolism of serum glycoproteins, a catabolic effect regulated by sialidase (Fig. 1.4) (Morell *et al.*, 1971).

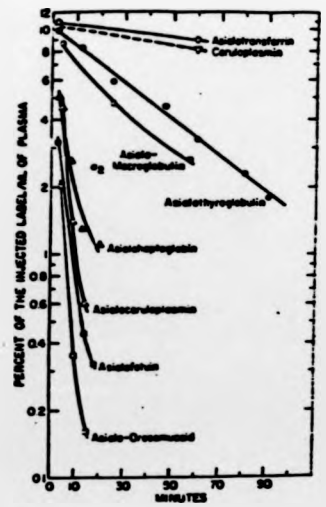


Fig. 1.4 Plasma survival times of tritiated asialoglycoproteins in the rat (Morell *et al.*, 1971)

After the removal of the sialic acid, a galactosyl or N-acetylgalactosamine-heterosaccharide core is exposed and this is believed to increase the susceptibility of asialoglycoproteins to phagocytic stimuli (Roseman, 1970) or possibly via the method described by Reutter et al., 1978 (Fig. 1.5) in the cell, effected by the lysosomes.

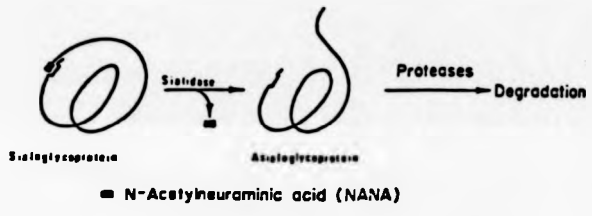


Fig. 1.5 Possible after event of sialidase action intracellularly (Reutter et al., 1978)

In the cell-to-cell interaction, removal of the sialic acid leads to increased cell adhesion, possibly in a similar manner as proposed by Roseman (1970), (including the action of the sialyltransferase). Therefore it can be inferred that, opposed to the aforementioned catabolic action of sialidase, it now participates in an anabolic process such as embryonic development and tumour formation (Jeanloz & Codington, 1976 and Preti et al., 1978).

Of the broad range of possibilities explored in trying to determine the biological function of sialidase upon endogenous substrates, a few have been chosen to be mentioned, in which the after-event of sialidase

action *viz* cell-to-glycoprotein and cell-to-cell interaction could be biologically significant.

Reproduction:- sialidase has been found to be part of the sperm acrosome by Srivastava *et al.*, (1970). Its role in fertilization is to reduce or block polyspermy. Sialidase has been shown to attack the hormones in reproduction (Brossmer & Walter, 1958; Sairam & Moudgal, 1971 and Yaginuma, 1972). This results in a greatly reduced uptake by the ovary, followed by lower biological activity.

Blood clot formation:- The role of adhesion of platelets to each other is an essential step in the formation of the haemostatic plug or an intravascular thrombolin deposit. Vermylen *et al.*, (1974) showed that sialidase treatment of factor VIII gave rise to strong platelet aggregation, not only in normal patients, but in haemophilic patients as well.

Interaction of hormones with target cells:- Changes in the sialic acid content in the plasma membrane of the cell have been found, in some cases, to block the ability of the cell to respond to some hormones (Hahn *et al.*, 1974; Hughes, 1979 and Haskar *et al.*, 1973).

Neurotransmission:- Increasing evidence indicates that sialic acid is involved in neuronal transmission. Several workers (Heilbronn, 1962, Augustinsson & Ekedahl, 1962 and Heilbronn & Cedergreen, 1970) have shown that cholinesterase contains sialic acid. Brodbeck *et al.*, (1973), observed that the removal of the sialyl residues increased its

specificity for acetyl choline, indicating a possible function for synaptic membrane-bound sialidase. Schengrund and Rosenberg (1970) and Yohe and Rosenberg (1978) have shown the presence of sialidase within the synapse and that it can act upon the endogenous lipid structure viz., the ganglioside effecting neurotransmission.

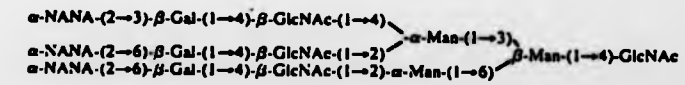
Immune response:- The greatest area of debate over the past few years pertaining to sialidase, has been its significance in the immune system. This enzyme has been implicated in both cellular and humoral immunity and the mechanism by which the immune response is stimulated is not clear. Some authors claim that the enzyme uncovers a highly immunogenic new antigen which may result in altered handling by macrophages (Barth & Singla, 1974 and Schmidtke & Simmons, 1973) while others claim that it stimulates individual participants of the immune response (Weiss et al., 1966, Lee 1968, Knop et al., 1978 and Galili & Schlesinger 1978). Yet again, Kuppers and Henney (1977) claim that the action of sialidase followed by that of galactose oxidase ($-CH_2OH \longrightarrow CHO$) induces cell-to-cell crosslinking, thereby altering the antigenicity of the lymphoid cell which in turn leads to T. lymphocyte proliferation.

1.3 Sialidase: Disease and Application

Disease associated with sialidase is manifested by either (i) an increase in sialic acid-containing compounds, (indicating sialidase deficiency), such as oligosaccharides, glycoproteins and lysosomal

enzymes in the urine, serum, fibroblasts, leucocytes and liver (Durand et al., 1977; O'Brien, 1977; Thomas et al., 1978 and Rapin et al., 1978), or (ii) an increase in sialic acid, (indicating sialidase hyperactivity), in the blood, urine (Merkhailova et al., 1968, & Mutton et al., 1977) and the after-effect of sialic acid removal from glycoproteins, glycolipids and cell surfaces (Heide, 1974 and Uhlenbruck & Rothe, 1974). The absence of normal sialidase activities has been listed under many disorders such as fucodosis, Mannosidosis (Lowden & O'Brien, 1979), gangliosidosis (Justice et al., 1977), I-cell disease (Minami et al., 1979), Mucolipidosis (Bach et al., 1979), Mucopolysaccharidosis (Leroy et al., 1971) and sialyloligosaccharidurla (Durand et al., 1977, and Kelly & Graetz, 1977). Because of its concurrence with the vast amount of disorders, be it primary or secondary, an attempt has been made to catergorise any effect caused by the primary deficiency of sialidase as "sialidosis" (Lowden & O'Brien 1979 and Thomas et al., 1979).

There are two types of sialidosis. The difference between them, besides the phenotypic features and intelligence, is that Type I accumulates sialyloligosaccharides in the urine (Durand et al., 1977; O'Brien, 1977; Thomas et al., 1978 and Rapin et al., 1978), whereas Type II accumulates sialylcompounds in the fibroblast (Spranger et al., 1977 and Spranger & Weidemann, 1970). The type of oligosaccharides isolated from the urine, which is increased 100 times more than normal, is derived from a common precursor which could resemble the compound below.



Of the oligosaccharides in the urine of the patient, more than 80% of the sialyl-linkages are of the α (2-6) configuration (Durand et al., 1977 and Strecker et al., 1977). Question, is this an iso-enzyme deficiency, as only the α(2-3) sialyl linkage is cleared? Sialidosis can be classified as an hereditary disease because it is transmitted as an autosomal recessive trait (Thomas et al., 1978 and O'Brien, 1978). Both parents of sialidosis patients have half the normal sialidase activity for α(2-3) and α(2-6) sialyl linkages (O'Brien, 1977).

In the other disorders lacking sialidase activity, such as I-cell and mucolipidosis III, the enzyme deficiency is only secondary since the removal of the sialic acid from the lysosomal enzymes still does not improve their uptake (Vladutiu et al., 1977).

Possible therapeutic effectors of sialidase should be explored, especially in view of the tragic consequences of debilitating myclonus in normally intelligent Type I patients. A possible approach could be the targetting of sialidase-loaded liposomes to the affected areas (Gregoriadis, 1976 and Finkelstein & Weissmann, 1978).

High levels of sialic acid in the blood and urine lead one to suspect hyperactive sialidase(s). This was suspected for coxarthrosis disease (Merkulera et al., 1968) and calculus disease (Mutton et al., 1977). For the former, enzyme activity was not investigated whereas in the latter activity was found to be normal. The disease hypercapnia and the resulting respiratory acidosis is believed to be the result of a released glycosidase activator which acts upon sialidase, as a loss of

one third of the sialic acid molecules per neuron has been found (Lowden & Wolfe, 1964).

Sialidase has been associated enpassing with many other diseases such as leukemia, (Kallee et al., 1964) Kwashiokor, (Ittyerah, 1970) and polyagglutinability (Lalezari & Al-Mondhiry, 1973), to mention but a few.

Although sialidase has not been unequivocally implicated in cancer, the following observations have been made:

(i) Schengrund et al., (1973) observed that transformed cells showed sialidase activity when gangliosides were used as substrate and that the activity, (of the malignant cells), parallelled the degree of oncogenicity of a series of herpes simplex virus-transformed hamster embryo fibroblasts (Schengrund et al., 1974).

(ii) That the distribution of sialic acid on malignant cells was concentrated in special zones, despite the fact that malignant cells have a lower total sialic acid level than normal cells.

(iii) Many human tumours caused by viral activity share antigens, perhaps because of their common viral etiology (Morton & Malmgren, 1968 and Rios & Simmons, 1976).

Because of the above, the question now arises as to whether it is possible that virally induced transformations strategically incorporate

viral sialidase into the transformed cell surface, thereby removing contiguous sialic acid residues and leaving pockets of unaffected, negatively charged residues. Further, does a similar *modus operandi* exist in the non-virally transformed cells?

Another disease attributed to viral sialidase is the autoimmune disease (Kolb-Bachofen & Kolb, 1979), in that the lymphocytes, once desialylated, are taken up by the liver against which the autoimmune response is then targetted. This is in keeping with two established observations: (i) that the autoimmune response is a pathological event and (ii) desialylation increases immunogenicity (Simmons *et al.*, 1971).

Foetuses and tumours share something in common - that is, they escape the immune attack brought about by the suspected masking effect of sialic acid and this is believed to prevent abortion (Granatek *et al.*, 1979). It has also been shown that the protozoa, *Trichomonas foetus* which is a producer of sialidase, leads to abortion in cows (Crampen *et al.*, 1979). The above observations (removal of sialic acid leading to an increased immune response), has become a tool in the hands of tumour researchers, who have demonstrated that tumour cells, treated *in vitro*, showed reduced tumouregenicity *in vivo* (Lindenmann & Klein, 1967; Sanford, 1967 and Sedlacek *et al.*, 1977). There have, however, been contradictions (Pimm *et al.*, 1978), thus sialidase-treated tumour cells used as a vaccine still remain speculative (Sanford, 1974).

Vaccination against influenza virus using pure viral sialidase has again aroused interest because it has now been found that antigenic changes are less frequent in the viral sialidase than in the viral haemagglutinin. The vaccine would therefore be usable for longer periods than the presently used inactivated whole-virus vaccine (Arora, 1979).

The diseases caused by sialidase-producing pathogenic bacteria are as numerous and as variegated as portrayed in their names, eg. Diplococcus pneumoniae, Corynebacterium diptheria, Corynebacterium acnes, Vibrio cholerae, Clostridium septicum and Neisseria meningitidis, as discussed previously and in the reviews of Muller (1974a & 1974b).

The action of bacterial sialidases upon serum glycoproteins, (Morell et al., 1971 and Schultze & Schwick, 1957) hormones, (Gottschalk et al., 1960 and Haksar et al., 1973) enzymes (Higashino et al., 1972 and Robinson & Pierce, 1964) and cell membranes (Kemp, 1970 and Vaheri et al., 1972) in vitro and in vivo have been discussed previously. The successful survival of these bacteria is dependent on their ability to adapt, of which the foremost is the ability of the sialidase to be induced. This removal of substrate and inducers from their host leads to their increased virulence and overall deleterious effects upon the host such as septicemia, lymphocyte stimulation, inflammatory response and thrombocytopenia (Muller, 1974a).

1.4 GENERAL PROPERTIES OF SIALIDASES

As sialidases are usually glycoproteins (Lazdins et al., 1972 and Kabayo & Hutchinson, 1977), the inevitable presence of microhetero - geneity will bedevil molecular weight determinations (Gottschalk, 1971). This is obvious in the broad range of molecular weights obtained for sialidases, even within a specific group.

1.4.1 Size And Shape

1.4.1.1 Viral sialidases

In determining the molecular weights of viral sialidases, the added problem of harsh isolation techniques, employing proteolytic enzymes, detergents and mechanical methods, resulted in an enzyme that may have suffered unsuspected degradation.

The molecular weights differ for the various myxovirus sialidases. Their sizes range between 1 to 2.8 x 10^5 daltons (Kendal et al., 1968; Maeno et al., 1970 and Allen et al., 1977), for the high molecular weight complex and 5 to 6 x 10^4 for the monomeric enzymes or subunits (Drzeniek, 1972).

Viral sialidase has been described as a square, box-shaped head with a long tail having a small knob at the end embedded in the viral coat

(Laver & Valentine, 1969; Wrigley et al., 1973). Removal of the head from the tail via proteolytic enzymes has given the B/Lee virus a molecular weight of 2.8×10^5 daltons, with four subunits of molecular weight 7.0×10^4 daltons each. Oxford (1973) and Lazdins et al., (1972) obtained values of 6.3×10^4 daltons per subunit whereas Wrigley et al., (1973) obtained a molecular weight of 4.8×10^4 daltons after proteolytic digestion. Lazdins et al., (1972) observed further that if he treated his monomers with trypsin as well, a carbohydrate-rich fragment was released, thereby reducing the monomer weight to 5.6×10^4 daltons resulting in the loss of its ability to aggregate again.

A thorough investigation into the molecular weight of the N2 virus sialidase was carried out by Groome et al., (1977) in which all the possible effects of the carbohydrate side chains of the enzyme were considered. They found that the molecular weight of the enzyme wa between 1.47 to 1.55×10^5 daltons as opposed to 2×10^5 daltons (Kendal & Kiley, 1973). Their sequential breakdown of this enzyme, first using 6M guanidine-HCl, halved the molecular weight to 66,000 (2 subunits) then, after irreversible reduction and the blocking of sulphydryl groups, the molecular weight was halved again to 33,500 daltons (4 subunits). They proposed the figure 1.6 below as a probable arrangement of the polypeptides in influenza sialidase.

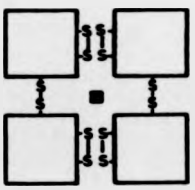


Fig. 1.6 The probable arrangement of polypeptides in the Influenza sialidase (Groome et al; 1977).

1.4.1.2 Bacterial sialidases

Bacterial sialidases are so far considered to be single polypeptide chains and there is no existing evidence of subunit structure (Rosenberg & Schengrund, 1976). Compared to viral sialidases, the bacterial enzymes are smaller. Their molecular weights are spread between 3.2×10^4 and 10×10^4 daltons. (Kabayo & Hutchinson, 1977; Myhill & Cook, 1972; von Nicolai et al., 1978; Balke & Drzeniek, 1969; Tanenbaum & Sun, 1971; Flashner et al., 1977 and Mohr & Schramm, 1960). Whether this is attributable to de Novo synthesis, remains to be answered. Molecular weight discrepancies lie within subgroups, for example V. cholerae sialidase has a size between 6.8×10^4 to 10×10^4 daltons (Mohr & Schramm, 1960 and Drzeniek, 1972). The differences may be due to analytical procedures, technique, different degrees of aggregation of the enzyme or microheterogeneity.

The existence of isoenzymes for bacterial sialidase as claimed by Tanenbaum & Sun (1971) and Nees et al., (1975) have raised doubts as to whether they are true isoenzymes or not. As it was shown that if the three "isoenzymes" isolated by Nees et al., (1975) were subjected to sodium dodecyl sulphate (SDS) or 8M urea, they migrated as a single band on polyacrylamide gels. The only evidence in favour of true isoenzymes was published by Ziegler et al., (1978). They found the two isoenzymes from V. cholerae showed differences in optimum pH and catalytic activities. Uchida et al., (1979) also reported two

isoenzymes for a A. ureafaciens differing in size and catalytic activity only towards gangliosides. No molecular weights for mammalian sialidases have been registered thus far.

1.4.2 pH Optimum And Km

The pH optimum of sialidases varies within the range of 3.5 to 7.0, depending on the substrate used and the state of purification (Gottschalk & Drzeniek, 1972 and Drzeniek, 1973). Schneir & Rafelson (1966) found that influenza virus Strain A_2/Japan 305/57 sialidase had a pH optimum of 4.5 with N-acetylneuraminosyl (α,2→6) lactose, whereas it was pH 6.5 for the action of the same enzyme on the isomeric substrate N-acetylneuraminosyl (α,2→3) lactose as substrate Uchida et al., (1979) observed a change of approximately 1.0 pH unit in going from N-acetylneuraminosyl- lactose to colominic acid for sialidase from a bacterial source. Whereas Thomas et al., (1979) and Sander et al., (1979) reported similar but less dramatic pH changes for mammalian sialidases using sialyl-oligosaccharides as substrates. Ada (1963) reported that the pH optimum shifted from 5.8 for the crude enzyme of chick embryo, to a lower pH optimum (4 - 5) for the purified enzyme.

Generally, the pH optimum ranges for viral and bacterial sialidases are 6-7 and 4.5-6 respectively (Gottschalk & Drzeniek, 1972 and Drzeniek, 1973) whereas the vertebrate sialidases exhibit two optimum pH ranges,

4-5 for the membrane bound enzymes (Ada, 1963; Mahadevan et al., 1967; Horvat & Touster, 1968; Tulsiani & Carubelli, 1970; Parker et al., 1979 and Schiller et al., 1979) and 5.2-5.8 for the soluble enzymes (Ada, 1963; Taha & Carubelli, 1967 and Tulsiani & Carubelli, 1970).

The Michaelis constants (Km) of sialidases, like most enzymes, are perturbed by the normal effectors of enzyme rates such as pH, temperature and ionic strength (Lipovac et al., 1973 and Barton et al., 1975).

When small substrates such as N-acetylneuraminyl-lactose are used, the Km's for viral, bacterial and mammalian sialidases range between 2 x 10^{-3} to 2 x 10^{-4} M (Gottschalk & Drzeniek, 1972). With larger molecular weight substrates such as glycoproteins and gangliosides, Km values of 10^{-5} and 10^{-6} M have been obtained (Schengrund & Rosenberg, 1970 and Tallman & Brady, 1973) (see Table 1.3).

Table 1.3

pH Optimum And Michaelis Constant(Km) Of Sialidases Directed Against Various Substrates

Source of Sialidase	pH Optimum	Substrate	Km M	References
Influenza A_2/Jap.305/57	6.5	(α,2 — 3)NANL [1]	2.00×10^{-4}	Schnaar & Rafelson, 1966
Influenza A_2/Jap.305/57	4.5	(α,2 — 6)NANL	1.00×10^{-3}	Schnaar & Rafelson, 1966
NDV	5.2	Fetuin	1.00×10^{-6}	Brostro- et al., 1971
Sendai virus	5.1	Fetuin	1.00×10^{-6}	Brostrom et al., 1971
V. cholerae	5.6	(α,2 — 3)NANL	1.00×10^{-3}	Ada et al., 1963
C. perfringens	4.5	(α,2 — 3)NANL	1.00×10^{-3}	Cassidy et al., 1965, 1966
Rat liver lysosomes	4.2	(α,2 — 3)NANL	1.98×10^{-3}	Horvat & Touster, 1968
Chicken chorioallantois membrane	4.5	(α,2 — 3)NANL	1.00×10^{-3}	Ada & Lind, 1969
Fibroblast	4.0	(α,2 — 3)NANL	1.1×10^{-3}	Frisch & Neufeld, 1979
Fibroblast	4.2	Fetuin	3.6×10^{-4}	Thomas et al., 1979

[1] NANL, N-acetylneuraminyl-lactose

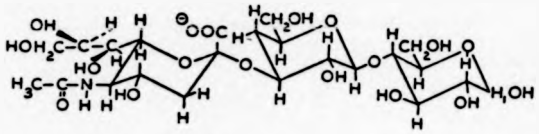
N-acetylneuraminyl-lactose was at one stage frequently used as the test substrate for brain sialidases but it has now proved misleading. Since brain sialidase exhibits reduced activity towards N-acetylneuraminosyl-lactose, the substrates of choice nowadays are di- and trisialogangliosides (monosialogangliosides and sialoglyco - proteins are normally not hydrolysed) (Liebovitz & Gatt, 1968). Gangliosides have been used as substrates for other sialidases as well and Km's vary from 9×10^{-6} to 1×10^{-4} (Tallman & Brady, 1973; Ohman et al., 1970; Schiller et al., 1979 and Parker et al., 1979.

1.4.3 Substrate Specificity

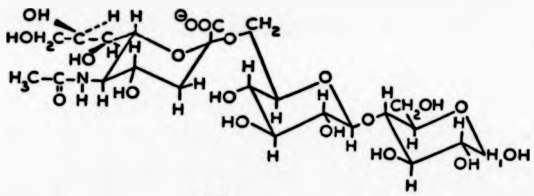
Sialidases are able to hydrolyse only α-O- ketosidically bound sialic acid (Kuhn and Brossmer, 1958 and Gottschalk, 1958), while the β-anomers are not split (Meindel & Tuppy, 1966 a,b and Yu & Ledeen, 1969). The α-S-ketosides, α-N ketosides and the naturally occurring compound cytidine-5-monophospho-N- acetylneuraminic acid (CMP-NANA) are resistant to sialidases (Khorlin et al., 1970 and Comb et al., 1959).

Three different types of α-ketosidic linkages have been found in sialic acid-containing substances. An (α,2→3) linkage was found in N-acetylneuraminosyl (α,2 → 3)-D-lactose (Fig. 1.7(i)) (Kuhn & Brossmer, 1959) and in a number of gangliosides (Wiegandt, 1968). An isomeric 6-sialyl-lactose (Fig. 1.7(ii)), namely N-acetyl - neuraminosyl (α,2→6)-D-lactose, in which the neuraminic acid is joined

to C-6 of the galactose molecules, was isolated from human milk (Kuhn & Brossmer, 1959).



(i)



(ii)

Fig.17 Isomeric sialyllactoses: i N-Acetylneuraminosyl (α, 2→3)-D-lactose.
ii N-Acetylneuraminosyl (α, 2→6)-D-lactose

The (α,2→6) linkage between N-acetylneuraminic acid and N-acetylgalactosamine was found in the disaccharide isolated from bovine submaxillary glycoprotein (Gottschalk & Graham, 1959). Sialic acid is joined by an (α,2→8) linkage to an adjacent second sialic acid molecule in a number of gangliosides (Wiegandt, 1968) in colominic acid (McGuire & Binkley, 1964) and in disialyl-lactose (N-acetylneuraminosyl (α ,2→ 8) -N-acetylneuraminosyl (α ,2→ 3)-D-lactose)(Fig. 1.8) (Kuhn & Gaughe, 1965).

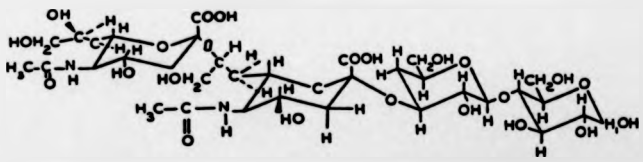


Fig.18 Disialyllactose: N-acetylneuraminosyl (α, 2→8)-N-acetylneuraminosyl (α, 2→3)-D-lactose

An additional type of linkage, namely an (α,2→4) linkage between sialic acid and galactose in α_1-acid glycoprotein has been reported (Jeanloz, 1960).

Experiments with bacterial enzymes have shown that C. perfringens (Cassidy, et al., 1965) or V. cholerae sialidase (Drzeniek & Gauhe, 1970) hydrolyse N-acetylneuraminosyl (α,2→3)-D-lactose twice as fast as N-acetylneuraminosyl (α,2→6)-D-lactose. Great differences in the hydrolysis of these two isomers were found when viral enzymes were used (Schneir & Rafelson, 1966). At the optimum pH values for the enzyme obtained from influenza virus Strain A_2 /Japan 305/57, the rate of hydrolysis for the 2-3-isomer was sixteen times faster than the 2-6-isomer.

Differences in the capacity to hydrolyse (α,2→3), (α,2→6) and (α,2→8) linkages by a number of viral sialidases were revealed by Drzeniek (1967 & 1970). The most specific viral sialidases tested were the enzymes of fowl plague virus (FPV) resembling influenza A viruses and Newcastle disease virus (NDV), a representative of parainfluenza viruses. Under standard conditions as laid down by Drzeniek (1973), FPV and NDV sialidase easily hydrolysed the (α,2→3) linkage of the N-acetylneuraminosyl (α,2→3)-D-lactose, whereas from the (α,2→6) isomer no more than 7% of NANA was liberated.

FPV and NDV sialidases, though indistinguishable in their action on (α,2⟶6) linkages, showed marked differences when acting on the (α,2⟶8) linkage. It was found that the total amount of NANA could be

removed from disialyl-lactose by brief incubation with NDV sialidase. This substance was resistant to the action of fowl plague virus enzyme (Drzeniek & Gauhe, 1970). Since disialyl-lactose contains two differently linked NANA molecules, it is evident that the (α,2-8) linkage is resistant to the action of FPV sialidase. In this substance, however, the second (α,2-3) linkage is not split off by this enzyme either. This behaviour demonstrates that viral sialidase is an exoenzyme, incapable of liberating a non-terminal sialic acid molecule.

The above oligosaccharides were easily hydrolysed by V. cholerae sialidase. The substrate specificity of V. cholreae and both viral sialidases is compiled in Table 1.4.

Table 1.4

Substrate Specificity of Sialidases. The sensitivity (+) or resistance (-) of different types of α-ketosidic linkages of N-acetylneuraminic acid towards Vibrio cholerae, Newcastle disease virus (NDV) and fowl plague virus (FPV) sialidases are compared.

Type of α-ketosidic linkage	Sialidase of			Reference
	Vibrio cholerae	NDV	FPV	
2→3	+	+	+	Drzeniek, 1967
2→4	+	-	-	Huang & Orlich, 1972
2→6	+	-	-	Drzeniek, 1967
2→8	+	+	-	Drzeniek & Gauhe, 1970

For a compound to be cleaved by sialidase, the following criteria have to be met (i) the carboxyl group of sialic acid must not be substituted (Gottschalk, 1962 and Yu & Ledeen, 1969), (ii) the nitrogen atom of sialic acid must not be substituted by groups other than N-acetyl or N-glycolyl (Meindel & Tuppy, 1966 and Faillard, et al., 1969), (iii) the polyhydroxy side chain of sialic acid should not be substituted with bulky or negatively charged groups and it should not be oxidized beyond C-8. (Schauer & Faillard, 1968, Holmquist, 1975b and Suttajit, 1970).

Slight differences in catalytic selectivity occur between bacterial and viral sialidases concerning substitution at C-4, C-6 and C-8. While viral (Influenza A_2/Singapore/57 virus) and bacterial [C. perfringens and V. cholerae] sialidases both catalyse C-7 and C-8 O-acetylated sialic acid, only viral sialidase can catalyse C-4 O-acetylated sialic acid (Schauer & Faillard, 1968 and Pepper, 1968).

Another factor which could possibly be considered to be a criterion for the hydrolysis of naturally occurring substrates such as gangliosides, is that the vicinal carbon atom, (normally C-4 of galactose), of the sugar partner to which sialic acid is linked, should not be substituted. This substitution sterically hinders the action of sialidase. Kuhn and Wiegandt (1963) have shown that the monosialoganglioside (Fig. 1.9) as well as oligosaccharides derived therefrom are resistant to V. cholerae, chicken retina and calf brain sialidase (Liebovitz Gatt, 1968 and Preti & Fiorilli, 1978). It is only after enzymic removal of galactose (linkage a) and

N-acetylgalactosamine (linkage b) that sialidase will split the ketosidic linkage c.

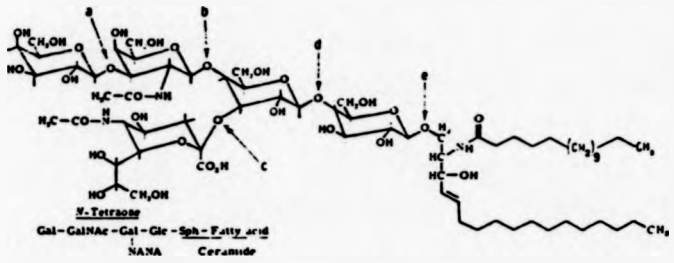


Fig 19 Enzymic degradation of monosialoganglioside (G_{M1}) from brain: (a) β-galactosidase, (b) β-N-acetylgalactosaminidase, (c) sialidase (d) β-galactosidase, and (e) β-glucosidase.

However, the sialidase from A. ureafaciens has been shown to cleave the sialic acid from G_{M1} without prior treatment with other glycosidases (Sugano et al., 1978, 1979).

Defined specificity will be discussed further in Chapter 4.

1.4.4 Activators And Inhibitors

Some metal ions are either activators or inhibitors of enzymes. However, from the reviews of Gottschalk and Drzeniek (1972), Drzeniek (1972 & 1973), Rosenberg and Schengrund (1976), and Holmquist (1975), it appears that there is no hard and fast rule about the effects of divalent or monovalent ions, especially the calcium ion on sialidases.

Most viral sialidases are activated by calcium ions which are often tightly bound to the enzyme. Bacterial sialidases on the other hand are somewhat indifferent to the influence of metal ions. Some are activated, inhibited or unaffected, eg. the sialidases of V. cholerae, D. pneumoniae and C. perfringens respectively (Mohr & Schramm, 1960; Hughes & Jeanloz, 1964 and Cassidy et al., 1965, 1966). Mammalian sialidases are generally unaffected by calcium ions (Kuratowska & Kubicka, 1967 and Schengrund & Nelson, 1975) but there are claims to the contrary Mahadevan et al., (1967) and Schengrund & Nelson (1975).

Though ethylenediaminetetracetate (EDTA) inhibits most viral sialidases except the Sendai virus enzyme (Popa & Repanovici, 1977), the effect on bacterial sialidases is ambivalent in that it inhibits the V. cholerae and not the C. perfringens enzyme (Boschman & Jacobs, 1965 and Popenoe & Drew, 1957). The EDTA inhibition could be overcome by the addition of calcium ions (Ada et al., 1961).

Viral sialidases are insensitive to heavy metal ions such as Fe^{++}, Zn^{++} and Hg^{++}. The bacterial enzymes again portray diverse behaviour for example, S. griseus enzyme is inhibited whereas the A. ureafaciens enzyme is not inhibited by Hg^{++} ions. (Kunimoto et al., 1974 and Uchida et al., 1979). Mammalian sialidases are generally inhibited by Hg^{++}, Cu^{++} and Zn^{++} (Tulsiani & Carubelli, 1970 and Yohe & Rosenberg, 1978).

The chloride ion was shown to inhibit V. cholerae and C.

perfringens sialidases (Brossmer et al., 1977 b, and Barton et al., 1975) whereas the ammonium ion inhibits the calf brain enzyme (Preti et al., 1974). Inhibitors of sialidases can be arbitrarily classified as analogues and derivatives of sialic acid or as substances with no direct resemblance to the substrate or product of the enzyme Viral sialidases can be inhibited by the product of their reaction namely NANA or by 2-deoxy-2,3 dehydro - sialic acids (dehydro-NANA) (Fig. 1.10).

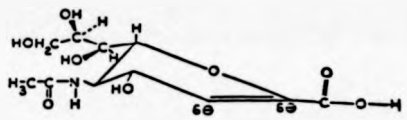


Fig. 1.10 2-Deoxy-2,3-dehydro-N-acetylneuraminic acid.

The competitive inhibition constant (Ki) observed for the above is in the range of 5×10^{-3} M to 5×10^{-6} M. (Walop et al., 1960; Drzeniek, 1970 a,b and Meindl et al., 1971).

For the S-ketoside, 2-Deoxy-2-p-nitrophenylthio-N-acetyl- α -D-neuraminic acid and the δ lactone, 3-Aza-2,3,4-trideoxy-4-oxo-D-arabino-octonic acid δ -lactone, complete inhibition is obtained at a concentration of 10^{-3}M (Khorlin et al., 1970).

