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DEGRADATION OF SERUM GLYCOPROTEIN BY DIPLOCOCCUS PNEUMONIAE

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BY DIPLOCOCCUS PNEUMONIAE

APPROVED BY

M R Shetlar

Irving T. Bever

[illegible]

Renyon H. Bradford

[illegible]

DISSERTATION COMMITTEE

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DEGRADATION OF SERUM GLYCOPROTEIN

BY DIPLOCOCCUS PNEUMONIAE

CHAPTER I

INTRODUCTION

It is well established that plasma proteins contain several carbohydrates firmly attached to the protein moiety. The major monosaccharide components of serum glycoproteins are now known to be galactose, mannose, glucosamine and sialic acid. Galactosamine and fucose are minor constituents of some serum glycoprotein. The average serum protein-bound carbohydrate levels of normal individuals are 114 ± 3 mg% for hexose, 71 ± 4 mg% for hexosamine, 62 ± 2 mg% for sialic acid, 8.9 ± 0.6 mg% for fucose and 0.226 ± 0.075 mg% for uronic acid (Shetlar, 1961). These carbohydrates are widely distributed in all major serum protein fractions with the possible exception of serum albumin, but are especially prominent in α_1 and α_2 globulin fractions.

Changes of the serum protein fractions and of the bound carbohydrates (glycoproteins) of these fractions are known to occur in many pathological conditions, including cancer, rheumatoid arthritis, rheumatic fever, myocardial infarctions, pyogenic infections, pneumonia and tuberculosis. Very little is known at the present time about the

metabolic alterations which lead to these changes, or even about the normal synthesis and destruction of glycoproteins in the animal body. Knowledge of the nature and size of the carbohydrate units and these linkages with amino acids is still incomplete.

For degrading the polypeptide chain, both chemical and enzymatic methods can be applied. However, enzymatic digestions are still more specific, and, therefore, more useful for a structural study. Similar reasoning can be applied for the hydrolysis of the polysaccharide chain. Proteolytic enzymes obtained from several biological sources demonstrating different specificities are available which will completely degrade the heteropolysaccharide chain of serum glycoprotein.

This investigation was undertaken for the purpose of finding a microorganism which would provide a source of enzymes for the degradation of heteropolysaccharide chains as a tool in structural studies of glycoproteins. A preliminary survey of a number of microorganisms revealed a surprising resistance of serum glycoproteins to bacterial destruction (Shetlar, 1959). Among those organisms tested, only *Diplococcus pneumoniae* was found to liberate carbohydrates, other than sialic acid, from serum glycoproteins.

CHAPTER II

LITERATURE REVIEW

Since this study concerns a search for a microorganism which will degrade serum glycoprotein and a structural study of glycoprotein using enzymes obtained from bacteria, this section of the dissertation will review, (1) Studies concerning the degradation of serum glycoprotein by microorganisms, and, (2) Studies dealing with the structure of glycoprotein.

Studies Concerning the Degradation of Serum Glycoprotein by Microorganisms

General

During the studies of antigen-antibody reactions with pneumococci, Benzancon and Griffon (1900) found that, upon incubation of *D. pneumoniae* with its antiserum, a voluminous white precipitate was obtained after sixteen hours of incubation, whereas a mixture of normal serum and pneumococci developed only a slight cloudiness. They noted that the precipitation was not due to the specific antigen-antibody reaction, but was due to the growth of bacteria during the incubation. Wadsworth (1903) also found that *D. pneumoniae* and certain other bacteria grew more abundantly in sera obtained from pneumonia patients than in normal

sera. Rosenow (1904) and Longcope (1905) showed that the precipitation described by Benzancon and Wadsworth was not a direct result of the bacteria and bacterial debris present, as was previously proposed, but rather was due to the production of unusually large quantities of acid which precipitated serum protein during the incubation. Longeope noted that this phenomenon could be found in sera from patients with pneumocococcal or streptococcal infections and acute articular rheumatism. He concluded that "there is some substance which makes its appearance in the blood stream under certain conditions and from which the pneumococci are capable of forming large quantities of acid." Since it is now well known that the serum glycoprotein level rises in various disease states, one may speculate that Longeope may have observed an elevation of glycoprotein in pathological sera.

Friedmann and Sutliff (1939) compared the lactic acid production of pneumococci in pneumococcal antisera and in normal sera. They found that the production of lactic acid in normal sera could be accounted for entirely by the free blood sugar consumed by the organism, and that the growth of the organism appeared to stop when the free blood sugar was exhausted. In pathological sera, they found that the gorwth continued at an apparently undiminished rate after the free blood sugar had been consumed. They also noted that the quantities of lactic acid produced in pathological sera were always greater than could be obtained from the free blood sugar. The production of acid paralleled the consumption of protein-bound polysaccharide in pathological sera. These results caused them to conclude that the phenomenon resulted from the presence of a

large quantity of protein-bound polysaccharide which supported the rapid growth of the organism.

The Discovery of Receptor Destroying
Enzyme (R.D.E.)

Around 1940 many workers showed that Influenza virus multiplied freely in the cells lining the amniotic and allantoic cavities of the chick-embryo. From the infected cells, large amounts of virus were liberated into the fluid, providing a very convenient source of virus for experimental work. Hirst (1941) observed that contamination of virus-infected allantoic fluid with chick-embryo blood produced firmly agglutinated erythrocytes. Later, Hirst (1942A) found that washed red cells from mature chickens were also agglutinated by fluids which contained virus, and he developed this hemagglutination reaction into an accurate method of titrating Influenza virus. Hirst (1942B) further demonstrated that Influenza virus was firmly adsorbed to chicken red cells at 4°C. and remained in the adsorbed state as long as 24 hours. When the temperature was raised to 37°C., spontaneous release of virus from the red cells was observed. Once the red cell reacted with virus, it could not be agglutinated again, but the reacting virus remained unchanged. The eluted virus particles retained their infectivity and their capacity to interact with red blood cells and were indistinguishable from fresh virus. Their agglutination of new red cells and release subsequent occured as before. Hirst recognized that this phenomenon presented the essential element of an enzyme action. The virus by some means functioned as an enzyme which destroyed some cell surface components

or "receptors", to which the virus particles were adsorbed. The virus itself was not changed in the process. It was believed at that time that: (1) viruses were simple nucleoproteins, (2) a virus itself did not have an independent metabolism, and, (3) the metabolic pathways of viruses were entirely derived from the host cell. Therefore, Hirst first postulated that the actual enzyme activity was not associated with the virus particle itself, but was due to the contamination of virus with host cell material. However, he was not able to show any receptor destroying activity in allantoic fluid freed of virus particles. This indicated that the enzyme activity was actually associated with virus particles. Influenza A, Influenza B, Mumps, Newcastle disease of fowls and Fowl plague produce the hemagglutination described by Hirst (Burnett, 1952).

Burnett and Stone (1947) obtained a soluble exoenzyme from the culture filtrate of *Vibrio cholerae* and *Clostridium welchii* which apparently caused irreversible changes of the receptor substance of red blood cells similar to that caused by Influenza virus.

Briody (1948) found that heating Influenza virus at 55°C. for 30 minutes destroyed the effect of the virus on receptors but left unaltered its capacity to produce hemagglutination. The virus which retains its full agglutination properties but has lost the capacity to destroy the cell receptor and be subsequently eluted from the red cell is known as an "indicator virus". It is now understood that such preparations have lost their enzymatic activity.

The discovery of the bacterial receptor destroying enzyme and

indicator virus supported Hirst's explanation of hemagglutination by virus as an enzyme action. The product liberated by receptor destroying enzymes was later found to be neuraminic acid and the name neuraminidase was given by Gottschalk to any enzyme with receptor destroying power. Neuraminidase is a carbohydrase which splits off the terminal neuraminic acid from the non-reducing end of disaccharides, oligosaccharides and polysaccharides (Gottschalk, 1957).

After Hirst's discovery of the receptor destroying properties of Influenza virus, many efforts were made to isolate "receptor" substance directly from red blood cells, but thus far no one has isolated a homogeneous substance from this source which will serve as a substrate for the enzyme. The realization that glycoprotein serves as a substrate of neuraminidase evolved gradually. Hirst (1948) first reported that periodate destroyed the receptor site of the red blood cell and suggested that the receptor may be a mucoid substance.

Later many workers (Ianni and Beard, 1948; Anderson, 1948; Gott-' Schalk and Lind, 1949; Burnett, 1942) found that a variety of mucoids, erythrocyte mucopolysaccharide, ovarian cyst mucin, serum glycoprotein, and sheep submaxillary mucoprotein inhibited hemagglutination by heat-inactivated Influenza virus. The inhibitory capacity of these mucoids was lost upon treatment with living (infective) virus or receptor destroying enzyme from bacterial sources. These results suggested that the mucoid or glycoprotein and the receptor site compete for the virus; this would provide a mechanism for the observed inhibition of hemagglutination. The soluble inhibitory mucoids were visualized as chemical

analogues of the cellular receptor. It is presently well established that sialoproteins (glycoproteins containing sialic acid at the non-reducing end) will serve as substrates for receptor destroying enzymes. The receptor is now considered to be bound sialic acid.

The discovery of receptor destroying enzymes facilitated the discovery of neuraminic acid and its specific localization in various glycoproteins. Usually new enzymes have been discovered using known substrates. In the case of neuraminidase, the change in the hemagglutinative property of red cells after reaction with virus was initially the only evidence suggesting the presence of an enzyme action. Therefore, investigators were encouraged to study the changes of the physiochemical properties of receptor destroying enzyme treated red blood cells.

Hanging (1948) and Stone and Ada (1950) were able to show changes in the mobilities of red blood cells in an electric field after treatment with enzyme. They suggested that the electrophoretic mobility of the neuraminidase treated red blood cell was markedly reduced because of some highly charged group. Perlman, Tamm and Horsfall (1952) treated a urinary mucoprotein which had inhibitory activity with active Influenza virus and observed a 20% reduction in its electrophoretic mobility at pH 6.8. Curtain and Pye (1955) showed that the receptor destroying enzyme of Vibrio cholerae reduced the electrophoretic mobility of several mucoproteins which are inhibitors of Influenza virus hemagglutination. Pye (1955) proposed that this property be utilized for the assay of the inhibitor content of mucin.

Klenk *et al.* (1955) isolated and crystallized the substance released from urinary mucoprotein by Influenza virus and showed that it was identical to the N-acetylneuraminic acid which he obtained from the same mucoprotein by acid hydrolysis. From Hirst's discovery in 1942 of enzyme activity in a virus to Klenk's isolation of crystalline neuraminic acid from an incubation mixture of mucoprotein and Influenza virus, it took thirteen years to identify the substrate and hydrolysis products of this enzyme. Later, klenk and Uhlenbruck (1958) again isolated and crystallized neuraminic acid after incubation of erythrocyte stroma with receptor destroying enzymes.

Heimer and Meyer (1956) reported that pneumococcal extracts contained neuraminidase activity which hydrolyzed sialic acid from bovine submaxillary mucin.

Schultze and Schwick (1957) incubated serum with filtrates of *Cholera vibrio* and *Clostridium perfringens* toxin and found a reduction of the electrophoretic mobility of α_1-globulin, α_2-macroglobulin and transferrin as indicated by immunoelectrophoresis. Popenoe and Drews (1957) postulated that this effect was due to the receptor destroying enzyme which hydrolyzed neuraminic acid from protein bound polysaccharide and decreased the electrophoretic mobility. Laurell (1959) also found that incubation of human sera with *D. pneumoniae*, *Streptococcus pyogenes* and *Pasteurella pseudotuberculosis* the electrophoretic mobility of some of the α- and β-globulins was reduced. They concluded that the organisms produced neuraminidase which hydrolyzed neuraminic acid from serum glycoprotein with the result that to decrease the

electrophoretic mobility of different serum fractions.

Neuraminidase from Influenza virus has been partially purified by Myron *et al.* (1961) by treatment of a virus or vaccine with trypsin to remove the remainder of the virus particle. Ada and French (1961) succeeded in crystallizing neuraminidase from a culture filtrate of *Vibrio cholerae*, using a procedure which employed methanol fractionation, adsorption and elution from human red cells, fractionation with ammonium sulfate and chromatography on columns of hydroxyl apatite.

Studies Dealing with the Structure of Glycoproteins

While the isolation of glycoprotein from different sources have made a comparable progress, structural studies on glycoproteins have been initiated only recently.

According to the Physiological Society and American Society of Biochemists, the carbohydrate-rich proteins of plasma are included in the sub-classification, glycoprotein, under the general class of conjugated proteins. Glycoproteins are, by this definition, "compounds of the protein molecule with a substance or substances containing a carbohydrate group other than nucleic acid", (Committee on Protein Nomenclature, 1908). In the glycoprotein molecule, the polypeptide chain and the carbohydrate chains are linked through stable chemical bonding. Carbohydrates in the glycoprotein can not be separated from the protein without drastic treatment with acid or alkali.

Our knowledge of the nature of the bonds linking the carbohydrate and protein is still in a primitive state. The difficulty experienced

in studies to determine the linkage between amino acid and carbohydrate may be explained by the following quotation (Gottschalk et al., 1962): "Throughout the animate world, nature adheres strictly to the glycosidic type linkage when joining sugar to sugar, and to the amide type of juncture when adding amino acid to amino acid. Yet, in the attachment of carbohydrates to peptide almost any of the functional groups of the latter may be involved, resulting in a variety of modes of linkage".

For a complete understanding of the structure of the carbohydrate moiety of glycoproteins, the following information is necessary: (1) the composition and sequence of the monosaccharides in the carbohydrate chain, (2) the amino acid residue linked to the carbohydrate chain, (3) the monosaccharide residue linked to the polypeptide chain, and, (4) the type of bonding between amino acid and monosaccharide.

The carbohydrate composition of various glycoproteins is fairly well established due to the rapid developement of analytical methods for various carbohydrates. Examples of the carbohydrate composition of some well studied serum glycoproteins may be found in Table I (Stary, 1959).

For the elucidation of the amino acid residue which is linked to the carbohydrate chain, preparation of small glycopeptides by slightly selectively hydrolyzing the protein moiety by proteolytic enzymes which do not alter the carbohydrate chain appears to be the best approach. There is a wide variety of proteolytic enzymes available from different sources to serve this purpose. However, enzymes which cleave the carbohydrate-protein bond and leave the polypeptide portion unchanged are not

TABLE I

COMPOSITION OF THE CARBOHYDRATE GROUP OF SOME SERUM GLYCOPROTEINS

		1	2	3	4	5	6
		Oroso-mucoid	Macro-globulin	Cerulo-plasmin	Hapto-globin II	γ	Fetuin
Molecular Weight	total.............	44,000	848,000	150,000	85,000	160,000	45,000
	polypeptidic part.	26,500	780,000	138,000	not det.	155,000	33,000
	carbohydrate part.	17,500	68,000	12,000	not det.	4,960	12,000
Carbohydrate in percent	total.............	41	8.4	7.7		3.1	26.3
	galactose.........	10.0	1.8	2.0		0.4	0.0
	mannose...........	5.0	1.8	1.0		0.7	9.5
	hexosamine*.......	13.3	2.9	2.3	6.7	1.3	9.4
	sialic acid*......	12.4	1.8	2.4	not det.	0.3	7.4
	fucose............	0.8	0.12	0.18	not det.	0.3	not det.
	hexuronic acid....	0.4	0.1	not det.	not det.	0.1	not det.
No. of carbohydrate residues per protein molecule	total.............	77	237	56		24	754
	galactose.........	22	85	16		3	-
	mannose...........	11	85	8		6	23
	hexosamine*.......	25	111	16	26	10	20
	sialic acid*......	17	99	14	not det.	1.5	11
	fucose............	2	6	2	not det.	2.0	not det.
	hexuronic acid....	1	6	not det.	not det.	1.0	not det.

*Expressed as an acetylated sugar

available. Acid or alkali hydrolysis is not applicable because both peptide and glycosidic bonds will be equally hydrolyzed. If one could find enzymes which preferentially hydrolyze the carbohydrate chain of glycoprotein and leave the protein moiety intact, the question of what carbohydrate is adjacent to the polypeptide chain might be answered. An effort to solve this problem is one of the main purposes of this dissertation.

To determine the type of likely linkages between amino acid and monosaccharide in glycoprotein, the best approach appears to be to isolate the amino acid-sugar complexes from glycoprotein and compare these to ones prepared by organic synthesis. Though this goal appears difficult to achieve at present, progress in this direction is to be expected in the future.

Up to the present time, the research concerning structural studies of glycoproteins has concentrated on the following proteins: γ-globulin, ovalbumin, serum α_1-acid glycoprotein (orosomucoid) and submaxillary gland glycoprotein. These are relatively easy to prepare in the purified state and their carbohydrate compositions have been well determined. Almost all the workers in this field have utilized proteolytic enzymes to degrade the polypeptide part of the glycoprotein, then isolated the carbohydrate rich peptides from the enzyme hydrolysate and attempted to characterize the amino acid-sugar linkage of glycopeptide.

Sequential Studies on Carbohydrate Chain in Glycoprotein

Few publications are found in the literature concerning the studies

of carbohydrate sequence in glycoproteins. Winzler (1958) found that partial acid hydrolysis of orosomucoid with 0.001 N HCl liberated sialic acid; hydrochloric acid at 0.0025 N liberated a small amount of galactose; whereas, at 0.005 N and 0.01 N both galactose and fucose were liberated. In order to liberate mannose and glucosamine, higher concentrations of hydrochloric acid were required. These findings suggested that sialic acid occupied the terminal position of the carbohydrate chains and had adjacent to it galactose and then fucose. Mannose and glucosamine apparently occupied an interior position in the carbohydrate chain.

