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Studies in the Electrophoresis of Rat Submandibular Gland Glycoproteins and the Isolation and Partial Characterization of the Major Rat Submandibular Gland Glycoprotein

by

Robert Alexander Kapitany

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements for the Degree

of Doctor of Philosophy

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Dedication

The author is indebted to Dr. Charles M. Dowse, who showed the author that it is possible to be a scientist, a scholar and a gentleman all at the same time. Without his constant example; that it is always possible to be better than one already is; that improvement is only accomplished by patience, diligence, honesty and forbearance; this work would never have been completed. The author therefore wishes to dedicate this thesis to him.

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Abstract

Previous investigations into the nature of submandibular gland glycoproteins have indicated a polydisperse nature. The present work concerns itself with the examination of this polydisperse character by means of gel electrophoresis and column chromatography. The results of this work indicated that the rodent submandibular gland contains a great many glycoproteins which were of a wide range of molecular weights. In addition, it appeared as if the soluble protein content of the gland was largely glycoprotein in nature. The evidence for this was that almost all of the bands and fractions prepared by electrophoresis and chromatography gave staining reactions characteristic of glycoproteins. As a result of this work, it became apparent that the water soluble fraction of the submandibular gland contained the rat submandibular gland mucin (RSM). This was isolated in a relatively homogeneous state by preparative column chromatography on Sephadex G-200 and the carbohydrate and amino acid content analyzed. The mucin (RSM) contained mannose, galactose, sialic acid, fucose, N-acetylglucosamine, N-acetylgalactosamine, and perhaps traces of glucose. It was 51% carbohydrate and 49% protein by weight. The amino acid content was unusual for a submaxillary mucin and the implications of this are discussed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

THE STATEMENT OF THE RESEARCH PROBLEM

It certainly appears from the literature, that much of the history of glycoprotein chemistry has centered around studies with submaxillary glycoproteins. Most notably, much research has been done on a specific glycoprotein of high carbohydrate content, submaxillary mucins from sheep and cattle (ovine submaxillary and bovine submaxillary mucin). There have been isolations of this molecular species from a wide variety of animal sources, including rat, hamster, dog, cat and pig. Interest in the structure of these molecules has been high. The literature in this area has been reviewed extensively a number of times, and a table summarizing these results has been presented in the Results and Discussions section on page 71A.

The mechanisms for the biosynthesis of these molecules has yet to be clearly demonstrated. The enzymes necessary for the elaboration and attachment of the oligosaccharide prosthetic groups have been demonstrated to be present in the glands of a few of the species[203-207] and the understanding of the assembly of the carbohydrate side chains is well under way.

However, several studies on submaxillary salivas[132,147, 138] have indicated more than one glycoprotein present in significant quantities. In preliminary studies on rat submaxillary glands, Phillips[131] and Ericson[161,167] have suggested there may be several glycoproteins of importance present within this tissue. Indeed, it is difficult to believe

otherwise. It was thought necessary to examine the soluble fractions obtainable from the rat submandibular gland to determine some estimation of its glycoprotein content, and some measure of their properties. Firstly, however, it became necessary to develop the extensive electrophoresis methodology necessary to begin the study. Since, earlier work in this laboratory and others[119,125,130,131] had indicated the sialic acid metabolism of the gland to be significant, emphasis was to be placed on the sialoglycoproteins especially. Subsequently, it became apparent that a single glycoprotein occupied a prominant position in the electrophoretic and chromatographic profiles. It was decided to attempt the isolation, purification and partial analysis of this molecule to determine if this was the "mucin" of the gland. Contained within this thesis are the results obtained from the examinations of these problems.

INTRODUCTION

This thesis concerns itself with glycoproteins. Furthermore, it concerns itself with a fairly strictly defined structural group of glycoproteins. In the most simple-minded sense, a glycoprotein is a protein to which is attached, by covalent linkage, one or more carbohydrate residues. However, to the first and any other approximation, due to the vast array of possible "glycoproteins" in animal, vegetable and microbial worlds, this definition, as a descriptive tool, is too general to be useful.