For the perineal enigma, namely bacterial sialidases, it is reported that the V. cholera enzyme exhibits a similar profile to the viral enzymes (Mohr & Schramm, 1962; Meindl & Tuppy, 1969 a,b, 1970 and

Khorlin et al., 1970). C. perfringens and A. sialophilus show no inhibition by NANA or dehydro-NANA. Furthermore, dehydro-NANA methyl ester is an effective inhibitor of the A. sialophilus sialidase whereas the V. cholerae enzyme is insensitive to this analogue (Gottschalk & Drzeniek, 1972; Rosenberg & Schengrund, 1976 and Miller et al., 1978). The membrane bound mammalian enzymes of the brain, heart and liver are inhibited by dehydro-NANA but uncertainty prevails as to whether or not NANA is an inhibitor (Sandhoff & Pallman, 1978; Parker et al., 1979 and Sander et al., 1979).

Inhibitors showing no structural analogy to sialic acid are either low molecular weight substances with different properties, or high molecular weight substances with polyanionic character. The action of these substances is not specific since they react not only with the sialidase molecule, but also with other proteins or substances often present in sialidase preparations.

The important examples of the low molecular weight substances are the glyoxals. The most active of these potent N-substituted oxamic acids for both influenza and V. cholerae sialidases, are phenyloxamic acid (Fig. 1.11(i)) N-(2-pyridyl) oxamic acid (Fig. 1.1(ii) N-(2-thiazolyl) oxamic acid (Fig. 1.11(iii)) and thio-oxanilic acid (Fig. 1.11(iv)) (Edmond et al., 1966). Structurally, they all have similarities to phenylglyoxal (Fig. 1.11(v) (Brossmer et al., 1977 a) (Fig. 1.11).

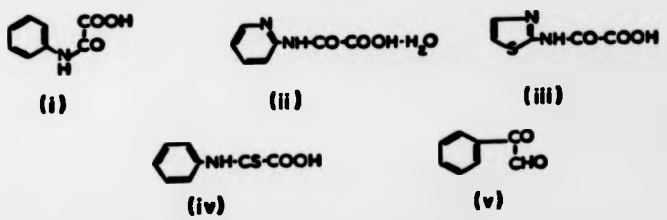


Fig. 1.11 N-substituted oxamic acid inhibitors of sialidase (Edmond et al., 1966)

How these compounds would react with the mammalian enzymes is not known.

The high molecular weight substances with polyanionic properties are very potent inhibitors (Drzeniek, 1966). Thus macromolecules such as dextran sulphate, ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and heparin, which are all anionic in nature, will inhibit most sialidases. This inhibition can be reversed by the addition of polycationic substances such as poly-L-lysine or protamine sulphate. Comparing the action of the different polyanions, it was concluded that the anionic groups present in the molecules inhibit in the following order: $RSO_4^- > RCOO^- > R_2PO_4^-$. Therefore the best inhibitor is expected to be dextran sulphate (Drzeniek 1970b , 1972). These effects have to be kept in mind when experiments are performed in the presence of polyanionic substances, eg. glycoproteins or nucleic acids.

A third type of inhibitor, Neuraminin, for (Influenza and NDV) sialidases, which is composed mainly of neutral sugar and lacks neuraminic acid, is still under study (Lin et al., 1977).

CHAPTER 2

ASSAY OF SIALIDASES

2.0 Introduction

One of the major drawbacks in research involving sialidase has been the lack of a standard assay procedure by which the activity of all sialidases can be determined. As a result it has been difficult to compare or meaningfully interpret the observed properties of different sialidases. Thus, the amounts or properties determined may reflect the substrate specificity of the enzyme which varies, with either the number of different linkages in the same substrate (Table 2.0),

Table 2.0

Naturally Occurring Substrates Of Sialidases

Substrate	Mol. Wt.	% NANA Content	N- Substitution	Type of α-ketosidic Linkage				Source	Reference
				2 ⟶3	2 ⟶4	2 ⟶6	2 ⟶8		
Sialyl-lactose	633	49	N-Acetyl	+	-	+	-	Bovine colostrum	Rosher & Palese 1975
Gangliosides	1,000	23-38	N-Acetyl	+			+	Bovine brain	Cumar et al 1963
Colominic acid	10,000	90-100	N-Acetyl	-	-	-	+	E. coli	Kimura 1966b
α,-acid glycoprotein	44,100	12.1	N-Acetyl	+	+	+	-	Human plasma	Rosher & Palese 1975
Fetuin	48,400	8.7	N-Acetyl N-Glycoyl	+	-	+	-	Foetal calf serum	Rosher & Palese 1975
Submaxillary mucin	1.2×10^6	21	N-Acetyl N-Glycoyl O, N-Diacetyl	+	-	+	-	Bovine submaxillary gland	Rosher & Palese 1975
Colominic acid	2.0×10^6	9-13	O, N-Diacetyl	+	Nil	Nil	Nil	Edible bird's nest	Rosher & Palese 1975

the limitation in the detection system (for example, low sensitivity, interference and reproducibility), or the differences in the purity of both the enzyme preparation and the substrates used.

Since sialidases hydrolyse substrates (eg. see Table 2.0) containing

α-ketosidically bound sialic acids, it is therefore possible to measure the activity of the enzyme by the decrease of bound sialic acid, by the increase of free sialic acid or by the determination of the free aglycone. The quantitive method used most frequently is the thiobarbituric acid (TBA) assay of Aminoff (1959, 1961) and Warren (1959, 1963), which allows the determination of free sialic acid in the presence of bound sialic acid. In this procedure free sialic acid is oxidized by sodium periodate to β-formylpyruvate (Paerels & Schut, 1965) which, in turn, reacts with thiobarbiturate to form a chromophore with a molar extinction of 57,000 at 549 nm in cyclohexanone (Fig. 2.0).

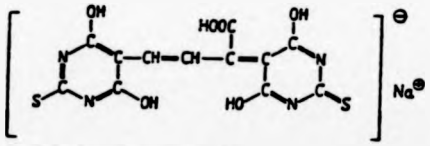


Fig. 2.0 Proposed structure of the chromophore generated during the thiobarbituric assay (Kuhn & Lutz, 1963)

For greater sensitivity Hammond and Papermaster (1976) modified the Warren method by extracting the chromophore in acidified butanol and reading the fluorescence at 550 nm. Free sialic acid can also be determined by using the coupled enzyme system of N- acetylneuraminate aldolase (N-acetylneuraminate pyruvate-lyase)and

lactate dehydrogenase (LDH) (Fig. 2.1).

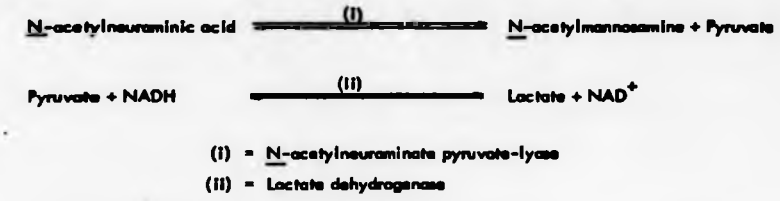


Fig. 2.1 Enzymic determination of free sialic acid

This enzymic method is based on the cleavage of NANA by N-acetylneuraminate pyruvate-lyase to pyruvate and the corresponding N-acetylmannosamine. The reaction was coupled to the reduction of pyruvate by LDH and NADH, which drove the aldolase cleavage to completion. The sialic acid concentration was estimated spectrophotometrically by either determining the initial rate of oxidation of NADH or the quantity of NADH oxidized at the end of the reaction (NADH is a function of sialic acid concentration) (Brunetti, et al., 1963 and Comb & Roseman, 1962).

Sweeley and co-workers (Sweeley & Walker, 1964 and Vance & Sweeley, 1967) and Rauvala and Karkkainen (1977) used gas-liquid chromatography (G-LC) in determining free sialic acid. In this procedure NANA and N-glycolylneuraminic acid (NGNA) could be measured simultaneously (Yu & Ledeen, 1970).

Radioactive methods have been developed for detecting sialidase activity at very low levels. A substrate,α_1-acid glycoprotein, was labelled at the C-8 of sialic acid with tritium after periodate oxidation (Schauer et al., 1976). After incubating the radioactive substrate with sialidase, the radioactive N- acetylanalogues liberated were dialysed and the activity of the low molecular weight

compounds counted. An ingenious method developed by Petitou et al., (1977) employed lymphoblastoid cells grown in ^{3}H-glucosamine (a precursor of sialic acid). This resulted in membrane bound ^{3}H-labelled sialic acid and was successfully used in the assay of membrane bound sialidase.

Determination of sialidase activity by measurement of the liberated aglycone was developed for the synthetic substrate methoxyphenyl neuraminic acid (2-(3-methoxyphenyl)-N-acetyl α -D-neuraminic acid) (Meindl & Tuppy, 1967) and for N-acetylneuraminosyl-lactose (NANL) (Holmquist, 1969). In the first procedure free phenol is determined by the folin-Ciocalteu (1927) reagent (Layne, 1957). Because this method cannot be used in the presence of high protein concentrations, an improved method was developed by Santer et al., (1978), using 4-aminoantipyrine [Fig. 2.2(iv)] in the presence of an oxidizing agent which forms a coloured quinone [Fig. 2.2(v)] with para unsubstituted phenols.

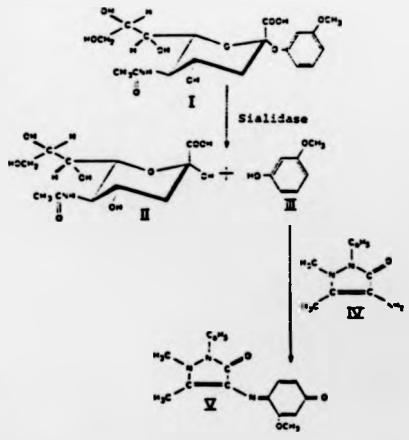


Fig. 2.2 Reactions involved in the assay of sialidase. 2-(3-Methoxyphenyl)-N-acetyl- α -D-neuraminic acid (I) is hydrolyzed by sialidase to yield NeuAc (II) and methoxyphenol (III). III and amino-antipyrine (IV) in buffer, pH 8.5, in the presence of an oxidizing agent, potassium ferricyanide, will yield a coloured quinone (V).

Another sialidase assay system based on measuring the aglycone moeity is that of Bhavanandan et al., (1975). In this method the N-acetylneuraminosyl (α,2-3)-lactit[^{3}H]ol, the tritiated borohydride reduction product of N-acetylneuraminyl- lactose, is digested with sialidase after which the tritiated neutral aglycone is separated from the rest of the hydrolysate by ion exchange chromatography.

A method patented by Thomas and Folger (1976) describes the assay of sialidase in which a fluorescent aglycone, namely methyllumbelliferone is liberated from the substrate 2-O-4-methylumbelliferyl-N-acetyl-α-D-neuraminic acid and its intensity determined (Fig. 2.3).

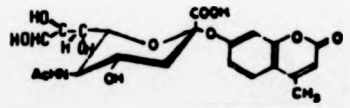


Fig. 2.3 Structure of the fluorogenic substrate, 2-O-4 methylumbelliferyl-N-acetyl- α -D-neuraminic acid, for sialidase

It is clear from the number of assay methods available that no one method is ideal to meet all situations. For example the thiobarbituric assay is subject to interference by a number of foreign substances in biological materials, such as 2-deoxyribose, sucrose and malonaldehyde (Warren, 1959; Aminoff, 1961; Horvat & Touster, 1968; Waravdekar & Saslaw, 1957 and Schneir, et al., 1970). Compounds that inhibit colour formation in Warren's procedure include L-fucose, acetaldehyde, DNA and nucleotides (Warren, 1959; Rosenberg & Einstein, 1972; Onodera et al., 1965 and Kuwahara, 1980). Further, free sialic acid exhibit different molar extinction coefficients (Aminoff, 1961 and Schauer & Faillard, 1968) in the thiobarbiturate procedure and ferrous ions at

concentrations of 30 μM to 10 m Molar decrease the colour yield in the Warren assay by 35 - 55% (Hartree & Brown, 1970).

The coupled enzymic system has its limitations in that the NANA and NGNA must not be O-acetylated and the system should be free of NADH oxidase activity.

As no one assay is suitable, being either tedious, insensitive, expensive or unreliable, we decided upon two assay systems; the Warren assay and a radioactive assay using α_1-acid glycoprotein as the substrate. The Warren assay proved suitable for moderate enzyme quantities in a fairly pure state. It was most unsuccessful when assaying for sialidase activity in the culture fluid or solutions containing chromophores that interfered with the absorbance reading at 549 nm. The assay using tritiated α_1-acid glycoprotein yielded unreproducible results though it could have been effective under conditions in which the Warren assay failed.

It was for these reasons we attempted an improvement of the radioactive assay and investigated the possibility of a radioimmunoassay (RIA) as it has long been known, though with scepticism (Bergman et al., 1962 and Barry, 1958), that sialic acid is an immunodeterminant sugar (Rule, 1965; Prat & Comoglio, 1976; Pogonowska- Goldhar et al., 1967; Apicela, 1976; Baker & Kasper, 1976; Egan et al., 1977 and Sela et al., 1975).

The techniques of RIA have expanded over the last two decades to include the detection of many biological substances. The reasons for such wide application of this technique are derived from its high sensitivity, specificity and precision compared to other analytical

methods. In a typical RIA, the antibodies (Ab) to the biological substance of interest (Antigen, Ag) are prepared and used to determine the concentration of the antigen, following observations in which the radiolabelled antigen (Ag*) molecules compete with the unlabelled antigen (Ag) molecules for a limited number of binding sites on the antibodies. Fig. 2.4 summarises the reactions involved in RIA (Berson & Yalow, 1968).

Fig. 2.4 Summary of the principles of a RIA

The mixture of Ab, Ag and Ag*, in which there is a fixed amount of Ab but a relative excess of Ag, is incubated to allow establishment of an equilibrium before the antigen-antibody complexes (Ag-Ab & Ag*-Ab) are separated from free unbound Ag* and Ab. The radioactivity of complexed or free Ag* or both are measured. When increasing amounts of unlabelled antigen are added to the assay, the limited binding sites of the antibody can bind less of the radiolabelled antigen (Ag*). A diminished binding of labelled antigen offers evidence for the presence of unlabelled antigen. The concentration of the unlabelled sample antigen can then be obtained by comparing the observed inhibition of labelled antigen binding with that obtained by standard solutions containing known amounts of unlabelled antigen.

Any compound can be measured by the RIA technique provided that (a) it can be made immunogenic, (b) it is available in a pure form, (c) it can be radioactively labelled and (d) there is a technique available to separate antibody-antigen complex from the free compound. Though both

labelled and unlabelled NANA are available commercially and sialic acid is an immunodeterminant sugar, (on its own it cannot raise antibodies), neither antibodies to sialic acid nor attempts to design a RIA for its determination have been reported.

2.1 Experimental Procedure

Definition of a sialidase enzyme unit:-

One unit, (u), of enzyme activity is defined as the amount that releases 1 μMol of NANA per minute under the reaction conditions used. The specific activity, (SA),is defined as units/mg.

2.1.0 Warren Assay

The enzyme (0,02-0,6 u) was incubated with 100μl of the substrate, fetuin or α_1 acid glycoprotein (19 mg/ml) in a total volume of 500μl in 0.05M sodium acetate buffer (pH 5.3 for S. griseus, pH 4.5 for C. perfringens and pH 5.1 for the V. cholerae sialidases). After 1 hr at 37°, the reaction mixture (200μl) was removed and dispensed into centrifuge tubes at 4° containing 100μl 10% phosphotungstic acid (in 12.5% H_2SO_4) (PTA) this was rapidly mixed and the precipitated protein removed by centrifugation (10,000 x g for 5 min .). The clear supernatant (S/N) was then used to determine the released sialic acid by the method of Warren (1959), except where otherwise stated.

2.1.1 Radioactive assay

2.1.1.0 Preparation of the radioactive substrate (Schauer et al., 1976)

Fetuin (10 mg) was dissolved in 4.0 ml of 0.1M sodium acetate buffer pH 5.6, containing 0.15M sodium chloride. To this solution cooled in ice, was added sodium metaperiodate (0.4 ml, 4.8μMol) and allowed to react in the dark. After exactly 8 min , (determined by trial experimentation), 200μl of 50% ethylene glycol was added. Ten minutes later the solution was dialysed against 0.15M sodium chloride in 0.05 sodium phosphate buffer pH 7.4 (2 x 5 lts) for 36 hr . To the dialysis residue was added 330μ l of sodium boro-[^{3}H]-hydride in 0.01M sodium hydroxide (661 m Ci/mMol). This was allowed to react for 30 mins at 21° 2 ml of unlabelled sodium borohydride (4 mg in 2.0 ml 0.01M NaOH) was added and the mixture was left to react for a further 30 min at the same temperature. The solution was then dialysed extensively against the following buffers 0,005M sodium phosphate, pH 7.4 (48 hr, 2 x 5 lts) and 0.001M sodium phosphate, pH 7.4 (120 hr , 5 x 5 lts). The non-diffusable material was lyophylized and thereafter resuspended in 1.0 ml, 0.001M sodium phosphate buffer, pH 7.4 and the specific activities of the tritiated fetuin and the tritiated sialic acid determined.

2.1.1.1 Determination of the specific activity of the tritiated sialic acid analogue by acid hydrolysis (100%)

Tritiated fetuin (50 μl) was hydrolysed in 450 μl of 0.01M H_2SO_4 for one hr at 80°. The protein was then precipitated with 10% phosphotungstic acid and centrifuged as previously described. The released sialic acid present in the S/N was determined by the Warren method and the radioactivity was determined by counting aliquots of the S/N using

Triton X-100 butyl PBD as the scintillant (Table 2.1).

2.1.2.2 Determination of the specific activity of the tritiated sialic acid analogue by enzymic hydrolysis

Tritiated fetuin (50 µl) was added to 0.005M sodium acetate buffer pH 5.1 (150 µl) containing V. cholerae sialidase (10 µl, 0.8 u). This was allowed to react for 1 hr at 37°. The reaction was stopped with 10% PTA as above and the free sialic acid and the radioactivity in the supernatant was determined as above (Table 2.1).

2.1.1.3 Trial application of the radioactive assay

V. cholerae sialidase (5 µl 0.002 u) was incubated at 37° with substrate, [^{3}H]fetuin, at varying concentrations (legend of Fig. 2.5) in 0.05M sodium acetate buffer, pH 5.1. 50 µl of reaction solution was removed at 15 min intervals and dispensed into centrifuge tubes at 4° containing 100 µl of 10% PTA. This was rapidly mixed, centrifuged and the radioactivity of the supernatant determined as previously described (Fig. 2.5). This assay procedure has been used where interfering chromophores hampered the Warren assay.

2.1.2 Radioimmunoassay (RIA)

2.1.2.0 Purification of colominic acid

The further purification of the commercially available colominic acid (see Fig. 2.11 for structure) was an adaptation of the methods of Barry

(1958), Kimura (1966a), Uchida (1973) and Alhadeef (1978). The colominic acid (1 gm) was dissolved in 8.0 ml bicine buffer, (8.0 ml) pH 8.0 containing 0.1M magnesium chloride, and to this was added ribonuclease A (0.8 ml, 1mg/0.1ml) and allowed to react for 12 hr at 21°. This was followed by the addition of deoxyribonuclease 1 (1mg/ml) and allowed to react as above. Pronase was then added after 12 hr and the digestion allowed to proceed for a further 16 hr at 37°. The solution was then made 90% with ammonium sulphate at pH 8.0 and left for 6 hr at 21°. This was then centrifuged for 2 hr at 15,000 rpm x g, the supernatant carefully removed and then dialysed, in spectrapor membrane tubing with a mol. wt cut off of 6,000-8,000, against 0.001M sodium phosphate buffer, pH 7.4 (6 x 5 lts), after which the non-diffusible material was lyophilized. The freeze dried material was made up to a concentration of 30% (w/v) in 0.02M sodium acetate buffer, pH 4.0, and then fractionated with ethanol at -10° (0-75% fractionation) and allowed to stand for 2 hr at -20°. The solution was centrifuged as above at -20° and the resulting precipitate extensively dialysed against 0.001M sodium phosphate buffer pH 7.4 and the dialysis residue lyophilized. Yield = 33%.

2.1.2.1 Sizing of colominic acid (generation of the hapten)

Purified colominic acid (250 mg) was dissolved in 16.0 ml of 0.05M NaOH and left overnight at 21° (Kimura 1966b). This was followed by dialysis as previously described and lyophilized.

The colominic acid was dissolved in 9.0 ml water to which was added 1.8 gm Dowex 50 - x 8[H^+] and allowed to hydrolyse for 16 hr at 50°. The hydrolysate was spun clear and freeze dried. The material was

resuspended in 300 µl 0.001M sodium phophate buffer, pH 7.4 and applied to a column (1.2 x 45 cm) containing Bio-Gel P-2 (fractionation range 100-1,800) pre-equilibrated with the same buffer. Fractions containing the colominic acid polymers of mol. wt 100-1,800 namely the Vi (Vi = internal volume within the gel particles) fractions were collected, these were freeze dried and applied to a Sephadex G-10 column (1.2 x 45 cm) and the fractions containing the polymer size of 700-1,800 the (Vo fractions, Vo = void volume surrounding the gel particles) were collected and freeze dried. This fraction, the hapten, now having a chain length of 3 - 4 NANA units long, was conjugated to bovine serum albumin (BSA).

The time of 16 hr for the hydrolysis was determined from a trial experiment in which samples were removed at hourly intervals for 24 hr and subjected to the above procedure. The Vo fractions from the Sephadex G-10 column was split and one half subjected to 0.01M H_2SO_4 hydrolysis at 80° and the NANA determined in both. It was found that at 16 hr 70% of the colominic acid had hydrolysed into chain lengths of 3 - 4 NANA units and that 16% was single NANA units, whereas at 24 hr 58% was single NANA units.

2.1.2.2 Conjugation of the hapten to BSA (formation of the BSA-NANA conjugate)

The methods of Erlanger and Beiser (1964) and Butler and Chen (1967) were used with slight modifications.

The hapten (15 mg) was dissolved in 0.9 ml, 0.05M sodium acetate buffer, pH 5.0, to which was added 0.9 ml (75 µMole) sodium meta periodate and allowed to react in the dark at 21° (and 200µl samples

were removed for the trial experiment at 0 - 9 hr). After 1.8 hr the reaction was stopped by the addition of 4 µl 1.0M ethylene glycol, 30 min later 0.273 ml BSA (28 mg/ml at pH 9-9.5 adjusted with 5% $K_2 CO_3$) was added and the pH adjusted to 9-9.5 and stirred for 1 hr. Sodium borohydride (273µl, 15 mg/ml) was added and set aside for 18 hr. The pH was readjusted to 8.5 with 1M NH_4OH and stirred for another hour. The BSA-hapten conjugate was precipitated with saturated ammonium sulphate in phosphate buffer, pH 8.0 (0-70% fractionation) and left overnight at 4°. This solution was centrifuged at 15,000 rpm x g for 30 min and the precipitate dialysed extensively against 0.001M sodium phosphate buffer pH 7.4 and freeze dried. The oxidation and conjugation times of 1.8 and 1.0 hr respectively giving the maximum attachment of hapten onto the BSA, 39%, was achieved by trial experimentation which was performed as above but with proportionately reduced samples (Fig. 2.6).

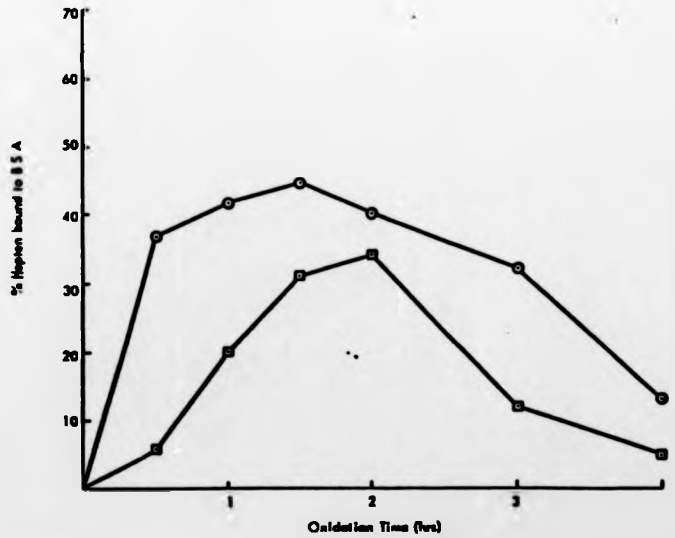


Fig. 2.6 The attachment of hapten to BSA at varying periodate oxidation times of the hapten and a fixed conjugation time of 1 hr.

o———o Indicates the amount of lysines reacted with hapten by the 2,4,6-trinitrobenzenesulphonic acid of Fields (1972). □———□ Indicates the amount of NANA associated with BSA by the method of Warren (1959) after acid hydrolysis and the determination of the free NANA.

were removed for the trial experiment at 0 - 9 hr). After 1.8 hr the reaction was stopped by the addition of 4 µl 1.0M ethylene glycol, 30 min later 0.273 ml BSA (28 mg/ml at pH 9-9.5 adjusted with 5% $K_2 CO_3$) was added and the pH adjusted to 9-9.5 and stirred for 1 hr. Sodium borohydride (273µl, 15 mg/ml) was added and set aside for 18 hr. The pH was readjusted to 8.5 with 1M NH_4OH and stirred for another hour. The BSA-hapten conjugate was precipitated with saturated ammonium sulphate in phosphate buffer, pH 8.0 (0-70% fractionation) and left overnight at 4°. This solution was centrifuged at 15,000 rpm x g for 30 min and the precipitate dialysed extensively against 0.001M sodium phosphate buffer pH 7.4 and freeze dried. The oxidation and conjugation times of 1.8 and 1.0 hr respectively giving the maximum attachment of hapten onto the BSA, 39%, was achieved by trial experimentation which was performed as above but with proportionately reduced samples (Fig. 2.6).

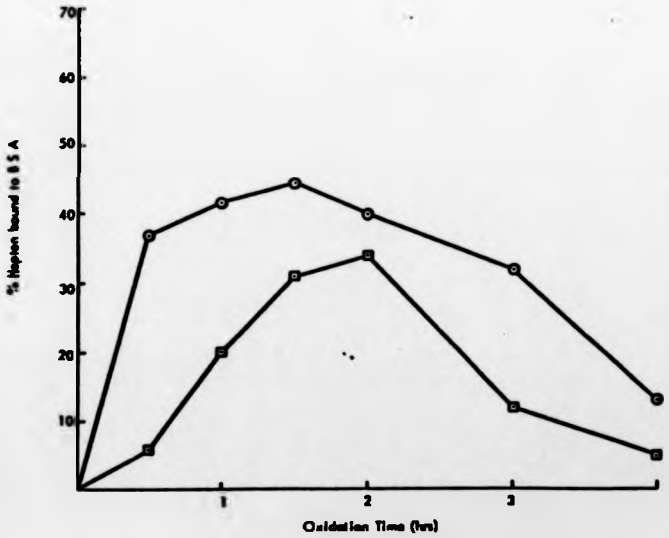


Fig. 2.6 The attachment of hapten to BSA at varying periodate oxidation times of the hapten and a fixed conjugation time of 1 hr.

⊙——⊙ Indicates the amount of lysines reacted with hapten by the 2,4,6-trinitrobenzenesulphonic acid of Fields (1972). □——□ Indicates the amount of NANA associated with BSA by the method of Warren (1959) after acid hydrolysis and the determination of the free NANA.

2.1.2.3 Immunization

A sheep was injected over a three month period with a total of 2.0 mg BSA-(NANA) conjugate, suspended in phosphate buffer and emulsified in Freunds Complete Adjuvant to a total concentration of 1 mg/ml. Fifteen days after the primary intramuscular injection, the sheep was boosted with 200 µl of antigen in complete Freund Adjuvant at multiple sites. Seven days thereafter a trial bleed was obtained. Injections were repeated every two weeks and bleedings obtained at intervals of seven days. A control bleed was obtained before the primary injection. Blood collected from the challenged sheep was allowed to clot overnight, the serum decanted and centrifuged (15,000 rpm x g) for 30 min at 5°. The antiserum obtained was subjected to various immunological tests.

2.1.2.4 Immunodiffusion

The double diffusion technique of Ouchterlony (1953) and Milford-Ward (1977) was carried out on glass slides, 8 x 8 cm, coated with 9.6 ml 1% agar (electrophoresis grade) in phosphate buffer pH 7.1. The wells were loaded as described in the legend of Fig. 2.7 and allowed to develop at 21° in a moist chamber for 36 hr . After this the gels were washed, dried, stained and destained as described (Ouchterlony & Nilsson, 1979). The precipitation lines produced by these secondary reactions appeared only between 70 and 90 days after the first immunization.

2.1.2.5 Equilibrium dialysis

Prior to equilibrium dialysis, the antibody (Ab) was purified by the

methods of Heide and Schwick, (1979) and Allen et al., (1977), and its purity judged by PAGE. A small amount of IgA was found in the major IgG active fraction.

The equilibrium dialysis was carried out in an apparatus similar to that described by Neuhoff and Kiehl (1969) (Fig. 2.8).

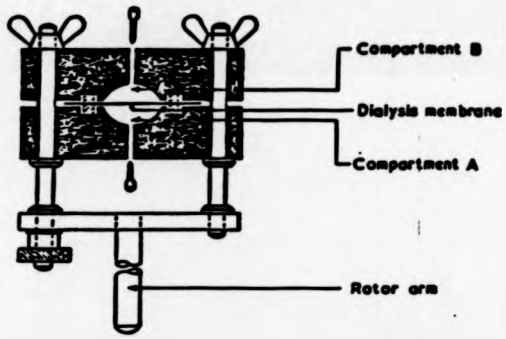


Fig. 2.8 Section through the main body of the equilibrium dialysis apparatus.

Compartments A & B each with a capacity of 450 µl were separated by a dialysis membrane. The exact constituents of these compartments are described below, but the compartment A will always contain the hapten (labelled or unlabelled) or the enzyme while compartment B will always contain the antibody except where equilibration time controls are run. Controls, as each point in the following experiments were set up in triplicate, with 10 µl of labelled ^{14}C NANA (245 m Ci/mM diluted 1/100) but without Ab's in compartment B. The radioactivity of each compartment was determined by counting the contents of both compartments using Triton X-100 butyl PBD as scintillant.

2.2.5.0 The determination of the binding capacity of the antibody by equilibrium dialysis

Each compartment A (1-16) contained 10 µl of labelled ^{14}C NANA plus 445 µl of PBS. Compartments B contained 0-300 µl of antibody (1 mg/ml) in a final volume of 450 µls PBS. This was allowed to equilibrate while rotating at 4 for 44 hr (total equilibration time) after which the radioactivity of each compartment was determined as previously described (Fig. 2.9).

2.1.2.5.1 The competition assay for sialic acid with the aid of equilibrium dialysis (development of a diagnostic plot for the sialidase activity)

Compartments A (1-16) contained 10 µl labelled ^{14}C NANA and varying concentrations of unlabelled NANA (0-50nMoles) while compartment B contained 250 µl of the Ab solution. The differences in volume were made up to 450 µl with PBS. This was allowed to equilibrate and the activity determined as above (Fig. 2.10).

A similar assay was set up with the following sugars, N-acetylgalactosamine, N-acetylglucosamine, D- mannose and D-glucose in place of the unlabelled NANA.

2.1.2.5.2 The radioimmunoassay for sialidase

Sialidase of unknown activity was incubated at 37° in the appropriate assay buffer with the substrate fetuin (ca 400nM). At set intervals (0-24 hrs) 10 µl was dispensed into compartment A containing 10 µl of

^{14}C NANA and 430 µl of PBS while compartment B contained 250 µl Ab and 200 µl PBS. This was allowed to equilibrate and the radioactivity determined as above. The decrease in the amount of radioactivity bound to the Ab is equivalent to the amount of unlabelled NANA released from fetuin by sialidase, and this amount is determined from the diagnostic plot as previously set up (Fig. 2.10) which is then converted to enzyme units.