Spiro (1962) determined the order of monosaccharide release from fetuin during both graded acid hydrolysis and selective enzymatic cleavage using several glycosidases, and concluded that sialic acid occupies the terminal positions in the carbohydrate units and that galactose is the sugar next in sequence. Some of the hexosamine residues are attached to the galactose, whereas the remainder of the hexosamine residues and mannose are located more interiorally. Eylar and Jeanloz (1962) utilized periodate oxidation of the α_1-acid glycoprotein, and arrived at similar conclusions to those described by Winzler (1958). It seems rather generally accepted that sialic acid always occupies the terminal position and that galactose is always next to sialic acid in serum glycoproteins. Mannose and glucosamine are in the interior portion of the carbohydrate chain.

Structural Studies on Glycoprotein in γ-Globulin

Rosevear and Smith (1958, 1961) digested human γ-globulin with papain. By employing procedures of ion exchange chromatography, ethanol precipitation and starch column electrophoresis, they isolated three very similar glycopeptides, with an average molecular weight of less than 5000. The evidence strongly suggested that two of these glycopeptides were derived from the third and supported the view that there was a single type of prosthetic carbohydrate unit bound by an ester or amide linkage to the γ-carboxyl group of aspartic acid. They suggested that the glycopeptide had the structure of Asp.(NH_2). Tyr. Glu. Asp. (CHO). The molar ratio of the carbohydrate moiety was 3 galactose, 5 mannose, 2 fucose, 8 glucosamine and 1 sialic acid. These ratios correspond reasonably well to the molar ratios of carbohydrates in the original γ-globulin. After exhaustive treatment of the glycopeptides with aminopeptidase and carboxypeptidase, a fraction was obtained which, upon hydrolysis with 6 N hydrochloric acid, contained only aspartic acid as a major amino acid component. This result strongly suggested that the linkage between carbohydrate and protein was through the terminal carboxyl group of aspartic acid.

Later, Nolan and Smith (1962) digested rabbit and bovine γ-globulin with papain and isolated glycopeptides from the papain hydrolysate. They were also able to show that in their glycopeptides aspartic acid was always attached to the carbohydrate. Jevons (1958) digested egg albumin with pancreatic protease and removed the free amino acids in

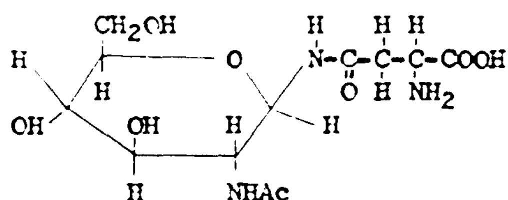
the digest with a Dowex-50 column. The carbohydrate rich fraction obtained by subsequent paper electrophoresis was shown to be a glycopeptide containing aspartic acid, leucine, glucosamine and mannose in a molar ratio of 1:1:2:4. Reaction of the glycopeptide with flurodinitrobenzene showed that the amine group of the aspartic acid was free and he concluded that the aspartic acid was linked through the carboxyl group to glucosamine.

Structural Studies on Ovalbumin

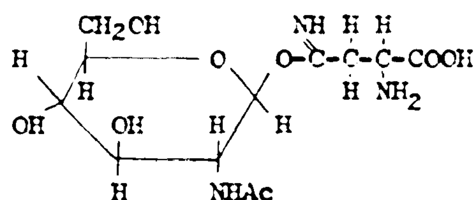
Johansen, Marshall and Neuberger (1958), subjected crystalline ovalbumin to various types of proteolytic digestion and purified the glycopeptide by Celite column chromatography and column electrophoresis. They obtained a glycopeptide with the sequence of Asp.-(carbohydrate)-Leu.-Ser.-Thr. The carbohydrates consisted of 5 mannose and 3 N-acetylglucosamine residues. Upon treatment of the glycopeptide with carboxypeptidase, leucine, serine and threonine were liberated and the remaining carbohydrate fragment still contained 1 mole of aspartic acid, suggesting that aspartic acid is linked to carbohydrate.

Cunningham, Neunke and Neunke (1952) and Neunke and Cunningham (1961) isolated a glycopeptide from ovalbumin digested with trypsin and cymotrypsin. The sequence of the glycoeptide was found to be Tyr.-Asp.-Leu.-Thr.-Ser. They were able to remove all of the amino acids except aspartic acid from the glycopeptide by carboxypeptidase. This achievement again suggested a carbohydrate to aspartic acid linkage. The authors further found that the amino group of aspartic acid was not substituted and that, upon mild acid treatment, one mole of ammonia per

mole of glycopeptide was liberated. Treatment of glycopeptide with hydroxylamine failed to separate the carbohydrate from the peptide, indicating that the linkage was not ester-like. From these data, an aspartyl glycosaylamine structure, analogous to glycinamide ribotide was tentatively proposed, (Figure A).



(Figure A)



(Figure B)

Marks and Neuberger (1961) synthesized N-(L-β-aspartyl)-β-D-glucopyranosylamine and N-(L-βaspartyl)-β-D-glucosamine to compare with derivatives obtained from egg albumin.

Bogdanov, Kaverzenva and Anderejeva (1962) succeeded in isolating the polysaccharide-aspartic acid fragment by short term acid hydrolysis. The polysaccharide-aspartic acid complex was free from mannose and contained only aspartic acid and glucosamine in equimolar quantities. The complex contained excessive amounts of nitrogen and an aldehyde group could not be demonstrated by qualitative chemical tests. They interpreted these results as evidence that the structure of the glucosamine-aspartic acid complex was similar to the one suggested by Neunke and Cunningham (1961).

Yamashina and Makino (1962) digested ovalbumin with <u>Streptomyces griseus</u> protease and isolated a polysaccharide-amino acid complex. The complex was composed of aspartic acid, ammonia, N-acetylglucosamine and mannose in the ratio of 1:1:3:5. Only aspartic acid was present in the

complex, indicating that aspartic acid must be involved in the linkage with carbohydrate. By partial acid hydrolysis, they were able to detect asparaginyl-glucosamine by paper electrophoresis. They found this compound was more sensitive towards alkali than towards acid. However, O-glycosylamine (N-phenyl-N-acetyl-2-amino-2-deoxy-glucosylamine) was very easily hydrolyzed by acid; on the other hand, the reduced glycosylamine was quite resistant to acid hydrolysis. From these results, the authors concluded that the complex did not have the exact glycosylamine type linkage proposed by Neunke and Cunningham and suggested an imino ether (imidic acid ester or imido ester) type linkage between aspartic acid and glucosamine. (Figure B, Isumi, K. 1962).

Structural Studies on Orosomucoid

Winzler (1958) found that hexosamine was more slowly liberated during acid hydrolysis than other carbohydrate components of orosomucoid, suggesting that hexosamine might be linked with protein. Similar to the structural studies of various glycoproteins mentioned above, many workers have tried to isolate the carbohydrate rich glycopeptide from orosomucoid following proteolytic digestion. However, orosomucoid is resistant to the action of proteolytic enzymes, due to the presence of sialic acid in the terminal position of the carbohydrate chain, although it can be easily digested by proteolytic enzyme after removal of sialic acid (Yamashina, 1956; Popenoe and Drew, 1957; Schmid, 1959). Several reports concerning isolation and purification of carbohydrate rich glycopeptides from proteolytic digestion of orosomucoid have appeared (Wenfeld and Tunis, 1961; Kamiyama and Schmid, 1961;

Isumi, Makino and Yamashina, 1961). The amino acids found in the glycopeptide were invariably aspartic acid, threonine and serine.

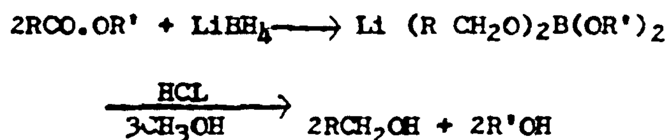
By prolonged and repeated digestion of sialic acid-free orosomucoid with Streptomyces griseus protease, Isumi, Makino and Yamashina (1962) were able to isolate a homogeneous glycopeptide having a molecular weight of 2800. The complex contained glucosamine, hexoses and fucose in a similar molar ratio to that contained in the parent glycoprotein. Aspartic acid and threonine were found to be separately bound to the oligosaccharide of the complex.

By an approach similar to that of Isumi et al. (1962), Eylar (1962) isolated a glycopeptide fraction whose sole amino acid was asparagine, based on his finding that the glycopeptide yielded one mole of NH_3 per mole of aspartic acid. From the fact that hydrolysis of DNP-glycopeptide gave DNP-aspartic acid and aspartic acid was not released from the glycopeptide by leucine aminopeptidase, the author postulated the presence of an aspartyl-glycosylamine linkage in this glycoprotein, as had previously been proposed by Johansen et al. (1961) for ovalbumin.

Structural Studies Concerning Bovine Submaxillary Glycoprotein

In studying the composition and structure of submaxillary gland glycoprotein, Graham and Gottschalk (1960) found that heating bovine submaxillary glycoprotein at 80°C. and pH 10.4, produced a 90% yield of a dialyzable disaccharide. The structure of this disaccharide was proven to be α-D-N-acetylneuraminyl (2,6) N-acetylgalactosamine. The

carbohydrate of bovine submaxillary gland glycoprotein consists almost exclusively of equimolar amounts of N-acetylneuraminic acid and N-acetylgalactosamine. N-acetylneuraminic acid always occupies the terminal position as indicated by its complete removal by neuraminidase from Vibrio cholerae (Graham and Gottschalk, 1960). Murphy and Gottschalk (1961A) reacted bovine submaxillary glycoprotein with $LiBH_4$ in tetrahydrofuran; at the end of reaction, the boron-intermediate complex formed was decomposed according to the following equation:



$$2RCO.OR' + LiBH_4 \longrightarrow Li\ (R\ CH_2O)_2B(OR')_2 \xrightarrow[3CH_3OH]{HCL} 2RCH_2OH + 2R'OH$$

They found the reduction of approximately 88% of the total dicarboxylic amino acid residues of the glycoprotein and release of unreduced disaccharide. For bovine submaxillary gland glycoprotein, reduction of 80% of the total dicarboxylic amino acid and 81% of the intact disaccharide were obtained (Murphy and Gottschalk, 1961). From these results, they concluded that the majority of the disaccharide of submaxillary gland glycoprotein is joined through a glycosidic-ester linkage to the β-carboxyl group of aspartyl and γ-carboxyl group of glutamyl residues respectively. A minor portion of this disaccharide was considered to be linked by an O-glycosidic bond to serine or threonine.

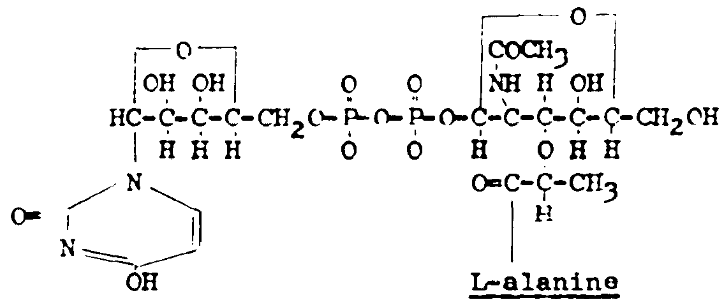
Structural Studies on Miscellaneous Glycoproteins

Masamune (1955) studied the blood group substances obtained from pig stomach mucus and provided evidence two types of carbohydrate-amino acid bonds. He suggested that one is an ether linkage between the

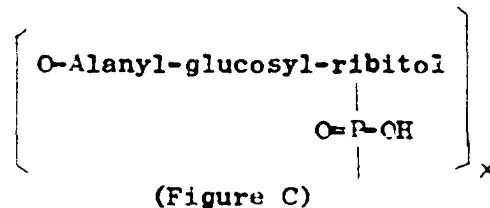
hydroxyl group of serine and a hydroxyl group of glucosamine or galactose, and the ether is a N-glycosidic linkage between the free amino group of a terminal aspartic acid and the reducing group of acetylglucosamine. Tsugita and Akabori (1959) digested crystalline Taka-amylase A with *Streptomyces griseus* protease. They isolated a glycopeptide by Dowex 50 resin chromatography and concluded that mannose was linked to the hydroxyl group of serine in the glycopeptide.

Bacterial cell wall substances. It is well established that bacterial cell walls contain lipid, polysaccharide, amino sugars and amino acid complexes. The cell walls of Gram-positive microorganisms apparently contain compounds of simpler structures than do Gram-negative microorganisms. They invariably contain D-alanine, D-glutamic acid and either lysine or diaminopimelic acid. They also contain N-acetylgucosamine, and N-acetylmuramic acid and phosphate ester. Strominger (1960) was able to establish that in *Staphylococcus aureus*, the molar ratio is as follows: acetylmuramic acid, 1; D-glutamic acid, 1; L-lysine, 1; D-alanine, 2; L-alanine, 1; acetylglucosamine, 2; and glycine, 4 or 5. Phosphorous is slightly larger than 1. He suggested that the cell wall may be composed of a repeating unit containing the above constituents and which is polymerized in an undetermined manner.

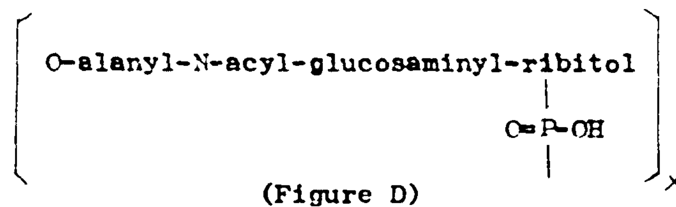
Park (1952) isolated three different compounds from penicillin treated *Staphylococcus aureus* and showed one of them was UDP-acetylglucosaminelactyl-alanine; in this amino acid sugar complex, lactic acid serves as the bridge between carbohydrate and amino acid.



Armstrong, Baddiley, Buchanan, Carss and Greenberg (1958) showed that cell walls of Gram-positive bacteria contain polymers of ribitol phosphate, called teichoic acids. They were able to show that the teichoic acids isolated from Lactobacillus arabinosus and Bacillus subtilis contain glucosyl residues jointed to ribitol and D-alanyl groups probably linked to the glucose (Figure C). In the teichoic acid from Staphylococcus aureus H, a N-acylglucosaminyl residue is present in place of glucose (Figure D).



(Figure C)



(Figure D)

Ghuysen (1961) isolated a disaccharide-peptide complex from the cell walls of Micrococcus lysodeikticus. He was able to show that the fragment 6-O-β-N-acetylglucosaminyl-N-acetylmuraminyl is joined to a peptide moiety consisting of 2 alanine, 1 glycine, 1 glutamic acid and

l lysine by an amide bond between the carboxyl group of N-acetylmuramic acid and the amino group of one of the alanine residues of the peptides.

Hochstraber (1961) isolated a ninhydrin positive substance from a formic acid hydrolysate of barley albumin treated with trypsin. He was able to prove that the structure of this compound was 4-L-alanyl-D-Xylo-pyranose.

CHAPTER III

MATERIALS AND METHODS

Microorganisms Tested for the Ability to Hydrolyze Glycoproteins

The following microorganisms were screened for their ability to hydrolyze glycoproteins: Bacillus subtilis, Bacillus megatherium, Corynebacterium diphtheriae, Corynebacterium bovis, Clostridium perfringens, Diplococcus pneumoniae 612, Escherichia coli, Neisseria gonorrhoeae, Proteus vulgaris, Pseudomonas aergineosa, Staphylococcus aureus, Streptococcus faecalis, Vibrio cholerae, Bacteriophage ØX 174, Influenza virus and Rous sarcoma virus. These microorganisms were obtained through the help of Dr. Florene Kelly, Department of Microbiology, University of Oklahoma Medical School and partly from the American Type Culture Collection, with the exceptions of Streptococcus faecalis, Bacteriophage ØX 174, Influenza virus and Rous Sarcoma virus. Streptococcus faecalis was a gift of Dr. John R. Sokatch, Department of Microbiology, University of Oklahoma Medical School; Bacteriophage ØX 174 was kindly provided by Mr. Bernard Bowman, Department of Microbiology, University of Oklahoma Medical School; Influenza virus and Rous Sarcoma virus were gifts of Dr. L. Vernon Scott, Department of Microbiology, University of Oklahoma Medical School.

The following screening procedure was performed in an effort to find microorganisms which would utilize or degrade the protein-bound hexoses of serum glycoprotein: pooled human, rat or bovine sera were sterilized by filtering through a Seitz filter. Two ml. of the serum was transferred to a sterile test tube and inoculated with the micro-organism to be tested. Duplicate tubes were prepared for incubations of 12, 24 and 48 hours at 37°C. A control serum seated in all respects except not inoculated with organism was incubated in the same manner. Sometimes 0.1 ml. of washed bacteria (approximately 2 x 10^{10} cells) were added directly to the serum samples. Following the incubation, the bacterial cells were removed by centrifugation and an aliquot taken for some determination of protein-bound hexoses by the tryptophan-sulfuric acid method as described by Shetlar *et al.* (1948).

From the results of screening, as will be discussed in Chapter V of this dissertation, it was found that *Diplococcus pneumoniae* 612 significantly was able to cause a decrease of serum protein-bound hexose. *Neisseria gonorrhoeae* is delicate and thus difficult to cultivate; therefore, *Diplococcus pneumoniae* 612 was chosen for extensive study. This organism was reported to be Type VI by the United States Public Health Service, Communicable Disease Center, Atlanta, Georgia.

Maintenance of the organism. The organism was maintained in the frozen stock by the following procedure: the organism from a blood agar slant was suspended in skimmed milk to make a heavy suspension, then quick-frozen by means of dry-ice and acetone and stored at -20°C. in the deep freezer. Viability was mainatined after three years of keep.