The history of the evolution of the definition has proceeded as follows. Hammersten[1] first applied the term "mucins" to a number of compounds which he and his students had isolated from a number of different sources. These compounds were differentiated from "true mucins" i.e. those from saliva, the respiratory tract, etc. by their solubilities in acids, precipitability and other physical properties. Later, Levene (1925)[2] classified all carbohydrate containing proteins (mucoproteins) into two groups as follows: chondroitin sulfuric acids and mucoitin sulfuric acids, erroneously assuming that all glycoproteins (mucoproteins) contained uronic acids and were sulfated. (For further discussion see Gottschalk, 1972).[14] Following this, Meyer (1938)[3], in possession of more information, proposed to distinguish two main groups as follows:

A. Mucopolysaccharides

(1) containing uronic acids

(a) Sulfate free

(b) Sulfate containing

(2) neutral mucopolysaccharides of known composition.

B. Glycoproteins containing protein-bound hexosamine in addition to other neutral sugars.

Meyer (1953)[4] later modified his scheme to include "mucoproteins" and "mucoids". Mucoproteins were defined as salts produced from the dissociable ionic binding of a mucopolysaccharide to a protein. He postulated a covalent bond between carbohydrate and protein for both his mucoid (which he defined as containing more than 4% hexosamine) and glycoprotein (which were defined as having less than 4% hexosamine) categories.

Other early classifications involving similar arbitrary criteria have come forth from Stacey (1946)[5], Blix (1951)[6], Masamune (1944)[7,8], Kent and Whitehouse (1955)[9], Bettelheim-Jevons (1958)[10] and others. (For discussion of these see Gottschalk (1972)[14] and Schmidt (1968)[19]).

A genuine improvement in the systematics of high molecular weight carbohydrate containing substances appeared with Jeanloz (1960)[11]. Since mucus is a purely physiological term, the prefix "muco" was dropped altogether from his chemical nomenclature in which high molecular weight complex carbohydrate substances are divided into 5 groups:

1. Pure polysaccharides
2. Compounds containing a carbohydrate component attached non-covalently to a polypeptide (polysaccharide protein complex)

3. Compounds containing a carbohydrate component attached covalently to a polypeptide (glycoproteins, glycopolypeptide, glycopeptides)
4. Compounds containing carbohydrate and lipid components
5. Compounds containing carbohydrate, lipid and polypeptide components.

The basis for the classification appears to be (especially for Jeanloz classes (2) and (3)) the type of linkage between the protein and the carbohydrate. Gottschalk (1962)[12] and Gottschalk et al., (1962)[13,14] have taken this assumption to task (for discussion of objections specifically directed against category 2 see Gottschalk (1972))[14] and proposed that these compounds be classified according to the characteristic structural features of the carbohydrate moieties. He has reviewed the observations that the homo- and hetero- polysaccharides such as chitin, hyaluronic acid, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan-sulfates, etc. are for the most part linear structures composed of small repeating units. In the glycosaminoglycuronoglycans the constituent hexosamine and hexuronic residues are arranged in an alternating fashion. Within one type of glycosaminoglycuronoglycan, the glycuronide linkages are as a rule all of the same type, and this is also true for the hexosamine residues.

In contrast, the carbohydrate prosthetic group of glycoproteins may contain 2-6 different types of sugars and it

certainly appears that a branched structure is quite common. Furthermore, the grouping of the sugar residues within the prosthetic group occurs as a distinct pattern, characteristic of the particular glycoprotein in question. There appears to date no evidence of the presence of a repeating unit as such[14,15]. It is true that one may find preferred sequences occurring; however, these in no way represent a serial repetition of particular sequences, in a manner analogous to that found for the glycosaminoglycuronoglycans.

In addition to differences in the overall arrangement and composition of the carbohydrate units between polysaccharide-proteins and glycoproteins, another major distinguishing feature is the chain length. The carbohydrate moiety of polysaccharide-proteins may range from 46 to 95 glycosidically linked monosaccharide units per chain[16,17]. For glycoproteins with branches, the main chain generally does not exceed 14-20 sugar residues.[14]

Schmid (1968)[19], in a fine review of the literature prior to 1966, uses the simplest definition possible for glycoproteins in order to include as many carbohydrate containing types of molecules as possible. His definition is merely an extension of the Gottschalk objection to the Jeanloz (1962) categories based on linkage. Although he accepts the evidence for covalent linkages in the mucopolysaccharides (see Gottschalk, 1972) he does not, however, attempt any differentiation on the basis of structure or biology.

Spiro (1970) uses, as his most recent definition, one indistinguishable from that of Schmidt (1968)[19] i.e. glycoproteins are "simply defined as proteins which have carbohydrate covalently attached to the peptide portion"[18]. Unfortunately, in his review he includes the mucopolysaccharides as a specialized group of glycoproteins. His discussion, however, clearly refers only to compounds conforming to the restricted glycoprotein category of Gottschalk (1972)[14]. Spiro[18] does point out that glycoproteins have been shown to contain some or all of a particular set series of sugars.