2.1.3 The Determination Of Bound Sialic Acid (with the use of equilibrium dialysis and sialidase)

In this procedure (experimental detail see legend of Fig. 2.11), the sialic acid containing macromolecules are allowed to incubate at 21° for 2 hr with the sialidase (specific activity 47u,50 µl) in compartment A and then allowed to equilibrate for 30 hr with Ab, before 5 µl of ^{14}C NANA is added and allowed to equilibrate for a further 44 hr and then the radioactivity determined as previously described (Fig. 2.11).

2.2 Results And Discussion

The Radioactive Assay For Sialidase

By controlling the periodate oxidation time during the synthesis of the tritiated fetuin substrate for the radioactive assay, we were able to achieve maximum labelling (104%) at C-8 of the NANA unit in fetuin instead of the C-7 NANA analogue which is not hydrolysed by sialidase (Suttajit, 1970). The 104% labelling at C-8 indicating no labelling at C-7 is questionable as the periodate oxidation is probably not sequential (Table 2.1).

Table 2.1

Determination Of Specific Activities Of Hydrolysis Product (NANA) Via Acid And Enzymic Released From The Substrate(s)

Specific Activities Of Substrate(s) And Product (NANA)	
Substrate, Product	Specific Activity (Ci/mMol)
^{3}H Fetuin(s)	332
^{3}H NANA (Acid hydrolysis)	24
^{3}H NANA (Enzymic hydrolysis)	25

However, this substrate proved reliable in that a proportional linear response in the release of product over a period of time (0-60 min) at various substrated concentrations and fixed enzyme concentration was achieved (Fig. 2.5).

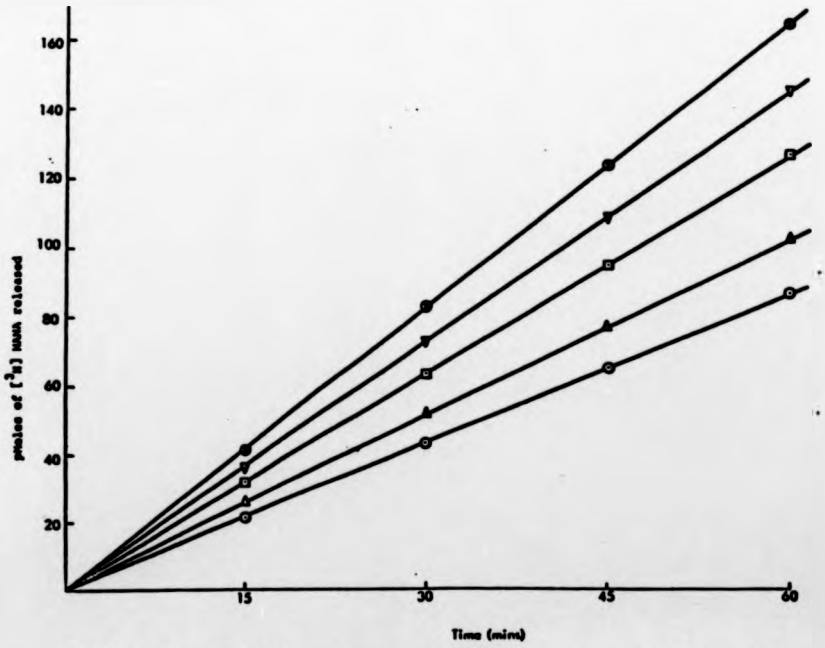


Fig. 2.5 The release of tritiated N-acetylneuraminic acid from [1] differing concentrations of [^{3}H]fetuin by V. cholerae sialidase

1 The varying concentrations of tritiated fetuin used: O—O, 90 p moles; Δ—Δ, 180 p moles; ▽—▽, 270 p moles; □—□, 360 p moles; ◇—◇, 450 p moles. The experimental detail is discussed in the text

Further, we could detect enzyme activities as low as 2×10^{-6} units and reproduce this result and results within the range of 2×10^{-6} to 16×10^{-5} in a linear fashion. This assay method, rapid and inexpensive, is very much more sensitive than the Warren assay and is not hampered by chromophoric substances, commonly associated with the impure enzyme solution, which interferes with the spectrophotometric method. It also does away with the unnecesary, time-consuming chromatographic and dialysis procedures normally accompanying radioactive assays (Bhavanandan et al., 1975 and Schauer, et al., 1976). Thus this assay fits the criteria of a good enzyme assay in that it can detect enzyme activity at low concentrations and accurately reproduce these results, a hallmark not attained by many of the radioactive assays for sialidase.

The Radioimmunoassay For Sialidase.

The authenticity and specificity of the BSA-NANA antiserum was tested by primary (equilibrium dialysis[1]) and secondary (immunodiffusion) reactions.

Immunodiffusion carried out with the antiserum cross reacted with sialic acid containing macromolecules such as α_1-acid glycoprotein, fetuin and mucin but once these sialic acids were enzymically removed from these macromolecules, this phenomenon disappeared, indicating the existence of antibodies specific for sialic acid (Fig. 2.7).

[1] Equilibrium dialysis was the system of choice besides being sensitive and the most common system at present for ligand binding studies. It separated the Ab from the enzyme substrate, which would cross-react with the Ab, and the enzyme solution which is frequently contaminated with proteases.

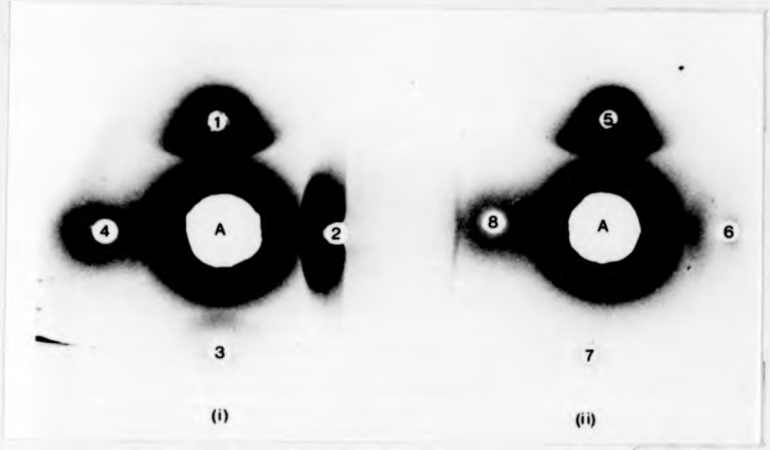


Fig. 2.7 (I) Immunodiffusion of sialic acid containing macromolecules and BSA

(II) Immunodiffusion of desialylated macromolecules (from above) and BSA.

Well A contained the BSA-NANA anti serum (30 µl)
Wells 1 & 5 contained BSA (10 µl of a 5mg/ml solution)
Well 2 contained fetuin (10 µl of 20mg/ml solution)
Well 3 contained α_1-acid glycoprotein (10 µl of 20mg/ml solution)
Well 4 contained fetuin (10 µl of 20mg/ml)+ NANA 50 µg)
Well 6 contained desialylated fetuin (20mg/ml)
Well 7 contained desialylated α_1 acid glycoprotein (20mg/ml)
Well 8 contained desialylated fetuin (10 µl of 20mg/ml) + NANA 50 µg

Desialylation was carried out by incubating each sialic acid-containing macromolecule (100mg) with V. cholerae sialidase (10 U) in assay buffer for 24 hr at 4°. This was then extensively dialysed, lyophilized and resuspended in PBS (20mg/ml)

Definitive evidence for the presence of NANA specific antibodies was obtained by equilibrium dialysis.

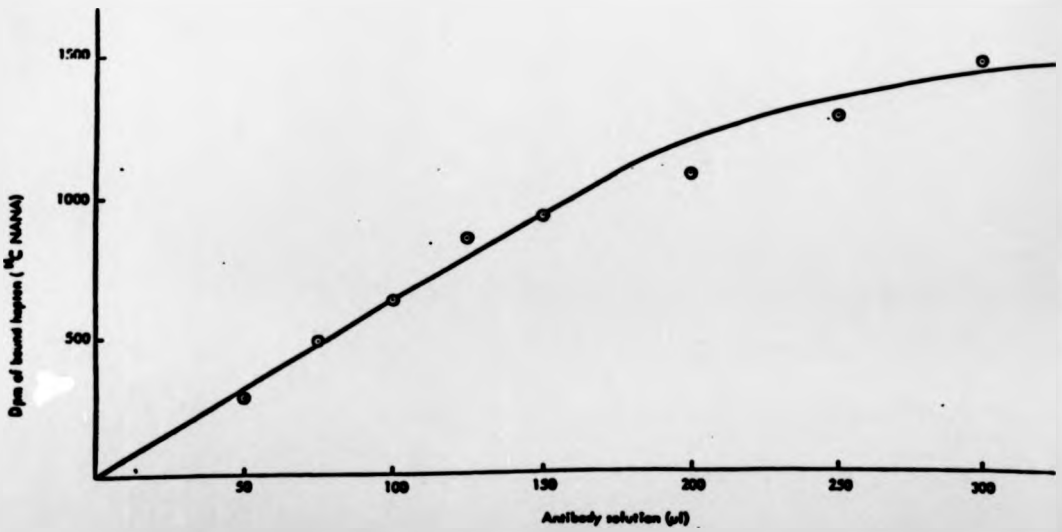
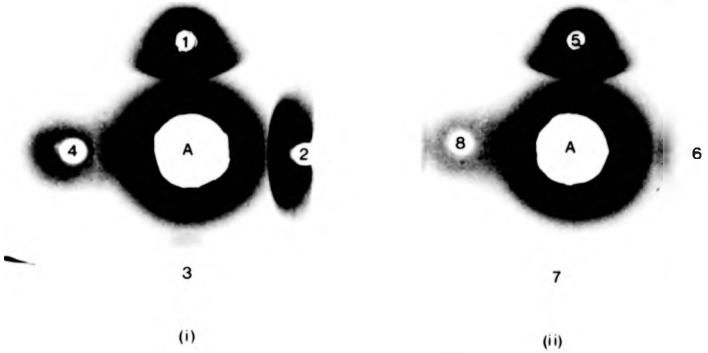


Fig. 2.9 The binding of the hapten, ¹⁴C NANA by varying concentrations of antibody solution via equilibrium dialysis. Experimental detail in text



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Well 2 contained fetuin (10 µl of 20mg/ml solution)
Well 3 contained α_1-acid glycoprotein (10 µl of 20mg/ml solution)
Well 4 contained fetuin (10 µl of 20mg/ml)+ NANA 50 µg)
Well 6 contained desialylated fetuin (20mg/ml)
Well 7 contained desialylated α_1 acid glycoprotein (20mg/ml)
Well 8 contained desialylated fetuin (10 µl of 20mg/ml) + NANA 50 µg

Desialylation was carried out by incubating each sialic acid-containing macromolecule (100mg) with V. cholerae sialidase (10 U) in assay buffer for 24 hr at 4°. This was then extensively dialysed, lyophilized and resuspended in PBS (20mg/ml)

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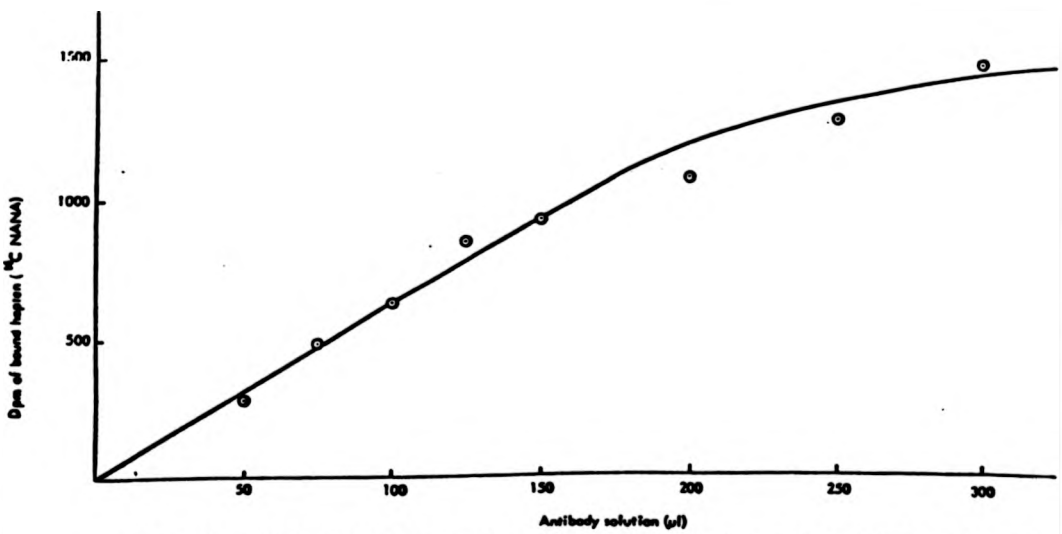


Fig. 2.9 The binding of the hapten, ^{14}C NANA by varying concentrations of antibody solution via equilibrium dialysis. Experimental detail in text

Fig. 2.9 shows that with increasing antibody concentration, increasing radiolabelled ^{14}C-NANA is bound. This strong binding capacity was not present in the preimmunization serum; it was a property of the immunoglobulin fraction of the antiserum. This specificity was further confirmed by observing a decrease in the amount of labelled ^{14}C-NANA bound to the antibody with increasing concentrations of unlabelled NANA (Fig. 2.10).

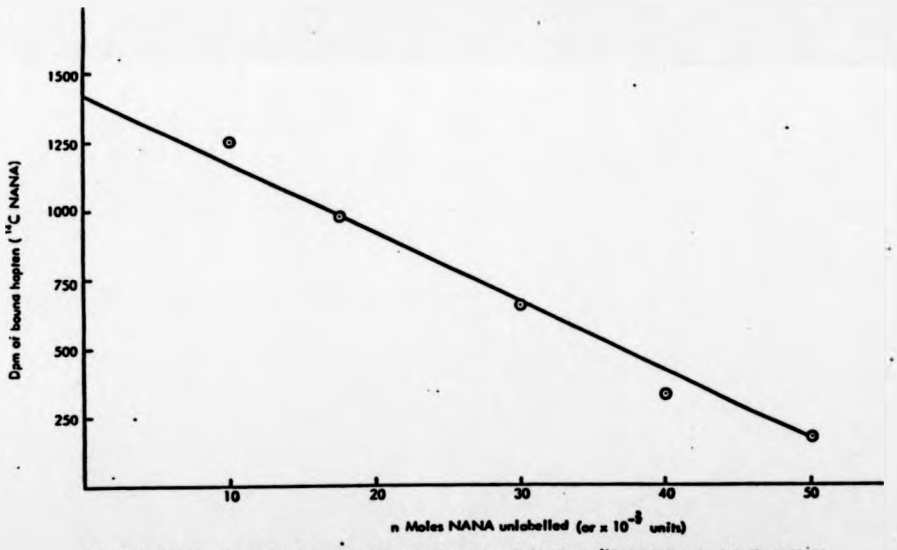


Fig. 2.10 Diagnostic plot for the competition assay between radiolabelled ^{14}C-NANA and unlabelled NANA

From a 5.0 mM solution of unlabelled NANA aliquots (0 - 10 µl) were dispensed into Compartment A of the equilibrium dialysis apparatus which also contained 10 µl of ^{14}C NANA (1/100 dilution) and the correct volume of PBS to give a total volume of 450 µl. Compartment B contained 250 µl of Ab solution and 200 µl PBS. This system was allowed to equilibrate for 44 hr while rotating at 4° after which the radioactivity of the solutions in each compartment was determined as described in the text.

The inhibition of binding by unlabelled NANA has formed the basis of the radioimmunoassay for sialidase, the enzyme releasing the sialic acid which competes with the C-NANA for the limiting binding sites on the BSA-NANA antibodies. With this assay unlabelled NANA in the pico molar range could be detected and with the aid of the diagnostic plot, it was possible to determine enzyme activities at 1 x 10^{-2} units (Fig.

2.10).

No inhibition of labelled hapten binding was observed in the presence of the sugars tested. This inhibition was the major disadvantage of our previous attempt (Kabayo, 1978) and also that of Smith and Ginsburg (1980). The reason for the success of eliminating this inhibition, we think, lies in the fact that the NANA units were linked directly to the BSA molecule, thereby excluding the intermediate polysaccharide chain which normally binds the NANA molecule to the protein backbone as seen in the structures of fetuin /Fig. 2.11 (v)/ and the sialyllactose /Fig. 2.11 (i)/ used by Kabayo (1978) and Smith and Ginsburg (1980), or the antibodies could be heterologous in nature. There is, however, a disadvantage associated with our method, which is its low titre. Although it is known that sialic acid generates a poor immune response (Simons & Rios, 1971; Simons et al., 1971 and Winzler, 1970), we suspect that by binding the NANA unit through the polyhydroxy side chain, we possibly reduced the response even further as the polyhydroxy side chain could be one of the antigenic determinant sites on the NANA molecule (Lisowka & Roelcke, 1973).

One bonus to develop from the basis of this assay is its capability to determine the mole to mole ratio of the number of NANA units per sialic acid containing macromolecule as was done for fetuin and α_1-acid glycoprotein (Fig. 2.12).

NeuNAc (α,2→3 (6)) Gal (β, 1→4) Glc

(i) Sialyl-lactose

NeuNAc (α, 2→3)

Gal (β, 1→3) GalNAc (β, 1→4) Gal (β, 1→4) Glc (β, 1→4)-Ceramide

(ii) Ganglioside GM_1 (Kuhn & Wiegant 1963 b)

NeuNAc (α, 2→6) GalNAc-peptide

(iii) Submaxillary mucin (Roelcke et al., 1978)

NeuNAc (α,2→*) Gal (β, 1→4) GlcNAc (β, 1→2) Man (α, 1→3 (6))

NeuNAc (α,2→*) Gal (β, 1→4) GlcNAc (β, 1→2) Man (β,1→4) GlcNAc (β,1→4) GlcNAc (β,1→4) Asn

NeuNAc (α,2→*) Gal (β, 1→4) GlcNAc (β, 1→4)

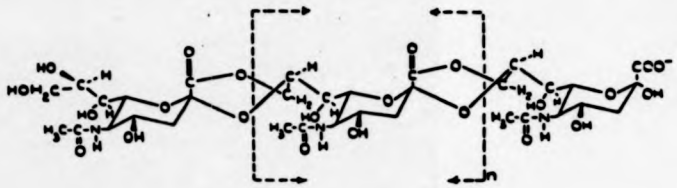
(iv) Structure of Heterosaccharide unit of -acid glycoprotein (Schmid, 1975)

(* Not resolved as yet, see Table 2.0)

NeuNAc (α,2→3) Gal (β,1→4) GlcNAc | Man
NeuNAc (α,2→3) Gal (β,1→4) GlcNAc | Man
NeuNAc (α,2→3) Gal (β,1→4) GlcNAc | Man
NeuNAc (α,2→3) Gal (β,1→4) GlcNAc | GlcNAc

GlcNAc - peptide

(v) Proposed structure of Heterosaccharide unit of fetuin (Spiro, 1964 and Sela et al., 1975)



(vi) Colominic Acid

Fig. 2.11 Structures of naturally occurring sialo-compounds

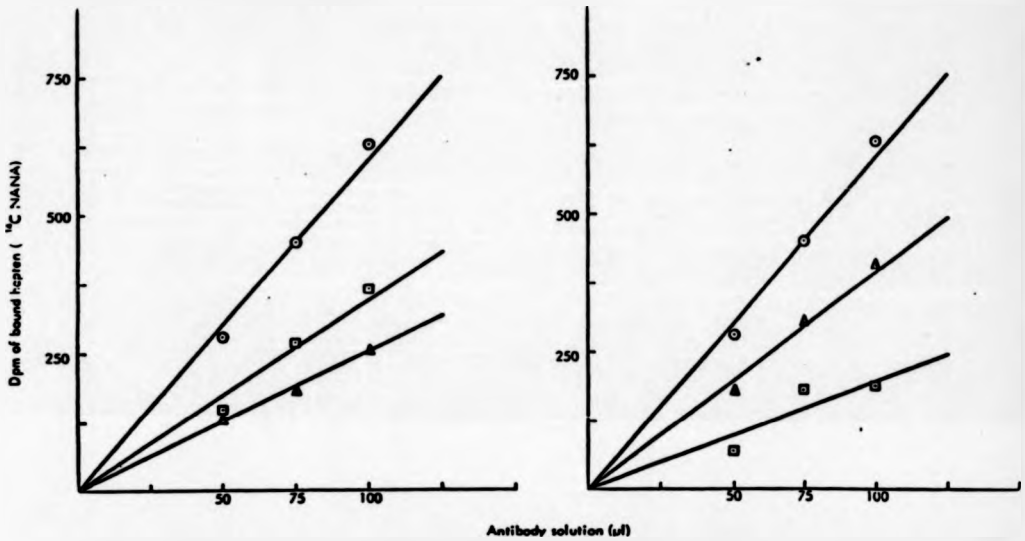


Fig. 2.12 Radioimmunoassay (Diagnostic plot) for the determination of the number of moles of NANA per mole of sialyl compound via equilibrium dialysis.

Of a 0.7 µM solution of extensively dialysed fetuin or α_1-acid glycoprotein in PBS, 0, 5, 7.5 and 10 µls were dispensed into Compartment A of the equilibrium dialysis unit to which was added 10 µl (0.6 Units) of V. cholerae sialidase. Compartment B contained either 50, 75 or 100 µl antiserum in PBS. The final volume in each compartment was 450 µl. After 30 hr, 5 µl of 1/100 ^{14}C NANA was added to Compartment A and the system allowed to equilibrate for 44 hr and the radioactivity determined in each compartment. Control compartments contained PBS and ^{14}C NANA only.

O———O in absence of unlabelled NANA

o———o in presence of labelled ^{14}C NANA and unlabelled NANA. Δ———Δ is the adduct of the bound ^{14}C NANA (dpm, O——O) in absence of unlabelled NANA - (minus) the amount of bound ^{14}C NANA in presence of cold NANA (o———o)

With greater care, increased controls and sialidase of high specific activity the mole to mole ratio of NANA bound by fetuin was determined to within 5-10% of the reported value (Bucher & Palese, 1975) while that of α_1-acid glycoprotein was with 15-25% (Bucher & Palese, 1975). Therefore with this method the mole to mole ratio can also be determined for biologically important molecules, such as interferon which, if pure, is available in only very small amounts (Morser et al., 1978).

In view of the increasing number of diseases associated with sialidases (Chapter 1), an assay as effective as the radioimmunoassay system is long overdue. Our RIA, though effective down to 10^{-2} units, requires an improvement in the specific activity of the radiolabelled ^{14}C NANA and

the antibody titre as well as a reduction in the assay time. However, despite these difficulties, the accuracy (delivered by equilibrium dialysis) in determining low levels of sialidase activity in plasma and biological fluids in close contact with diseased and/or infected organs and in which sialidase plays an primary role, is still possible with this radioimmunoassay.

CHAPTER 3

PURIFICATION OF SIALIDASES

3.0 Introduction

The purification of bacterial sialidases has followed the time honoured, standard lines of approach for protein purification; thus, with the exception of affinity chromatography, very little change has occurred in the basic methodology in recent years, as can be gathered from table 3.0.

Because the majority of bacterial sialidases are extracellular enzymes, inducible and stable for long periods at the purification temperatures [4° - 8°], (Ada & French, 1959 and Ada et al., 1961), it would appear to be the easiest to purify and should, theoretically, yield a pure, stable enzyme as opposed to the unstable and often impure preparations of viral or mammalian enzymes which are normally membrane associated.

After removing the cells, the basic procedure in purifying soluble proteins such as the bacterial sailidases normally follows the pattern described below in which each step exploits a certain protein characteristic.

The first stage in purifying these enzymes involves concentrating the large volumes of culture filtrate. The most common method involves salt fractionation (ammonium sulphate), which has been used by Rosenberg et al., (1960), Cassidy et al., (1965, 1966), Balke and Drzeniek (1969) and Schramm and Moore (1959) for the concentration of the culture

Table 3.0

Major Stages In The Purification Of Bacterial Sialidases

Source of Sialidase	Sequential Stages Of Purification	References
A. sialophilum	Ammonium sulphate fractionation, DEAE-cellulose ion-exchange chromatography, ultrafiltration, Sephadex G-200 gel exclusion chromatography	Flashner, et al., 1977
A. ureafaciens	Ultrafiltration, ammonium sulphate fractionation, affinity chromatography, ammonium sulphate fractionation	Uchida et al., 1977
C. diptheriae	Ammonium sulphate fractionation, Sephadex G-200 partition chromatography	Jamieson, 1966
C. diptheriae	Ammonium sulphate fractionation only	Jagielski, 1969
C. diptheriae	Zinc chloride fractionation, biogel P-60 gel exclusion chromatography	Vertiev & Ezepchuk, 1972
C. perfringens	Ammonium sulphate fractionation, Sephadex G-25 partition chromatography	Cassidy et al., 1965, 1966
C. perfringens	Affinity chromatography	Cuatrecasas & Illiano, 1971
D. pneumoniae	Sepharose 68 gel permeation chromatography, affinity chromatography	Brossmer et al., 1978
K. streptococcus	Ammonium sulphate fractionation, DEAE-cellulose ion-exchange chromatography	Hyono & Tanaka, 1967
Lactobaccillus bifidius	Ammonium sulphate fractionation, Sephadex G-75 gel permeation chromatography	von Nicholai & Zilliken, 1972
pneumococcus	Ammonium sulphate fractionation, heat denaturation, DEAE-cellulose, CM-cellulose and CM-Sephadex ion-exchange chromatography	Tanenbaum et al., 1970
pneumococcus	Ammonium sulphate fractionation, DEAE-Sephadex ion-exchange chromatography, Sephadex G-100 gel exclusion chromatography	Stahl & O'Toole, 1972
S. griseus	Ammonium sulphate fractionation, DEAE-cellulose, CM-cellulose ion-exchange chromatography, Sephadex G-100 gel exclusion chromatography	Kunimoto et al., 1974
S. albus	Ammonium sulphate fractionation, DEAE-cellulose ion-exchange chromatography, Sephadex G-75 gel permeation chromatography	Myhill & Cook, 1972
V. cholerae	Erythrocyte adsorption, Kieselguhr	Burnet & Stone, 1947
V. cholerae	Methanol fractionation, erythrocyte adsorption, ammonium sulphate fractionation, hydroxyl apatite adsorption	Ada & French, 1957, 1959
V. cholerae	Ammonium sulphate fractionation, DEAE-cellulose ion-exchange chromatography, ammonium sulphate fractionation	Rosenberg et al., 1960
V. cholerae	Affinity chromatography	Cuatrecasas & Illiano 1971
V. cholerae	Affinity chromatography	Brossmer et al., 1977

filtrate containing the sialidase. Ultrafiltration, a fairly recent technique in large scale preparation of proteins, has now become the method of choice over salt fractionation as a concentration procedure. Uchida et al. (1977) have been the only group so far to use ultrafiltration as a concentration step for culture filtrate containing sialidase.

The second stage in the purification of soluble proteins normally involves ion exchange chromatography. The principle feature /underlying this form of chromatography is represented in fig. 3.0 and 3.1.

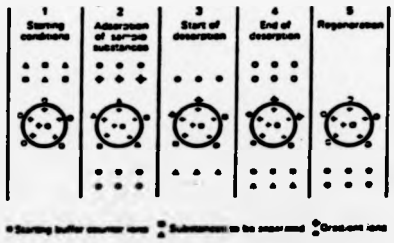


Fig. 3.0 Principle of ion-exchange chromatography

Stage 1 shows the ion exchanger in equilibrium with its counter ions. Sample substances are about to enter the ion exchanger bed. At stage 2 the counter ions have been exchanged for sample substances. After this adsorption a gradient is applied. Desorption of one sample species occurs at stage 3. This substance is exchanged for gradient ions and is therefore eluted from the ion exchanger. At stage 4 the remaining sample substance is exchanged for gradient ions and eluted, after which regeneration may be started. The gradient ions are exchanged for counter ions in stage 5 and the ion exchanger is thus regenerated and ready for re-use.

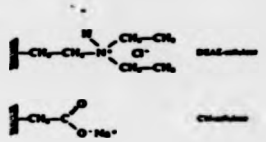


Fig. 3.1 The structures of the two common cellulose ion-exchange resins.

In the purification of sialidases, ion exchange chromatography has been a popular choice by the researchers listed in table 3.0: it has been

best exploited by Kunimoto *et al.* (1975) (Fig. 3.2) in the purification of *S. griseus* sialidase because this enzyme has a very high pI (7.9–8.0), which is far above that of most proteins.

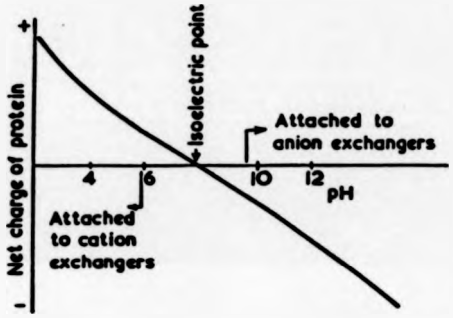


Fig 3.2 The net charge of *S. griseus* sialidase as a function of pH. The pH ranges in which the enzyme is bound to anion and cation exchangers (Kunimoto *et al.*, 1975).

The latter stage is often followed by gel permeation chromatography. The principle is founded on the separation of molecules on the basis of their molecular size and shape and it utilizes the molecular sieve properties of a variety of porous materials. The popular choice of gel for sialidase purification is Sephadex G-100, which has a fractionation range (molecular weight) of 4,000 to 150,000, within the molecular weight range of the bacterial sialidases. This procedure was used as a final purification step by Vertiev and Ezepchuk (1972), von Nicolai and Ziliken (1972) and Kunimoto *et al.* (1975).

Some authors chose affinity chromatography (Fig. 3.3) as a final step in the purification of the enzyme. The purification of a macromolecule by affinity chromatography exploits its most unique property, namely biological specificity. In the case of an enzyme, its three-dimensional structure is such that the active site and regulatory

site(s) are accessible to only a small number of compounds (ligands) which may be either substrates or effectors of a reversible or irreversible type. It is this specific recognition of these sites which forms the basis for affinity chromatography of enzymes. Purification is possible provided that the affinity of the site for the ligand is high, that the binding is reversible, and that at the experimental conditions chosen no reaction occurs after the ligand is attached to the site.

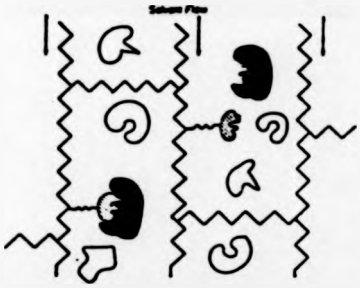


Fig. 3.3 Diagrammatic illustration of the principle of affinity chromatography

Three strands of the crosslinked polymer matrix are shown. The spacer arm is shown in bold, attaching the ligand (shaded) to the polymer matrix. Various roughly globular protein molecules are shown diffusing between the strands of the polymer but only the enzyme molecules (shaded) have a binding site for the ligand. One enzyme molecule is shown actually binding to the immobilized ligand. Note that the attachment of ligand to the spacer arm is made at a point where there is no interaction with the enzyme.

Basically, the technique consists of covalently attaching the ligand, (which is normally a reversible competitive inhibitor or substrate), to a suitable insoluble matrix in such a way that its potential for combination with the enzyme is not impaired (Fig. 3.4).

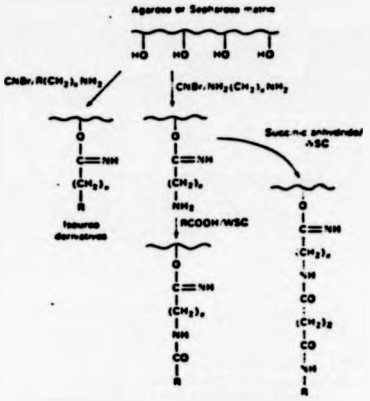


Fig. 3.4 Some possible synthetic routes for the attachment of the ligand to agarose or Sepharose. R is the ligand to which the macromolecule will bind and WSC is a water-soluble carbodiimide. For simplicity, reaction at only one of the hydroxyl groups is indicated.

A solution of the impure enzyme is then applied to a column of the liganded-matrix immersed in the correct buffer medium and the enzyme is selectively retained. Impurities which are not bound are eluted and the enzyme is subsequently displaced by elution with a solution of the substrate, or any compound which has a higher affinity for the enzyme than the ligand, in a medium of different pH and/or ionic strength.