Media and cultivation of organism. For the production of pneumocococcal cells in large quantity, the organism was first transferred from the stock culture to a blood agar slant. After growing, the cells were transferred to 20 ml. of trypticase soy broth containing 10% human plasma and incubated at 37°C. for 8 hours. A large volume of medium was inoculated with the 8 hour culture at a culture medium ratio of 1:10. The inoculum was used if microscopic examination showed only Gram-positive diplococci and a blood agar smear revealed exclusively the characteristic glistening, pinpoint, grey colonies. Incubation was carried out at 37°C. and as soon as the growth reached the stationary phase (usually 16-20 hours) the cells were harvested by using a Sharple centrifuge or a Servall RC2 centrifuge with a continuous flow system. The bacterial cells were washed three times with 0.85% NaCl solution to remove the contaminated nutrients and lyophilized. The lyophilized cells were stored at -20°C. until used.

Preparation of cell-free extract. About 1 gm. of dried cells were suspended in 50 ml. of 0.02 M phosphate buffer, pH 6.6, containing 0.08% NaCl and subjected to sonic disintegration for 30 minutes with a Raytheon, magnetostrictive oscillator (250 Watt, 10 kc) or a Bronwill-Blackstone Biosonic Ultrasonic Probe. After centrifuging at 10,000 xg for 10 minutes at 4°C. in order to remove cell debris, the precipitate was discarded and the cell-free extract dialyzed against phosphate buffer for 6 hours with constant stirring and with several changes of buffer to remove endogeneous dialyzable nutrients. The dialyzed, cell-free extract was used as the enzyme source.

Substrates Used

Normal and Pathological Sera

Normal human sera were obtained shortly before use from healthy individuals and from patients with cancer and rheumatoid arthritis. Rat serum was collected by cardiac puncture, following ether anesthesia. Rabbit serum was obtained by cardiac puncture, following intraperitoneal injection of sodium pentobarbital (nembutal). Bovine sera were obtained from beef blood which was purchased from a local slaughterhouse.

Preparation of Radioactive Serum

Glucosamine-1-C-14 was injected intraperitoneally into rats (200-300 gms, 20 μc each) or rabbits (1.3-1.7 kg, 60 μc each). Blood was obtained by cardiac puncture 3 hours following injection for the rats and 6 hours for the rabbits.

Glycoproteins

1. Cohn fraction VI. Both human and bovine glycoproteins (Cohn fraction VI) were purchased from Pentex Inc., Illinois.

2. Seromucoid. Seromucoids from various animals were prepared according to the method of Goa (1960) with slight modification. One volume of serum was diluted with one volume of 0.9% NaCl. Serum protein other than seromucoid was then precipitated by the addition of two volumes of 1 M perchloric acid. The precipitate was immediately filtered. The filtrate subjected to exhaustive dialysis was then

lyophilized.

3. Orosomucoids. Human and bovine α_1-acid glycoprotein (orosomucoid) were prepared from corresponding Cohn fraction VI, by using carboxymethyl cellulose column chromatography according to the method described by Bezkorovainy and Winzler (1961).

4. Bovine submaxillary gland glycoprotein. This glycoprotein was obtained from Wilson & Co., Inc., Chicago, Illinois.

5. Neuramin-lactose was a gift from Dr. Raul E. Trucco.

6. Ovomucoid was purchased from Mann Research Laboratories, New York.

Chemicals

1. N-acetylneuraminic acid was synthesized according to the method of Conforth, Firth and Gottschalk (1958), and also purchased from California Corporation for Biochemical Research, Los Angeles, California.

2. O-Nitrophenyl β-D-galactopyranoside was purchased from the Sigma Chemical Company, St. Louis, Missouri.

3. p-Nitrophenyl-N-acetyl- β-D-glucosaminide was a kind gift of Dr. P. G. Walker, Department of Biochemistry, Institute of Orthopaedics, Royal National Orthopaedic Hospital, Stanmore, Middlesex, England.

Chemical Analysis

Determination of Bound Hexose

Quantitative determination of protein-bound hexoses was performed

by the tryptophan-sulfuric acid method of Shetlar, Foster and Everett (1958). An equimolar mixture of galactose and mannose was used as a standard.

Hexosamine Determination

Total hexosamine was determined by the method of Boas (1953), employing Elson and Morgan's acetylacetone reagent and Ehrlich's reagent (2.67% p-dimethyl aminobenzaldehyde in 1:1 mixture of absolute ethanol and concentrated hydrochloric acid) after removal of interfering substances (largely amino acids) by Dowex 50 resin. Glucosamine hydrochloride was used as a standard.

Glucosamine and Galactosamine Determination

Glucosamine and galactosamine can be easily separated and quantitatively determined by the Beckman, Amino Acid Analyzer, using the short column technique as described by Spackman, Stein and Moore (1958). The type 15A resin which size is approximately 25-30 μ used in this column was purchased from Beckman Spinco Co. Citrate buffer containing 0.2 N sodium ion, pH 5.28, is automatically controlled at 30 ml. per hour. The temperature of this column is maintained at 50°C. Glucosamine and galactosamine peaks always appear before the lysine peak in about 40 minutes. The glucosamine peak precedes the galactosamine peak by 10 minutes.

N-acetylglucosamine Determinations

Free N-acetylglucosamine was determined by the Morgan-Elson

reaction as modified by Leissig, Strominger and Leloir (1955).

Fucose Determinations

Winzler's modification (1954) of Dische and Shettle's (1958) cysteine-sulfuric acid method was used for the determination of protein-bound fucose.

Sialic Acid Determinations

Sevennerholm's (1957) resorcinol-hydrochloric acid method was used for the determination of protein-bound sialic acid. Free sialic acid was determined by the thiobarbituric acid method of Warren (1959).

Total Protein Determinations

Total protein was determined by the biuret method or by using Folin's phenol reagent as described by Lowry *et al*. (1951). Lab-Trol of Dade reagents, Inc., Miami, Florida, was used as a standard.

Amino Acid Analyses

The protein samples were hydrolyzed with 6.0 N HCl in sealed, thick-wall tubes at 110°C. for 22 hours. Quantitative determinations of amino acids were carried out by Beckman Automatic Amino Acid Analyzer as described by Spackman, Stein and Moore (1958).

Electrophoretic Studies

Paper-strip electrophoresis techniques, as previously described by Shetlar *et al*. (1956), was carried out by using Spinco hanging strip electrophoresis cells. Three mm. filter paper was used for all work.

Electrophoresis was conducted at constant current, 5 milliamperes for 8 strips, using barbital buffer, 0.075 M, pH 8.6, at approximately 25°C. for 16 hours. Eight microliters for protein and 30 microliters for glycoprotein were made of each serum sample. For estimation of protein, the strips were developed with bromophenol blue. For glycoprotein studies, the strips were subjected to the periodic acid Schiff reaction, modified from the method of Koiw and Gronwall (1952). The protein strips were quantitated with Spinco Analytrol Model RB, using 500 mu interference filters. The glycoprotein strips were similarly evaluated with the same instrument with 550 mμ interference filters. The total area under the protein curve was equated to the serum protein as determined by the biuret reaction, and the area under the glycoprotein curve was similarly made equal to the total serum glycoprotein determined by the tryptophan-sulfuric acid method.

Chromatography of Sugars

Qualitative identification of neutral sugars was made by paper chromatography, using Whatman No. 1 filter paper and a solvent system containing butanol-pyridine-0.1 N HCl (5:3:2). Carbohydrates were detected by dipping the paper in o-amino-biphenyl, as described by Gordon, Thornburg and Werum (1956). The samples were demineralized by an electric desalter (Research Specialties Co., Calif.) if necessary.

Glycoprotein or glycopeptide was prepared for chromatographic identiiication of hexoses by an initial hydrolysis with 2.0 N HCl at 100°C. for 18 hours in a tightly closed, screw-capped tube. The

hydrolysate was mixed with charcoal and filtered through Whatman No. 5 filter paper and the filtrate taken to dryness in a vacuum oven. The residue was taken up in 1 ml. of water and again taken to dryness. After storing *in vacuo*, over soda lime, for 24 hours, the residue was dissolved in 0.1 ml. of 10% isopropanol in water prior to chromatography.

For chromatographic studies of hexosamine, glycoprotein or glycopeptide was hydrolyzed with 4 N HCl for 16 hours. The material was then dissolved in 1.0 ml. of water and passed through a column of Dowex 50 x 12 resin, in the hydrogen form. After washing the column with water to remove neutral sugars, 0.3 N HCl was added to elute amino sugars and some amino acids. This latter solution was taken to dryness and dissolved in 0.1 ml. of water prior to chromatography. The chromatograms were developed in a pyridine:ethyl acetate:water (1:24:0.8) system.

Ultracentrifugation

Ultracentrifugation studies on the homogeneity of glycoproteins were carried out by Spinco Model E analytical ultracentrifuge at 59,780 rpm.

CHAPTER IV

EXPERIMENTAL RESULTS

Preliminary Survey for Microorganism which Will Degradate Serum Protein-Bound Hexose

As noted in Chapter II, some microorganisms (e.g., Influenza virus, *Clostridium welchii*, *Vibrio cholerae*) are able to produce neuraminidase and thus split off sialic acid from glycoproteins. Prior to this work, however, no microorganism was known with certainty to have an ability to utilize hexoses and other bound carbohydrates of serum glycoprotein. The results of the preliminary survey are summarized in Table II. Among those organisms tested, only *Diplococcus pneumoniae* 612 and *Neisseria gonorrhoeae* were shown to cause detectable decrease of the protein-bound hexoses in serum. The decrease was most prominent and consistent after incubation with *D. pneumoniae*. *Corynebacterium diphtheriae* and *Corynebacterium enzymicum* also caused some decreases in protein-bound hexose, but the results were not consistent.

After a 20 hour incubation of mammalian serum inoculated with *D. pneumoniae*, the pH decreased from 7.4 to 5.5 and the observed turbidity was associated with a large quantity of pneumococcal cells detected by

TABLE II

PER CENT DECREASE OF PROTEIN-BOUND HEXOSE
AFTER INCUBATION OF MICROORGANISMS
WITH HUMAN SERA FOR 24 HOURS

Microorganisms Tested	% Decrease of Bound Hexoses
Bacillus subtilis	0
Bacillus megatelium	0
Corynebacterium diphtheriae	0-5
Corynebacterium enzymicum	0-5
Corynebacterium bovis	0
Clostridium perfringens	0
Diplococcus pneumoniae 612	15-30
Escherichia coli	0
Neisseria gonorrhoeae	5-10
Proteus vulgaris	0
Pseudomonas aerugenosa	0
Staphylococcus aureus	0
Vibrio cholerae	0
Bacteriophage ØX 174	0
Influenza virus	0
Rous Sarcoma virus	0

microscopic examination following Gram-staining of an aliquot. No turbidity was found in the control serum sample (not inoculated with D. pneumoniae) under similar conditions. In contrast to D. pneumoniae, no appreciable growth and less striking pH changes (from 7.4 to 6.6), could be found in the serum sample inoculated with Neisseria gonorrhoeae. Due to these observations and because of the difficulty of cultivating Neisseria gonorrhoeae, Diplococcus pneumoniae 612 was chosen for subsequent studies.

Further Studies on the Degradation of Serum Glycoprotein by Diplococcus Pneumoniae 612

Degradation of Protein-Bound Hexose by Pneumococci

Diplococcus pneumoniae 612 was used for further studies of the degradation of protein-bound hexose in normal and pathological human sera and rat and bovine sera. Table III presents the changes of protein and protein-bound hexose of various sera after incubation with D. pneumoniae for 20 hours at 37°C. These data indicate that D. pneumoniae cause significant decreases of protein-bound hexose of human (both normal and pathological), rat and bovine sera. These sera with higher glycoprotein (cancer, arthritis and rat sera) show more striking decreases in bound hexoses after incubation with the organism. D. pneumoniae also cause some decrease in total serum protein content, suggesting that the organism may have proteolytic activity. Fresh serum

TABLE III

CHANGES OF SERUM PROTEIN AND GLYCOPROTEIN AFTER INCUBATION WITH *DIPLOCOCCUS PNEUMONIAE* 612

Conditions	Total Protein Gm./100 ml.		Total Glycoprotein Mg./100 ml.	
	Organism	Control	Organism	Control
Human, Normal (4)*	6.64±.07**	6.93±.08	99 ± 3.1	117±3.5
Human, Carcinomatosis	4.75	5.04	134	174
Human, Breast Cancer	7.12	7.62	152	176
Human, Rheumatoid Arthritis	6.10	6.66	129	180
Human, Rheumatoid Arthritis	5.87	6.19	158	182
Rat	5.40	5.65	146	172
Bovine	6.46	7.13	130	151

*Number of Cases

**Standard Error

from some individuals was found to be resistant to pneumococcal action; however, after heating this serum at 60°C. for 1 hour, it could be degraded by _D. pneumoniae_. This suggests that the phenomenon may be due to inhibitors, which may be proteins.

Electrophoretic Studies of Serum after Incubation with _D. pneumoniae_

After degradation of serum glycoprotein by _D. pneumoniae_ was demonstrated by chemical analysis, the glycoprotein changes in different serum fractions was investigated. Paper strip electrophoresis of mammalian serum samples indicated that pneumococcal effects were exerted largely on the α_1-globulin fraction. Figure I is a typical electrophoretic study showing the change obtained with arthritis serum after incubation with _D. pneumoniae_. A significant decrease of the α_1-globulin band as demonstrated by staining for protein or glycoprotein is noted in the samples incubated with the organism. This disappearance of α_1-globulin band in the electrophoretic pattern may result from: (1) reduction in the mobility of this fraction, due possibly to the loss of some highly charged compound (e.g., sialic acid), or, (2) a preferential degradation of α_1-globulin by _D. pneumoniae_. The first possibility cannot be easily excluded because in some instances the serum protein fractions did appear to show retardation of electrophoretic mobility after incubation. Figure II is the electrophoretic pattern of cancer serum after incubation with _D. pneumoniae_. It shows some decrease in electrophoretic mobility of the α_2- and β-globulin

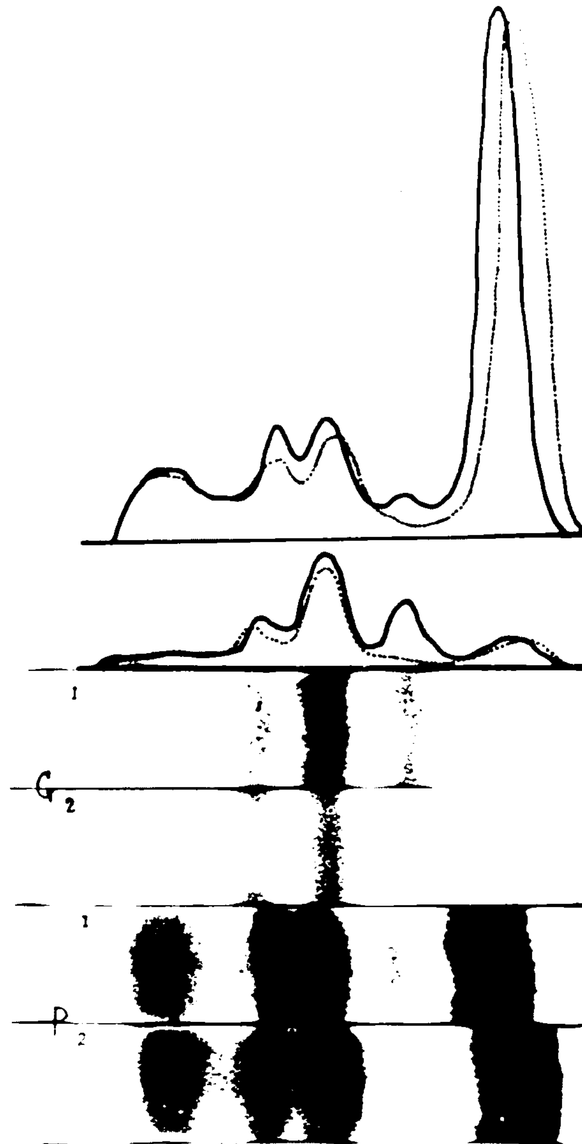


Figure I. Alterations of electrophoretic patterns of human serum after incubation with Diplococcus pneumoniae. The lower two strips (marked "p") are stained for protein with bromophenol blue. No. 1 is of control, incubated without organism; No. 2 is a strip of the sample after incubation with microorganism. Upper strips (marked "G") are corresponding strips stained by the periodic-acid-Schiff procedure for glycoprotein, (strip No. 2). Above both strips are the densitometer tracings of these patterns. Protein tracings (from "p") are at top. In both cases, the control tracings are shown by solid lines and those from samples incubated with the organism are shown as dotted lines.

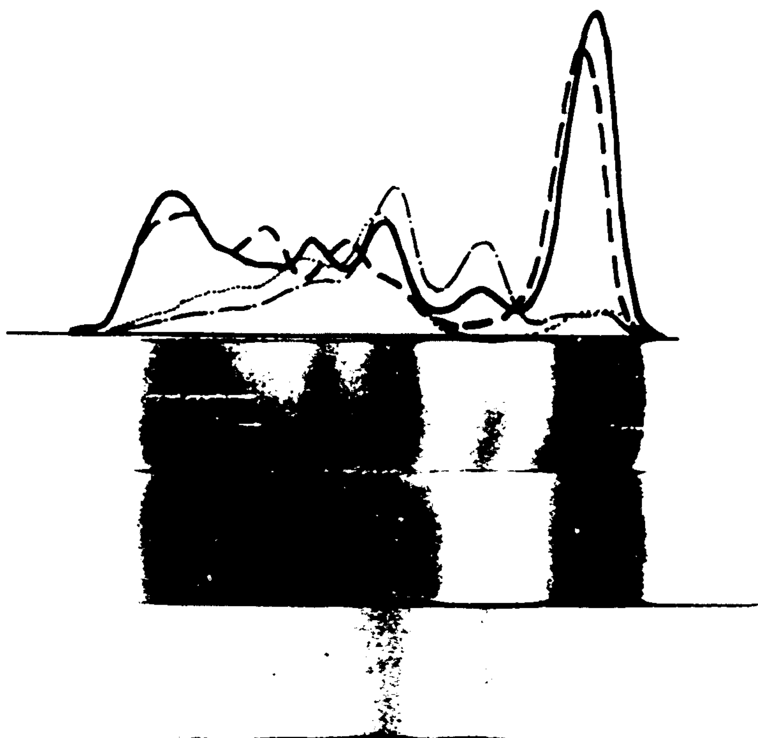


Figure II. Decrease in electrophoretic mobility of the α_1-, α_2-, and β-globulin fractions of human cancer serum after incubation with *Diplococcus pneumoniae*.

fractions. Although disappearance of α_1-globulin fraction can be seen after incubation, the appearance of a faint band in preceeding the α_2-globulin band is also noted.