Affinity chromatography applied to the purification of bacterial sialidases has seen the use of substrates, such as α_1-acid glycoprotein (Geisow, 1975), colominic acid (Uchida *et al.* 1977) and fetuin (Kabayo & Hutchinson, 1977), the product NANA (Holmquist & Ostman, 1975) and inhibitors of no structural resemblance to the substrate or product, such as *N*-(p-aminophenyl) oxamic acid (Cuatrecasas & Illiano, 1971) and *N*-(4-nitrophenyl) oxamic acid (Brossmer *et al*, 1977), as ligands attached to a solid support. Spacer arms were used when NANA and the inhibitors were used as ligands. Most of these

affinity columns have displayed specific adsorption except that of Cuatrecasas and Illiano (1971), in which contaminating proteins also adhered.

Many unsettling reports have appeared in the literature questioning the purity of the bacterial sialidases as prepared by the aforegoing procedures. For example, immunologically distinct sialidase and the toxin of Corynebacterium diptheria have been found by Moriyama and Barksdale (1967) to move together on CM-cellulose and, presumably, to have similar molecular weights. Commercial preparations of sialidase, made according to methods similar to those described above, have been further purified by ion-exchange chromatography (Hatton & Regoeczi, 1973) to remove proteases, and an earlier study has described (Kraemer, 1968) the presence of cytotoxic, haemolytic and phospholipase (White & Mellanby, 1969) activities still remaining in commercial preparations of sialidase from C. perfringens prepared ostensibly by a method similar to that described by Cassidy et al., (1965). Rood and Wilkinson (1974), Huang and Aminoff (1974) and Den et al., (1975) pointed out that the enzyme preparation purified from the culture filtrate of C. perfringens by means of Cuatrecasas's affinity column still contained a number of other enzymes and toxins.

As our research centred on the comparison of the active site of pathogenic and non-pathogenic sialidases, it was essential that the enzymes, to be compared, were completely free of contaminating proteins or polysaccharides.

Our initial method of purification was that of Kunimoto et al., (1975). We found that this procedure yielded small amounts of impure enzyme and was therefore still unsatisfactory. By including an affinity

step using either the α_1-acid glycoprotein or fetuin-bound matrix, the resultant enzyme solution displayed new low molecular weight protein bands on SDS- polyacrylamide gel electrophoresis (PAGE). We suspected that low levels of contaminating proteases in the still impure sialidase solution were digesting the protein ligand. When we used the N-(4-nitrophenyl) oxamic acid affinity column of Brossmer et al., (1977) we achieved an improvement in the purity of the enzyme but still more than one protein band was present in the analytical polyacrylamide gels.

Because of the above reasons, a new method of purification was investigated to improve not only the purity but the yield and rate of purification. Conditions selected were of such a nature that it could be used for all three enzymes, namely S. griseus, C. perfringens and V. cholerae sialidases.

3.1 Experimental Procedure

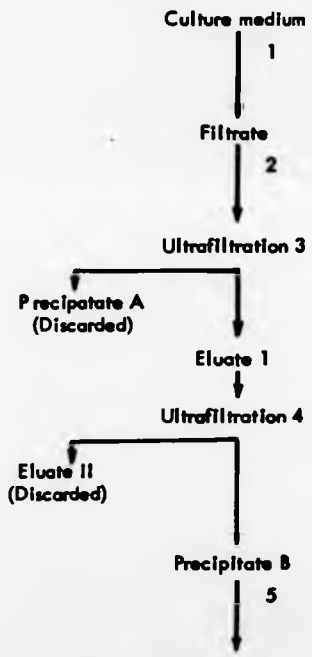
Calcium phosphate gel was prepared by the method of Keilin and Hartree (1938). The Amicon Matrex Blue A and Red A were activated by washing with 4 bed volumes of 8M urea, 0.5M sodium hydroxide followed by 40 bed volumes of loading buffer, namely 0.05M sodium acetate buffer pH 5.0. The preparation of the N-(4- nitrophenyl) oxamic acid coupled via 1,6 diaminohexane to Sepharose 4B (NNPOA-AHS4B) was prepared by the methods of Aschan (1885), Marcus and Balbinder (1972), Cuatrecasas (1970) and Brossmer et al., (1977). Fetuin was purified from foetal calf serum via the method of Spiro (1960).

3.1.0 Isolation Of Sialidase From The Cell Culture (S. griseus)

S. griseus MB395-A5 (obtained from The American Culture Collection) was agitated under aerobic conditions as previously described by Augustus (1977) and Kabayo (1978). The culture fluid containing the sialidase was separated from the cells after 72 hr , by filtration under vacuum through a buffer-washed Celite 545 bed on an 18.5 cm Greens Hyduro filter paper. After filtration, the bed was washed with 0.01M sodium phosphate buffer pH 7.0 and 0.025M potassium chloride. The cell free culture filtrate was adjusted to pH 7.0 with 1.0M sodium phosphate buffer and EDTA was added to a concentration of 0.05 Molar.

3.1.1 Ultrafiltration (UF)

Selective ultrafiltration was carried out in a ChemLab C2L (which had been modified to a 10 litre capacity), stirred ultrafiltration cell fitted with an Amicon XM50 (50,000 mol. wt cut off) ultrafiltration membrane. Ten litres were introduced into the cell and the system pressurized with air (50 p.s.i.). After concentrating the first 10 litres, the remaining 7.0 litres were concentrated and finally washed with 1.0 litre 0.001M sodium phosphate buffer pH 7.0. The non-diffusible material, precipitate A, was assayed and discarded while the filtrate 1 was reintroduced into the ultrafiltration cell, this time fitted with a G10T (10,000 mol. wt cut off membrane). The resultant eluate 11 was assayed and discarded, while the precipitate B, retained in the cell, was resuspended in 1.0 litre of 0.001M sodium phosphate buffer pH 8.0 and diafiltration (DF) was carried out with a further 5.0 litres of the latter buffer [Fig. 3.4(i)].



1 Culture medium was filtered through a buffered Celite 545 bed.

2 Adjusted to 0.01M sodium phosphate buffer, pH 7.0 and 0.025M KCl

3 Using a 50 000 m.wt. cut off membrane
p.s.i. = 50, flow rate/h = 800 ml

4 Using a 10.000 m.wt. cut off membrane. The concentrate was washed extensively with 0.001M sodium phosphate buffer pH 7.0.
p.s.i. = 50, flow rate/h = 600 ml

5 Lyophilized, resuspended in 0.001M sodium phosphate buffer, pH 8.0 and adsorbed to calcium phosphate

Fig 3.4(i) The initial steps in purification of S. griseus sialidase by ultrafiltration through a series of graded ultrafiltration membranes

3.1.2 Calcium Phosphate Gel Treatment

The precipitate B, resulting from the concentration-diafiltration procedure, was dissolved in 0,001M sodium phosphate buffer, pH 8.0 and stirred with calcium phosphate gel (ratio of wet gel to protein, 50:1). After 1.0 hr stirring at 4°, the suspension was spun for 10 minutes at 10,000 x g and the gel precipitate retained. This was then suspended in 20 ml 0.05M sodium phosphate buffer pH 8.0, allowed to stir for 30 min then centrifuged as above. The supernatant was discarded (for *S. griseus* sialidase preparation only) and the gel precipitate resuspended in 0.4M sodium phosphate buffer at 21° and stirred for 30 min after which it was spun again. The supernatant was retained. (The trial experiment of adsorbtion and desorbtion is elaborated upon in the legend of fig. 3.5). The supernatant from the above was diafiltered with 0.05M sodium acetate buffer, pH 5.0 in an Amicon ultrafiltration cell 52 fitted with an Amicon PM10 (10,000 mol. wt cut off membrane).

3.1.3 Affinity Chromatography On Amicon Matrex Blue A: (Affinity Column 1)

Prior to the use of the Matrex Blue A as an affinity column, inhibition studies were carried out with the ligand namely F3GA (reactive blue). (See legend of fig. 3.7 for method and fig. 3.6(i) for F3GA structure).

The concentrate from the previous step ($Ca_3(PO_4)_2$ step) in 0.05M sodium acetate buffer, was applied at a flow rate of 0.3 ml per minute to a chromatographic column (1 x 14 cm) containing the Amicon Matrex Blue A equilibrated with the same acetate buffer. After the sample was applied and allowed to equilibrate at zero flow for 1 hr, the column was washed with the loading buffer until a zero absorbance reading was

obtained at 280nm. The affinity column was then charged with a stepwise gradient, of 1.0M sodium chloride in acetate buffer, followed by a 1.5M sodium chloride acetate buffer. The enzyme containing fractions were diafiltered as above with 0,05M sodium acetate buffer pH 5.0. The trial experiments with Blue A and Red A are expressed in the legends of fig. 3.8.

3.1.4 Affinity Chromatography 11 On NNPOA-AHS4B

As in the case of Matrex Blue inhibition studies were carried out with NNPOA (see legend of fig. 3.10 for method).

The diafiltered concentrate from the above stage was applied to the NNPOA-AHS4B affinity column (3 x 22 cm) previously equilibrated in 0,05M sodium acetate buffer, pH 5.0. The flow rate and the washing procedure was similar to the Matrex blue affinity column. After achieving zero absorbance at 280nm a stepwise gradient was applied using 1.0M sodium chloride in acetate buffer. (See legend of fig. 3.9). The enzyme containing fractions was extensively dialysed against 0,001M sodium phosphate buffer pH 6.8 and then concentrated.

Hydroxylapatite Column Chromatography

The enzyme solution was loaded onto an hydroxylapatite column (1.4 x 26 cm) and washed until zero adsorbance at 280nm was achieved and then eluted with a linear gradient of 0.05M to 0.8M sodium phosphate pH 6.8 (Table 3.1).

The commercial preparation of C. perfringens was further purified via Calcium phosphate gel, Amicon Matrex Blue A, NNPOA-AHS4B affinity and hydroxylapatite column chromatography. The elution of C. perfringens sialidase from calcium phosphate occurred in one step using 0.3M sodium phosphate and the elution from the Amicon Matrex Blue A was with 1.0M sodium chloride in acetate buffer (see figures and legends of Fig. 3.5, 3.8 and 3.10).

The V. cholerae sialidase obtained from Burroughs Welcome was further purified via Amicon Matrex Blue A, NNPOA-AHS4B affinity and hydroxylapatite column chromatography (Fig. 3.11). The elution of the V. cholerae from NNPOA-AHS4B was a two step elution of 0.8M NaCl and 1.2M NaCl, which gave rise to V. cholerae sialidase 1 and V. cholerae sialidase 11. The 1 and 11 enzyme fractions were loaded separately onto the hydroxylapatite column as described previously and eluted via a stepwise gradient of 0.4M and 0.5M sodium phosphate, pH 6.8 (Fig. 3.11).

3.1.6 Molecular Weight Determination

3.1.6.0 By Polyacrylamide gel electrophoresis (PAGE)

The molecular weights of the sialidases were determined by SDS-polyacrylamide (10%) gel electrophoresis using the method of Weber and Osborne (1969): 15μg - 100 μg of enzyme was loaded per gel. The BDH molecular weight markers compromising a mixture of oligomers of molecular weights in the range 14,300-85,000 were used as described by the manufacturers (BDH handbook 1979). After electrophoresis the gels

were stained with Coomassie blue and destained by diffusion (Weber & Osborne, 1969) [Fig. 3.12 (i + ii) and Table 3.2].

3.1.6.1 By gel permeation chromtography

The molecular weight of the sialidases were also determined by gel filtration on a Sephadex G-100 column (2.2 x 50 cm) following the procedure of Andrews (1965). The column was calibrated with five proteins of known molecular weight. Two milligrams of each of these proteins (0.5 mg sialidase) was dissolved in 2.0 ml 0.1% dextran and the solution eluted (40ml/hr) from the column with 0.2M phosphate buffer, pH 7.0, containing 0.05M potassium chloride, 4.0 ml fractions were collected. The elution volume of each protein was determined by monitoring the absorbance of the eluted fraction at 280nm. The elution peak of the sialidases were identified by its enzyme activity (fig. 3.13).

3.1.7 Determination Of Enzyme Purity

The purity of the enzyme was determined by the method outlined for SDS-PAGE (Weber & Osborne, 1969), the only difference being that 15 µg as well as 100 µg enzyme loads were run per gel (Fig. 3.12i).

Assay of sialidase activity is discussed in Chapter 1.

3.1.8 Assay Of Protease Activity

The protease activity was assayed according to the method of Sekine *et al.*, (1969). The reaction mixture, containing enzyme preparation, distilled water and 1.5% casein in McIlvaine buffer, pH 6.0, was incubated at 37 for 10 min . The reaction was stopped by adding 3.0 ml of 0.44M trichloroacetic acid (TCA). The amount of TCA soluble material showing a positive reaction to the Folin reagent was determined colorimetrically (Lowry *et al.*, 1951).

3.1.9 Assay Of Other Enzyme Activities

The N-acetylneuraminic acid aldolase acivity was assayed according to the method described by Comb and Roseman (1962). For the assay of phospholipase C, the reaction conditions described by Pastan *et al.*, (1968) were employed with egg phosphatidyl choline as substrate (Rood & Wilkinson, 1974) and phosphoryl choline liberated by this reaction was determined by the method of Lowry and Tinsley (1974).

Glycosidases were assayed according to the methods of Uchida *et al.* (1977).

Haemolysin activity was determined as described by Geisow (1975).

3.2.10 Protein Determination

Protein concentration was determined by the method of Lowry *et al.*, (1951) with bovine serum albumin (BSA) as a standard.

3.2.11 Glycoprotein Detection

Gels fixed overnight in an aqueous solution, 40% ethanol, 5% glacial acetic acid (v/v) were oxidised with 0.7% periodic acid at room temperature for 2 hr. The gels were rinsed with distilled water and treated with 0.5% sodium metabisulphite in 5% aqueous acetic acid for 1 - 1.5 hr. until colourless in order to destroy excess periodic acid. The gels were rinsed and to each tube was added an equal volume of acidic dimethyl sulphoxide solution (0.60 ml conc. HCl/l DMSO) and freshly prepared dansylhydrazine solution (2 mg/ml DMSO). The tubes were stoppered, mixed and incubated for 2 hr at 60 . The dansylhydrazine solution was poured off and a solution of $NaBH_4$ in DMSO (5 ml, 0.2 mg $NaBH_4$ /ml DMSO was added to each tube and incubated at room temperature for 30 minutes. The gels were then rinsed with distilled water and destained overnight in 1% glacial acetic acid (v/v) with several changes (Eckhardt et al., 1976).

After destaining, the fluorescent-labelled glycoproteins can be visualised with long range ultraviolet light.

A fetuin sample was tested in parallel with enzyme as a check on procedures and, in certain cases, the gels were sliced longitudinally and stained simultaneously for protein and glycoprotein.

3.2 Results And Discussion

During the large scale preparation of this enzyme we endeavoured to improve the enzyme yield over the first concentration step, which originally involved an ammonium sulphate fractionation step which proved to be costly not only in expense but also in manhours; the batchwise technique appeared the most suitable. However, our attempts with various adsorbents such as DEAE-, CM-cellulose and C-G 50 ion exchange resins as well as calcium phosphate gel, showed no significant adsorbtion of the enzyme. With the advent of relatively cheap ultrafiltration cells which could be modified, we have been successful in not only concentrating and diafiltering the enzyme solution but also in removing 97% of the contaminating protein with a loss of only 16% of sialidase (Table 3.1). This has proved to be the most effective step in the entire large scale purification of S. griseus sialidase.

When calcium phosphate gel was added to the concentrated enzyme, (S. griseus), as well as the commercial C. perfringens enzyme solution in 0,001M sodium phosphate buffer, pH 8.0, we obtained a positive adsorbtion of the enzyme as opposed to the expected negative adsorbtion (Colowick, 1955). It was found that this step proved essential for the removal of viscous material associated with S. griseus sialidase which could not be removed as easily by our previous purification methods (Kunimoto et al., 1974 and Kabayo, 1978). Further, we could selectively remove contaminating protein with a lower affinity for calcium phosphate with 0,05M sodium phosphate, whereas the S. griseus sialidase could be removed with 0,4M sodium phosphate. This same selective desorbtion could not be achieved with C. perfringens sialidase, although the adsorbtion was selective. However, a 9-fold

Table 3.1

Purification Of Sialidases From S. griseus, C. perfringens And V. cholerae

Procedure	Vol. (ml)	Conc. (units/ml)	Total Units	Protein (mg/ml)	Specific Activity (units/mg)	Yield (%)	Purification
Streptomyces griseus							
Culture filtrate, post Celite	18,500	0.0056	103.6	1.100	0.005	100.00	1.00
Ultrafiltration, (50,000 m.wt.) eluate 1	19,000	0.0046	87.4	0.768	0.006	84.40	1.20
(10,000 m.wt.) ppt. B. retained	130	0.7000	91.0	4.000	0.175	87.80	35.00
Calcium phosphate, desorbed	60	1.4850	89.1	3.090	0.480	86.00	96.00
Affinity column I	20	3.9000	78.0	1.230	3.170	75.30	634.00
Affinity column II	60	1.1500	69.0	0.240	4.800	66.60	960.00
Hydroxylapatite, Fraction I	6	11.2000	67.2	0.800	14.000	64.90	2800.00
Fraction II	6	2.2000	13.2	0.250	8.800	12.74	1760.00
Clostridium perfringens							
Commercial preparation	78	9.0000	702.0	1.070	8.400	100.00	1.00
Calcium phosphate (desorbed)	20	30.0000	600.00	0.400	75.000	85.50	8.92
Affinity column I	40	15.0000	600.00	0.085	176.000	85.50	21.00
Affinity column II	35	17.1400	600.00	0.097	176.700	85.50	21.00
Hydroxylapatite	10	52.5000	525.00	0.210	250.000	74.80	29.70
Vibrio cholerae							
Ultrafiltration. (10,000 m.wt.) ppt. B. retained	12	75.0000	900.00	2.000	37.500	100.00	1.00
Affinity column I	10	81.5000	815.00	1.600	51.000	91.00	1.36
Affinity column II, Fraction 1	6	75.0000	450.00	0.530	141.510	50.00	3.77
Fraction II	6	46.7000	280.00	1.000	46.700	31.00	1.24
Hydroxylapatite, Fraction I	5	80.0000	400.00	0.480	166.670	44.40	4.44
Fraction II	5	28.0000	140.00	0.320	87.500	15.50	2.33

purification was achieved for the latter enzyme over that step (Table 3.1 and Fig. 3.5).

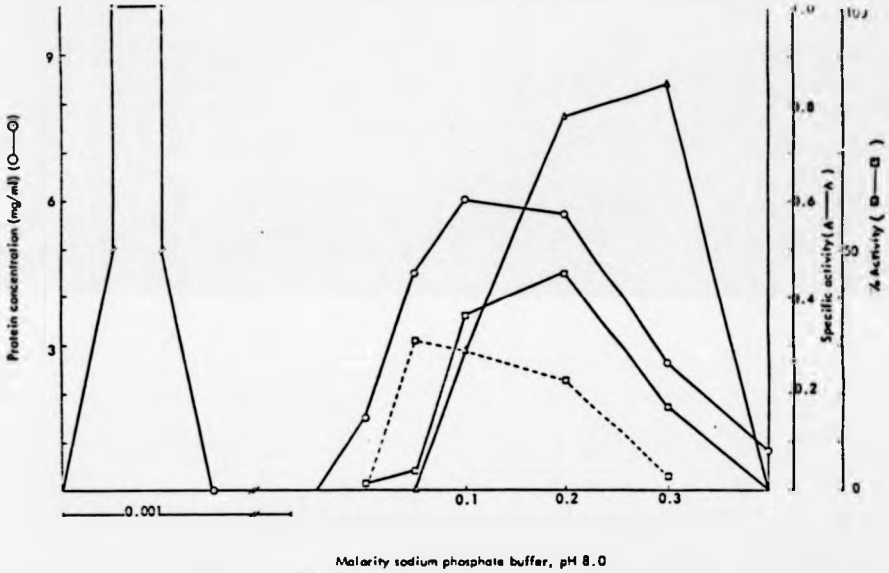


Fig. 3.5 The elution profile of S. griseus (——) and C. perfringens (-----) sialidase from calcium phosphate gel with increasing sodium phosphate buffer concentration. Protein concentration (O——O), specific activity (Δ——Δ) and % activity (○——○).

Residue B resulting from the S. griseus culture filtrate, after UF and DF (see Table 3.1 for charge) was suspended in 20 ml 0.001M sodium phosphate buffer, pH 8.0, to this was added calcium phosphate gel (ratio of gel to protein, 50:1). The suspension, after stirring for 1 hr at 4°, was centrifuged at the same temperature at 10,000 x g for 10 mins. The supernatant (S/N) was retained while the precipitated gel was suspended in 20 ml 0.025M sodium phosphate buffer, pH 8.0, stirred for 30 mins. and centrifuged as above. The S/N was again kept and the precipitate resuspended in 0.05M buffer, stirred for 30 mins at 4° and then centrifuged. This procedure of resuspension of the precipitated gel in increasing concentrations of sodium phosphate, stirring, centrifuging and retaining the S/Ns and gel precipitates was continued up to a concentration of 1.0M sodium phosphate. The resultant S/Ns were dialysed against the assay buffer before determining the enzyme specific activity. The same procedure was adopted for the further purification of the commercial preparation of C. perfringens sialidase.

Since polyanionic substances such as sulphonated dyes inhibit sialidases (Drzeniek, 1966), it was in our interest to investigate the cheap, commercially available Matrex Blue A and Red A which possess immobilized polycationic ligands Cibacron blue F3GA and Procion red HE3B [Fig. 3.6(i) and (ii)] respectively, as a possible affinity column for sialidases. Inhibition studies carried out on V. cholerae sialidase with the ligand F3GA, revealed that it was a non-competitive inhibitor ($K_i = 1.125 \times 10^{-3}$M). (Fig. 3.7). When used as a purification

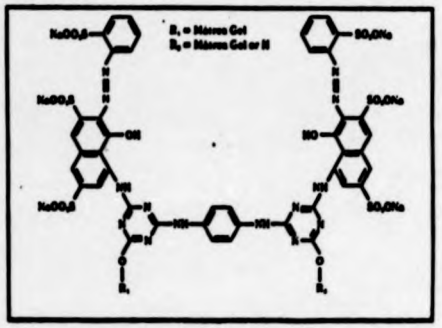


Fig. 3.6(i) Partial Structure of Mātrex Gel Red A.

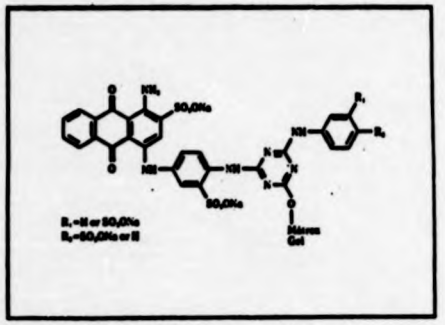


Fig. 3.6(ii) Partial Structure of Mātrex Gel Blue A.

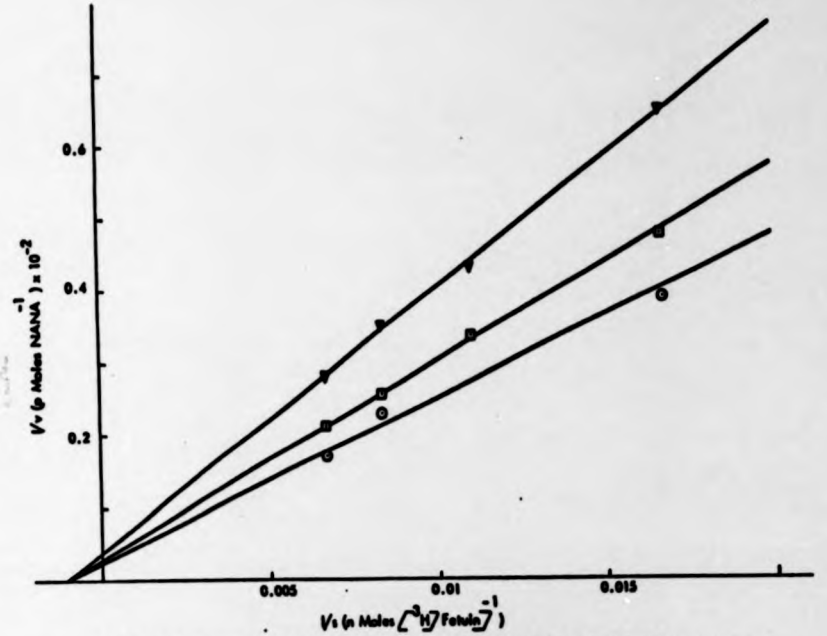


Fig. 3.7 Lineweaver-Burk (1934) plot of the effect of F3GA (0nMoles ●—●, 0.3nMoles ▫—▫ and 0.6nMoles ▽—▽) on the activity of V. cholerae sialidase.

Enzyme (10 µl) was added to 240 µls of 0.05M sodium acetate buffer, pH 5.1 containing the substrate ^{3}H-Fetuin at different concentrations (0 - 150nMoles) and final concentrations of F3GA. After incubation at 37° for 30 mins 50 µls were dispensed from the reaction tube into a precooled centrifuge tube to which 50 µl of cold PTA was added. It was immediately mixed and centrifuged. 50 µls of the S/N was then counted for radioactivity. Controls in the absence of enzyme were set up simultaneously.

step, the Matrex Blue A was as effective as Red A but required a lower salt concentration to remove the enzyme (Fig. 3.8).

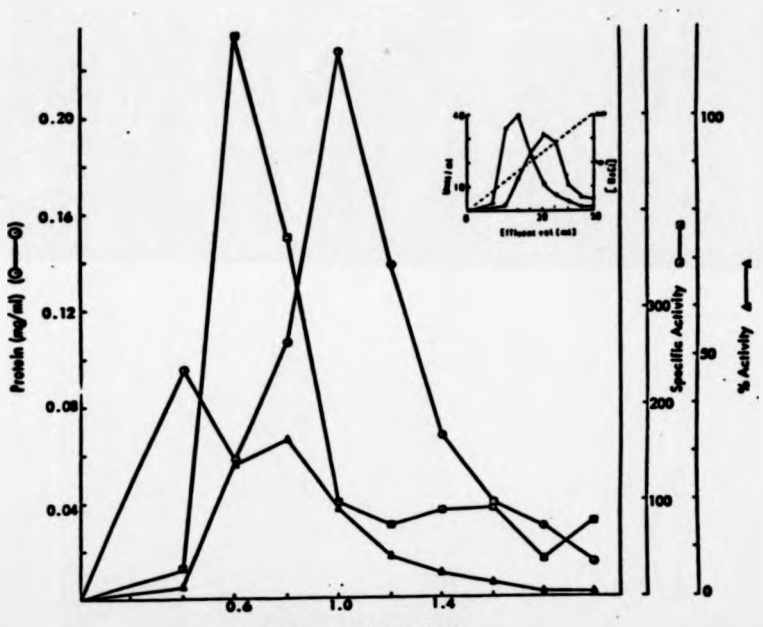


Fig. 3.8 The elution profile of C. perfringens sialidase from Amicon Matrex Blue A with increasing sodium chloride concentration. Protein concentration (O—O), specific activity (□—□) and % activity (Δ—Δ)

The concentrated-dialysed enzyme fraction from the calcium phosphate gel stage dissolved in 20 ml 0.05M sodium acetate buffer was applied to a chromatographic column containing the Matrex dye as described in the text. After equilibration and washing out the unbound protein (A_{280} = 0) the column was eluted with a linear gradient of sodium chloride (0 - 2.0M). 5 ml fractions were collected and dialysed against the appropriate assay buffer before determining the specific activity.

A similar trial run for S. grieus and V. cholerae was carried out.

Inset shows the elution profile of C. perfringens sialidase from both Amicon Matrix Blue A o—o and Red A Δ—Δ .

Matrex Blue A cannot be labelled as a true affinity column for sialidase since the ligand, being a non-competitive inhibitor in that it interacts with the binding site, does not have any influence on the affinity of the enzyme for the substrate (Mahler & Cordes, 1971).

Nevertheless, it has proved to be a very effective step in the purification of the three sialidases increasing the purification 1 - 6 fold (Table 3.1).

NNPOA-AHS4B was shown to be a specific adsorbent for all three sialidases. Although no increased purification was observed for the C. perfringens sialidase, the column still adsorbed the enzyme and could be eluted with 1M sodium chloride, whereas 6% of the total S. griseus sialidase activity was found in the breakthrough (fig. 3.9) (no adsorbtion occurred when this 6% was reloaded on a new NNPOA-AHS4B column).

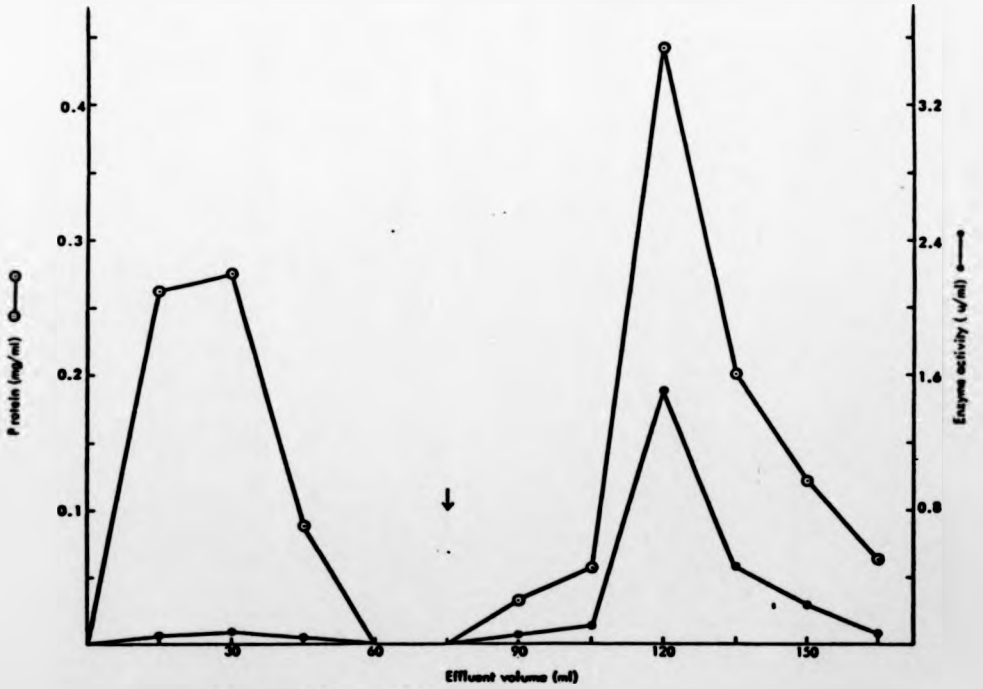


Fig. 3.9 The elution profile of S. griseus sialidase from NNPOA-AHS4B affinity column. Protein concentration (O——O) and enzyme activity (•——•).

The column was charged and eluted (arrow) as described in the text. Fractions (15 ml) were collected and dialysed against the appropriate assay buffer before enzyme activities were determined. A similar procedure was used for C. perfringens sialidase whereas the V. cholerae enzyme was eluted with 0.8M and 1.2M sodium chloride in buffer.

In the case of the V. cholerae enzyme, two enzyme peaks were observed, one at elution with 0.8M and the other at 1.2M sodium chloride concentrations (Table 3.1). NNPOA was shown to be a competitive inhibitor of sialidase from S. griseus, C. perfringens and V. cholerae (Brossmer, 1977 a) with Ki's of 2.48 x $10^{-4}M$, 3.15 x $10^{-4}M$ and 2.3 x $10^{-4}M$ respectively (Fig. 3.10). This, and the high molarity of salt required to remove the enzyme from the column, illustrates the strong adsorbtion of the sialidases. The column was effective in removing N-acetylneuraminate pyruvate lyase (EC4.1.3.3), a NANA requiring enzyme, from the protein solution as well by passing through the column without adsorbing to the affinity gel.

The use of hydroxylapatite as a final step in the purification of all three sialidases proved crucial to our purposes because of the following reasons: (i) It improved the purification, (ii) it separated the two forms of S. griseus and V. cholerae sialidase (fig. 3.11) and (iii) it removed contaminating sugars that leached from the previous affinity column, a common source of contaminating sugars which affects structural studies, one of the themes of this thesis. These preparations have been adjudged free of the contaminating enzyme activities tested for.

With this newly developed method, we were able to increase the S. griseus sialidase yield by ca. three-fold (6.3 mg) over our previous method, though still less than Kunimoto's et al., (1975), but with an increased specific activity. The yield of the V. cholerae sialidase prepared by our method could not be compared but the highest specific activity so far reported is 133 (Ada et al., 1961). We were able to improve on this with the aid of the NNPOA-AHS4B affinity gel and hydroxylapatite. Further, with the aid of the latter two

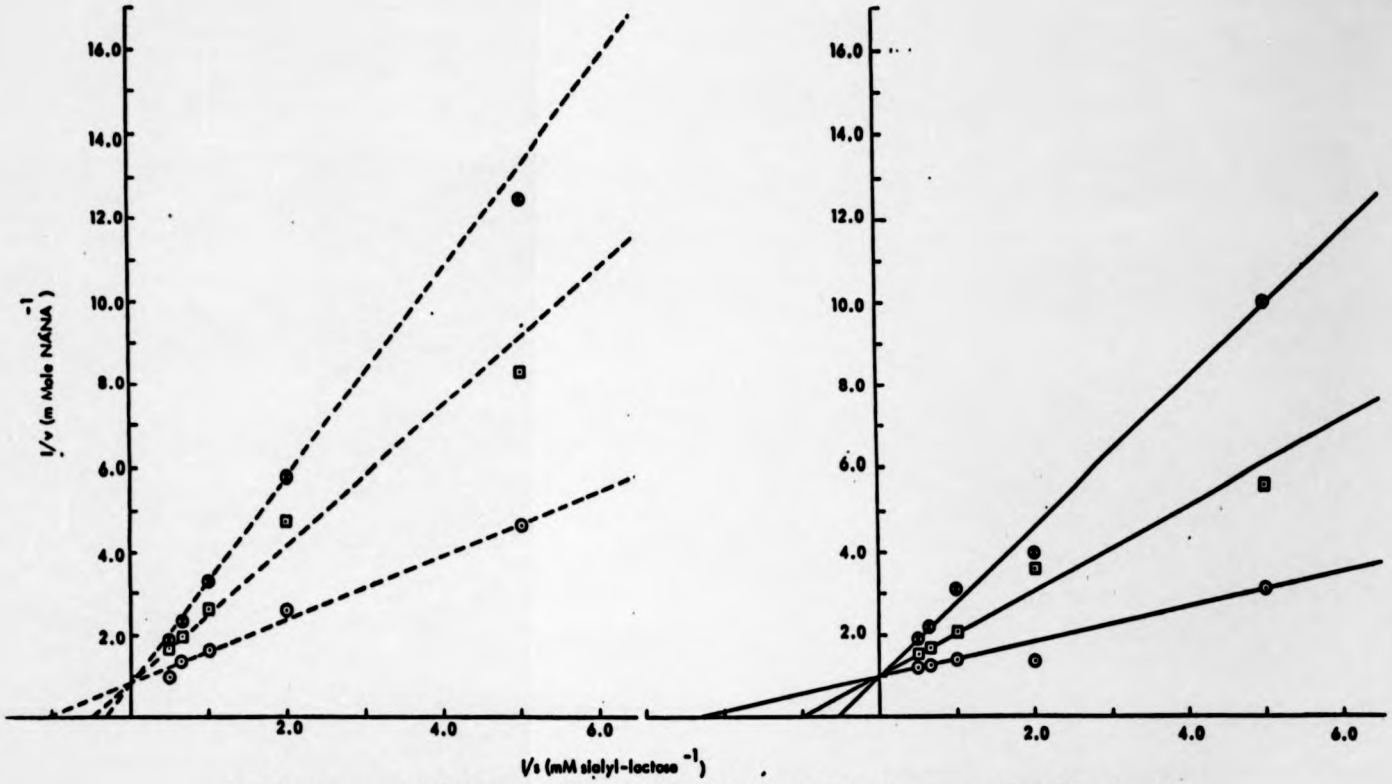


Fig. 3.10 Lineweaver-Burk (1934) plot of the effect of NNPOA(0mMoles ⊙—⊙, 0.4mMoles ⊡—⊡ and 0.8mMoles ⊞—⊞) on the sialidase activity from S. griseus (——) and C. perfringens (----).

Enzyme (10 µl) was added to 490 µls of 0,05M sodium acetate buffer (pH 5.3 for S. griseus and pH 4.5 for C. perfringens sialidases) containing the substrate, sialyl-lactose at varying concentrations (0 - 2mM) and fixed concentrations of NNPOA. After incubation at 37° for 1 hr the reaction was stopped with PTA (100 µl) and 200 µls removed to determine the enzyme activity via the Warren method.

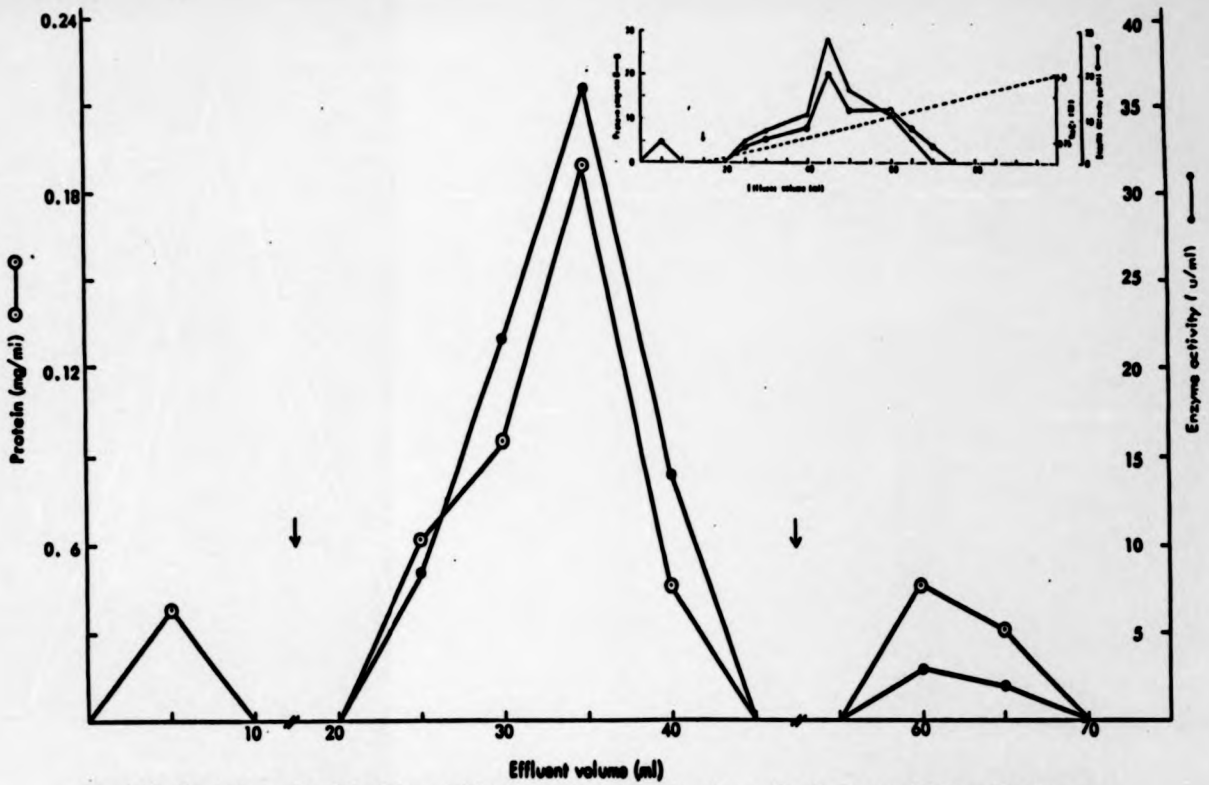


Fig. 3.11 The elution profile of *V. cholerae* sialidase from an hydroxylapatite column. Protein concentration ⊙——⊙ and enzyme activity (•——•).

The column was charged with *V. cholerae* sialidase I (see Table 3.1) and eluted with a stepwise gradient of 0.4M and 0.5M sodium phosphate (arrows). Fractions were collected and dialysed against 0.05M sodium acetate buffer, pH 5.1 before the enzyme activities were determined. The insert shows the elution profile of *V. cholerae* sialidase in the presence of a linear gradient (0.001 - 1.0M) of sodium phosphate buffer pH 6.8.

chromatographic steps, we were also able to separate the two isoenzymes. The determination of the molecular weights of glycoproteins is no simple problem as these compounds are known to behave in an anomalous manner under most techniques used for its mol. wt estimation (Segrest et al., 1971 and Hughes 1976). It is for this reason that a combination of methods were used to estimate the mol. wts of the sialidases. By our methods (SDS-PAGE, 10%) fig. 3.12 (i + ii) and table 3.2 and gel filtration chromatography, fig. 3.13, we were able to approximate the molecular weights of the sialidases within 1,000-2,000 mol. wt units. The molecular weights obtained, 32,000 (S. griseus 1) 57,000 (C. perfringens) and 69,000 (V. cholerae 1), were those used throughout the forthcoming experiments.

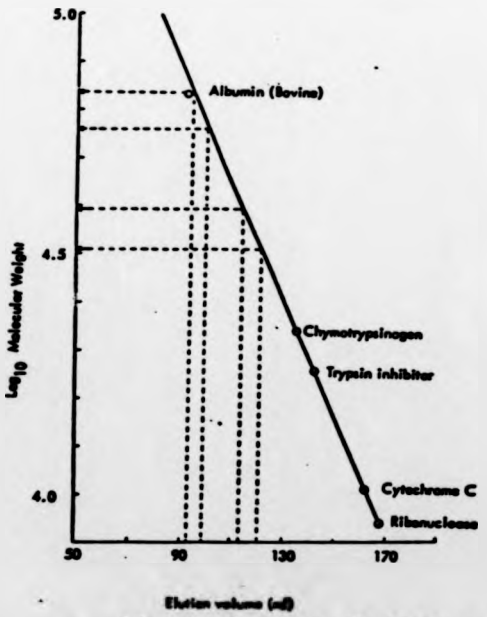


Fig. 3.13 Determination of the molecular weights of the sialidases by gel filtration chromatograph as described in the text.

The mol. wt. obtained for the various sialidases were: 32,000 (S. griseus 1), 57,100 (C. perfringens), 69,500 (V. cholerae 1) and 59,000 (V. cholerae 1)

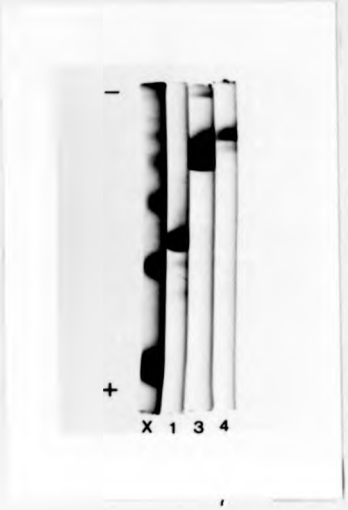


Fig. 3.12 (i)

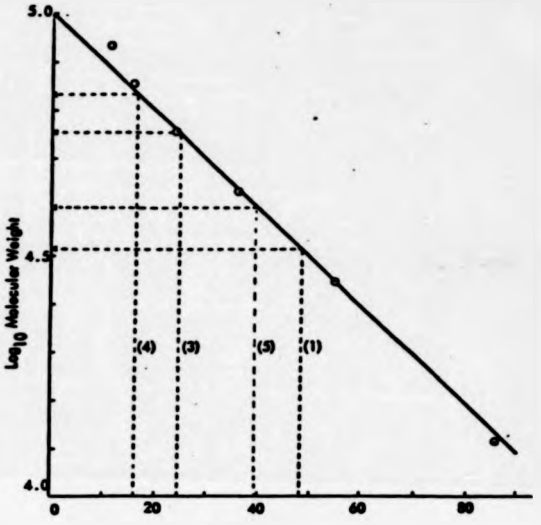


Fig. 3.12 (ii)

Fig. 3.12 (i & ii) Table 3.2 Determination of the molecular weights of the sialidases by SDS-PAGE (10%).

The calibration line fig. 3.12 (ii) for the mol. wt. markers (molecular weight range 14,300 - 71,500, fig. 3.12 (i) X) was obtained as described in the text. The % electrophoretic mobility obtained from fig. 3.12 (i) for the appropriate sialidases are listed in table 3.2

Table 3.2

The Molecular Weight Determination Of The Sialidases By SDS-Polyacrylamide Gel Electrophoresis

Band In order of decreasing mobility	Molecular weight	Molecular weight (Log_{10})	Distance of band from start (cm)	Electrophoretic mobility (%)
Bromo phenol blue (reference)			8.6	100.00
Monomer	14,300	4.1553	7.3	85.00
Dimer	28,600	4.4564	4.6	54.90
Trimer	42,900	4.6325	3.0	35.00
Tetramer	57,200	4.7574	2.0	23.62
Pentamer	71,500	4.8543	1.3	15.73
Hexamer	85,800	4.9335	9.0	11.00
(1) S. griseus sialidase I	32,700	4.515	4.17	48.50
(2) S. griseus sialidase II	Nd	Nd	Nd	Nd
(3) C. perfringens	56,500	4.752	2.10	24.40
(4) V. cholerae sialidase I	70,000	4.834	1.40	16.20
(5) V. cholerae sialidase II	40,000	4.602	3.37	39.18

Nd Not determined

Fig. 3.12 (i)

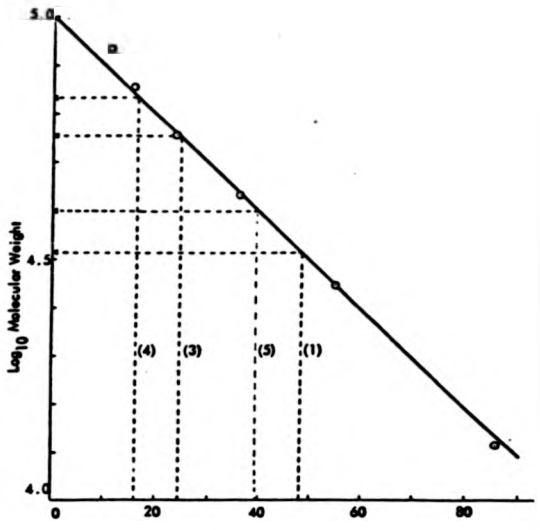


Fig. 3.12 (ii)

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Trimer	42,900	4.6325	3.0	35.80
Tetramer	57,200	4.7574	2.0	23.62
Pentamer	71,500	4.8543	1.3	15.73
Hexamer	85,800	4.9335	9.8	11.00
(1) *S. griseus* sialidase I	32,700	4.515	4.17	48.38
(2) *S. griseus* sialidase II	Nd	Nd	Nd	Nd
(3) *C. perfringens*	56,500	4.752	2.10	24.40
(4) *V. cholerae* sialidase I	70,000	4.834	1.40	16.20
(5) *V. cholerae* sialidase II	40,000	4.602	3.37	39.18

Nd Not determined

As the aim of our project was not to characterize the various sub-forms of sialidases per bacteria, we however determined the molecular weight of the V. cholerae 11 sialidase; it was found to be approximately 40,000. We suspect it is the isoenzyme described by Ziegler et al., (1978) in which no molecular weights are given. The mol. wt of the second S. griseus enzyme was not determined.

CHAPTER 4

ACTIVE SITE STUDIES ON SIALIDASES (CHEMICAL MODIFICATION - ENZYME MECHANISM)

4.0 Introduction

Whether we consider enzymes or immune-, transport-, receptor- and contractile-proteins, the key step in their biological function is the specific recognition and binding of a ligand (substrate, antigen, hapten, transported ligand, hormone, regulatory substance, etc.). A small and well defined portion of the total protein surface is presumed to be involved in this selective, high affinity interaction with the specific ligand, and is referred to as the binding site. The binding site (or active site in an enzyme), is taken to include those side chains and peptide bonds which are in direct physical contact with the substrate (perhaps through intervening water molecules) and other side chains or peptide bonds that, although not in direct contact with the substrate, perform a direct function in the catalytic process.

The complete understanding of how enzymes carry out their catalytic function requires a detailed knowledge of how this active site is constituted, and this then has become the key problem in the area of enzyme (protein) structure-function analysis.

The central question in the study of reaction mechanisms concerns the structure of the transition state and the nature of the intermediates for the reaction under consideration. In the case of enzyme-catalysed

reactions, a knowledge of the structure of the transition state involves not only an understanding of the geometry of the substrate molecule but also the three dimensional conformation of the enzyme. The high catalytic efficiency and marked specificity exhibited by enzymic reactions imply the participation of several distinct functional groups of the enzyme in the catalytic process. This implication has been fully borne out by, for example, the X-ray diffraction studies of lysozyme (Imoto et al., 1972), chymotrypsin (Kraut, 1971) and carboxypeptidase (Hartsuck & Lipscomb, 1971), which established the involvement of several groups, usually distant from one another, along the polypeptide backbone but near one another in space in the associated catalytic processes. This fact, together with structural complexities inherent in the chemistry of molecules of the size of proteins, suffices to indicate that a precise definition of the transition-state, structure for enzymic reactions is a formidable task. Nevertheless, the mutually supportive investigations concerning protein structure and the various facets of enzyme activity have, in several cases, including the three enzymes mentioned above, brought the goal within reach. For the great preponderance of proteins for which the three-dimensional structure is unknown, one must be content to work towards less sophisticated goals.

The immediate goals in the study of the mechanism of enzyme-catalysed reactions may be formulated as follows: (i) the elucidation of the pathway for enzyme-catalysed reactions, (ii) the identification of the amino acids involved in substrate binding and in bond making and bond breaking reactions, (iii) determination of their approximate arrangement in space and (iv) development of a rationale, through assigning specific catalytic roles to the groups involved, that explains the velocity of enzyme catalysed reactions to within an order of magnitude. It is within the context of this approach (especially

(ii) and (iv)), that the succeeding part of the chapter is couched.

The identification of the amino acids present in the active site of enzymes is clearly of major importance for the understanding of the basic facets of the mechanism of enzyme-catalysed reactions. Several methods, of varying utility, have been employed in attempts to identify at least some of the residues that constitute these active sites. Some of these methods involve (i) chemical modification of specific groups on the substrate molecule and determining whether they are essential for enzymic activity and, by so doing, it can be deduced which amino acid groups in the enzyme would be likely to react with the essential group on the substrate. (ii) Chemical modification of the enzyme with group specific reagents[1] (iii) affinity labelling (iv) X-ray diffraction studies on enzyme-substrate or enzyme-inhibitor complexes.

Besides deducing which amino acids are involved in the active site with the aid of the substrate, important information can also be obtained regarding the action of the enzyme (Chapter 1).

4.0.1 Chemical Modification

With a few exceptions, most of the advances made in the field of chemical modification of protein structure during the last twenty years, have resulted from increased reagent specificity and selectivity. Carefully designed reagents could be used to probe details of reaction mechanisms and to detect the changes in protein conformation which accompany function. Some of the types of reactions that are used for amino acid modification are given in table 4.0

[1] The term group-specific represents a level of optimism not often achieved in practice; group-selective describes more accurately the reality since the successful chemical modification of a single type of functional group in a protein, to the total exclusion of other types, is the exception more than the rule.

Table 4.0

Reactions For Modification Of Functional Groups In Proteins

Functional Group	Reactions
Amine of lysine	Acylation, alkylation, arylation, reaction with carbonyls
Guanidinium of arginine	Condensation with dicarbonyls
Carboxyl of aspartic and glutamic acids	Esterification, amide formation with carbodiimides, reduction
Thiol of cysteine	Oxidation, arylation, alkylation, β-elimination, heavy metal derivatives
Imidazole of histidine	Alkylation, diazonium coupling, iodination, oxidation, photooxidation
Thioether of methionine	Oxidation, alkylation
Phenol of tyrosine	Acylation, alkylation, iodination, nitration oxidation, diazonium coupling
Indole of tryptophan	Alkylation, formylation, oxidation, ozonlysis
Hydroxyl of serine and threonine	Esterification, phosphorylation, alkylation.

Most of these reactions are non-specific; they occur with more than a single residue or even a single type of residue. Fortunately, certain residues, because of their microenvironment within the three-dimensional structure of the protein, may exhibit reactivities different from those expected on the basis of studies with model systems. They often participate in biological function and, in fact, the structural features generating function are closely related to those giving rise to unusual chemical reactivity. Because of this unique reactivity they can be modified selectively, thereby facilitating structure-function correlation studies.

Moreover, it became possible to employ a variety of reagents to modify the same residue. With tyrosine, for example, reactions can be carried out either on the phenolic oxygen or at the 3- position of the aromatic

ring (Riordan & Sokolovsky, 1971). Structural circumstances that activate one of these sites usually activate the other as well. Depending on the nature of the 3- position substituent, the pK of the phenolic hydroxyl group can vary over several units. This provides a means to explore the pH dependence of function as it relates to degree of ionization of the specific tyrosine in question.

Hartley (1960) identified the "seryl proteases" by virtue of the fact that they all were inactivated by diisopropylphosphofluoridate (DFP), a reagent that phosphorylates a specific seryl residue at the active site of these enzymes. DFP cannot be called a seryl reagent, however, since it does not react with seryl residues in general but only with those having a neighbouring histidine residue at the active site of certain enzymes. On the other hand, a large number of reagents has been used to identify the "sulphydryl enzymes". These enzymes are inactivated by heavy metal ions such as Ag^{+} and Hg^{++}, p-mercuribenzoate (PMB), N-ethylmaleimide (NEM) and iodoacetate among others. However, in many of these instances, loss of activity is not due to the selective modification of an active site cysteine residue but rather to effects on structure (Vallee & Riordan, 1969). Thus, classification of proteins based on functional site residues is operationally convenient but can be mechanistically misleading. Therefore, with all protein chemical modifications, it is important to understand the specificity of the reagent employed and its effects on protein structure before making functional deductions.

The chemical modifications discussed below are the systems of choice used in our investigations. Group-specific reagents have been used extensively for site specific modification of native proteins. For such a purpose, the modification is performed on the native protein (or in a

solvent which does not irreversibly alter the conformation of the protein). The concentrations of the modifying reagents are generally considerably lower than those used for the total modification of a given side-chain and the conditions of reaction are suitably chosen, bearing in mind the stability characteristics of the protein under study.

In the attempt to ascertain which amino acids participate in the binding or catalysis in the active site of sialidases, group selective reagents of varying specificity have been used over the years with controversial results (Table 4.1).

It is yet to be established whether the apparent disagreement in the results of these studies reflect on differences in the (i) mechanisms of action for sialidases of various origins, (ii) limitations of the techniques employed for chemical modification, (namely, side reactions, effectiveness of the modification reactions and interferences in the determination of enzyme activity), (iii) structures of the enzymes, (iv) substrates used for enzyme assays.

Preliminary studies inclined towards the resolution of these queries have been carried out in our laboratory by Kabayo (1978) on S. griseus sialidase and Groundwater (1979) on V. cholerae sialidase. This thesis reports on the comparative studies, by chemical modification and amino acid analysis, carried out on the active sites of sialidases obtained from pathogenic (V. cholerae and C. perfringens) and non-pathogenic (S. griseus) sources under identical conditions (namely, temperature, time, pH substrates and chemical modification reagents) and the precursory investigation into

Table 4.1

Chemical Modification Of Sialidases (1957-1979)

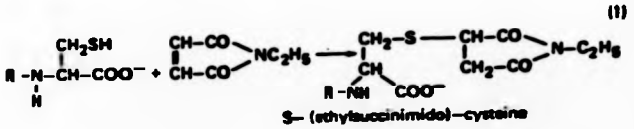
Enzyme	Reagent	Amino Acid Residue Modified	Percentage Residual Activity	References
S[a]	Hg^{2+}	Cyst	0	Kunimoto et al., 1974
C[b]	Hg^{2+}	Cyst	0	Kunimoto et al., 1974
V[c]	Hg^{2+}	Cyst	0	Rosenberg et al., 1960
V	Hg^{2+}	Cyst	100	Helenquist, 1975 (a)
Au[d]	Hg^{2+}	Cyst	91	Uchida et al., 1979
AV[e]	Hg^{2+}	Cyst	0	Ada, 1963
S	Iodoacetic acid	Cyst	100	Kunimoto et al., 1974
C	Iodoacetic acid	Cyst	100	Saito et al., 1979
Au	Iodoacetic acid	Cyst	100	Wang et al., 1978
Au[f]	Iodoacetic acid	Cyst	92	Uchida et al., 1979
S	p-CMB[1]	Cyst	91	Kunimoto et al., 1974
C	p-CMB	Cyst	0	Kunimoto et al., 1974
C	p-CMPS[2]	Cyst	100	Saito et al., 1979
Au	p-CMB	Cyst	94	Uchida et al., 1979
AV	p-CMB	Cyst	0	Ada, 1963
C	NEM[3]	Cyst	99	Saito et al., 1979
Au	NEM	Cyst	100	Wang et al., 1978
AV	NEM	Cyst	85	Ada, 1963
C	NBS[4]	Tryp	0	Brachmayer, 1972
Au	NBS	Tryp	2	Uchida et al., 1979
Au	NBS	Tryp	0	Wang et al., 1978
IV[g]	NBS	Tryp	100	Hoyle, 1969
S	EDC[5]	Asp-Glu	25	Kabaya, 1978
Au	EDC	Asp-Glu	100	Wang et al., 1978
S	Phenylglyoxal	Arg	10	Kabaya & Hutchinson, 1977
IV	p-HPG[6]	Arg	20	De Bruck et al., 1957
IV	Glyoxal	Arg	100	Hoyle, 1969
Au	2,3 Butanedione	Arg	100	Wang et al., 1978
S	2,4 Pentanedione	Arg	4	Kabaya & Hutchinson, 1977
S	TNBS	Lys	100	Kabaya & Hutchinson, 1977
Au	TNBS	Lys	89	Wang et al., 1978
Au	O MS[7]	Lys	100	Wang et al., 1978
S	Rose Bengal	His	100	Kabaya & Hutchinson, 1977
S	DPC[8]	His	100	Kabaya & Hutchinson, 1977
IV	DSA[9]	His	0	Hoyle, 1969

a S. griseus sialidase
b C. perfringens sialidase
c V. cholerae sialidase
d A. ureafaciens sialidase
e Avian sialidase
f A. sialophilus sialidase
g Influenza virus sialidase
1 p-Chloromercuribenzoic acid
2 p-Chloromercuriphenyl sulphonate
3 N-Ethylmaleimide
4 N-Bromosuccinimide
5 1-Ethyl-(3 dimethylaminopropyl-)carbodiimide
6 p-hydroxyphenylglyoxal
7 O-Methylisourea
8 Diethylpyrocarbonate
9 Diazotised sulphanilic acid

the function of the carbohydrate moieties of these glycoproteins.

4.0.1.0 Modification of sulphydryl groups

Of the long list of sulphydryl reagents N-ethylmaleimide (NEM) (Riordan & Vallee 1972) and 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) (Janatova et al., 1968 and Ellman, 1959) were the reagents of choice (Fig. 4.0 (i) and (ii).



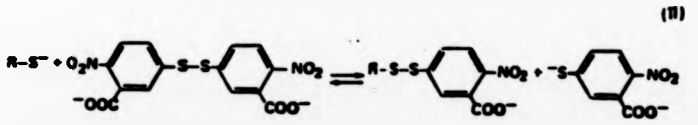


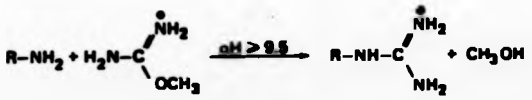
Fig. 4.0 (i) & (ii) Reactions of protein thiol groups with (i) N-ethylmaleimide and (ii) 5' 5' -dithiobis-(2 nitrobenzoic acid). (R, represents the protein group).

NEM, besides being specific, is known to form a stable adduct with the thiol group, whereas thiol groups from the derivatives of organomercurials such as p-chloromercuribenzoate (p-CMB) (Bradbury & Smyth, 1973) are readily regenerated. With the use of DTNB we were also aware that incomplete conversion of protein sulphydryl groups to the mixed disulphide leaves open the possibility of intramolecular reactions leading to the formation of disulphide bonds.

4.0.1.1 Modification of amino groups

Lysyl residues may be converted to the homoarginine derivative by treatment of proteins with O-methylisourea at alkaline pH (Hughes et al., 1949) [Fig. 4.1 (i)]. At high pH values, (eg. pH 10.5), the guanidation of primary amino groups takes precedence over the S-methylation of thiols (Banks & Shafer, 1972). Another advantage of this method is the α-amino groups react much more slowly with this reagent than do ε-amino groups (Plapp et al., 1971).

2,4,6-Trinitrobenzenesulphonate (TNBS) [Fig. 4.1 (ii)] initially used by us, (although its reaction in alkaline conditions is restricted to and ε-amino acids in proteins), also reacts with sulphydryl groups forming unstable thiol derivatives (Fields 1972).



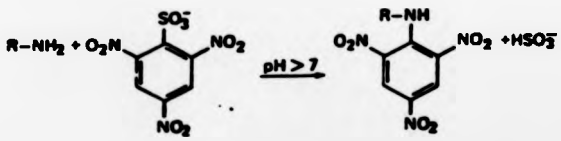
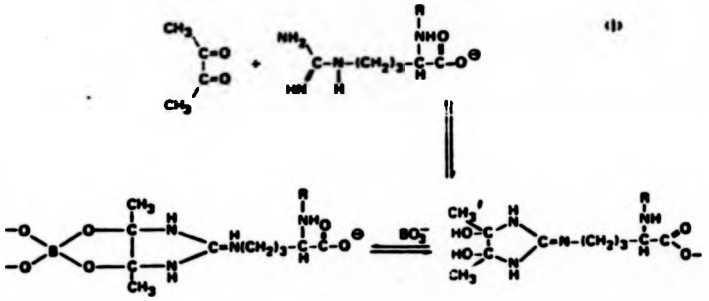


Fig. 4.1 (I) & (II) Reactions of protein amino groups with (I) O-methylisourea and (II) 2,4,6-trinitrobenzenesulphonate. (R, represents the protein).

4.0.1.2 Modification of guanidium groups

Procedures for the modification of arginine residues in proteins, under mild conditions, are based on their reaction with 1,2- or 1,3-dicarbonyl compounds (Cohen, 1968, Means & Feeney, 1971, Yankeelov, 1972 and Riordan, 1979).



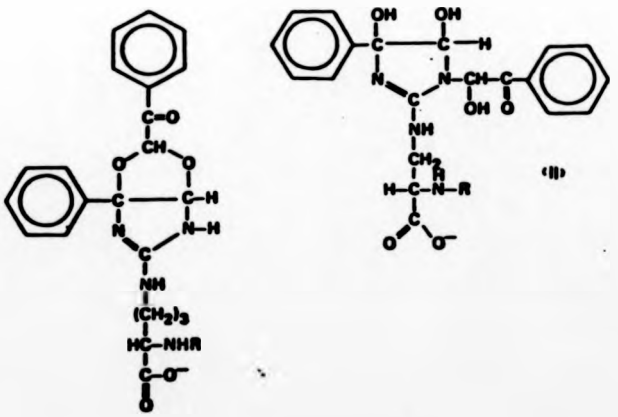


Fig. 4.2 (I) & (II) Reaction of protein guanidinium groups with (I) 2,3 butane dione and (II) phenylglyoxal yielding the alternative structures for the phenylglyoxal-arginine adduct. (R, represents the protein).

Following the procedures outlined by Riordan (1973) and Daemen and Riordan (1974) the specific modification of arginine residues is achieved when using 2,3-butanedione [Fig. 4.2 (i)]. Phenylglyoxal (PG), first introduced as an arginyl reagent by Takahashi in 1968, reacts with these residues under mild conditions to give a product that contains two phenylglyoxal moieties per guanidino group [Fig. 4.2(ii)].