Changes in Bound Hexose in Different Protein Fractions of Human Serum after Incubation with *D. Pneumoniae*

Data presented in Table IV demonstrate the changes of bound hexose in different electrophoretic fractions of human serum samples after incubation with *D. pneumoniae*. These are detected by staining the paper electrophoretic strips with periodic-acid-Schiff reaction for bound hexoses.

After incubation with *D. pneumoniae*, the α_1-globulin fraction is significantly decreased to less than 20% of that found in the control serum, whereas there are no significant changes in albumin, α_2-, or β-globulin fractions. The decrease in total bound hexoses between control serum and serum after incubation is 32 mg%, a value which agrees well with the amount of glycoprotein lost from the α_1-globulin fraction (30 mg%).

Although chemical analysis and paper electrophoresis studies indicate a decrease of protein-bound hexose in the α_1-globulin of the serum after incubation with *D. pneumoniae*, it was still not possible to conclude that the decrease of the serum protein-bound hexose was entirely attributable to the disappearance of α_1-globulin fraction as observed by paper electrophoresis.

TABLE IV

EFFECT OF D. PNEUMONIAE ON SERUM GLYCOPROTEIN FRACTIONS*

	Mg% Bound Hexose of					
	Total Serum	Albumin	Globulin			
			α_1	α_2	β	γ
After Incubation	134	17	7 (0-16)	55	30	25
Control	166	17	37 (19-62)	57	31	22

*1 normal subject, 2 patients with cancer, 2 patients with severe rheumatoid arthritis

Incubation of Serum Containing Radioactive Glycoprotein with D. pneumoniae

After intraperitoneal injection of glucosamine-1-C-14 into rats and rabbits, radioactivity was rapidly incorporated into serum glycoprotein. The majority of the radioactivity was found associated with α_1-, α_2-, and β-globulins. Table V shows the distribution of radioactivity in rat serum after injection of glucosamine. The radioactivity in different fractions is roughly correlated with the hexosamine content and is highest in the seromucoid and α_1-globulin fractions. Serum containing glycoprotein labeled by administration of glucosamine-1-C-14 was incubated with D. pneumoniae and the radioactivity of fractions determined. This experiment was designed to evaluate the extent of the α_1-globulin degradation. Table VI shows the changes in radioactivity obtained in different fractions after incubation with pneumococci for 20 hours.

After incubation, aliquots of the serum were placed on planchets for radioactivity determination with a gas-flow counting unit. The radioactivity in different protein fractions was also determined with this instrument after excising out the protein bands from electrophoresis strips after staining with bromophenol blue. Table VI shows that, incubation of the rat serum with pneumococcal cell resulted in a 16% decrease in radioactivity (from 1001 to 838 c/m/10 μl) disappeared. If the organism only hydrolyzed the glucosamine from the protein-bound state to the free form, no radioactivity change would be expected since

TABLE V

RADIOACTIVITY IN SERUM FRACTIONS AFTER ADMINISTRATION OF GLUCOSAMINE-1-C-14 TO RAT (SHETLAR ET AL., 1961)

	C/m/mg Protein	Hexosamine Content (%)
Total Protein	2,900	1.1
Seromucoid	8,900	10.0
α_1-globulin	7,900	6.3
α_2-globulin	5,900	4.2
β-globulin	2,900	1.9
γ-globulin	1,100	1.5
Albumin	400	0.06

TABLE VI

CHANGES OF RADIOACTIVITY IN RAT SERUM PROTEIN FRACTIONS AFTER INCUBATION WITH *DIPLOCOCCUS PNEUMONIAE*

	C/m/10 µl Total Activity	Counts Per Minute in* Albumin	Globulin α_1	α_2	β	γ	Sum of Activity in Different Fractions, c/m/10 µl
Organism	838	8	162	229	128	45	572
Control	1001	36	547	235	211	33	1062

*10 µl of the serum was used for electrophoresis.

the sample had not been subsequently subjected to dialysis or other procedure which would remove monosaccharide. One would postulate that the 16% decrease in radioactivity observed following incubation might result through a process whereby protein-bound C-14-containing substances were metabolized to volatile C-14-labeled compounds, for example, protein-bound C-14-glucosamine were hydrolyzed and subsequently metabolized to $C^{14}O_2$.

A recovery of approximately 100% was obtained when control serum was separated by paper strip electrophoresis (recovery based on summation of the radioactivity present in individual protein fractions). In contrast, only 68% of the radioactivity could be recovered after serum incubated with pneumococci was subjected to a similar electrophoretic process (summation of radioactivity in individual fractions was 572 cpm/10 µl whereas the total serum value was 838 cpm/10 µl.). These data suggest that, in the control serum, radioactive glucosamine was present in protein-bound form. Incubation with D. pneumoniae yield some free radioactive glucosamine which had a greater electrophoretic mobility than any serum protein fraction and, therefore, had moved off the paper strip. The radioactivity was highest in α_1-globulin, considerable radioactivity was also associated with β-and α_2-globulin. Only slight radioactivity was associated with albumin or γ-globulin fractions. After incubation, disappearance of radioactivity was most prominent in the α_1-globulin fraction (decrease about 70%) and to a less extent in the β-globulin fraction. There was no appreciable changes in α_2- and γ-globulin fractions. Although the radioactivity

level in albumin was not very high, almost all of the initial activity was gone after incubation. This may be due to: (1) incomplete electrophoretic separation of albumin from α_1-globulin, which is subsequently degraded by the organism, (2) the radioactivity detected in the albumin may be associated with the so called "pre-albumin", a fraction rich in bound carbohydrates which may be hydrolyzed by the organism.

A similar study was made using rabbit serum obtained from animals administered glucosamine-C-14. The serum was subjected to paper electrophoresis after incubation and the radioactivity distribution in different protein fractions recorded by the Baird Atomic Accessories Scanogram III (windowless 4π Chromatogram Scanner). Results are shown in Figure III. This figure also shows the very high activity present in α-globulin fraction of control serum (α_1- and α_2-globulin are not well separated in this experiment). After incubation with <u>D. pneumoniae</u> about 80% of the total radioactivity, estimated from the area under the curve, disappeared. The loss in radioactivity was most striking in the α_1-globulin fraction but decreases in radioactivity in other fractions also can be noted. In this experiment, dried intact pneumococcal cells were used instead of growing the organism on the serum and the decrease in total radioactivity was more striking. An alternative explanation for the relatively greater decreases in radioactivity is that rabbit serum is more easily degraded by <u>D. pneumoniae</u> than is rat serum.

From the above experiments, it would appear that: (1) disappearance of the electrophoretically separated α_1-globulin fraction demonstrated as protein or glycoprotein after incubation of the serum sample with

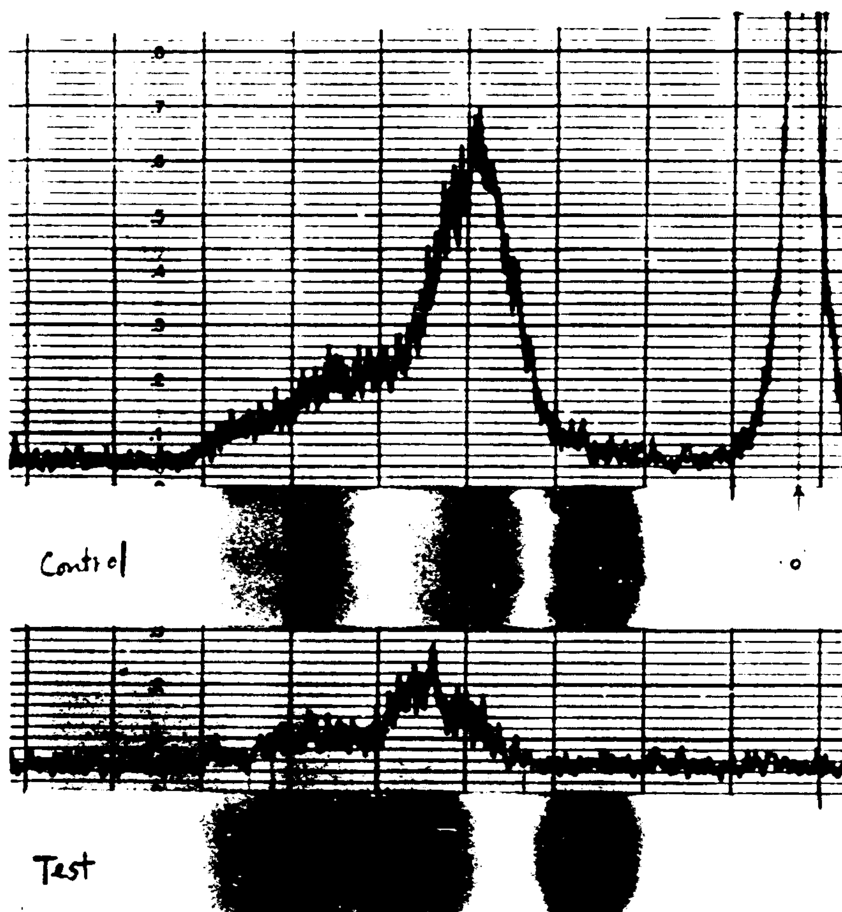


Figure III. Changes of radioactivity in rabbit serum protein fractions after incubation with Diplococcus pneumoniae. The control strip is of the serum sample before incubation; the strip which is marked "test" is after incubation with pneumococcal cells for 20 hours. Both strips were stained for protein with bromophenol blue. Above both strips are the corresponding radioactivity scans of the radioactivities in different fractions. The first peak to the right is due to a spot of glucosamine-1-C-14 placed on the strip after electrophoresis for reference purpose.

D. pneumoniae was due to extensive degradation of this fraction by the organism, (2) some reduction in electrophoretic mobility and decreases in radioactivity in α_1, α_2, and β-fractions were also noted, and, (3) the organism is able to remove both protein-bound hexose (indicated by the tryptophan-sulfuric acid method) and protein-bound hexosamine.

Changes of Various Protein Bound Carbohydrates in Sera after Incubation with Diplococcus Pneumoniae

Results of previous experiments indicated that D. pneumoniae was able to cause decreases of both the protein-bound hexose and protein-bound hexosamine of mammalian serum. The following experiment was performed to observe changes in three of the major serum protein-bound carbohydrates (sialic acid, hexoses and hexosamine) produced by incubation of serum with D. pneumoniae for 20 hours at 37°C.

As indicated in Table VII, D. pneumoniae are able to hydrolyze hexose, hexosamine and sialic acid from serum glycoprotein. The observation that sialic acid appears in relatively greater amounts than the other carbohydrates may be explained by the fact that sialic acid is frequently, if not always, present in the terminal position of the polysaccharide chain and thus may be more easily hydrolyzed by the organism. Pneumococcal neuraminidase activity has been reported by Heimer and Meyer (1956) and by Oncley, Eylar and Schmid (1958).

TABLE VII

CHANGES OF PROTEIN-BOUND CARBOHYDRATES AFTER INCUBATION OF HUMAN SERA WITH <u>D. PNEUMONIAE</u>

Condition	Hexose mg./100 ml.		Hexosamine mg./100 ml.		Sialic Acid mg./100 ml.	
	Control	Organism	Control	Organism	Control	Organism
Normal (4)*	117±3.5**	99±31	75±3.2	66±2.2	66±4.0	44±3.0
Carcinomatosis	174	134	89	62	86	52
Rheumatoid Arthritis I	180	129	92	67	90	47
Rheumatoid Arthritis II	182	158	96	64	86	47

*Number of Cases

**Standard Error

Detection of Free Carbohydrates after Incubation of Serum with Pneumococci

It has been shown that *D. pneumoniae* cause striking decreases in protein-bound hexoses, hexosamine and sialic acid of mammalian sera. An attempt was, therefore, made to analyze free monosaccharides in the sera after incubation with *D. pneumoniae*. Among the major bound carbohydrates (sialic acid, hexosamine and hexoses, including galactose and mannose), free sialic acid and free hexosamine are easily distinguished from their bound form. Free sialic acid may be assayed by Warren's thiobarbituric acid method (1959). This method is based on the observation that sialic acid, but not bound sialic acid, can be oxidized with periodate ion to form formyl pyruvate, which in turn reacts with thiobarbituric acid to produce a red color. Bound hexosamine is found as the N-acetylated form in glycoproteins. Thus, if hexosamine (bound) is hydrolyzed enzymatically, a free N-acetylated hexosamine, which may be determined by the Morgan and Elson reaction, would be obtained. The amounts of free sialic acid and free N-acetylhexosamine detected in the serum after incubation with *D. pneumoniae* are presented in Table VIII. Some disparity was observed between the loss of protein-bound carbohydrates and the recovery of monosaccharides in the serum after incubation with *D. pneumoniae*, suggesting, as one alternative, that *D. pneumoniae* may be able to metabolize monosaccharides. This hypothesis was further studied by incubating 1 ml. of a solution containing 0.1% each of sialic acid, N-acetyl glucosamine and galactose in 0.05 M

TABLE VIII

RELATIONSHIP BETWEEN APPEARANCE OF FREE SUGARS AND DISAPPEARANCE OF PROTEIN-BOUND CARBOHYDRATES AFTER INCUBATION WITH D. PNEUMONIAE*

	Bound Carbohydrates mg. per 100 ml.			
	Control	After Incubation	Difference	Free Carbohydrate mg. per 100 ml.
Hexosamine	96	64	32	19**
Sialic Acid	86	47	39	22

*Serum from human subject with severe arthritis

**Converted to glucosamine from N-acetylglucosamine

phosphate buffer at pH 6.5 with approximately 0.1 mg. of dried pneumococcal cells. Five to ten percent of the carbohydrate lost from the protein-bound fraction could not be recovered in the free carbohydrate fraction at the end of 1 hour. Pneumococcal cells were then suspended in phosphate buffer, disrupted by sonic oscillation and the particulate fraction removed by ultracentrifugation at 105,000 xg for 20 minutes. This cell-free extract retained a substantial portion of its capacity to hydrolyze carbohydrate from serum glycoprotein but had much less activity in degrading monosaccharides than did the intact cells.

Incubation of Isolated Glycoprotein with Pneumococcal Extract

Incubation of Bovine Orosomucoid with *D. Pneumoniae*

It has been previously shown that the quantity of α_1-globulin demonstrable by paper strip electrophoresis is substantially decreased when serum samples are incubated with pneumococcal cells or pneumococci are grown in the serum. Orosomucoid, a glycoprotein present in the α_1-globulin fraction, has a very high bound carbohydrate content and has been relatively well studied. Orosomucoid was, therefore, chosen as a substrate for further studies of monosaccharide liberation by *D. pneumoniae*. Bovine orosomucoid, chosen for this study largely because the starting material is cheaper and easier to obtain, was prepared by the method of Bezkororainy and Winzler (1961), using bovine glycoprotein (Cohn Fraction VI) as a starting material. The orosomucoid prepared

was found to be homogeneous by paper electrophoresis (at pH 8.6 and 4.5) and ultracentrifugation. Figure IV shown the ultracentrifugal pattern of bovine orosomucoid at a concentration of 10 mg. per ml. of 0.2 M NaCl and demonstrates a single symmetrical peak, indicating the homogeneity of the preparation. The chemical composition of this bovine orosomucoid is presented in Table IX. The carbohydrate composition of bovine orosomucoid is very similar to that of human orosomucoid. About 40% of this glycoprotein is carbohydrate. Both glucosamine and galactosamine, in a molar ratio of 13:1 as determined by amino acid analyzer, have been observed, as shown by Figure V. Aspartic acid and glutamic acid are the amino acids present in greatest quantity in bovine orosomucoid, although almost all the common amino acids found in nature have been reported.

Upon incubation of bovine orosomucoid with pneumococcal extract for 3 hours, free sialic acid, galactose and N-acetylglucosamine were detected in the incubation mixture by chemical analysis and paper chromatography. The result of this experiment suggests that D. pneumoniae contain several glycosidases which hydrolyze sialic acid, N-acetylglucosamine and galactose from bovine orosomucoid.