This product is stable at acid pH's and dissociates to regenerate arginine only on prolonged incubation in neutral and alkaline media. Besides this reagent being specific for arginine at pH 5.5 and 7.0 as described by Takahashi (1968, 1977a,b), it can also be made radioactive, and the ^{14}C-phenylglyoxal (Riley & Gray, 1947) can be obtained commercially. Thus the reagent offers the advantage of direct quantitation of the total number of arginyl residues in the protein or, by differential labelling, determine the presence of binding or catalytic arginine residues in the active site.

4.0.1.3 Modification of carboxyl groups

The raction most exploited for the modification of these residues involves the coupling of nucleophiles, such as glycine methyl ester to the carboxyl groups in the presence of water soluble carbodiimide eg. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Carraway & Koshland, 1972).

The activation reaction is performed with the carbodiimide in the presence of high concentrations of nucleophiles. The latter displaces the carbodiimide group from the initially formed carbodiimide-carboxyl adduct, as shown in Fig. 4.3

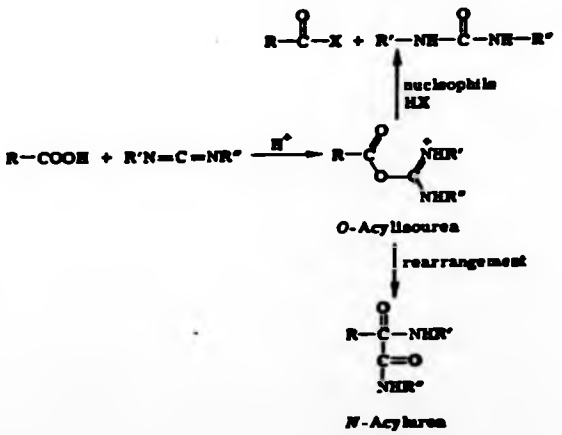


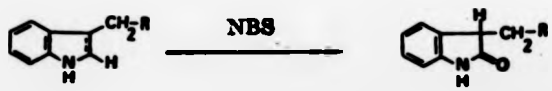
Fig 4.3 Reaction of protein carboxyl groups with carbodiimide followed by (I) a nucleophilic attack (HX) or (II) rearrangement. (R, represents the protein, R' and R" represent alkyl groups)

Water and other added nucleophiles compete with the intramolecular rearrangement of the O-acylisourea to N-acylurea. Attack by water regenerates the carboxyl group, whereas attack by other nucleophiles leads to an acyl nucleophile derivative of the carboxyl group. At pH 4.8 and high nucleophile concentrations in the presence of excess carbodiimide, the formation of the acyl nucleophile is essentially quantitative (Hoare & Koshland, 1967; Carraway & Koshland, 1972).

It should be noted that the carbodiimide reacts with both sulphydryl and tyrosyl OH groups as well as with carboxyl groups (Carraway & Triplett, 1970 and Carraway & Koshland, 1968). Tyrosine can be regenerated by treatment with hydroxylamine but successful regeneration of thiol groups has not been reported.

4.0.1.4 Modification of tryptophan residues

Oxidative bromination by N-bromosuccinimide (NBS) has been widely used for the modification of tryptophan residues giving rise to an oxindole moiety [Fig. 4.4].



indole oxindole

Fig. 4.4 Reaction of protein tryptophanal residue with N-bromosuccinimide (NBS) (R, protein).

As NBS is an extremely reactive reagent, side reactions with tyrosine, methionine, cysteine and sugars as well as peptide bond cleavage is possible if conditions are not stringently controlled (Spande & Witkop, 1967).

At low reagent to protein tryptophan ratios, in 50% aqueous acetic acid, it was found that 2-(2-nitrophenylsulphenyl)-3-methyl-3-bromo-indolenine (BNPS-skatole) reacts selectively with tryptophan residues converting these also to oxindole derivatives. Methionine is concomitantly converted to the sulphoxide and at high concentrations of reagent, selective cleavage of peptide bonds can occur (Fontana, 1972).

4.0.1.5 Modification of carbohydrate groups

The chemical modification pursued was not only confined to amino acid side chains but included carbohydrate side chains of sialidases as well because it is apparent that the carbohydrate moiety in glycoproteins play two important roles. The first is of a physicochemical order and concerns protein protection against proteases, the movement of ions and small molecules in tissues in the membrane regions. The second is of a biological order, the basis of which is essentially the notion of recognition of signals brought by the carbohydrate groups and consequently the notion of specific structure of these groups (Winterburn & Phelps, 1972; Montreuil, 1975; Hughes & Sharon, 1978).

The removal of carbohydrate moieties from most glycoenzymes (enzymes having carbohydrate residues covalently linked in their molecular structure (Pazur et al., 1969)), have been shown to have no effect on

their catalytic activities (Pazur et al., 1965; Lee & Hager, 1970; Yasuda et al., 1970) whereas a few have either displayed an increase or decrease in enzyme activity (Emmelot & Bos, 1965; Dziembor et al., 1970; Varute & Patil, 1971).

The principle linkages found between the carbohydrate and protein components of glycoenzymes are of the N-glycosyl and the O-glycosyl types (Pazur & Aronson, 1972). The components of the former linkage are normally asparagine covalently bound to N-acetylglucosamine and the latter involves either serine or threonine, linked either to N-acetylgalactosamine, mannose or glucose (Marshall, 1974). The mild procedures normally employed to investigate the significance of these residues involve the use of either glycosidases, periodate oxidations or β-elimination reactions (Fig. 4.5) (Spiro 1972; Li & Li, 1977; Zinn et al., 1977).

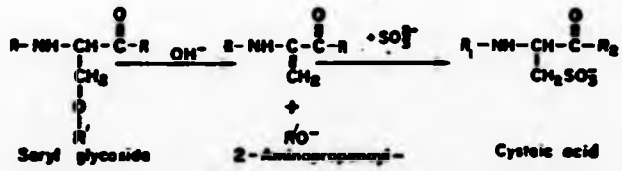


Fig. 4.5 Reaction of a seryl glycoside with alkali (β-elimination) in the presence of sulphite. (R, represents the protein; R', represents the carbohydrate moiety).

A fairly recent procedure for deglycosylating glycoproteins is the use of anhydrous hydrogen fluoride. According to Mort and Lamport (1977), it is possible to cleave all the linkages of neutral and acidic sugars or the O-glycosidic linkages leaving the N-glycosidic linkage and peptide bonds intact.

For the purpose of confirming that the chemical modification was absolutely specific, amino acid analysis is normally carried out on the protein before and after treatment. In the event of this not being possible for reasons such as (i) insufficient protein sample, (ii) the lack of the formation of an acid stable adduct with the group selective reagent or, (iii) the protein is a glycoprotein, (Eastoe, 1972; Niall, 1973 and Niall et al., 1973) cross checking of the results with different group selective reagents is essential and, where peptide bond cleavage is suspected, gel or paper electrophoresis would be an essential step.

Although it has been suggested that the ultimate procedure in determining the mechanism of enzyme catalysis, by chemical means, would be by affinity labelling (Glazer et al., 1975) the chemical modification process using group selective reagents still accounts for most of the papers published in this area. This is because of several reasons: firstly, minimal commitment of resources is necessary to initiate such studies and secondly, most of these site specific reagents are readily available and finally, affinity labels usually have to be synthesized - often by difficult routes. Although a successful site specific modification with these reagents is significantly less likely than with well defined affinity labels, many more experiments with these compounds are attempted and the results cross checked with other group selective reagents.

4.1 Experimental Procedure

4.1.0 Synthesis Of [2-^{3}H] Acetophenone (the starting material for phenyl[2-^{3}H]glyoxal)

To anhydrous tetrahydrofuran (2.5 ml) and diisopropylamine (0.49g, 4.9mMol) under an argon atmosphere was added 1.9 ml of 2.5M-n-butyllithium (standardized by the method of Kofron & Baclawski, 1976) in hexane and the mixture stirred for 30 min below 0°. Acetophenone (0.27g, 2.25mMol) and tetrahydrofuran (1 ml) were then added and the mixture stirred for a further 15 min. below 0° after which hexamethylphosphoramide (0.9 ml, 5mMol), was added. After 20 min , trifluoro [^{3}H] acetic acid (0.273 ml, 2.4mMol), prepared by treating ^3H_2O (5 Ci/ml; sp. radioactivity approx. 100mCi/mMol) with an equimolar amount of trifluoroacetic anhydride, was added and the reaction stirred for 2 hr at 21°. The reaction mixture was then acidified with 1M-HCl at 0° and the aqueous solution extracted with 3 x 20 ml of light petroleum (b.p. 40°-60°). The organic extracts were combined, washed with 5 x 10 ml of 1M-HCl, dried and evaporated in vacuo to give [2-^{3}H] acetophenone (0.27g), which was not purified further but was oxidized immediately.

4.1.1 Synthesis Of Phenyl[2-^{3}H]glyoxal

To a solution of sublimed SeO_2 (0,27g, 2.4mMol) in dioxan (0.84 ml) and water (4.5 1) at 50° was added [2-^{3}H]acetophenone (0,27g, 2.25mMol) and dioxan (0.5 ml). The mixture was heated at 80° for 6 hr and filtered through cotton wool, which was then washed with dioxan (20 ml). The

filtrate and washings were evaporated in vacuo and dry ethanol (2 ml) was added to the resulting oily residue. After being left for 2 days at 5°, the supernatant was carefully removed from the red precipitate that had formed and was evaporated in vacuo. The oily residue was dissolved in ethyl acetate (0.5 ml) and the phenyl[^{3}H]glyoxal purified by preparative t.l.c. with petrol/ethylacetate (1.1, v/v) as solvent. The phenyl[^{3}H]glyoxal was eluted from the silica with ethylacetate (50 ml), which was then concentrated in vacuo to 0.5 ml. The phenyl[^{3}H]glyoxal was rechromatographed as described earlier and the ethylacetate removed in vacuo to leave an oily residue. Final traces of ethylacetate were removed by repeated (5x) dissolution of the residue in ethanol (50 ml) and evaporation in vacuo. Unlabelled phenylglyoxal was synthesized in a similar manner.

4.1.2 Trial Experiments With The Phenyl[2-^{3}H]glyoxal

(i) Inactivation: The alkaline phosphatases and lactate dehydrogenase (LDH) were treated at 25° with various amounts of a solution of unlabelled phenylglyoxal hydrate (16 mg) in ethanol (100 µl) as described in table 4.2. Care was taken to use the same buffers for the inactivation as were for the assay. Samples were removed after fixed times and assayed. After inactivation, the modified enzymes were dialysed at 4° for 36 hr against the buffers indicated (3 x 5 litres), after which portions were removed and assayed.

(ii) Protection by substrate: The three enzymes in the presence of their substrates were treated with unlabelled phenylglyoxal hydrate as described in table 4.3 and then dialysed against buffer. The modified enzymes were then treated with 100-fold molar excess of phenyl[2-^{3}H]-glyoxal for 2 hr, the protein was precipitated on glassfibre discs with

trichloroacetic acid (Yang & Schwert, 1972) and their ^{3}H content determined.

4.1.3 Modification Of Amino Acid Residues In Sialidases

4.1.3.0 Active site protection and experimental conditions

In these experiments the sialidases were incubated with N-acetyl-neuraminic acid (NANA, 170 mg/ml) (S. griseus and V. cholerae enzymes) or sialyl-lactose (NL, 480 mg/ml) (C. perfringens enzyme) at molar ratios given in table 4.5 for 1.0 min in the specific buffers before treatment with the group specific reagents. In all cases, controls (including non-protective reactions), were set up as described under specific headings with omission of either the group specific reagent or enzyme. All the reactions were carried out at 37°.

4.1.3.1 Modification of arginine residues

4.1.3.1.0 (a) By phenylglyoxal

This was carried out on the three sialidases as described in the legends of fig. 4.8 (i) and (ii) for both the unprotected active centre and protected active centre.

4.1.3.1.1 By phenyl[2-^{3}H]glyoxal (Augustus & Hutchinson, 1979).

The active sites of the three sialidases were protected as described

above before the enzymes were treated with excess phenylglyoxal. The total volume of the reaction mixture was 150 µl. The excess unreacted phenylglyoxal and active site protector were removed by dialysis and the enzymes were treated with 100-fold molar excess of phenyl[2- ^{3}H]-glyoxal. After 2 hr the ^{3}H content of the protein was determined as previously described.

4.1.3.1.2 By 2,4, butanedione (Riordan & Scandurra, 1975)

The three sialidases from S. griseus, C. perfringens and V. cholerae (50, 20 and 25 µg respectively), were incubated in 0.125M sodium borate buffer pH 7.5 in the dark, containing butanedione (8.6 mg/100 µl) at molar ratios listed in table 4.4, the final volume being adjusted to 150 µl. After 1 hr the reaction mixture was diluted (10x) and the enzyme activity determined by the method of Warren (1959).

4.1.3.2 Modification of carboxyl residues

The methods of Carraway and Koshland (1972) and Braun et al., (1977) were used. The reagents and reaction conditions for both the unprotected and protected modification are stated in the legends of fig. 4.9 (i) and (ii) and tables 4.4 and 4.5.

4.1.3.3 Modification of tryptophan residues

4.1.3.3.0 By N-bromosuccinimide (Spande & Witkop, 1967)

Solutions of the three sialidases in sodium acetate buffer (2.0 ml, pH

4.0) were treated with N-bromosuccinimide (NBS concentrations Table 4.4). The reaction was dialysed after 1 hr against the appropriate assay buffer and the specific activities determined. Aliquots of the non-diffusable material were subject to polyacrylamide (10%) gel electrophoresis.

The protection of the active sites of the enzymes were carried out with sialyl-lactose in all three sialidases at concentrations listed in table 4.5.

4.1.3.3.1 By 2-hydroxy-5-nitrobenzylbromide (Fehske et al., 1978)

2-hydroxy-5-nitrobenzylbromide was added to the three enzymes (molar ratios given in table 4.4) in sodium acetate buffer (1.0 ml 0.1M pH 4.0) and the solutions incubated with shaking for 2 hr. The insoluble products were removed by centrifugation and the supernatants dialysed against the appropriate assay buffer after which the specific activities were determined. As above, aliquots were also subjected to polyacrylamide gel electrophoresis.

4.1.3.4 Modification of sulphydryl groups (Riordan & Vallee, 1972)

The three sialidases were incubated with N-ethylmaleimide (NEM) in sodium phosphate buffer (1.0 ml, 0.1M pH 7.0) for 1.5 hr. (Table 4.4). Aliquots were removed at various time intervals and the reaction stopped by the addition of 2-mercaptoethanol at twice the molar excess of NEM. The samples were dialysed against phosphate buffer before determining the specific activities.

4.1.3.5 Modification of lysine residues

4.1.3.5.0 By 2,4,6-trinitrobenzenesulphonic acid (Fields, 1972)

To solutions of the three enzymes in 0.1M sodium phosphate buffer (2.0 ml, pH 7.0) were added recrystallized 2,4,6 trinitrobenzenesulphonic acid at various concentrations (molar ratios Table 4.4). Samples of the reaction mixture were removed at fixed time intervals and the reaction was stopped by the addition of excess lysine. These were dialysed before determining the specific activity.

The modification was carried out in the presence of NANA and NL as previously described (molar ratios are represented in Table 4.5).

4.1.3.5.1 By O-methylisourea (Habeeb, 1972)

To solutions of the sialidases in 0.2M glycine NaOH buffer (2.5 ml, pH 10.5) was added a freshly prepared aqueous O-methylisourea (0.6M, pH 10.5). The molar ratios are given in table 4.4. The solutions were incubated for 24 hr at 6° during which time aliquots were removed, dialysed against sodium phosphate buffer pH 8.5 for 36 hr. They were then assayed for sialidase activity.

4.1.3.6 Isolation of the active site peptide

The overall strategy adopted for the isolation of the active site peptide of the three sialidases is illustrated in figure 4.6.

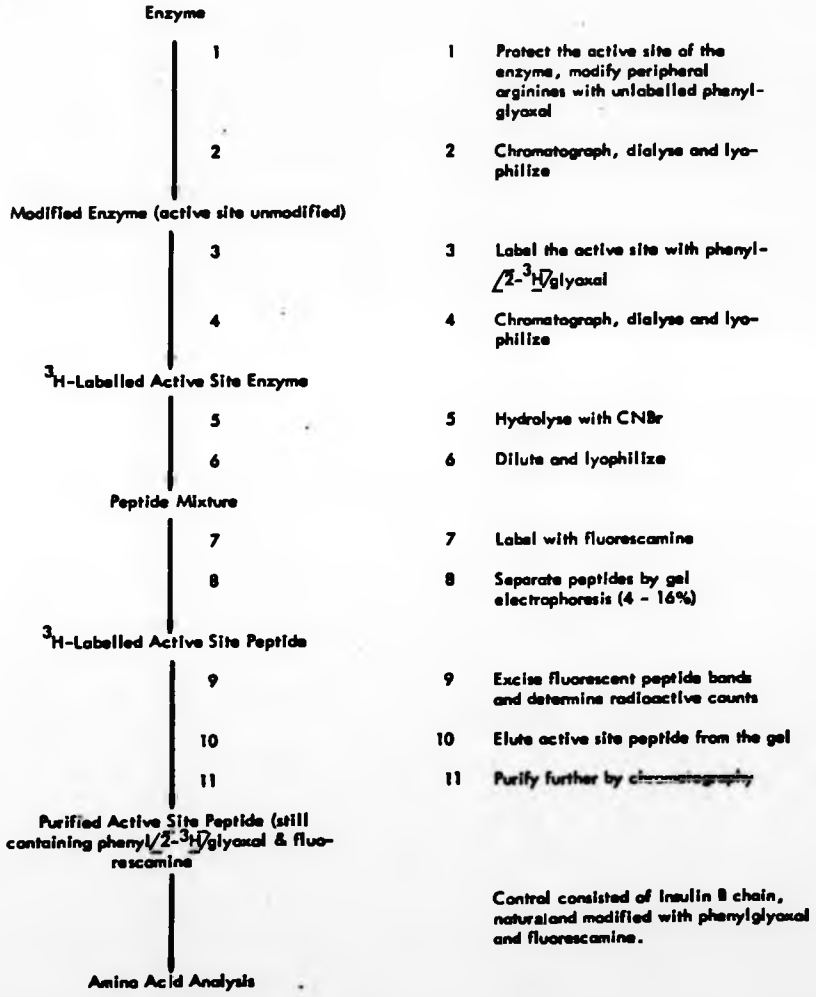


Fig. 4.6 Strategy adopted for the isolation, identification and purification of the active site peptides in *S. griseus*, *C. perfringens* and *V. cholerae* sialidases

Differential labelling with phenylglyoxal (unlabelled and labelled)

Differential labelling of the enzymes (500μg each) was carried out as described previously under the heading, "modification of arginine residues", the only difference being that the excess reagent was removed by gel-chromatography (Sephadex G-25, column dimension 1.2 x 45 cm), followed by dialysis and lyophilization.

Cynogenbromide (CNBr) cleavage of the modified enzymes

The modified enzymes were suspended in 70% formic acid (400 μl) to which solid CNBr was added (molar excess of 300). Half the required CNBr was added at zero time and the remainder after 12 hr and the reaction was allowed to proceed for 24 hr at 21° (Kurosky et al., 1976, Gross, 1967 and Augustus, 1977). The reaction was then diluted (10 x) with distilled water and lyophilized.

Detection and purification of peptides

The CNBr-peptides were dissolved in 0.05M borate buffer, pH 8.1, containing 50% (v/v) glycerol (100 μl) and freshly prepared fluorescamine (5 μl, 30 mg/ml acetone) was added while shaking vigorously on a vortex mixer. The reaction mixture was allowed to stand at 21° for 10 min (Rosemblatt et al., 1975) and applied to a polyacrylamide gel (discontinuous 4-12-16%). Electrophoresis was carried out in the dark as described earlier. The peptides were visualized under longwave ultraviolet light. The fluorescent peptides were excised and samples counted to determine the radioactivity. The radioactive peptide was

extracted from the macerated gel by diffusion at 37°, in 0.005M sodium bicarbonate buffer containing 0.05% SDS and a bacteriostat. After 24 hr the acrylamide fragments were removed by centrifugation and the supernatant lypholized (Weiner et al., 1972). The peptides were subjected to gel permeation chromatography (Sephadex G-10 and Bio-Gel P-2) in 35% formic acid. The peptide containing fractions were further purified by descending paper chromatography (Whatman 54) the solvent being butanol-acetic acid water (5:2:3 v/v). After locating the peptide, eluting and lyophilizing it, it was subjected to amino acid analysis. The controls consisted of (i) phenylglyoxal modified arginine, (ii) fluorescamine modified lysine, (iii) natural insulin B chain and (iv) phenylglyoxal and fluorescamine modified insulin B chain.

4.1.3.7 Modification of carbohydrate residues in sialidases

Periodate oxidation of C. perfringens sialidase (and its action on different substrates) (Steward et al., 1977)

The reagents and methods are described in the legend of figure 4.17 (i) and (ii).

4.1.3.7.0 Periodate oxidation of the three sialidases

For the method see the legends of figure 4.17 and 4.16 (i + ii).

Periodate oxidation of V. cholerae sialidase for polyacrylamide gel electrophoresis

V. cholerae sialidase (1 mg) was allowed to react with 0.1M sodium metaperiodate (450 µl) in 0.1M sodium acetate buffer, pH 4.5 at 4° in the dark for 24 hr. The reaction was stopped by the addition of 50% ethandiol (50% v/v). This was mixed very rapidly and continued to react for 30 min. and then dialysed against 0.001M sodium phosphate buffer, pH 7.0 (48 hr, 2 x 5 lts). The retentate was lyophilized and subjected to SDS-polyacrylamide (10%) gel electrophoresis, after which the gel was halved longitudinally and stained for protein and carbohydrate as described in Chapter 3. The control contained no periodate.

4.1.3.7.1 β-elimination on V. cholerae sialidase for polyacrylamide gel electrophoresis (Simpson et al., 1972; Birken & Canfield, 1977)

V. cholerae sialidases (1 mg) was reacted with 0.2M sodium sulphite in 0.1M NaOH (500 µl) for 24 hr. A control containing sialidase (0.5 mg) in the absence of sodium sulphite was set up. The reactions were stopped by the addition of glacial acetic acid (10 µl) and dialysed, lyophilized, electrophoresed and stained as above. The reaction was carried out at 25°.

β-elimination with [^{35}S] sodium sulphite on V. cholerae sialidase (Simpson et al., 1972; Birken & Canfield, 1977)

V. cholerae sialidase (6.9 g, 10 µl) suspended in 0.1M NaOH (90 ul) containing [^{35}S] sodium sulphite (10 µl, specific radioactivity 0.8 Ci/ Mole, prepared by the method of Tanase & Shikata,(1976) was allowed to react for 10 hr at 25°. The reaction was stopped by the addition of glacial acetic acid (10 µl). The solution was lyophilized resuspended in 20% trichloroacetic acid (TCA) (10 µl) and deposited on Whatman GCF discs and washed in vacuo with 20% TCA (500 ml) and the radioactivity associated with the disc determined.

4.2 Results And Discussion

Synthesis of phenyl[2- ^{3}H]glyoxal

The synthesis of phenyl[1- ^{14}C]glyoxal is unsatisfactory as the material which is prepared by the oxidation of [1- ^{14}C]acetophenone, is expensive and comparatively difficult to synthesize. (Dauben et al., 1950). The synthesis of [2- ^{3}H]acetophenone was achieved by a development of a method introduced for the alkylation of aliphatic carboxylic acids (Pfeffer & Silbert, 1970). In our synthesis, the ^{3}H originates from $^{3}H_2O$ which is easily converted into [^{3}H]CF_3COOH, thereby considerably decreasing the cost of isotopically labelled acetophenone. Under our labelling conditions, few side reactions occur and hence the [2- ^{3}H]acetophenone can be obtained in almost quantitative yield. The position of isotopic exchange was confirmed by ^{1}H n.m.r. spectroscopy with [2-^{2}H] acetophenone prepared in an analogous manner from trifluoro [^{2}H] acetic acid. The signal due to the acetyl group at 2.6 p.p.m. was greatly diminished as it is now deuterated.

The [2-^{3}H] acetophenone was oxidized by SeO_2 to yield phenyl[2-^{3}H]-glyoxal (76%, based on E_{250nm} of 9000 litre $mol^{-1}cm^{-1}$ in ethanol) with specific radioactivity 12.2 mCi/mMol. The phenylglyoxal ran as a single U.V.-absorbing spot on silica t.l.c. with petrol/ethyl acetate (1:1, v/v) as developing solvent with the same mobility as authentic phenylglyoxal. The ^{1}H n.m.r. phenyl[2-^{3}H]glyoxal was identical with that of authentic material with a singlet at 9.55 p.p.m. due to the aldehydic proton. This singlet was absent in the n.m.r. spectrum of phenyl[2-^{2}H]glyoxal, prepared as described above from [2-^{2}H]aceto-phenone, confirming the position of isotopic substitution. The phenyl[2-^{3}H]glyoxal decomposed slowly (30% in 3 months) when stored as a 1M solution in dry ethanol at 4°. Little decomposition occurred when the phenylglyoxal was stored as an oil or as the hydrate. The latter compound could be obtained by crystallization from a concentrated aqueous solution at 4°.

The oxidation of acetophenone by SeO_2 proceeds via the formation of an enol selenite ester that rearranges and then decomposes to phenylgly-glyoxal, two protons being lost during the reaction sequence (Fig. 4.7) Corey & Schafer, 1960).

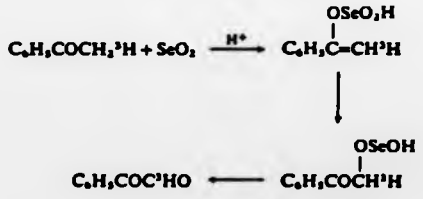


Fig. 4.7 Reaction sequence during the synthesis of phenylglyoxal

In the synthesis of phenyl[2- ^{3}H]glyoxal, the retention of ^{3}H may be assisted by favourable isotope effects operating in the two proton-abstraction steps.

Trial use of phenyl[2- ^{3}H]glyoxal

The phenyl[2-^{3}H]glyoxal was used to determine the number of arginine residues in the active centres of two alkaline phosphatases and lactate dehydrogenase. The interaction of the enzymes with unlabelled phenylglyoxal was investigated over a molar range (phenylglyoxal/enzyme) of $0.7 \times 10^{3} - 10^{5}$ (Table 4.2), assaying at fixed time intervals until the inactivation was constant. The enzymes were then dialysed and reassayed and in all cases the inactivation was irreversible with molar ratios of 3×10^{3} or greater. The rate of inactivation was less at pH 5.3 than at 7.5. Once the conditions for rapid inactivation had been determined, the inactivation was carried out on the enzymes in the presence of substrates. The concentrations of the latter substrates derived from the turnover number for each enzyme and were chosen so as to provide maximum protection of the active site. After 5 min the substrates and excess phenylglyoxal were removed by dialysis and the enzymes were treated with phenyl[2-^{3}H]glyoxal (Table 4.3). The number of molecules of phenyl[2- ^{3}H]glyoxal that bound to each enzyme molecule was then determined. This value is twice the number of arginine residues modified (Takahashi, 1968) and hence the number of arginine residues at or near the active centres of the enzymes can be calculated. The values obtained for calf intestinal (1.8) and *E. coli* (2.1) alkaline phosphatases and lactate dehydrogenase (3.3) are close to that (2) determined by Daemen and Riordan (1974) for *E. coli* alkaline phosphatase and 3 for beef heart lactate dehydrogenase as

Table 4.2

Inactivation of enzymes with phenylglyoxal

Enzyme	Buffer	Slow inactivation		Rapid inactivation	
		Molar ratio of phenylglyoxal to protein	Time for 75% inactivation (min)	Molar ratio of phenylglyoxal to protein	Time for 100% inactivation (min)
E. coli alkaline phosphatase[1] (mol.wt. 89,000)	0.125M-$NaHCO_3$ (pH 7.5)	1×10^3	256	3.3×10^3	60
Calf intestine alkaline phosphatase[1] (mol.wt.140,000)	0.125M-$NaHCO_3$ (pH 7.5)	1×10^3	240	3.3×10^3	60
C. perfringens sialidase (mol.wt. 56,000)	0.1M-Sodium acetate (pH 5.3)	6×10^4	180	1.6×10^5	96
Beef heart lactate dehydrogenase[2] (mol.wt. 144,000)	0.1M-Sodium phosphate (pH 7.4)	7×10^2	53	5.0×10^3	18

1 Assayed by the method of Ghosh & Fishman (1968)

2 Assayed by the method of Reeves & Fimognari (1963)

Table 4.3

Protection of active sites of enzymes during rapid labelling by phenylglyoxal followed by [^{3}H]phenylglyoxal

The buffers used were the same as in Table 4.2

Enzyme	Substrate	Molar ratio of phenylglyoxal to enzyme	Molar ratio of substrate to enzyme	Time for reaction (min)	Molar ratio of phenyl[2-^{3}H] - glyoxal bound to enzyme	Number of arginine residues/active site
Alkaline phosphatase[1] (E. coli)	Phenyl phosphate	1×10^5	10^5	5	4.23	2.12
Alkaline phosphatase[1] (calf intestine)	Phenyl phosphate	1×10^5	10^5	5	3.56	1.80
Lactate dehydrogenase[2]	Sodium pyruvate	2×10^4	7×10^4	1	6.62	3.31

1 In the presence of $MgCl_2$ (5 mol/mmol enzyme)

2 In the presence of NADH (20 mol/mmol enzyme)

determined by Yang and Schwert (1974).

Modification Of The Amino Acid Residues In Sialidases

The effect of group specific chemical modification reagents on the three sialidses are listed in table 4.4 and illustrated in figures 4.8 (i) and 4.9 (i), while table 4.5 and figures 4.8 (ii) and 4.9 (ii) respectively, list and illustrate effects of the same reagents on the three sialidases in the presence of the active site protecting agents.

Treatment of the three sialidases with N-ethylmaleimide did not cause any significant diminution of enzymic activity, suggesting that sulphydryl groups are not catalytically important. These findings are in agreement with those of Mohr (1960) and Holmquist (1975a) for the V. cholerae sialidase, Bachmayer (1972) for C. perfringens sialidase, and Wang et al., (1978) and Uchida (1979) for both Arthrobacter species. However, Kunimoto et al., 1974 have shown that while sulphydryl groups are not catalytically important for the S. griseus sialidase, the C. perfringens enzyme is inactivated by p-chloromercuribenzoate - also known to react with other amino acids (Riordan & Vallee, 1972). We therefore conclude that sulphydryl groups are not essential for catalytic activity.

Previously, Kabayo and Hutchinson (1977) have observed that the sialidase from S. griseus is unaffected by the lysine modifying reagent trinitrobenzene sulphonic acid. We now observe that partial inactivation does occur when the sialidases of C. perfringens (25%) and V. cholerae (20%) are treated with this reagent whereas Wang et

Table 4.4

Effect Of Group Specific Reagents On Sialidases

Enzyme	Reagent	Molar Ratio Reagent/Enzyme	Time	% Residual Activity
S[a]	Phenylglyoxal	1.2×10^3	1	14
C[b]	Phenylglyoxal	3.0×10^3	1	25
V	Phenylglyoxal	2.5×10^3	1	8
S	Butanedione	9.0×10^2	1	25
V	Butanedione	9.0×10^3	1	15
C	Butanedione	9.0×10^3	1	35
S	EDC[1] + glycine ester	1.0×10^4	4	2
C	EDC + glycine ester	1.0×10^4	4	5
V	EDC + glycine ester	1.0×10^4	4	17
S	NBS[2]	40	1.0	2.4
C	NBS	50	1.0	10
V	NBS	60	1.0	30
S	HNB[3]	6.0×10^3	2.0	20
C	HNB	6.0×10^3	2.0	10
V	HNB	6.0×10^3	2.0	3
S	NEM[4]	1.0×10^3	1.5	100
C	NEM	1.0×10^3	1.5	100
V	NEM	1.0×10^3	1.5	100
S	TNBS[5]	1.0×10^3	2.0	100
C	TNBS	2.0×10^3	2.0	75
V	TNBS	5.0×10^3	2.0	80
S	MIU[6]	1.0×10^3	24	95
C	MIU	1.0×10^3	24	100
V	MIU	1.0×10^3	24	100

a S. griseus sialidase mol. wt. = 32,000
b C. perfringens sialidase mol. wt. = 56,000
c V. cholerae sialidase mol. wt. = 69,000
2 N-bromosuccinimide
3 5-hydroxy-5 nitro benzyl bromide
4 N-ethylmaleimide

Table 4.5

Effect Of Group Specific Reagents On Sialidases With Active Site Protection

Enzyme	Reagent	Molar Ratio Reagent/Enzyme	Protecting Agent	Molar Ratio Protecting Agent/Enzyme	Time	% Residual Activity
S	Phenylglyoxal	3.1×10^3	NANA[1]	1.6×10^3	1.0	88
C	Phenylglyoxal	3.5×10^3	NL[2]	1.3×10^5	1.0	94
V	Phenylglyoxal	3.0×10^3	NANA	1.3×10^4	1.0	60
S	EDC	1.0×10^4	NANA	3.8×10^4	4.0	88
V	EDC	1.0×10^4	NL	4.8×10^5	4.0	84
C	EDC	1.0×10^4	NANA	6.1×10^5	4.0	74
S	NBS	40	NL	1.4×10^3	1.0	67
V	NBS	50	NL	1.4×10^4	1.0	79
C	NBS	40	NL	1.4×10^4	1.0	57
C	TNBS	2.0×10^3	NL	2.4×10^5	2.0	69
V	TNBS	5.0×10^3	NL	3.0×10^4	2.0	82

1 N-acetylneuraminic acid
2 Sialyl-lactone

al., (1978) observed only 11% inactivation for the Arthrobacter enzyme. If the modification of the C. perfringens and V. cholerae sialidase is modified by trinitrobenzenesulphonic acid in the presence of substrate, approximately the same percentage of inactivation is achieved as in the absence of protecting agent (substrate). This suggests that this could be because of a change in the tertiary structure of these enzymes due to the modification of non-specific lysine residues. Further, when the modification is carried out with O-methylisourea, virtually no loss of enzyme activity is observed for the three enzymes. Since O-methylisourea reacts only with ϵ-amino groups of lysine residues at pH 10.5, (Habeed, 1972) and not with N-terminal amino groups, we therefore concluded that lysine residues are not important in the catalytic function.

The most pronounced inactivation of all three sialidases was obtained with reagents which modify arginine, carboxylic and tryptophan amino acid residues.

In order to ascertain the location, function and number of catalytically active or binding amino acid residues in or near the active sites of these enzymes, the modification reactions were carried out in the presence of a competitive inhibitor or a substrate of the enzymes as these should protect the vicinity of the active site.

The enzymic activities of all three sialidases were maintained if we preincubated the enzymes with an inhibitor or substrate before treatment with phenylglyoxal (Fig. 4.8 (ii)), suggesting that arginine residues are in or near the active sites of the sialidases. This was in agreement with De Brock's (1957) results, in which influenza sialidase was inhibited by diols.

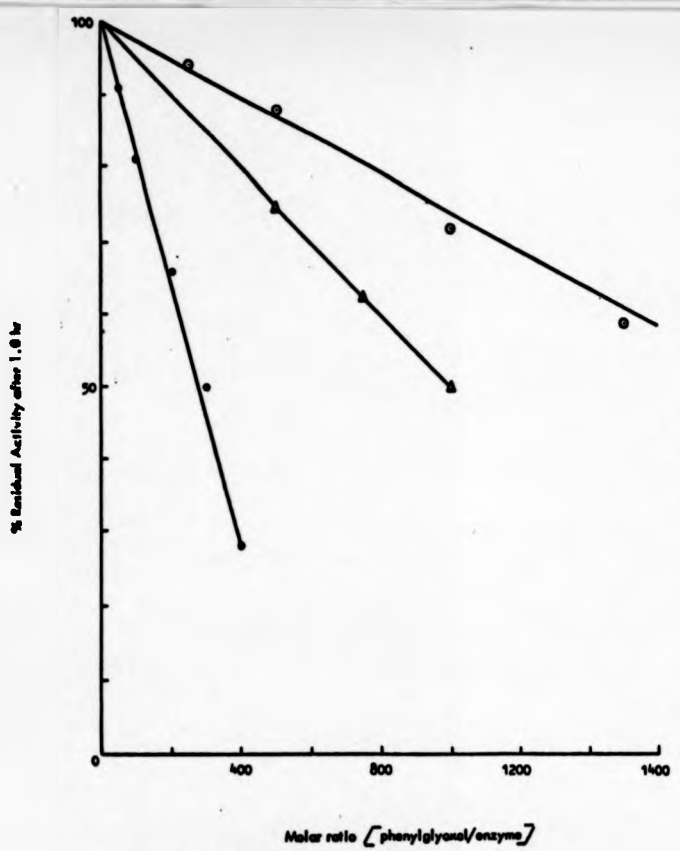


Fig. 4.8 (I) Inactivation of sialidases by phenylglyoxal.

Sialidases from S. griseus (174 µg, o—o), C. perfringens (56 µg, ⊙—⊙) and V. cholerae (15 µg, Δ—Δ) in 0.1M N-ethylmorpholinium acetate buffer pH 8.0 were treated with varying amounts of phenylglyoxal in ethanol, the final volume being adjusted to 50 µl with acetate buffer. After 1 hr., the reaction was terminated by adding arginine (at half the initial molar concentration of phenylglyoxal). After 15 min. the appropriate enzyme assay buffer (360 µl) was added and the enzymic activity determined as described in the text.

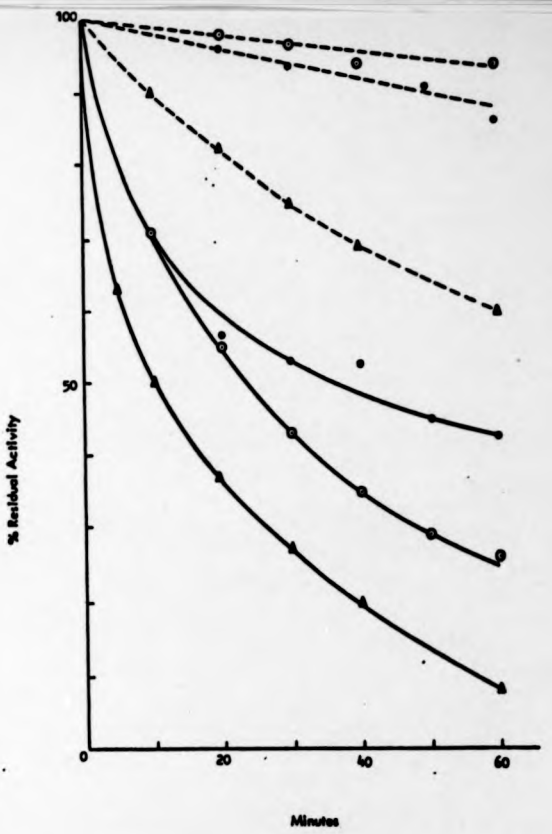


Fig. 4.8 (II) Inactivation of sialidases by phenylglyoxal in the presence of active site protecting agents.

The reaction was set up as in Fig. 4.8 (I) but in the presence of NANA and NL at concentrations listed in table 4.5. Aliquots were removed at fixed time intervals, the reaction stopped and the activities determined as above. The enzyme in the presence of protecting agent is represented by (----). The remaining symbols are represented as in Fig. 4.8 (I).

These results were further substantiated by using phenyl[2-^{3}H]glyoxal (Augustus & Hutchinson, 1979) when it was shown that two arginine residues in the C. perfringens sialidase were protected against modification by unlabelled phenylglyoxal in the presence of sialyl-lactose (followed by dialysis and reacting with tritiated phenylglyoxal, differential labelling). In addition to this, it was shown that for the sialidases from S. griseus and V. cholerae, one arginine residue is protected in each case (Table 4.6).

Table 4.6
Labelling Of The Active Sites Of Sialidases With Phenyl[2-^{3}H]glyoxal

Enzyme source	% Enzymic activity after dialysis	Molar ratio of phenyl[2-^{3}H]-glyoxal bound to enzyme	Number of arginine/Residues
S. griseus	83	1.90	0.85(1)
C. perfringens	85	3.74	1.85(2)
V. cholerae	70	1.60	0.80(1)

1 mole of arginine combines with 2 moles of phenylglyoxal (Takahashi, 1968). The numbers in brackets indicate the number of arginines labelled corrected to the nearest whole number.

All three sialidases were inactivated by treatment with glycine ethyl ester hydrochloride and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Fig. 4.9(i)). However, only incomplete protection was afforded by preincubation with N-acetylneuraminic acid (sialidases from S. griseus and V. cholerae) or sialyl-lactose (sialidase from C. perfringens) (Fig. 4.9 (ii)). As most of the amino acids in these enzymes are acidic amino acid residues, (Kabayo & Hutchinson, 1977; Wang et al., 1978 and Geisow, private communication). This loss of activity may be due to modification of carboxyl groups remote from the active site which would cause changes to the tertiary structure of the enzymes. The very large molar excess of modifying reagents required for inactivation suggests that many carboxyl groups may be involved in the activation process. Support for the requirement for intact carboxyl

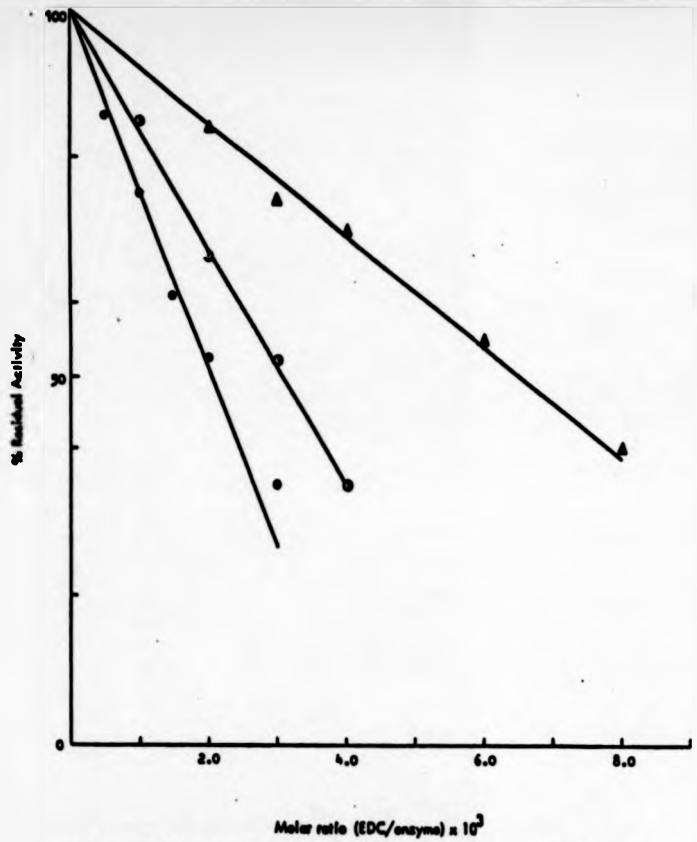


Fig. 4.9 (I) Inactivation of sialidases by 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and glycine ethyl ester.

Solid EDC was added to sialidases (obtained from S. griseus 15 µg, o—o C. perfringens, 50 µg, ⊙—⊙ and V. cholerae, 50 µg Δ—Δ) in buffer (1.0 ml, pH 5.0) containing glycine ethyl ester (1.5mM). The pH was maintained at pH 5.0 by automatic titration with 1M HCl. After 1 hr the reaction was stopped by the addition of 1.0M sodium acetate buffer pH 5.0. The samples were dialysed and the specific activity determined as described.

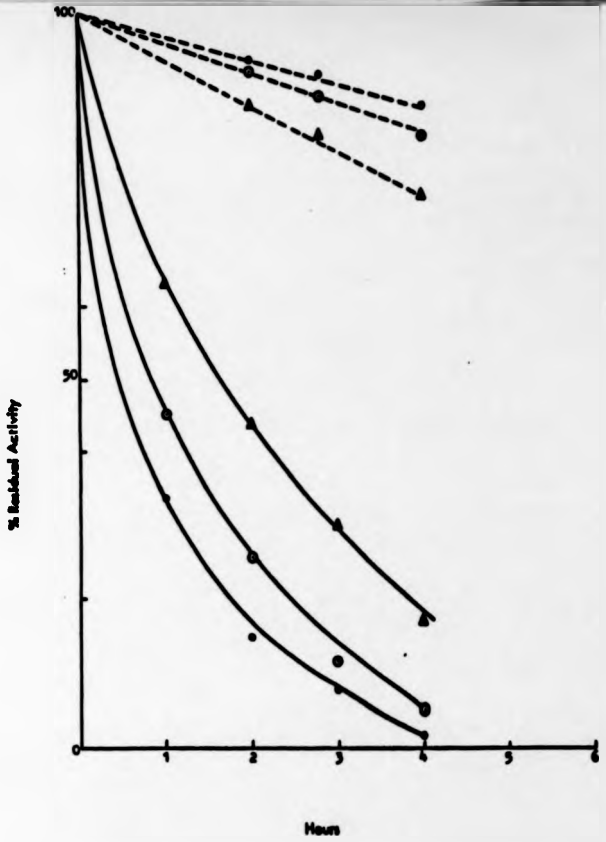


Fig. 4.9 (II) Inactivation of sialidases by 1-ethyl-3(3 dimethyl-aminopropyl) carbodiimide hydrochloride and glycine ethyl ester in the presence of active site protecting agents.

The reaction was set up as in Fig. 4.9 (I) but in the presence of NANA or NL at concentrations listed in table 4.5. Aliquots were removed, dialysed and the specific activity determined. The enzyme in the presence of protecting agent is represented by (----). The remainder of the symbols are represented as in Fig. 4.9 (I).

groups for full activity in V. cholerae sialidase is provided by Holmquist (1975b), who completely inactivated the enzyme using ethyl diazoacetate even in the presence of acetic acid which suggests that some carboxyl groups in this enzyme must be very reactive.

When the sialidases from S. griseus and C. perfringens were incubated with an active site agent (substrate sialyl-lactose in both cases - Table 4.5) prior to treatment with N-bromosuccinimide, their enzymic activities were decreased by 27% and 30% respectively. However preincubation with N-acetylneuraminic acid did not protect the V. cholerae sialidase from N-bromosuccinimide (70% loss of activity) (Groundwater, 1978) but, in the presence of sialyl-lactose, the loss of activity was reduced to 37%. As N-acetylneuraminic acid is capable of completely protecting an essential arginine in the active site of V. cholerae sialidase, the failure to protect the enzyme from inactivation by a tryptophan-modifying reagent suggests that the crucial tryptophan(s) do not occur near the active site or are in relatively exposed positions on the edge of the active site, namely at a position where it probably binds with the aglycone moiety. The inactivation of the three sialidases in the absence of protecting agent was also observed when using 2-hydroxy-5-nitrobenzyl bromide (Table 4.4). The inactivation by N-bromosuccinimide of sialidases from C. perfringens (Bachmayer, 1972) and A. sialophilus (Wang et al., 1978) in the absence of active site protecting agents has been reported and we confirm these observations with the sialidases from S. griseus, C. perfringens and V. cholerae.

The results of the modification studies described here for sialidase obtained from pathogenic and non-pathogenic sources indicate that these three enzymes have common amino acid residues necessary for their

activity, namely arginine, tryptophan and carboxylic amino acid residues.

Amino Acid Analysis

The amino acid analysis was performed on the peptides modified by phenylglyoxal, on the three enzymes, obtained by the procedure outlined in figure 4.6.

In our initial attempt at characterizing the CNBr-peptides obtained from S. griseus sialidase by polyacrylamide gel electrophoresis, problems were encountered, the major problems being insolubility of some of these peptides and the loss of others by diffusion during the staining and destaining of the gels. This indicated the presence of small peptides. Further, these peptides could not be detected by the ultraviolet spectroscopic method available for ion exchange and permeation chromatography, because of low peptide yields and the low ultraviolet absorbance of the peptide.

Because the primary aim was to sequence the active site peptide manually, it was decided to bypass the characterization step and label the active site directly via the method outlined in figure 4.6, but without the fluorescamine tag. Only one major radioactive peak was revealed by subjecting the 2 mm gel slices to radioactive counting. When this peak was checked for peptide purity by subjecting it to descending paper chromatography, two ninhydrin spots were observed, indicating either an impurity or a very closely accompanying peptide in the vicinity of the radioactive peptide in the gel. The latter was confirmed by following the procedure outlined in figure 4.6 but with the inclusion of the

fluorescamine label. The gels further revealed that the molecular weights of the active site peptides of the three sialidases were similar (smaller than 670 as they were migrating ahead of the bromophenol blue marker, mol. wt. 670) (Fig. 4.10).

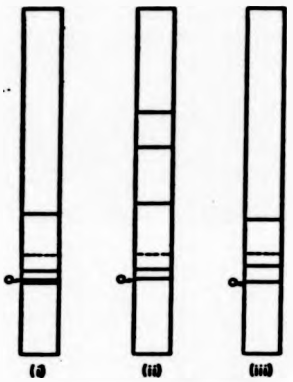


Fig. 4.10 Schematic representation of the fluorescent soluble CNBr-peptides of S. griseus (i) C. perfringens, (ii) and V. cholerae (iii) sialidases. (----) bromophenol blue marker (O—) radioactive peptide.

Similar modification experiments were performed on the insulin B chain (as it has only one arginine and one lysine) (Fig. 4.11).

H-Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-

Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr-OH

Fig. 4.11 Amino acid sequence of the insulin B chain (mol)

Hence we were able to determine the effect of the phenyl[2-^{3}H]glyoxal modification in the vicinity of the active site but were unable to pursue the sequence analysis because of the fluorescamine blocked

N-terminal residue and secondly, the results of histidine and glycine were suspect because of the presence of unknown contaminants.

Nevertheless, the results in table 4.7 confirmed the presence of arginine being involved in the active site of the three sialidases. Further, the number of arginines in the active site of S. griseus and V. cholerae sialidase verified our previous labelling experiments but not that of C. perfringens sialidase where we determined two arginines by labelling and one by amino acid analysis. The reason for the latter is unknown and open to speculation.

The amino acid analysis of these active sites further revealed the presence of glutamic acid the other probable amino acid involved in the catalytic mechanism (the phenomenon of protein folding is not overlooked). It does not, however, reveal the presence of the third type of amino acid involved in the active site, namely tryptophan, as this was not determined.

Table 4.7

Amino Acid Analysis[1] Of The Active Site Peptides Obtained From S. griseus, C. perfringens & V. cholerae sialidases

Amino Acid	S[a]	C[b]	V[c]
Threonine	0.66(1)	0.80(1)	0.50(1)
Serine	0.50(1)	0.58(1)	0.54(1)
Glutamic acid	1.40(1)	1.30(1)	0.80(1)
Proline	0	0.50(1)	0
Glycine	Nd	Nd	Nd
Histidine	0.60(1)	Nd	Nd
Arginine	0.70(1)	0.90(1)	1.20(1)
N-terminal	Nd	Nd	Nd

[1] Amino acid analysis was performed by Dr J E Fox, Macromolecular Analysis Service, Birmingham University.

a S. griseus sialidase

b C. perfringens sialidase

c V. cholerae sialidase

Nd Not determined (refer to text)

Number in parenthesis indicates nearest whole number

Thus, before any function is assigned to these essential active site residues, (viz. arginine, tryptophan and carboxylic acid), two major dictates (by the enzyme) have to be fulfilled by the substrate: (i) the terminal carboxyl group must be free (Brossmer & Holmquist, 1971 and Holmquist & Brossmer 1972) and (ii) the polyhydroxy side chain must be intact and unsubstituted for maximum enzyme activity (Schauer & Faillard, 1968). Because of these requirements, two different types of binding residues appear to be required, one of which offers an electrostatic interaction to the anionic substrate (Brossmer et al., 1974), the other offering a hydrophobic interaction. From our investigations, the only positively charged residue which can bind electrostatically to the free carboxyl group of the substrate is arginine. This is highly favourable as there are many enzymes acting in this manner on negatively charged substrates, of which the prime example would be carboxypeptidase A (Lipscomb et al., 1968). The hydrophobic interaction on the other hand, can be afforded by the tryptophan residues as it has been shown that these tryptophans can participate in the carbohydrate-protein binding interaction in some carbohydrate-protein binding proteins eg. lysozyme (Blake et al., 1967), α -mannosidase (Paus, 1978) and lectin (Privat et al., 1976). Thus it is possible that a hydrophobic side chain such as tryptophan could interact with the polyhydroxy side chain of the substrate and, further to this, a reduction of the polyhydroxy side chain by periodate oxidation to C-8 leads to a decrease in enzyme activity and further oxidation to C-7 leads to total loss of enzyme activity (Suttajit, 1970), indicating that the loss of the polyhydroxy side chain of the substrate diminishes the stabilizing hydrophobic interaction with tryptophan.

The unassigned function of the carboxylic amino acid residue leads

us to speculate on its role as a catalytic residue as opposed to a binding residue. Factors favouring this catalytic role of the carboxylic amino acid residues are based on the following observations that (i) the α-ketosidic bond in the substrate can be cleaved by an acid catalysed reaction. (ii) The pH (4.5 - 6) dependence of the enzyme catalysed reaction infers the presence of a dissociable group in the protonated form to act as a proton donating species. As histidine (pKa 6.0) has been ruled out as a catalytic site residue (Kabayo, 1978 and Groundwater, 1978), the only other group within that pKa range are the carboxylic amino acid residues. (iii) The polyhydroxy side chain of NANA α-ketosides modified with anionic groups are neither substrates nor inhibitors of the enzyme, inferring the presence of free carboxyl groups in the active centre of sialidase as well as the presence of an active site pocket and the line of approach of the substrate (Fig. 4.12) (Holmquist, 1975b). (iv) Besides our report showing that carboxyl groups are involved in the active site, Holmquist (1975a) and Geisow (private communication), have shown by experimentation that carboxyl residues play an essential role in the active site of sialidases. (v) The catalytic mechanism of glycosidases such as lysozyme (Blake et al., 1967), sucrose-isomaltase (Braun et al., 1977) and Aspergillus wentii β-glucosidase (Bause & Legler, 1973) proceeds via the action of carboxylic amino acid residues.

The most convincing evidence which would support the involvement of carboxylic amino acid residues in the active site of sialidases has to meet the criteria established by Cordes and Bull (1974) i.e. that the acid catalysed hydrolysis of glycosidic and ketosidic bonds, a unimolecular reaction involving the fission of the carbonyl-carbon-oxygen bond, must generate the cyclic carbonium of oxocarbonium ion as an intermediate. Meindl and Tuppy (1969b) have reported such a compound

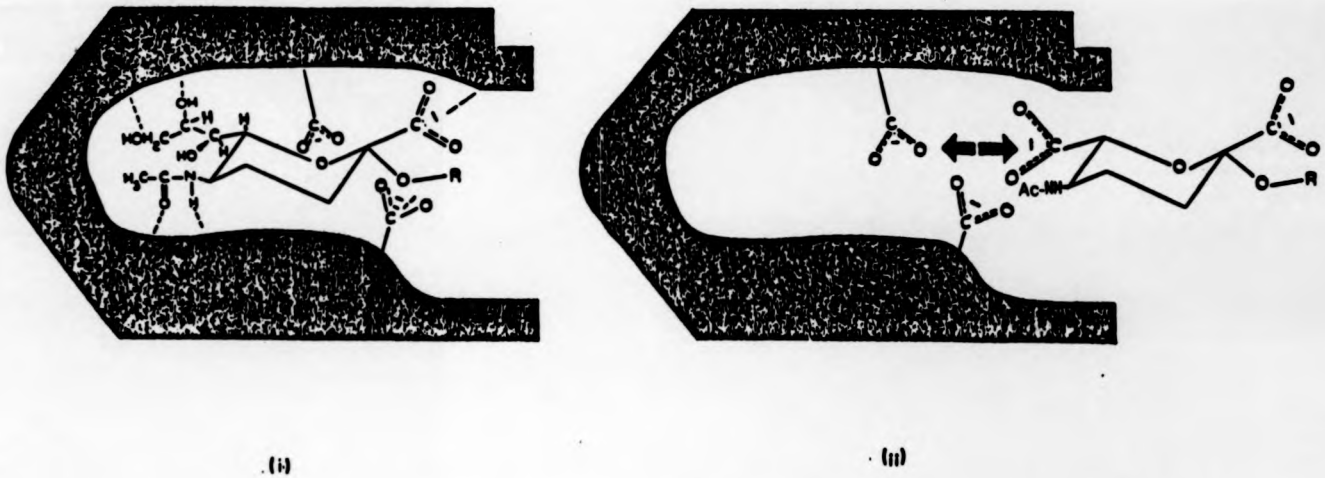
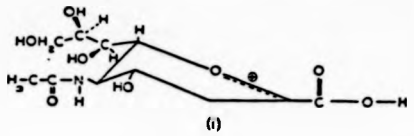
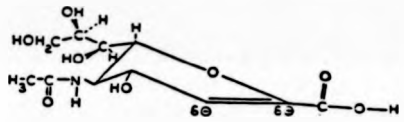


Fig. 4.12 Attempted illustration of (I) the α-ketoside of N-acetylneuraminic acid situated in a hypothetical active centre of sialidase, and (II) repulsive forces between sialidase and the α-ketoside of N-acetylneuraminic acid carrying an anionic site instead of the polyhydroxy side chain.

that resembles the oxocarbonium intermediate (Fig. 4.13 (i)) namely 2,3- dehydro-2-deoxy-N-acetylneuraminic acid (Fig. 4.13 (ii)), which inhibits V. cholerae sialidase very strongly thus functioning as a model of a possible transition state structure.



(i)



(ii)

Fig. 4.13 (i) Structure of the cyclic oxocarbonium ion of N-acetylneuraminic acid.

Fig. 4.13 (ii) Structure of the 2-Deoxy-2, 3 dehydro-N acetylneuraminic acid

Because of this strong evidence and those stated above (i - v), one can propose that the carbonyl-carbon-oxygen cleavage goes via the initial steps outlined by (A) Cordes and Bull (1974) [(i) protonation of the oxygen atom, followed by the rate determining formation of an oxocarbonium ion (specific acid catalysis) (ii) concerted proton transfer and cleavage of the carbonyl-carbon-oxygen bond with the formation of an oxocarbonium ion (general acid catalysis), (iii) attack by nucleophiles], (B) a further formulation of the above for a stepwise catalytic sequence for sialidases via the cyclic oxocarbonium ion by Holmquist (1975b)(Fig. 4.14) and (C) the conclusion by Miller et al., (1978) that whatever the mechanism, the geometry at the C-2 of NANA must pass through planarity

during the course of the reaction (substrate distortion).

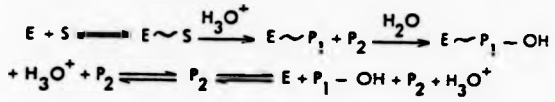


Fig. 4.14 Reaction sequence as formulated by Holmquist 1975b P_1 represents the cyclic oxocarbonium ion, P_1 − OH the free N-acetylneuraminic acid, P_2 the released aglycone, E the enzyme, and S the substrate.

Thus a precedent for the proposed mechanism involving the above steps can be found in one of the proposed mechanisms of lysozyme (Imoto et al., 1972) which involves a distortion of the saccharide moiety to its unstable half chain conformation, general acid catalysis and a charge-charge stability interaction. Therefore in the case of these three sialidases, the mechanism could possibly proceed as outlined in figure 4.15 (i - iv) in which the C-1 (carboxyl group) of the substrate binds to the arginine electrostatically producing a substrate distortion which facilitates the hydrolysis by a carboxylic amino acid residue which acts as a general acid catalyst by transferring a proton to the oxygen bridge. The formation of the positively charged oxygen bridge facilitates the rupture of the C-2 to oxygen linkage with the resultant formation of the C-2 carbonium ion in the sialic acid. The transition state for the formation of this carbonium ion should be stabilized by the proximity of a charged carboxylate group. The hydrolysis is completed by the replacement of the leaving group by a water molecule, a reversal of the first step, to give the products. The release of the free sialic acid is affected by the rapid anomerization to its β-anomer (Friebolin et al., 1980).

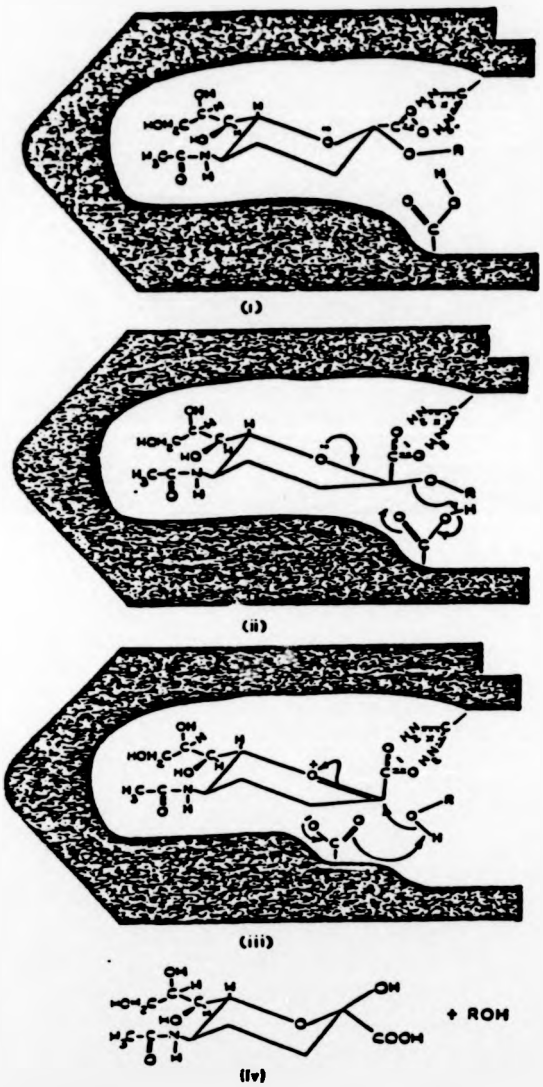


Fig. 4.15 (i – iv) Represents a schematic proposal of a catalytic reaction sequence explained in the text (▬, enzyme).

The S_N1 mechanism which we have proposed (fig. 4.15) is based solely on our chemical modification studies and supporting evidence reported in the literature. However, one has to be cognizant of the other possible mechanisms akin to that of lysozyme, illustrated in figures 4.15A (i) - (iv) (Fig. 4.15A iii is similar to our proposed mechanism). Therefore, in view of these possible mechanisms and the fact that no experiments, (other than the chemical modification reactions), have been carried out by us which were directed purely at the enzyme mechanism, it would be naive to conclude that figure 4.15 represents the true mechanism.

Future experiments would therefore have to be carried out to ascertain whether or not our proposed mechanism (fig. 4.15),or those represented in figure 4.15A or a yet undiscovered mechanism is the proper set of catalytic events for sialidase. These experiments could possibly employ the following techniques and ideas,` some of which have been previously applied to lysozyme (Imoto et al., 1972).