Incubation of Bovine Submaxillary Gland

Glycoprotein with D. Pneumoniae

The previous experiments indicated that D. pneumoniae were able to hydrolyze sialic acid, galactose and N-acetylglucosamine from bovine orosomucoid. An experiment was therefore performed to observe whether

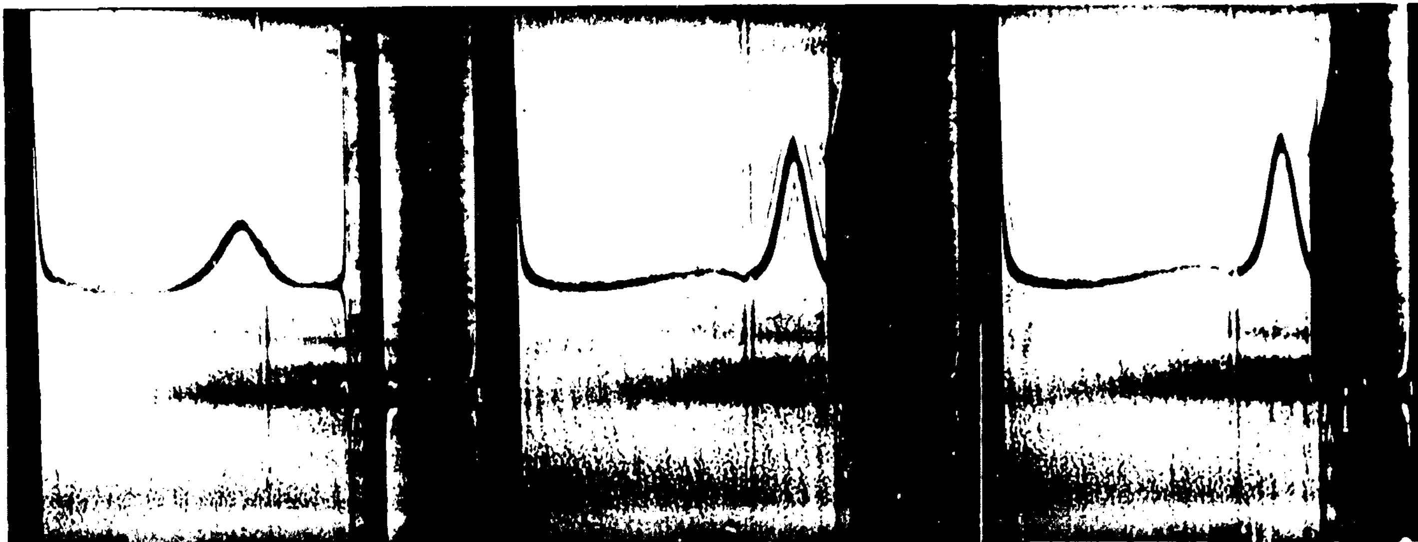


Figure IV. Ultracentrifugal pattern of bovine orosomucoid. The pictures shown were taken at 11, 14 and 78 minutes after commencement of run. Sedimentation proceeds from left to right.

TABLE IX

CHEMICAL COMPOSITION OF BOVINE OROSOMUCOID

	Percent Dry Weight
Lysine	4.06
Histidine	1.21
Arginine	2.76
Aspartic acid	5.34
Threonine	3.32
Serine	1.68
Glutamic acid	8.28
Proline	1.82
Glycine	1.26
Alanine	1.67
½ Cystine	0.82
Valine	2.09
Methionine	0.40
Isoleucine	1.93
Leucine	3.64
Phenylalanine	2.94
Tyrosine	3.10
Hexose*	14.1

TABLE IX - Continued

	Percent Dry Weight
Hexosamine**	11.3
Sialic acid	14.0
Fucose	0.5

*Consists of both mannose and galactose, as detected by paper chromatography.

**Consists of both glucosamine and galactosamine in a molar ratio of 13:1, as determined by amino acid analyzer.

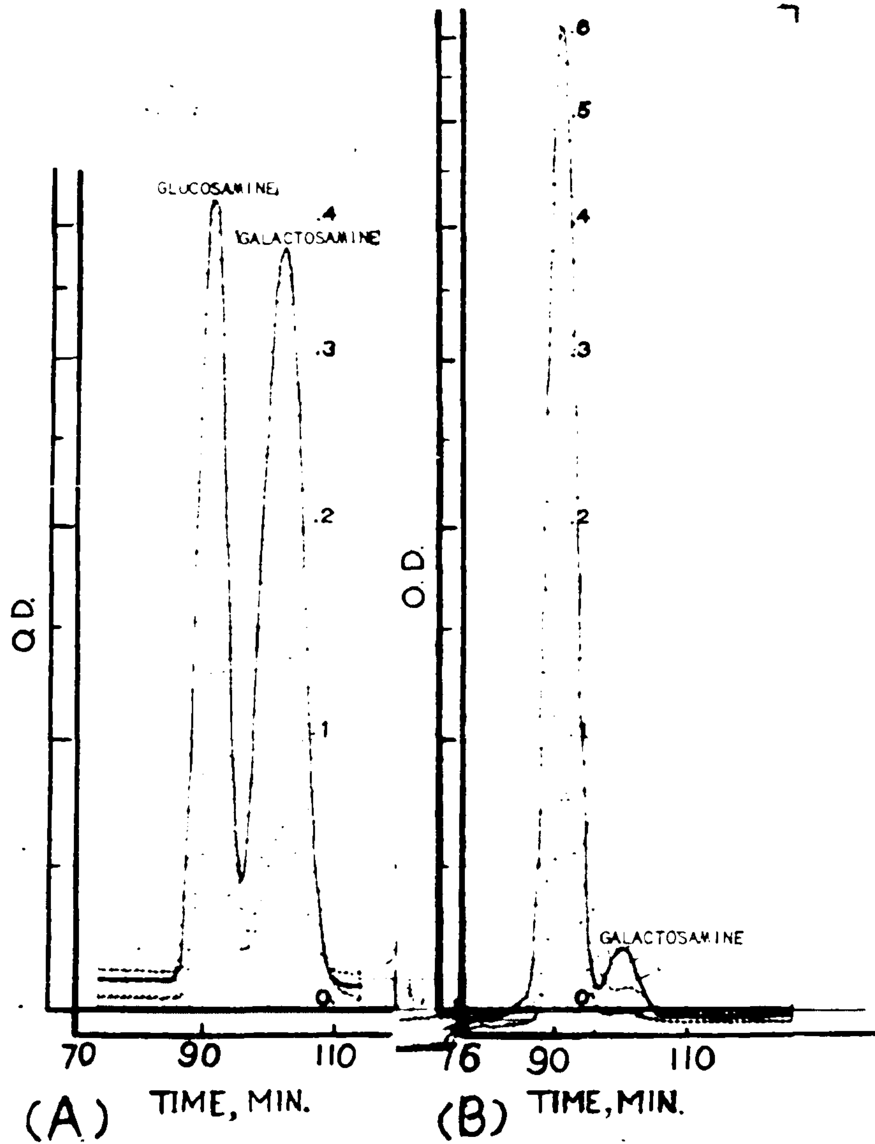


Figure V. Separation of Amino Sugars from Bovine Orosomucoid Hydrolysate by Amino Acid Analyzer. (A) Standard; (B) amino sugars from the hydrolysate of bovine orosomucoid.

D. pneumoniae were able to hydrolyze carbohydrates from other glycoproteins. Bovine submaxillary gland glycoprotein was chosen because this glycoprotein contains a large amount of sialic acid, N-acetylgalactosamine and appreciable N-acetylglucosamine and galactose (Niizawa and Pigman, 1959). Several spots were detected after paper chromatography of the incubation mixture of this glycoprotein with pneumococcal extract. Spots corresponding to sialic acid, galactose and N-acetylglucosamine and a strong spot somewhat above the N-acetylglucosamine reference spot were noted. No authentic N-acetylgalactosamine was available for reference purposes. After removal of glycoprotein by alcohol precipitation, the incubation mixture was hydrolyzed with 2 N HCl for 3 hours to remove the acetyl group from the N-acetylated amino sugars. The presence of free galactosamine and glucosamine was verified by the amino acid analyzer. Figure VI is a chromatogram showing the presence of both glucosamine and galactosamine in the incubation mixture, this indicating that pneumococcal extract was able to hydrolyze galactosamine as well as glucosamine from glycoprotein.

Incubation of Ovomucoid with
Pneumococcal Extract

According to Gottschalk and Ada (1955), ovomucoid contains 1.4% galactose, 4.3% mannose and 14% glucosamine, but no fucose, galactosamine or sialic acid.

Figure VII is the paper chromatogram showing the appearance of N-acetylglucosamine, galactose and mannose in the incubation mixture after

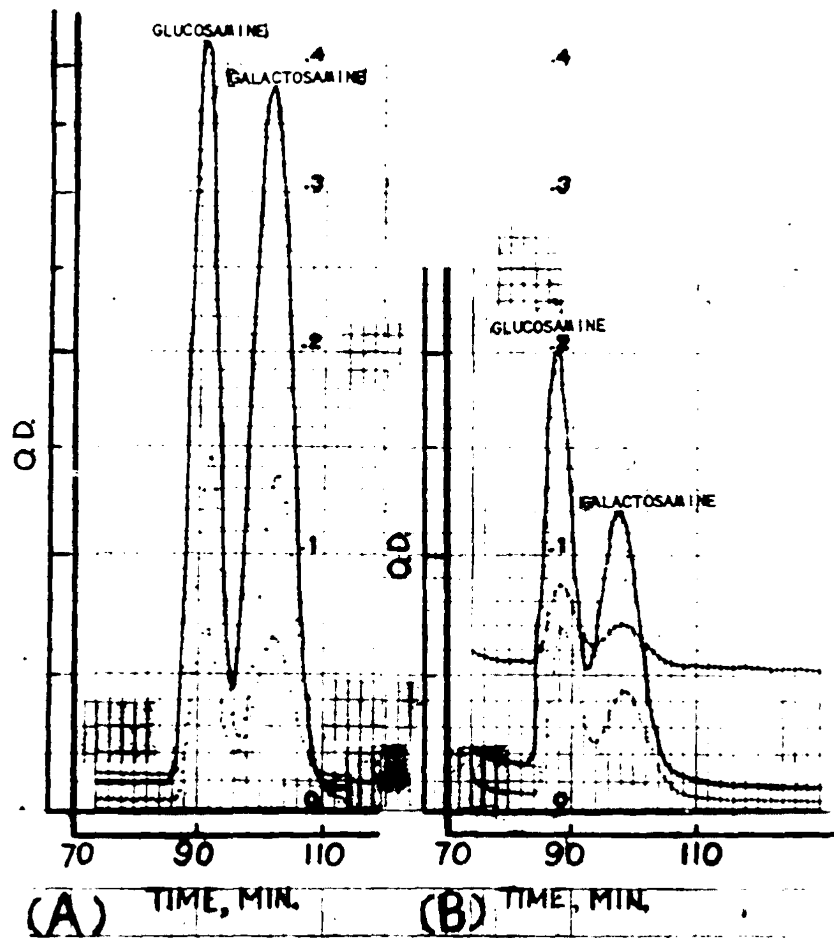


Figure VI. Liberation of glucosamine and galactosamine from bovine submaxillary gland glycoprotein by D. *pneumoniae*. (A) Standard; (B) Incubation mixture.

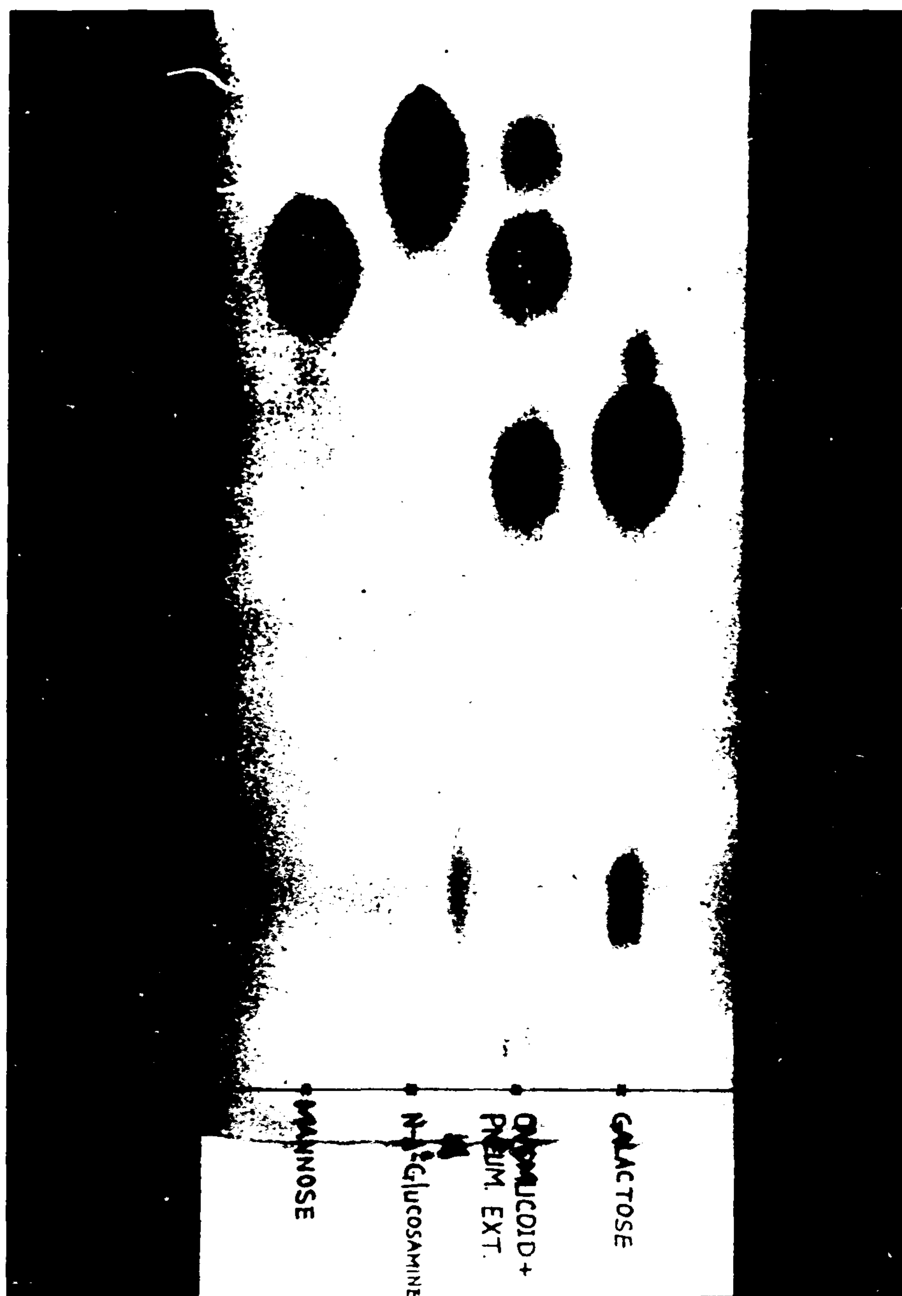


Figure VII. Liberation of monosaccharides from ovomucoid by _D. pneumoniae_.

incubation of ovomucoid with pneumococcal extract for five hours. The result again indicates that *D. pneumoniae* contains several glycosidases which enable this organism to hydrolyze carbohydrates from different glycoproteins.

Incubation of Pneumococci with Miscellaneous Glycoproteins

Pneumococcal extract hydrolyzed sialic acid and hexoses from sheep follicle-stimulating hormone and destroyed most of the biological activity of this hormone (Cahill, 1961). Incubation of glycopeptide prepared from bovine β-globulin (Yang, 1962) with this organism resulted in liberation of glucosamine, galactose and mannose as detected by paper chromatography. *D. pneumoniae* were also able to hydrolyze hyaluronic acid to produce N-acetylglucosamine and glucuronic acid. This suggests that the organism must have either β-glucosaminidase or β-glucuronidase in addition to hyaluronidase, since the end product of hyaluronidase is known to be β-glucuronido-N-acetylglucosamine.