(i) NMR to show whether or not:- (a) an inversion of configuration occurs at the anomeric carbon atom during hydrolysis. If this does not occur, it then eliminates mechanism i of figure 4.15A. (b) The binding is a function of pH. (c) A chemical shift of the acetamido methyl protons occurs during the catalytic mechanism. If no change occurs, it then eliminates mechanism

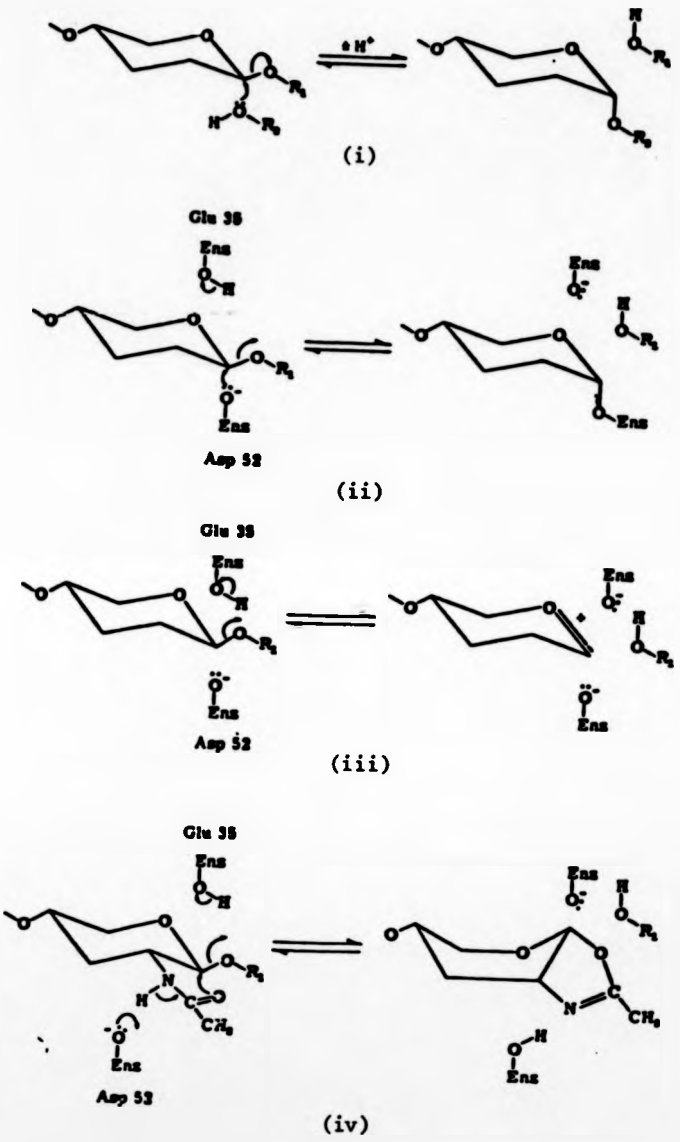


Fig. 4.15A Four proposals for the catalytic mechanism of lysozyme which could fit the catalytic spectrum for sialidase (Imoto et al., 1972).

iv of figure 4.15A. (d) A distortion of the ring occurs, employing the relationship between the coupling constant and the dihedral angle at C2. If distortion does occur, it would favour mechanism iii of figure 4.15A.

(ii) Affinity labelling, whereby the ligand could be covalently attached to the proton donating species, further confirming the active amino acid(s).

(iii) Secondary isotopic effects to ascertain whether or not the k_H/k_D ratio is 1.33 or 1.03, the former favouring mechanism iii i.e. S_N1 reaction and the latter mechanism ii, i.e. an S_N2 reaction.

(iv) A trapping system to determine whether or not a covalent ES complex is formed. If so, it would favour the S_N2 mechanism.

(v) X-ray crystallography and model building studies of the enzyme, enzyme-substrate and enzyme-inhibitor complexes to confirm the constellation of the active site residues.

With the aid of the above experiments and the aforegoing results, it should be possible to conclude the catalytic mechanism of sialidase.

Modification of the carbohydrate moieties

The effect of periodate oxidation on the enzyme activity of the three sialidases as represented if figure 4.16 shows an overall loss in activity (S. griseus, 100%; C. perfringens, 90%; V. cholerae, 45% when the substrate fetuin is used [Fig. 4.16 (i)]) but in an irregular manner. When the substrate α_1-acid glycoprotein is used, a total loss of enzyme activity is observed [Fig. 4.16 (ii)] in a similar fashion to that quoted above. This irregularity was further investigated on C. perfringens sialidase, using substrates of varying molecular weights, carbohydrate content and sialic acid content, as well as types of α_1-glycosidic linkages (Table 2.0, page 43).They all showed an overall drop in enzyme activity with a similar irregular profile (Fig. 4.17), except for colominic acid and sialyl-lactose in which, after 7 hr, there was a rise after an initial loss in enzyme activity. Further, substrates having a sialic acid content of greater than 20% showed an increase (greater than 100%), in enzyme activity within the first half-hour of periodate oxidation.

Although the result, (loss of activity), is contrary to the majority of results published for glycoenzymes showing no loss of enzyme activity on removal of carbohydrate units, the only other explanation (other than the protection against proteases or side reactions as periodate can modify amino acids (Clamp & Hough, 1965 and Spies & Chambers, 1949), is that the carbohydrate units participate in the maintenance of the three-dimensional structure of the enzyme. This, however, leads us to suspect that, because the drop in enzyme activity proceeds in a sequential fashion, the collapse in the three-dimensional structure therefore also proceeds via the same sequential fashion. Thus, the first stage in the collapse could block the approach or make the active

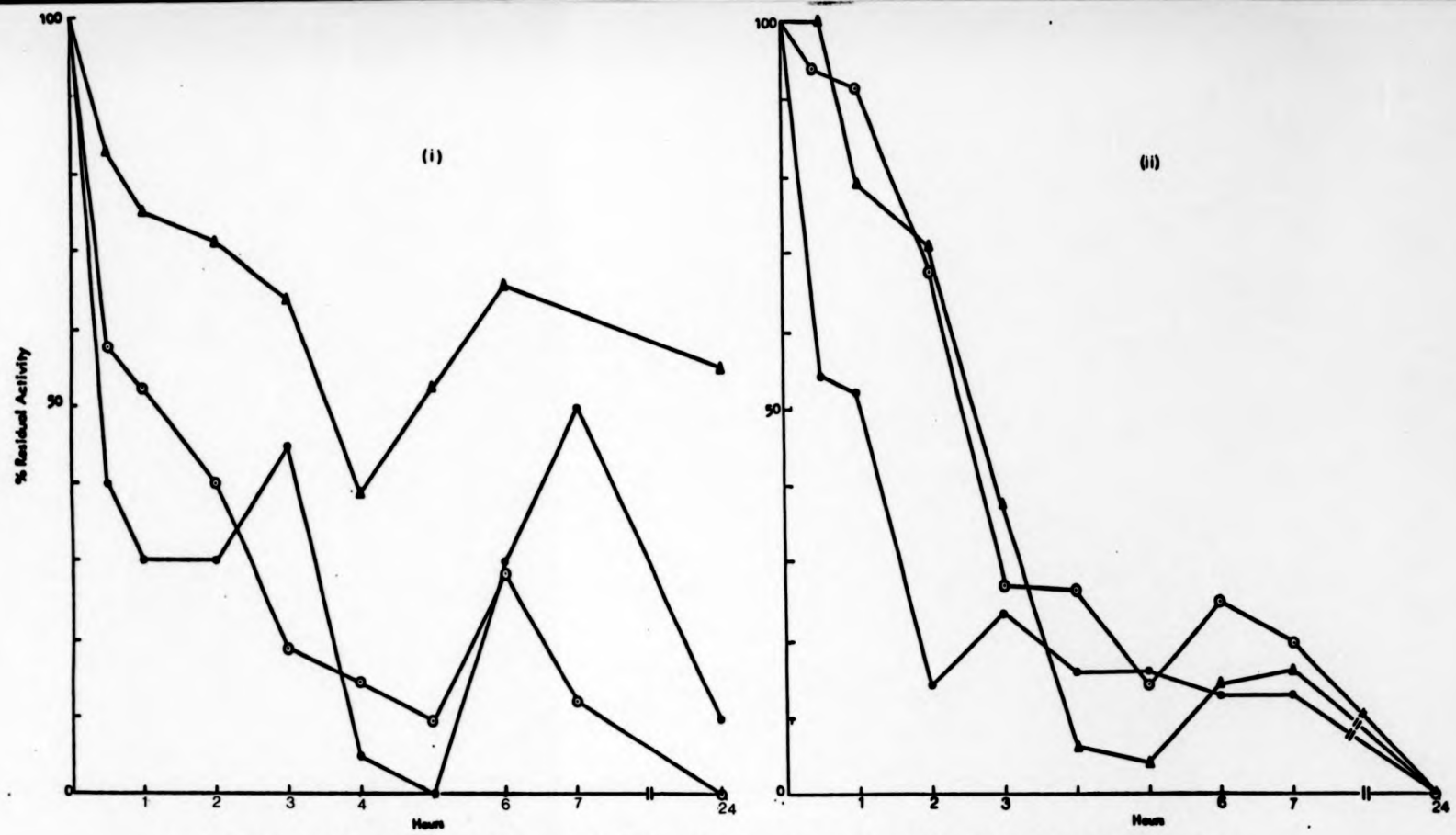


Fig. 4.16 (I) & (II) The effect of periodate oxidized sialidases (S. griseus, ●—● , C. perfringens ⊙—⊙, V. cholerae ▲—▲) assayed with (I) fetuin (10 mg/ml) (II) α_1-acid glycoprotein.

S. griseus (0.6 mg), C. perfringens (0.3 mg) and V. cholerae (0.2 mg) sialidases were incubated with periodate at various molar ratios (960, 1600 & 3100 respectively) and the experimental procedure carried out as in the legend of Fig. 4.17

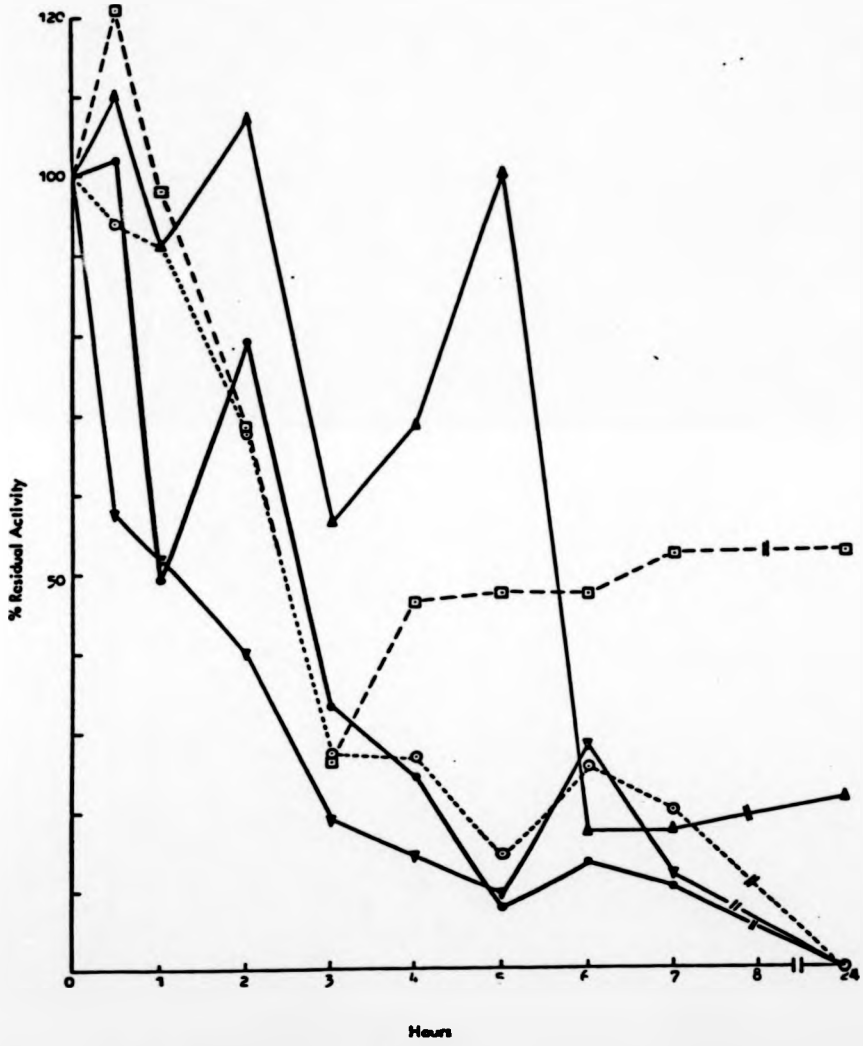


Fig. 4.17 The effect of periodate oxidized C. perfringens sialidase assayed with various substrates. ⊙—⊙, α$_1$-acid glycoprotein 10 mg/ml; ○——○ Bovine submaxillary mucin 5mg/ml; □---□ colominic acid 10 mg/ml; ▽—▽ Fetuin 10 mg/ml; △—△ sialyl-lactose 3.8 mg/ml.

C. perfringens sialidase (8.3 mg) in 0.1M sodium acetate buffer pH 4.5 (50 µl) was allowed to react with sodium metaperiodate (450 µl, molar ratio of IO_4^- : enzyme (1670:1 in the dark at 4°. Aliquots (50 µl) were removed at fixed time intervals and added to 40 µl of ethanediol (30% v/v). This was vigorously mixed for 30 sec. and then subjected to dialysis against the appropriate assay buffer. These aliquots were then assayed for enzyme activity using different substrates. Controls were set up in a similar manner but in the absence of periodate.

site less accesible to large molecular weight or bulky substrates than to that of a smaller molecular weight or less bulky substrate, such as sialyl-lactose.

When the β-elimination was performed on the V. cholerae sialidase, there followed a rapid loss of enzyme activity. This tends to favour the idea that the carbohydrate moieties participate in the three-dimensional structure of the enzyme. When the β-elimination was carried out with [^{35}S]-Na_2SO_3, [^{35}S] was incorporated, revealing the presence of O-glycosidic linkages to serine or threonine and from the amount of radioactivity bound, the presence of four such linkages could be deduced, which indicates that there exists the possibility of either a ser(thr)-N-acetylgalatosamine, ser (thr)-mannose or a ser(thr)-glucose linkage, (Marshall, 1974).

Gel electrophoresis of the enzyme subjected to β-elimination revealed by carbohydrate staining sugar moieties still attached to the enzyme. This suggests that besides O-glysidic linkages there probably exist N-glycosidic linkages as well or it could infer that the β-elimination reaction was incomplete. Further gel electrophoresis of the alkali and periodate modified enzyme revealed a decrease in the molecular weight of the enzyme (Fig. 4.18) and on carbohydrate staining it also disclosed the presence of carbohydrate moieties still attached to the protein backbone. Thus it is apparent that the two methods, i.e. periodate oxidation and β-elimination do not remove all the carbohydrate moieties from the V. cholerae

sialidase but those moieties which are modified or removed affect the activity and molecular weight of the enzyme.

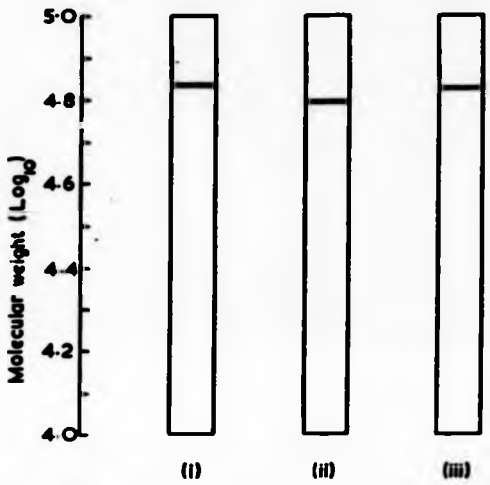


Fig. 4.18 Schematic representation of the polyacrylamide gel electrophoresis of V. cholerae sialidase subjected to (ii) periodate oxidation and (iii) β-elimination. Control (i)

In summary, our results reveal that:

(i) the synthesis of phenyl[^{3}H]glyoxal with a specific activity much higher than that of commercial ^{14}C phenylglyoxal, is possible:

(ii) with the aid of the phenyl[^{3}H]glyoxal the active sites of S. griseus and V. cholerae sialidase both have one arginine present whereas C. perfringens sialidase has two.

(iii) that the active sites of all three enzymes require arginine, tryptophan and carboxylic acid amino acid residues for full enzymic activity.

(iv) The arginine and tryptophan residues appear to be involved in

binding of the substrate whereas the carboxylic amino acid residues participate in the catalytic reaction possibly via acid catalysis involving the generation of an oxocarbonium ion as an intermediate.

(v) By amino acid analysis, the presence of arginine in the active site is confirmed.

(vi) By preliminary investigation that the carbohydrate moieties are more likely to be involved in maintaining the three-dimensional structure of the enzyme as opposed to being catalytically involved

(vii) That there exists O-glycosidic protein-carbohydrate bonds in the V. cholerae sialidase.

(viii) That removal of these carbohydrate moieties affect the catalytic and molecular weight of the enzymes.

binding of the substrate whereas the carboxylic amino acid residues participate in the catalytic reaction possibly via acid catalysis involving the generation of an oxocarbonium ion as an intermediate.

(v) By amino acid analysis, the presence of arginine in the active site is confirmed.

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(vii) That there exists O-glycosidic protein-carbohydrate bonds in the V. cholerae sialidase.

(viii) That removal of these carbohydrate moieties affect the catalytic and molecular weight of the enzymes.

APPENDIX I

Sources of Materials

N-acetylgalactosamine	Sigma (London) Chemical Co., Poole, Dorset, U.K.
N-acetylglucosamine	Sigma (London) Chemical Co., Poole, Dorset, U.K.
N-acetylneuraminic acid	Sigma (London) Chemical Co., Poole, Dorset, U.K.
N-acetyl[4,5,6,7,8,9-^{14}C]neuraminic acid	The Radiochemical Centre, Amersham, Buckinghamshire, U.K.
α_1-acid glycoprotein	Scottish National Blood Transfusion Association, Edinburgh, U.K.
Alkaline phosphatase (E. coli)	Sigma (London) Chemical Co., Poole, Dorset
Alkaline phosphatase (Bovine)	Boerhinger, Mannheim, G.F.R.
Bio-Gel P-2	Bio-Rad Laboratories, Bromley, Kent, U.K.
Bovine serum albumin	Miles Laboratories, Maidenhead, Buckinghamshire, U.K.
N-bromosuccinimide	Pierce and Warriner (UK) Ltd., Chester, Cheshire, U.K.
2, 3-Butanedione	Aldrich Chemical Company, Gillingham, Dorset, U.K.
n Butyl-lithium	Ventron Alpha Products, Beverley, MA., U.S.A.
Carboxymethyl cellulose	Whatman Ltd., Maidstone, Kent, U.K.
Colominic acid, sodium salt	Koch Light Laboratories, Colnbrook,

	Buckinghamshire, U.K.
Cyanogen bromide	Eastman Kodak Co., Rochester, New York, U.S.A.
Dansylhydrazine	Sigma (London) Chemical Co., Poole, Dorset, U.K.
DEAE-cellulose	Whatman Ltd., Maidstone, Kent, U.K.
Deoxyribonuclease 1	Sigma (London) Chemical Co., Poole, Dorset, U.K.
Di-isopropylamine	Aldrich Chemical Company, Gillingham, Dorset, U.K.
Dowex 50 - X8[H^+]	Sigma (London) Chemical Co., Poole, Dorset, U.K.
1-ethyl-3-(3-dimethylaminopropyl)carbodiimide	Sigma (London) Chemical Co., Poole, Dorset, U.K.
N-ethylmaleimide	Sigma (London) Chemical Co., Poole, Dorset, U.K.
N-ethylmorpholine	B.D.H. Chemicals Ltd., Poole, Dorset, U.K.
Fluorescamine	Sigma (London) Chemical Co., Poole, Dorset, U.K.
Glycine ethylester hydrochloride	Sigma (London) Chemical Co., Poole, Dorset, U.K.
^3H_2O	The Radiochemical Centre, Amersham, Buckinghamshire, U.K.
Hexamethylphosphoramide	Aldrich Chemical Co., Gillingham, Dorset, U.K.
Hydroxylamine hydrochloride	B.D.H. Chemicals Ltd., Poole, Dorset, U.K.
2 Hydroxy-5-nitrobenzylbromide	Sigma (London) Chemical Co., Poole, Dorset, U.K.

Lactate Dehydrogenase	Boerhinger, Mannheim, G.F.R.
Matrex Blue A & Red A	Amicon Corporation, Lexington, MA., U.S.A.
O-Methylisourea	Aldrich Chemical Co., Gillingham, Dorset, U.K.
Molecular weight markers	B.D.H. Chemicals Ltd., Poole, Dorset, U.K.
Mucin	Sigma (London) Chemical Co., Poole, Dorset, U.K.
Phenylglyoxal	Koch Light Laboratories, Colnbrook, Buckinghamshire, U.K.
Phosphatidyl choline	Sigma (London) Chemical Co., Poole, Dorset, U.K.
PM-10 membranes	Amicon Corporation, Lexington, MA., U.S.A.
Ribonuclease A	Boerhinger, Mannheim, G.F.R.
Sephadex G-10	Pharmacia Fine Chemicals, Uppsala, Sweden
Sephadex G-25	Pharmacia Fine Chemicals, Uppsala, Sweden
Sephadex G-100	Pharmacia Fine Chemicals, Uppsala, Sweden
Sepharose 4B	Pharmacia Fine Chemicals, Uppsala, Sweden
Sialidase from C. perfringens	Sigma (London) Chemical Co., Poole, Dorset, U.K.
Sialyl-lactose	Sigma (London) Chemical Co., Poole, Dorset, U.K.
Sodium borohydride	Hopkins and Williams, Romford, Essex

Sodium-[^{3}H]-borohydride	The Radiochemical Centre, Amersham, Buckinghamshire, U.K.
Spectrapor membrane tubing	Spectrum Medical Industries, Terminal Annex, L.A., U.S.A.
[^{35}S] Sulphur	The Radiochemical Centre, Amersham, Buckinghamshire, U.K.
Thiobarbituric acid	Sigma (London) Chemical Co., Poole, Dorset, U.K.
2,4,6-trinitrobenzenesulphonic acid (TNBS)	Sigma (London) Chemical Co., Poole, Dorset, U.K.
XM-50 Membranes	ChemLab, Hornchurch, Essex, U.K.

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PUBLICATIONS

1) The Synthesis of phenyl[2-^{3}H] glyoxal
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Biochem. J. (1979) 177, 377

2) Modification of Essential Amino Acids in the Active Site of Sialidases
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The Synthesis of Phenyl[2-^{3}H]glyoxal

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A simple inexpensive method has been developed for the synthesis of [2-^{3}H]acetophenone, which has been converted into phenyl[2-^{3}H]glyoxal. The latter compound has been used to modify arginine residues in alkaline phosphatase from two sources, and also a sialidase.

Phenylglyoxal will selectively modify arginine residues in proteins (Takahashi, 1968) and the sites of modification can be studied by using phenyl-[1-^{14}C]glyoxal (Armstrong *et al.*, 1976), which can be prepared by the oxidation of [1-^{14}C]acetophenone by SeO_2 (Riley & Gray, 1947). However, [1-^{14}C]-acetophenone is expensive to purchase and is comparatively difficult to synthesize (Dauben *et al.*, 1950). We have synthesized [2-^{3}H]acetophenone by a development of a method introduced for the alkylation of aliphatic carboxylic acids (Pfeffer & Silbert, 1970). In our synthesis the ^{3}H originates from ^3H_2O, considerably decreasing the cost of the isotopically labelled acetophenone. Moreover, under our labelling conditions few side reactions occur and hence the [2-^{3}H]acetophenone can be obtained in high yield. Oxidation of the latter compound with SeO_2 leads to the formation of phenyl[2-^{3}H]glyoxal and we have demonstrated the use of this reagent in the modification of arginine residues in some model enzymes.

Materials and Methods

n-Butyl-lithium was obtained from Ventron Alpha Products, Beverly, MA, U.S.A. and standardized by the method of Kofron & Baclawski (1976). Di-isopropylamine and hexamethylphosphoramide were obtained from Aldrich Chemical Company, Gillingham, Dorset, U.K. Phenylglyoxal was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Alkaline phosphatase (EC 3.1.3.1) from *Escherichia coli*, and sialidase (EC 3.2.1.18) from *Clostridium perfringens* were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. The latter was purified by affinity chromatography by the method of Brossmer *et al.* (1977), followed by the method of Geisow (1975). Calf intestinal alkaline phosphatase was obtained from the Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K.

Synthesis of [2-^{3}*H*]*acetophenone*

To anhydrous tetrahydrofuran (2.5ml) and di-isopropylamine (0.49g, 4.9mmol) under an Ar atmosphere was added 1.9ml of 2.5M-n-butyl-lithium in hexane and the mixture stirred for 30min below 0°C. Acetophenone (0.27g, 2.25mmol) and tetrahydrofuran (1ml) were then added and the mixture stirred for a further 15min below 0°C after which hexamethylphosphoramide (0.9ml, 5mmol) was added. After 20min, trifluoro[^{3}H]acetic acid (0.273ml, 2.4mmol), prepared by treating ^3H_2O (5Ci/ml; sp. radioactivity approx. 100mCi/mmol) with an equimolar amount of trifluoroacetic anhydride, was added and the reaction stirred for 2h at room temperature (21°C). The reaction mixture was then acidified with 1M-HCl at 0°C and the aqueous solution extracted with 3×20ml of light petroleum (b.p. 40–60°C). The organic extracts were combined, washed with 5×10ml of 1M-HCl, dried and evaporated *in vacuo* to give [2-^{3}H]acetophenone (0.27g), which was not purified further, but was oxidized immediately. The position of isotope exchange was confirmed by ^{1}H n.m.r. spectroscopy with [2-^{2}H]acetophenone prepared in an analogous manner from trifluoro[^{2}H]acetic acid. The signal due to the acetyl group at 2.6p.p.m. was greatly diminished.

Synthesis of phenyl[2-^{3}*H*]*glyoxal*

To a solution of sublimed SeO_2 (0.27g, 2.4mmol) in dioxan (0.84ml) and water (4.5μl) at 50°C was added [2-^{3}H]acetophenone (0.27g, 2.25mmol) and dioxan (0.5ml). The mixture was heated at 80°C for 6h and filtered through cottonwool, which was then washed with dioxan (20ml). The filtrate and washings were evaporated *in vacuo* and dry ethanol (2ml) was added to the resulting oily residue. After being left for 2 days at 5°C, the supernatant was carefully removed from the red precipitate that had formed and was evaporated *in vacuo*. The oily residue was dissolved in ethyl acetate (0.5ml) and the phenyl-[^{3}H]glyoxal purified by preparative silica t.l.c. with petrol/ethyl acetate (1:1, v/v) as solvent. The phenyl-[^{3}H]glyoxal was eluted from the silica with ethyl acetate (50ml), which was then concentrated *in vacuo* to 0.5ml. The phenyl[^{3}H]glyoxal was rechromato-

graphed as described above and the ethyl acetate removed *in vacuo* to leave an oily residue. Final traces of ethyl acetate were removed by repeated (5 ×) dissolution of the residue in ethanol (50ml) and evaporation *in vacuo*. Yield of phenyl[2-^{3}H]glyoxal was 0.23g (76%, based on $\varepsilon_{250\,nm}$ of 9000 litre·mol^{-1}·cm^{-1} in ethanol) with sp. radioactivity 12.2mCi/mmol. The phenylglyoxal ran as a single u.v.-absorbing spot on silica t.l.c. with petrol/ethyl acetate (1:1, v/v) as developing solvent with the same mobility as authentic phenylglyoxal (R_F = 0.33). The ^{1}H n.m.r. phenyl[2-^{3}H]glyoxal was identical with that of authentic material with a singlet at 9.55 p.p.m. due to the aldehydic proton. This singlet was absent in the n.m.r. spectrum of phenyl[2-^{2}H]-glyoxal, prepared as described above from [2-^{2}H]-acetophenone, confirming the position of isotopic substitution. The phenyl[2-^{3}H]glyoxal decomposed slowly (30% in 3 months) when stored as a 1M solution in dry ethanol at 4°C. Little decomposition occurred when the phenylglyoxal was stored as an oil or as the hydrate. The latter compound could be obtained by crystallization from a concentrated aqueous solution at 4°C.

Reaction of phenylglyoxal with enzymes

(*a*) *Inactivation.* The alkaline phosphatases and sialidase were treated at 25°C with various amounts of a solution of phenylglyoxal hydrate (16mg) in ethanol (100μl) as described in Table 1. Care was taken to use the same buffers for the inactivation as were used for the assay. Samples were removed after fixed times and assayed. After inactivation, the modified enzymes were dialysed at 4°C for 36h against the buffers indicated (3 × 5 litres), after which portions were removed and assayed.

(*b*) *Protection by substrate.* The three enzymes in the presence of their substrates were treated with unlabelled phenylglyoxal hydrate and then dialysed against assay buffer as described in Table 2. The modified enzymes were then treated with a 100-fold molar excess of phenyl[2-^{3}H]glyoxal for 2h, the protein was precipitated on glass-fibre discs with trichloroacetic acid (Yang & Schwert, 1972) and their ^{3}H content determined.

Discussion

The oxidation of acetophenone by SeO_2 proceeds via the formation of an enol selenite ester that

Table 1. *Inactivation of enzymes with phenylglyoxal*

Enzyme	Buffer	Slow inactivation: Molar ratio of phenylglyoxal to protein	Slow inactivation: Time for 75% inactivation (min)	Rapid inactivation: Molar ratio of phenylglyoxal to protein	Rapid inactivation: Time for 100% inactivation (min)
E. coli alkaline phosphatase* (mol.wt. 89000)	0.125M-$NaHCO_3$ (pH7.5)	10^3	256	3.3×10^3	60
Calf intestine alkaline phosphatase* (mol.wt. 140000)	0.125M-$NaHCO_3$ (pH7.5)	10^3	240	3.3×10^3	60
C. perfringens sialidase† (mol.wt. 56000)	0.1M-Sodium acetate (pH5.3)	6×10^4	180	1.6×10^5	96

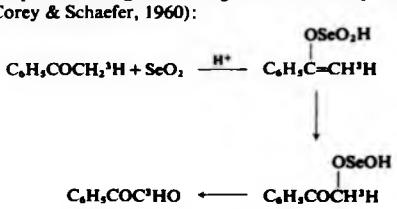
* Assayed by the method of Ghosh & Fishman (1968).
† Assayed by determining sialic acid released from sialyl-lactose by the method of Warren (1959).

Table 2. *Protection of active sites of enzymes during rapid labelling by [^{3}H]phenylglyoxal*
The buffers used were the same as in Table 1.

Enzyme	Substrate	Molar ratio of phenylglyoxal to enzyme	Molar ratio of substrate to enzyme	Time for reaction (min)	Molar ratio of phenyl[2-^{3}H]glyoxal to enzyme	Number of arginine residues/active site
Alkaline phosphatase* (*E. coli*)	Phenyl phosphate	10^5	10^5	5	4.23	2.12
Alkaline phosphatase* (calf intestine)	Phenyl phosphate	10^5	10^5	5	3.56	1.8
Sialidase	Sialyl-lactose	10^4	1.6×10^4	5	3.74	1.85

* In the presence of $MgCl_2$ (5mol/mmol of enzyme).

rearranges and then decomposes to phenylglyoxal, two protons being lost during the reaction sequence (Corey & Schaefer, 1960):



In the synthesis of phenyl[2-^{3}H]glyoxal, the retention of ^{3}H may be assisted by favourable isotope effects operating in the two proton-abstraction steps.

The phenyl[2-^{3}H]glyoxal was used to determine the number of arginine residues in the active centres of two alkaline phosphatases and a sialidase. The interaction of the enzymes with unlabelled phenylglyoxal was investigated over a molar range of phenylglyoxal/enzyme of 10^3–10^5 (Table 1), assaying at fixed time intervals until the inactivation was constant. The enzymes were then dialysed and reassayed and in all cases the inactivation was irreversible with molar ratios of 3×10^3 or greater. The rate of inactivation was less at pH 5.3 than at 7.5. Once the conditions for rapid inactivation had been determined, the inactivation was carried out on the enzymes in the presence of substrates. The concentrations of the latter enzymes were derived from the turnover number for each enzyme and were chosen so as to provide maximum protection of the active site. After 5 min the substrates and excess phenylglyoxal were removed by dialysis and the enzymes were treated with phenyl[2-^{3}H]glyoxal (Table 2). The number of molecules of phenyl[2-^{3}H]-glyoxal that bound to each enzyme molecule were then determined. This value is twice the number of arginine residues modified (Takahashi, 1968) and hence the number of arginine residues at or near the active centres of the enzymes can be calculated. Our values for calf intestinal (1.8) and *E. coli* (2.1) alkaline phosphatase are close to that (2) obtained by Daeman & Riordan (1974) for *E. coli* alkaline phosphatase. We found that approx. 2 arginine residues are involved in the active site of the sialidase from *C. perfringens*. No data on the involvement of arginine residues in the active site of this enzyme have been reported, although we have observed (Kabayo & Hutchinson, 1977) that arginine residues are essential for the activity of sialidase from *Streptomyces griseus*.

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