Studies of Glycosidase Activities in Pneumococcal Extract

Neuraminidases

The neuraminidase activity of pneumococcal extract was determined directly by Warren's thiobarbituric acid method for free N-acetylneuraminic acid liberated in the incubation mixture. Bovine orosomucoid

bovine submaxillary gland glycoprotein and neuramin-lactose were used as substrates. The reaction was carried out using 1 mg. of bovine submaxillary glycoprotein (containing 180 μg sialic acid), 1 mg. of orosomucoid (containing 110 μg sialic acid), or 50 ug of neuramin-lactose in a final volume of 0.2 ml.; 20 um phosphate buffer, pH 6.0, and pneumococcal extract. After incubation at 37°C., the thiobarbituric acid assay for liberated sialic acid was carried out directly on the vessel contents. A 10 to 15 fold purification of neuraminidase from pneumococcal extract was accomplished, with 40% recovery, by collecting the fraction which precipitates with ammonium sulfate concentrations between 30 and 50 percent saturation. Table X shows the liberation of sialic acid upon incubation of various substrates with pneumococcal extract. Orosomucoid appears to be a better substrate than bovine submaxillary gland glycoprotein. Apparently the pneumococcal neuraminidase activity is similar to that of Influenza virus (McCrea, 1957) and _Clostridium perfringens_ (Popenoe and Drew, 1957), in that it is not stimulated by calcium ion. In contrast, cholera neuraminidase is stimulated by calcium ion (Burnet and Stone, 1947). With bovine orosomucoid as substrate, maximal enzyme activity was obtained between pH 6.5 and 7.0 (Figure VIII). The neuraminidase activity was found to be more stable under acidic conditions than in alkaline. About 80% of the activity could be recovered after incubation with 0.05 N HCl at 4°C. for 16 hours. However, the activity was rapidly destroyed when the pH was above 10. Figure IX expresses the linear relationship between the liberation of sialic acid per hour from bovine orosomucoid and the

TABLE X

NEURAMINIDASE ACTIVITY OF PNEUMOCOCCAL EXTRACT

Additions	Mg. Sialic Acid Released Per mg. Protein per Hour
Pneumococcal extract	0.2
Partially purified enzyme	0.1
Bovine orosomucoid	0.1
Bovine submaxillary gland glycoprotein	0.3
Bovine submaxillary gland glycoprotein plus pneumococcal extract	27.5
Bovine orosomucoid plus pneumococcal extract	45.6
Bovine orosomucoid plus pneumococcal extract plus 10 mM $CaCl_2$	46.2
Neuramin-lactose plus pneumococcal extract	35.4
Bovine orosomucoid plus partially purified enzyme	464
Bovine orosomucoid plus partially purified enzyme plus 10 mM $CaCl_2$	461

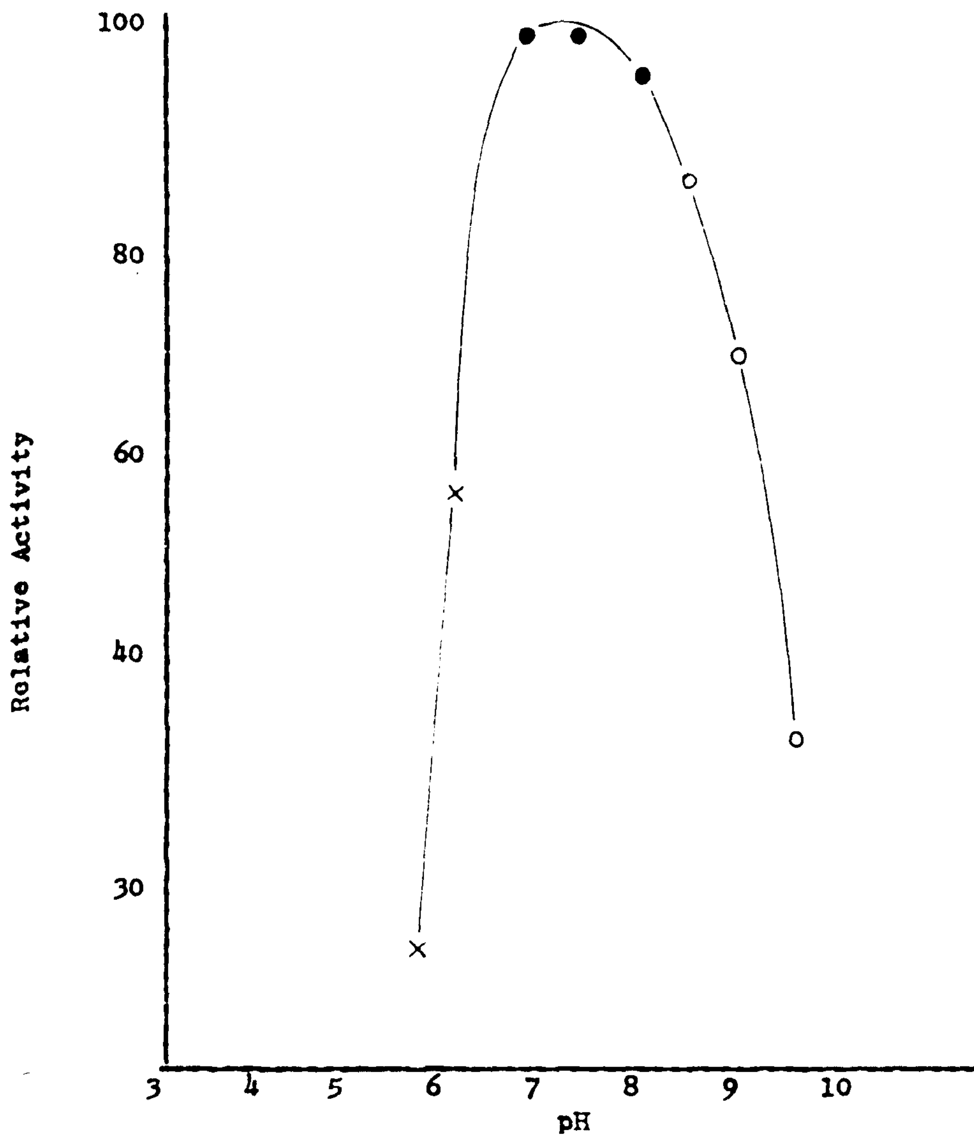


Figure VIII. Effect of pH on Neuraminidase activity. The buffers used were as follows: acetate (x), phosphate (•) and borate (o); the conditions of the assay are described in the text.

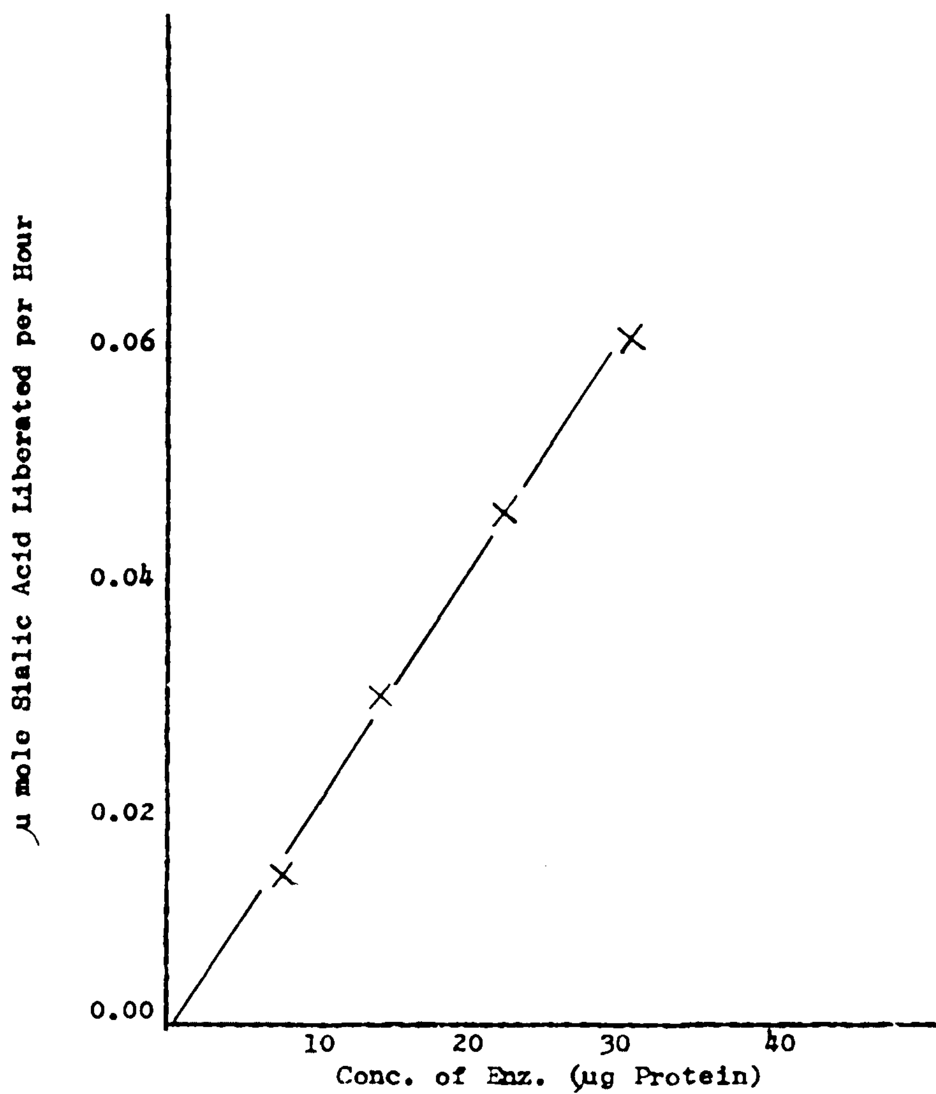


Figure IX. Neuraminidase activity as a function of enzyme concentration.

protein concentration of the cell-free extract added.

Glucosaminidase

β-glucosaminidase activity in pneumococcal extract was assayed by using p-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate. Solutions of enzyme (0.1 ml.) and 2 u moles of p-nitrophenyl-N-acetyl-glucosaminide were mixed in a total volume of 1.0 ml. of 0.05 M phosphate buffer, pH 6.0 and incubated for one hour at 37°C. Liberated p-nitrophenol was estimated spectrophotometrically after the addition of 2.0 ml. of 0.25 M sodium carbonate, as described by Borrooah _et al._ (1961). Effect of enzyme concentration on the β-glucosaminidase activity is expressed in Figure X. Figure XI shows the effect of different pH values, over the range 5.2 to 9.6, on the rates of hydrolysis of p-nitrophenyl-N-acetyl-β-D-glucosaminide. Maximum activity was found between pH 6.0 and approximately 8.0, which is significantly higher than that of testis β-glucosaminidase (optimum pH 4.5, Woollen _et al._, 1961). Though pneumococcal extract was found to hydrolyze N-acetylgalactosamine from bovine submaxillary gland glycoprotein, the estimation of activity of galactosaminidase was limited by a lack of suitable substrates.

Galactosidase

β-Galactosidase. The β-galactosidase activity in _D. pneumoniae_ was first recognized during the estimation of neuraminidase activity using neuramin-lactose as substrate. When the incubation mixture of neuramin-lactose and pneumococcal extract was chromatographed on paper, both glucose and galactose spots were detected in addition

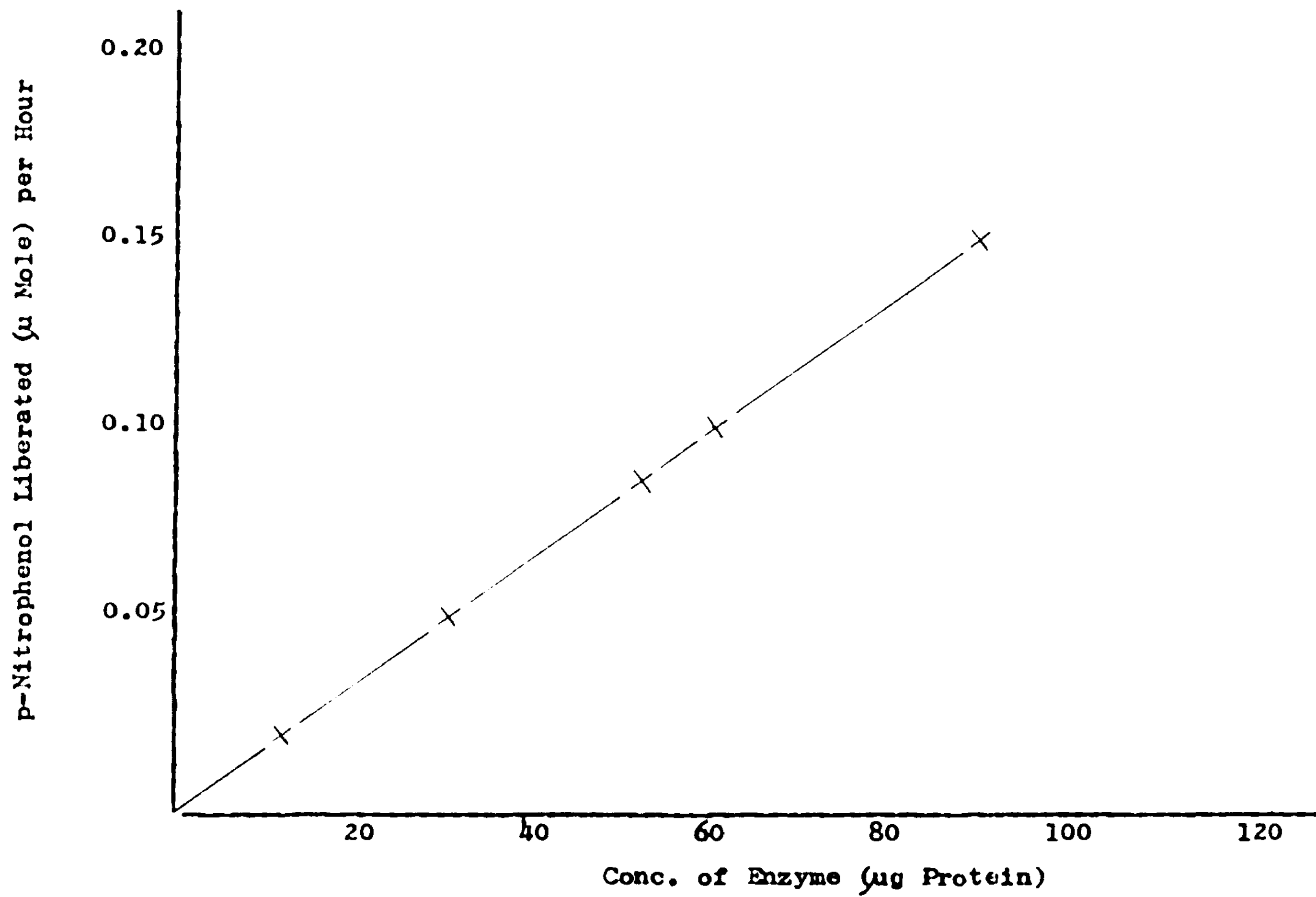


Figure X. β-N-acetylglucosaminidase activity as a function of enzyme concentration.

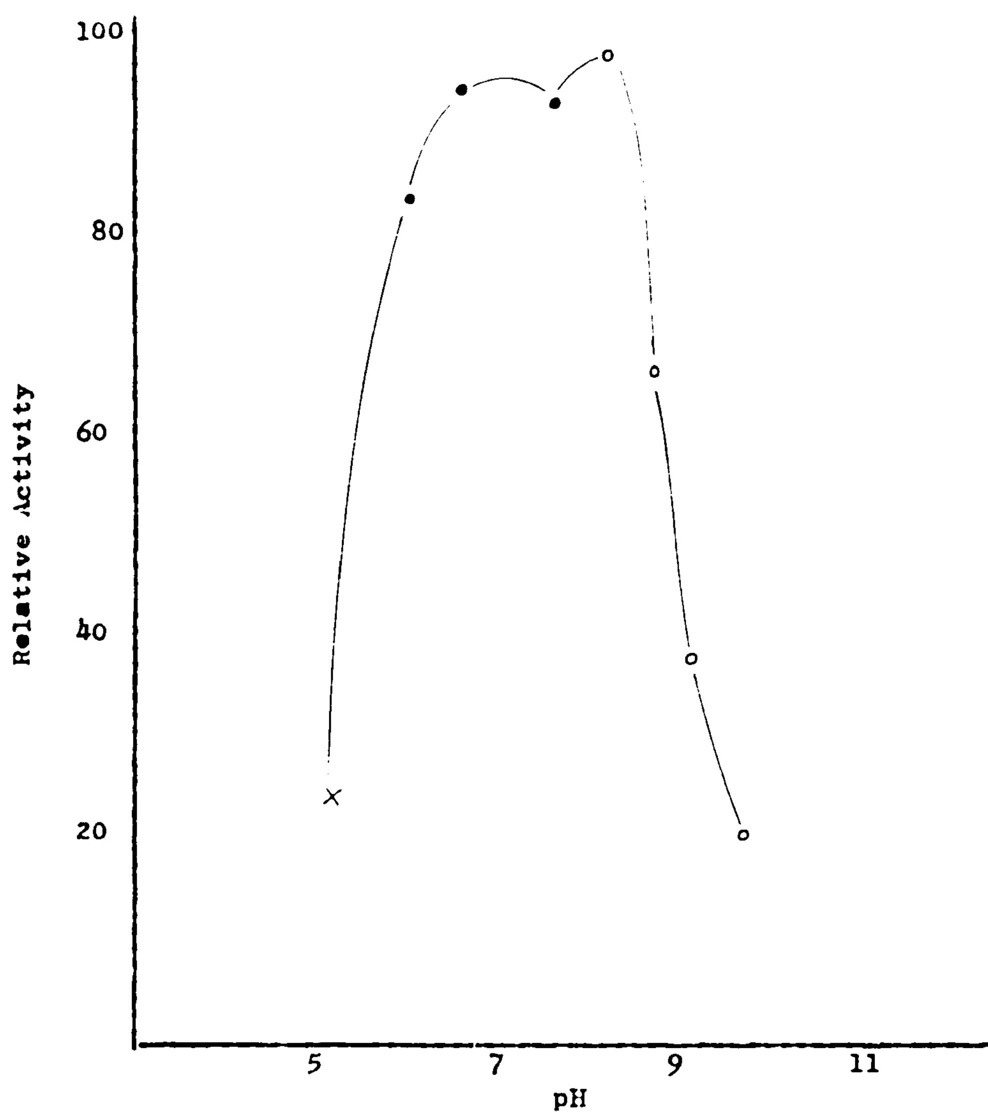


Figure XI. Effect of pH on β-N-acetylglucosaminidase activity. The buffers used were as follows: acetate, (x); phosphate, (•); and borate, (o); the conditions of the assay are described in the text.

to sialic acid spot. The β-galactosidase activity of pneumococcal extract was further confirmed by incubating lactose with pneumococcal extract. When 1 ml. of 0.05 M lactose in 0.05 M phosphate buffer, pH 6.5, was incubated with 0.1 mg. of pneumococcal extract for 30 minutes, the appearance of glucose and galactose was noted (Figure XII).

Some properties of this enzyme were also studied by using a chromogenic substance, o-nitrophenyl-β-D-galactoside, as a substrate. Reaction tubes containing 3.5 ml. of 0.05 M phosphate buffer, pH 6.0, and 0.5 ml. of o-nitrophenyl-β-D-galactoside (0.01 M) were equilibrated in a 37°C. bath and 1 ml. of pneumococcal extract added at the appropriate dilution. After incubation at 37°C. for 1 hour, the liberated chromogenic glycone, o-nitrophenol, was estimated spectrophotometrically after adding 5 ml. of 0.2 M Na_2CO_3. Figure XIII shows the effect of pH on β-galactosidase activity. The optimum pH is in the range of 6.0 to 6.5. The dependence of β-galactosidase activity upon the amount of pneumococcal extract added is presented in Figure XIV.

<u>α-Galactosidase</u>. After β-galactosidase activity of pneumococcal extract was recognized, the question arose as to whether the extract contained α-galactosidase activity. Melibose 6-(α-D-galactosido)-D-glucose was used as a substrate. After incubating 1 ml. of 0.05 M melibiose in 0.05 M phosphate buffer, pH 6.5, with 0.1 mg. pneumococcal extract for 30 minutes, both glucose and galactose were detected in the incubation mixture by paper chromatography (Figure XII), suggesting that the extract also contains α-galactosidase activity.

Figure XII. Presence of α- and β-galactosidase in pneumococcal extract. GLU - Glucose; GAL - Galactose; LACT - Lactose; MEL - Melibiose; EXT - Pneumococcal cell-free extract.

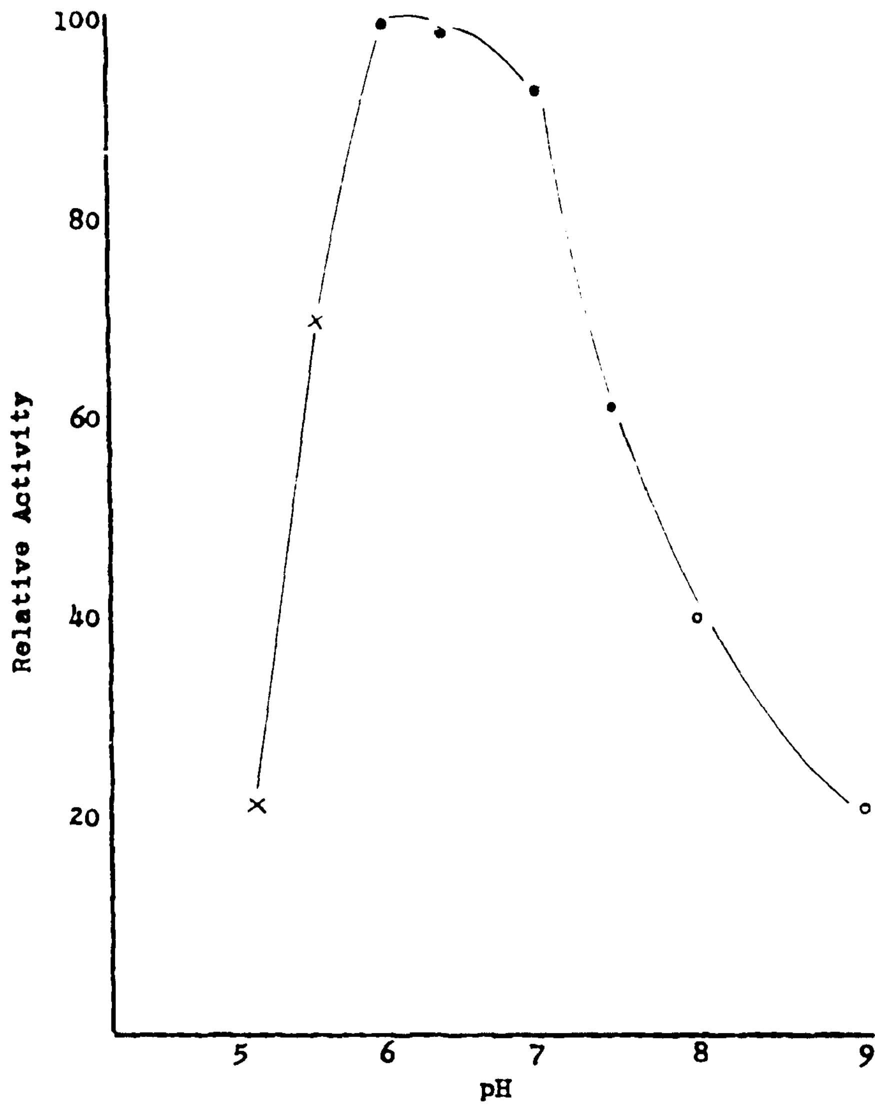


Figure XIII. Effect of pH on β-galactosidase activity. The buffers used were as follows: acetate, (x); phosphate, (•); and borate (o); the conditions of the assay described in the text.

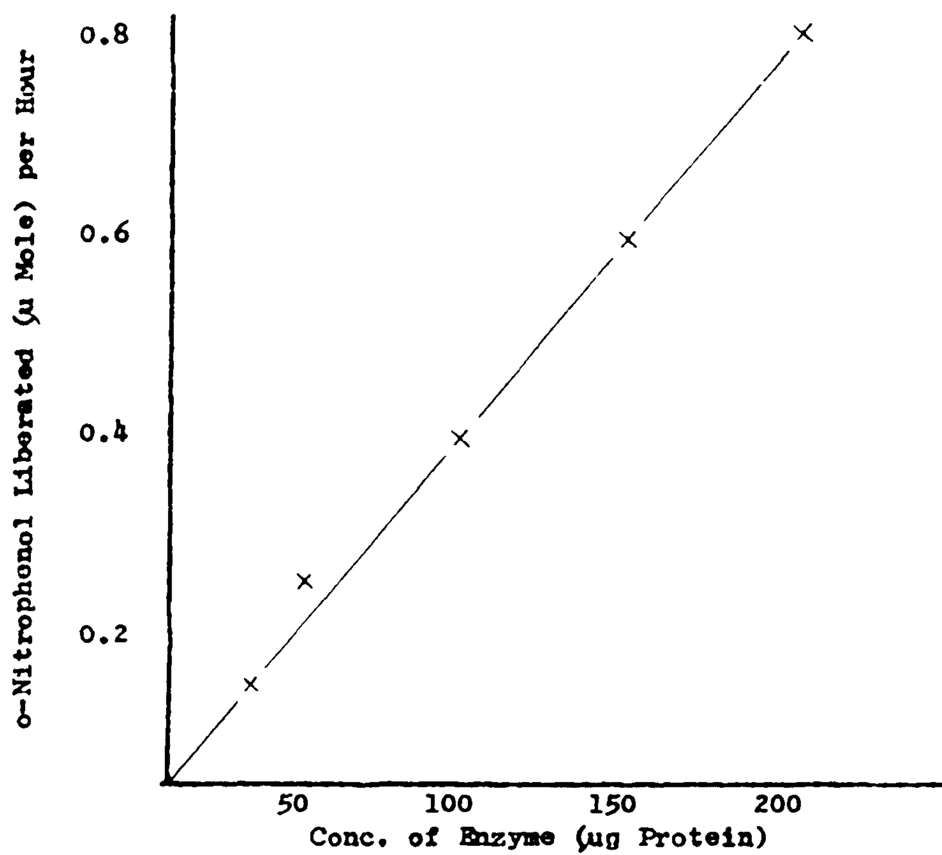


Figure XIV. β-galactosidase activity as a function of enzyme concentration.

Structural Studies of Bovine Orosomucoid

As indicated previously, pneumococcal extract contains several glycosidases, which enable this organism to hydrolyze the carbohydrates from various glycoproteins. It appears possible, therefore, to study the sequential arrangement and the linkages of the monosaccharide constituents of bovine orosomucoid by using pneumococcal extract as a tool. A highly purified sample of bovine orosomucoid was prepared, following the procedure described previously.

Graded Hydrolysis of Carbohydrates by Pneumococcal Extract

In order to determine the rate of release of monosaccharides by pneumococcal extract, 250 mg. of orosomucoid was dissolved in 50 ml. of a cell-free extract in 0.05 M phosphate buffer, pH 6.5, at ice bath temperature. Five milliliters (containing 25 mg. orosomucoid) of the mixture was then pipetted into test tubes, stoppered with rubber stoppers and incubated in a 37°C. water bath. The tubes were removed at suitable time intervals and heated in a boiling water bath for 5 minutes to stop the enzyme action. Precipitated protein was removed by centrifugation and the clear supernatant used for chemical analysis. In each run the crude extract, without added orosomucoid, served as control. After stopping the enzyme reaction, 2 ml. of the reaction mixture was used for determinations of free sialic acid and N-acetylhexosamine. The three remaining milliliters of reaction mixture were dialyzed against 100 ml. of distilled water for 24 hours at 4°C.

Both dialyzable and non-dialyzable fractions were lyophilized. The lyophilized dialysate was dissolved in 3 ml. of distilled water and demineralized with an electric desalter (Research Specialities Co., California). The desalted dialysate was evaporated to 0.5 ml. in the vacuum oven and used for paper chromatographic studies of free sugar and for determinations of dialyzable hexose. Bound sialic acid, hexose and hexosamine were determined on the lyophilized non-dialyzable fraction.

Results of the serial quantitative studies of the incubation mixtures are recorded graphically in Figures XII, XV and XVI. As indicated in Figure XV, sialic acid is rapidly released from orosomucoid until 3 hours, in which 84% (7.4 μ mole) of the total sialic acid was present as free sialic acid. At 12 hours, 7.8 μ mole of free and 0.9 μ mole of bound sialic acid (expressed as N-acetylneuraminic acid) were present. The sum of these two components may be compared with the original 8.0 μ mole of bound sialic acid. On the other hand, as indicated in Figure XVI, hexosamine was released at a much slower rate, only 12% (1.9 μm) being found as free N-acetylglucosamine at 3 hours and 45% (7.1 μm) at 12 hours. Somewhat different figures for liberated glucosamine were found if calculated from the bound hexosamine analysis. In this case, 59% (9.4 μm) of the original bound hexosamine had been lost at 12 hours. This may indicate further breakdown of the acetyl hexosamine by the extract after liberation. The amount of free hexose found was even lower than that for hexosamine; furthermore, the disappearance of bound hexose was somewhat faster than that of bound hexosamine

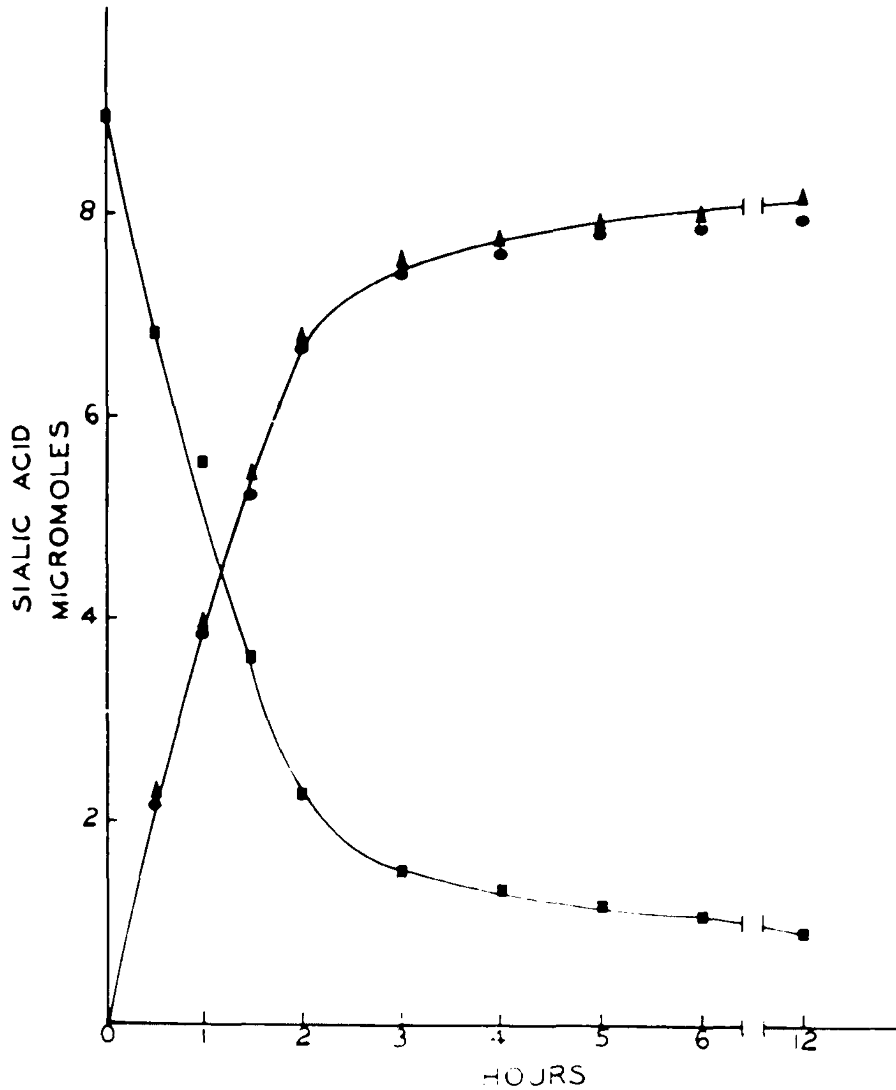


Figure XV. Liberation of sialic acid from bovine orosomucoid by pneumococcal extract. -▲-▲- indicates the released sialic acid as determined by direct analysis; -●-●- indicates the released substance as determined by diference between the original bound figure and the bound figure at the indicated time; -■-■- indicates the change of the bound component.

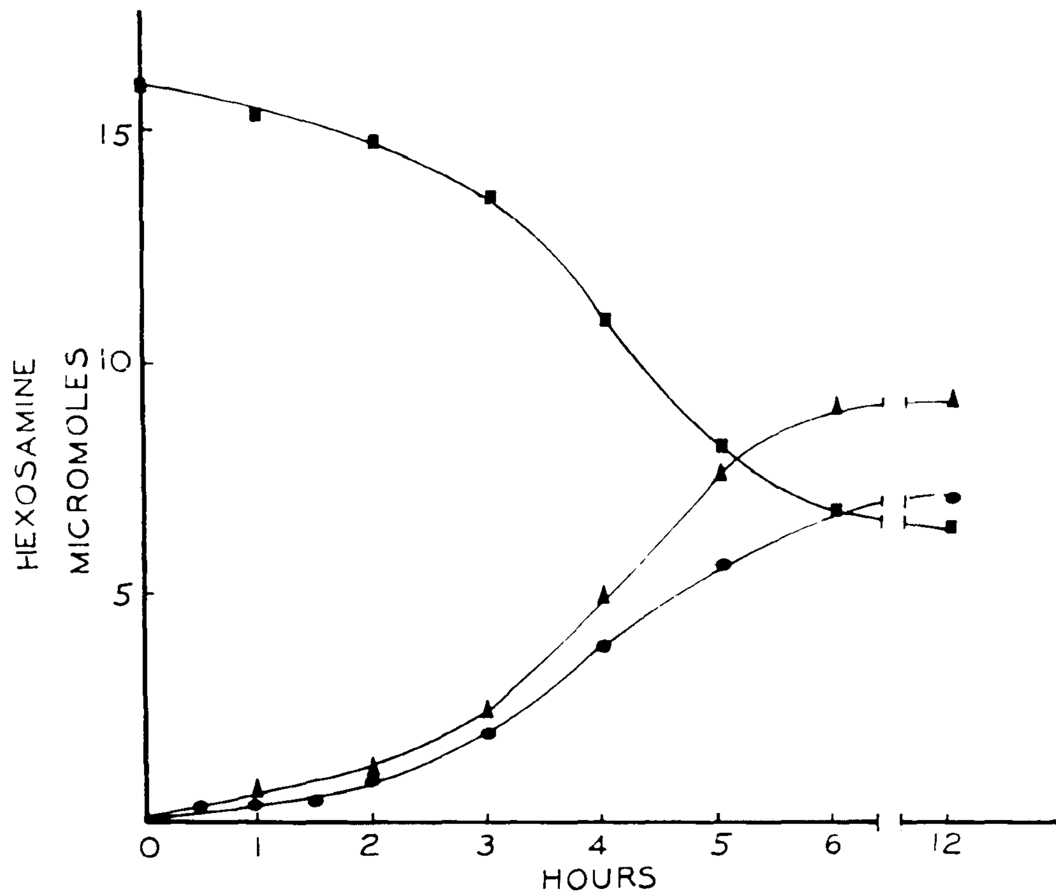


Figure XVI. Liberation of hexosamine from bovine orosomucoid by pneumococcal extract.—▲—▲—indicates released sialic acid as determined by direct analysis;-●-●-indicates released substance as determined by difference between the original bound figure and the bound figure at the indicated time;-■-■-indicates the change of the bound component.

(Figure XVII), suggesting definite degradation of hexoses after release from the glycoprotein.

Confirmation and elucidation of the above observations were made by paper chromatographic studies of the incubation mixture dialysates. As indicated in Figure XVIII, after 30 minutes, incubation, a spot with the mobility of N-acetylneuraminic acid was found; however, no other carbohydrate staining areas were present. At 3 hours, strongly staining spots with the mobilities of N-acetylneuraminic acid and galactose and a weaker spot with the mobility of N-acetylglucosamine were found. At 6 hours, an additional weak spot with the mobility of mannose was present and, 12 hours had increased in intensity. In the 3, 6 and 12 hour samples, a spot with a very limited mobility occurred and may be due to dialyzable oligosaccharide.

Prolonged Digestion of Bovine Orosomucoid
with Pneumococcal Extract

In addition to studying the monosaccharides released at various periods of time, it is valuable to obtain information concerning the carbohydrates still attached to the peptide portion of the glycoprotein after prolonged digestion of orosomucoid with pneumococcal extract. This experiment was intended to obtain information about the carbohydrate which is in the most interior portion of the carbohydrate sequence and is, therefore, probably attached to the polypeptide chain. Orosomucoid was incubated with pneumococcal extract for 60 hours at 37°C. A pH of 6.0 was selected for the incubation mixture, since only

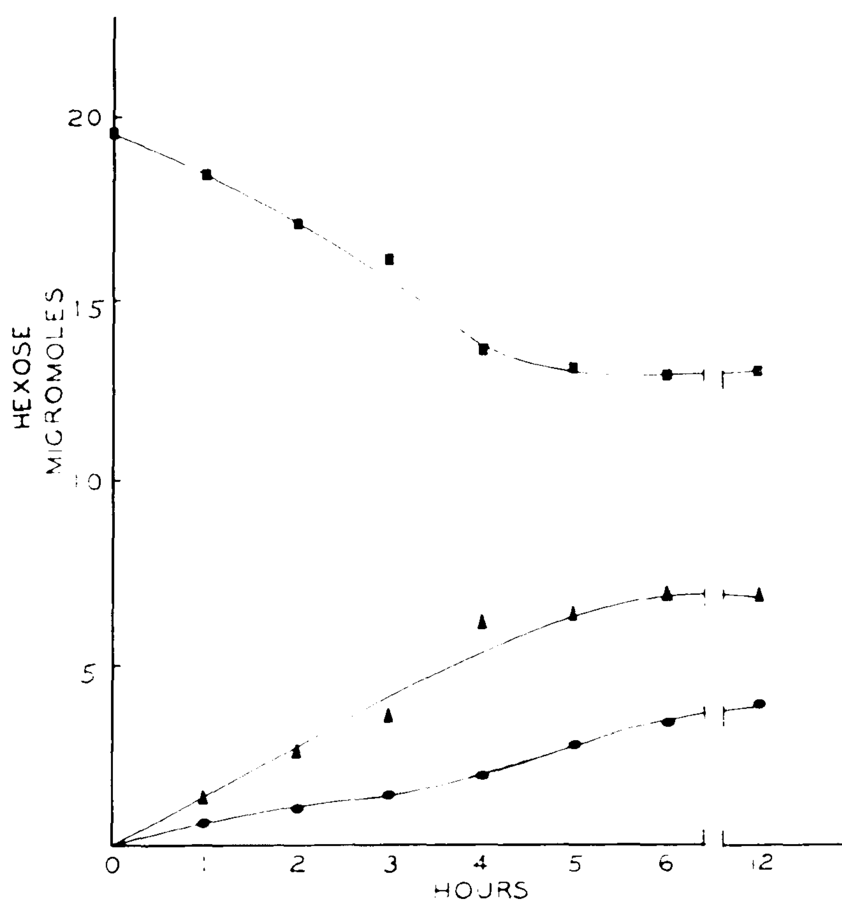


Figure XVII. Liberation of hexose from bovine orosomucoid by pneumococcal extract.-▲-▲-indicates the released sialic acid as determined by direct analysis;-●-●-indicates the released substance as determined by difference between the original bound figure and the bound figure at the indicated time;-■-■-indicates the change of the bound component.

Figure XVIII. Paper chromatograph of the carbohydrate constituents of dialysates from incubation mixtures of orosomucoid and extract from Diplococcus pneumoniae. A standard solution of sugars was placed on each side of the chromatographic strip. Position of the standard sugars are indicated as follows: 1 - N-acetyneuraminic acid; 2 - galactose; 3 - mannose; and 4 - N-acetylglucosamine. Times of incubation are indicated at the bottom of the chromatograph, i.e., 30 minutes, 3, 6 and 12 hours. Note the appearance of NANA at 30 minutes, galactose and NAG at 3 hours and mannose at 6.

slight proteolytic activity was found at this pH. The optimum pH for the proteolytic activity is 7.5 to 8.0. After incubation, the mixture was subjected to exhaustive dialysis and the non-dialyzable fraction used for qualitative determination of carbohydrates by paper chromatography. The result of this experiment revealed that only mannose and glucosamine were still found attached to the nondialyzable peptide of the glycoprotein after prolonged digestion of bovine orosomucoid with pneumococcal extract. Though glucosamine was the only amino sugar detected by paper chromatographic technique, galactosamine was also detected by using the amino acid analyzer. The ratio of glucosamine to galactosamine is 17:1, as compared with 13:1 determined by the same procedure in the original orosomucoid. Galactosamine content was lower after digestion with _D. pneumoniae_. The result may suggest that some galactosamine residues are present in the carbohydrate sequence not nearest to the polypeptide chain and more glucosamine is present in the neighbor of polypeptide chain.

Proteolytic Digestion of Bovine Orosomucoid

After the finding that mannose and glucosamine were the carbohydrates nearest to the polypeptide chain in the bovine orosomucoid, it was thought valuable to obtain information concerning the amino acids which are nearest to the carbohydrate chain. In order to achieve this purpose, orosomucoid was digested with proteolytic enzyme to remove as much of the peptide portion of the complex as possible, and the nondigestible part analyzed for amino acids. Proline in the protein molecule interferes with complete hydrolysis of protein by most proteo-

lytic enzymes. Hill and Schmid (1961) employed a combination of papain, leucine amino peptidase and prolinase to achieve the complete enzymic hydrolysis of proteins. However, isolation and purification of prolinase from swine kidney is laborious and time consuming. A proteolytic enzyme from *Streptomyces griseus* (commercial name, "Pronase") was selected for digesting bovine orosomucoid because this enzyme has a very wide specificity, including proline splitting activity (Nomoto and Narahashi, 1960). Sialic acid in orosomucoid interferred with the proteolytic digestion; therefore, orosomucoid free of sialic acid was prepared by hydrolysis of 0.1% of bovine orosomucoid in 0.05 N H_2SO_4 for 1 hour at 80°C. Sulfuric acid was removed by dialysis and the sialic acid-free orosomucoid obtained by lyophilization. More than 95% of the sialic acid and 60% of the fucose in the bovine orosomucoid was removed by this process. One hundred mg. of sialic acid-free bovine orosomucoid in 10 ml. of 0.1 M phosphate buffer, pH 7.4, was digested for 60 hours by 1 mg. Pronase (contains 45 units of *Streptomyces griseus* protease, Nomoto and Narahashi, 1960). The proteolytic digests were dialyzed in Visking dialyzing tubing at 4°C. against distilled water for 60 hours. The nondialyzable fraction was analyzed for amino acid composition. Table XI indicates that after prolonged digestion of bovine orosomucoid with *Streptomyces griseus* protease, the peptide moiety contained aspartic acid, threonine, serine and glutamic acid in relatively high percentage. The ratio of glucosamine to galactosamine is 13:1 (as in the original glycoprotein), suggesting that there was no degradation of carbohydrate chain during proteolysis.

TABLE XI

AMINO ACID AND AMINO SUGAR CONTENT OF BOVINE OROSOMUCOID AFTER DIGESTION WITH *STREPTOMYCES GRISEUS* PROTEASE

Amino Acid	Micromoles per mg. Glycopeptide
Aspartic Acid	0.23
Threonine	0.15
Serine	0.21
Glutamic Acid	0.23
Proline	0.08
Glycine	0.06
Alanine	0.08
Valine	0.07
Methionine	0.02
Leucine	0.08
Tyrosine	0.03
Lysine	0.06
Histidine	0.05
Arginine	0.05
Glucosamine	0.16
Galactosamine	0.013

Digestion of Bovine Orosomucoid with Pneumococcal Extract and *Streptomyces Griseus* Protease

In order to obtain further information concerning the amino acid-carbohydrate complex, 100 mg. of bovine orosomucoid was digested with 5 mg. pneumococcal extract for 60 hours at pH 6.5. The pH of the pneumococcal digest was adjusted to pH 7.4 by KOH, then 0.5 M phosphate buffer, pH 7.4, added to a final concentration of 0.05 M. The mixture was again digested with *Streptomyces griseus* protease for 60 hours, then dialyzed and lyophilized and the dialyzable fraction analyzed for amino acids and amino sugars by the amino acid analyzer. The results of this experiment are presented in Table XII. Substantial increases of glucosamine and glutamic acid after acid hydrolysis may indicate that these two are the components responsible for the linkage between polypeptide and polysaccharide chains.

TABLE XII

AMINO ACIDS AND AMINO SUGARS IN PNEUMOCOCCAL AND PROTEOLYTIC DIGESTS OF BOVINE OROSOMUCOID

	Before Acid Hydrolysis µM/mg. (a)	After Acid Hydrolysis µM/mg. (b)	Ratio b/a
Glucosamine	0.0050	0.0960*	19.2
Galactosamine	0.0050	0.032	6.4
Lysine	0.231	0.337	1.5
Histidine	0.054	0.066	1.2
Arginine	0.044	0.092	2.1
Aspartic Acid	0.093	0.187	2.0
Threonine	0.081	0.181	2.2
Serine	0.098	0.136**	1.3
Glutamic Acid	0.021	0.341	15.2
Proline	0.016	0.106	6.6
Glycine	0.096	0.136	1.4
Alanine	0.211	0.201	0.9
Valine	0.145	0.134	0.9
Methionine	0.071	0.060	0.8
Isoleucine	0.183	0.196	1.0
Tyrosine	0.163	0.171	1.0
Phenylalanine	0.131	0.142	1.1

*Corrected for free N-acetylglucosamine before acid hydrolysis

**Corrected for 10% destruction during acid hydrolysis

CHAPTER V

DISCUSSION

A survey concerned with microorganisms which will degrade serum glycoprotein indicated that Diplococcus pneumoniae 612 was able to cause significant decreases in protein-bound hexoses of human, rat and bovine sera. Previously, no microorganism was known to remove carbohydrates other than sialic acid from serum glycoprotein. Incubation of mammalian serum with D. pneumoniae resulted in decreases of 15 to 30% of the protein-bound hexose as determined by the tryptophan method. Paper electrophoretic studies indicated that these changes were largely restricted to the α_1-globulin fraction. Disappearance of total glycoprotein, preferentially from the α_1-globulin and glycoprotein fraction was noted, after incubation with D. pneumoniae. It is known that incubation of serum samples with organisms which contain neuraminidase will cause retardation in electrophoretic mobility of α_1- and α_2-globulin fractions due to their loss of sialic acid (Laurell, 1959). D. pneumoniae was known to contain neuraminidase activity (Heimer and Meyer, 1956). Therefore, the disappearance of the α_1-globulin fraction in sera after incubation with pneumococci might be due to changes of the mobility of this fraction associated with loss of sialic acid. This possibility was ruled out by incubating this organism with rat or rabbit

serum in which the glycoproteins were labeled with glucosamine-1-C-14. Disappearance of radioactivity in the α_1-globulin fraction was observed, suggesting that the degradation of the α_1-globulin fraction by this organism also involved removal of glucosamine.

Preferential utilization of the protein-bound carbohydrates contained α_1-globulin fraction can not be explained by postulating that only this fraction contains those carbohydrates which are necessary for the growth of this organism. Since it is known that sialic acid, hexoses and hexosamines are distributed in α-$_1$, α-$_2$ and β fractions, the requirement of some unknown factor from this fraction for the growth of this organism is still possible. The explanation may rather be that the enzymatic removal of the carbohydrates in the α_1-globulin is somehow less protected by the protein moiety. The α_1-globulin fraction is a complex mixture which contains protein, glycoprotein and lipopolysaccharide.

Heimer and Meyer (1956) and Oncley _et al._ (1958) reported the liberation of sialic acid from varius glycoproteins by _D. pneumoniae_. In the present study, it was found that, in addition to sialic acid, _D. pneumoniae_ hydrolyze hexoses, hexosamine and fucose from mammalian serum. The pneumococcal extract was found to be able to hydrolyze sialic acid, galactose, N-acetyl-glucosamine, N-acetyl-galactosamine and in some instances mannose from various glycoproteins, including bovine orosomucoid, bovine submaxillary gland glycoprotein, orosomucoid, sheep follicle stimulating hormone.

The ability of pneumococci to hydrolyze various carbohydrates from serum glycoprotein was further explained by the fact that pneumococcal cell-free extract contains neuraminidase, β-glucosaminidase, α-galactosidase and β-galactosidase as determined by studies with chromogenic substrates. The optimum pH of pneumococcal neuraminidase was between 6.5 to 7.0, a range very similar to that optimal for neuraminidase of Influenza virus (Myron _et al._, 1961); but signficantly higher than for the neuraminidase from other bacteria. The optimal pH of neuraminidase from _Clostridium perfringens_ is between pH 5.0 and 5.5 (Popenoe and Drew, 1957) and between 5.0 and 6.0 for crystalline neuraminidase from _Vibrio cholerae_ (Ada _et al._, 1961). Results reported in the present study showed that pneumococcal β-glucosaminidase was in the range 6.0 to 8.0. The optimal pH of the β-glucosaminidase of ram testis has been reported to be between 4.4 and 4.7 (Woollen _et al._, 1961), a value strikingly lower than that of pneumococcal β-glucosaminidase. The optimal pH of the β-galactosidase in pneumococcal extract was found to be between 6.0 and 6.5 which is very close to the value reported by Kuby and Lardy (1953) for β-galactosidase in _Escherichia coli_. Pneumococci also contain α-galactosidase activity which has not been previously reported. Although β-galactosidase has been extensively studied due to its genetic importance, α-galactosidase is relatively unexplored and further studies should prove interesting. α-Galactosidase in pneumococcal extract seems to be more stable to heat than β-galactosidase. Upon incubation of pneumococcal extract at 55°C. for 15 minutes, about 90% of the β-galactosidase, but only

30% of the α-galactosidase activity was lost.

Bovine orosomucoid contains about 60 percent protein and 40 percent carbohydrates. The carbohydrate composition of this glycoprotein was found to be 11% sialic acid, 14% hexose and 11% hexosamine. Since pneumococcal extract contains neuraminidase, β-galactosidase and β-glucosaminidase, the cell-free extracts of this organism were used for the structural studies of this glycoprotein. Analysis of the monosaccharides liberated at various periods during the contolled by hydrolysis of bovine orosomucoid with pneumococcal extract revealed that sialic acid was rapidly released from the glycoprotein. This observation is in agreement with the postulation that sialic acid is the terminal group of this glycoprotein (Winzler, 1958). Assuming that the terminal sialic acid must be removed before the adjacent carbohydrate residue may be hydrolyzed from the glycoprotein, consideration of the serial paper chromatographic studies indicate that the next residue of the carbohydrate moiety is galactose, followed by N-acetylglucosamine (Figure XVIII). However, since 41% of the hexosamine remained bound, following 12 hours of incubation, the carbohydrate moiety probably included a second glucosamine residue. By analogous reasoning, mannose is further from the terminal group than either galactose or glucosamine. Only 33% was removed of the bound hexose from the glycoprotein after 12 hours, indicating that many of the hexose residues are beyond the sialic acid-galactose-glucosamine sequence. These results are similar to those obtained in the graded acid hydrolysis of fetuin reported by Spiro (1962). By dilute acid hydrolysis of fetuin, Spiro found that galactose

in fetuin is released much more rapidly than either the hexosamine or mannose and also that hexosamine is released more rapidly than mannose. Eylar and Jeanloz (1962) oxidized human sialic acid-free orosomucoid at 0°C. to 4°C., pH 4.75, and suggested that polysaccharide units of human orosomucoid possess a total of 16 branches, terminated by the sequence N-acetylneuraminidyl-)2-4) β-B-D-galactosyl-(1-4)-2-acetamido-2-deoxy-D-glucose.

In order to obtain the information about what carbohydrates are the nearest to the polypeptide chain, bovine orosomucoid was subjected to prolonged digestion with pneumococcal extract. After 60 hours of digestion, it was found that mannose and glucosamine are still attached to the polysaccharide, confirming previously reported observations of other workers. Winzler (1958) and Spiro (1962), using graded acid hydrolysis of human orosomucoid and fetuin, concluded that hexosamine and mannose were the carbohydrates attached to the polypeptide chain. By periodate oxidation of sialic acid, free human orosomucoid, Eylar and Jeanloz (1962) also concluded that the inner core of the polysaccharide units of human orosomucoid is composed of mannose and/or glucosamine.

To obtain information about the amino acids linked to the polysaccharide chain, bovine orosomucoid was digested with Streptomyces griseus protease for 60 hours. Glutamic acid, aspartic acid and serine were found to remain attached to the non-dialyzable polysaccharide fraction, suggesting that one of these amino acids is linked to carbohydrate. In ovalbumin and human orosomucoid, aspartic acid has been reported to

be linked to the glucosamine; however, in bovine orosomucoid, the possibility that glutamic acid might be linked to the carbohydrate still exists. The results of amino acid analysis of protease and pneumococcal digests of bovine orosomucoid indicated that glucosamine and glutamic acid increase more than fifteen times after acid hydrolysis (Table XII). This result strongly suggest that these two components are present in the bound form before acid hydrolysis.

CHAPTER VI

SUMMARY

1. A strain of Diplococcus pneumoniae was found to degrade the serum glycoprotein which migrates on paper electrophoresis with the α_1-globulin fraction. Incubation of mammalian serum with pneumococci resulted in decreases in protein-bound sialic acid, glucosamine, hexoses and fucose.

2. Most of the α_1-globulin fraction demonstrable by paper strip electrophoresis disappeared after incubation of mammalian serum with D. pneumoniae.

3. Pneumococcal extract liberated various monosaccharides from several isolated glycoproteins, including bovine orosomucoid, bovine submaxillary gland glycoprotein and ovomucoid.

4. Pneumococcal extract contains neuraminidase, β-glucosaminidase and α- and β-galactosidases.

5. Bovine orosomucoid which was shown to migrate as a single peak in both paper electrophoretic and ultracentrifugal analysis was isolated from Cohn Fraction VI. Both carbohydrate and amino acid composition of this glycoprotein were studied.

6. The controlled graded hydrolysis of bovine orosomucoid by pneumococcal extract indicated that sialic acid occupies the terminal

position of the carbohydrate chain, galactose is next to the sialic acid and glucosamine and mannose are in the inner part of this glycoprotein.

7. After prolonged digestion of bovine orosomucoid with pneumococcal extract, mannose and some glucosamine were still attached to the peptide chain.

8. Upon digestion of bovine orosomucoid with *Streptomyces griseus* protease, glutamic acid, aspartic acid, threonine and serine were most abundant in the non-dialyzable fraction.

9. Increases of glutamic acid and glucosamine after hydrolysis of dialyzable fractions of pneumococcal and protease digests of bovine orosomucoid indicated that glutamic acid and glucosamine may be responsible for the linkage between polypeptide and carbohydrate chains in the bovine orosomucoid.

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