Montgomery[20] in his section on the definition and classification of glycoproteins states quite generally that glycoproteins are "biopolymers having amino acids and sugar residues covalently linked to each other". He does, however, recognize that two types of glycoproteins do in fact exist. One type has a protein core to which a few oligosaccharide chains are attached. This type he recognizes as having a low molecular weight ("frequently less than 100,000"). He describes the other type as being of much higher molecular weight, and is mainly carbohydrate in content. The structure presented was a small protein core to which is attached hundreds of small oligosaccharide chains. The sugars D-galactose, D-mannose, D-glucose, L-fucose, D-xylose, L-arabinose, the N-Acetylhexosamines, the various sialic acids and hexuronic acids, D-glucuronic and L-Iduronic acid can be found in the carbohydrate groups of glycoproteins as defined by Schmidt[19],

Spiro[18] and Montgomery[20].

Gottschalk[12,14] has proposed two groups of high molecular weight carbohydrate containing compounds:

A. Heteropolysaccharide-proteins (glycosaminoglycuronoglycans),

B. Glycoproteins.

The first group is characterized by long branching chains of a large number of monosaccharide residues (40) and consists of a serially repeating disaccharide or some similar structural unit.

The second category, "glycoproteins" are defined, "as conjugated proteins containing as prosthetic group(s) one or more heterosaccharide(s), usually branched, with a relatively low number of sugar residues; lacking a serially repeatint unit and bound covalently to the polypeptide chain". Furthermore, "in view of (a) the variation from glycoprotein to glycoprotein in number, composition and size of the heterosaccharide chain, (b) the diversity of the composition of the protein moiety and (c) the widely varying carbohydrate content, it would appear that the lack of a serially repeating unit, the low number of sugar residues in the heterosaccharide, the branching of the heterosaccharide and the covalency of its linkage to protein are the only structural features common to all glycoproteins of animal origin" (Gottschalk 1972)[14]. Glycoproteins are then defined, for the operational purposes of this thesis, according to Gottschalk (1972)[14],

"as conjugated proteins containing as prosthetic group(s) one or more heterosaccharide(s), usually branched, with a relatively low number of sugar residues, lacking a serially repeating unit and bound covalently to the polypeptide chain".

Furthermore, it has now been suggested possible to limit the possible sugars appearing in glycoproteins to the following:

A. Neutral Hexoses

glucose, galactose, mannose

B. Hexosamines

N-acetylglucosamine, N-acetylgalactosamine,

A-acetylmannosamine

C. Terminal sugars

L-fucose (6-deoxygalactose) and the sialic acid.

Xylose, arabinose and the hexuronic acids, being constituents of mucopolysaccharides and non-animal glycoproteins are excluded.

This definition excludes mucopolysaccharides, peptidoglycans and bacterial cell-wall protein polysaccharides as well as bacterial glycoproteins. Recognition is given to limitations of the above definition. However, the author is in agreement with Gottschalk (1970)[14], that a definition that is less exclusive and less explicit can be confusing. The author also respects the suggestion of Gottschalk (1972)[14], that sufficient information is now available that a rigorous definition of this kind can be, at least in large part, scientifically justified.

This definition then includes:

1) Plasma glycoproteins
2) Immunoglobulins
3) Urine Tamm and Horsfall glycoproteins
4) Glycoproteins (hormones)
5) Enzymes (glycoproteins)
6) Egg white glycoproteins
7) Mucins, especially salivary mucins
8) Collagens, aortic glycoproteins, acid glycoprotein from bone
9) Extracellular membrane protein
10) Cellular membrane glycoproteins

as listed by Spiro (1970) and any other molecular structures which agree with the definition so proposed.

Furthermore, in keeping with the suggestion of Montgomery[20], glycoproteins as defined by Gottschalk[14], can be separated into 2 categories:

i) glycoproteins of low carbohydrate content
e.g. ovomucoid and α acid glycoprotein
and
ii) glycoproteins of high carbohydrate content
e.g. salivary and epithelial mucins.

GLYCOPROTEIN STRUCTURE

Little chemical understanding of glycoprotein structure was forthcoming prior to 1940. The literature of that period is extensively reviewed by Gottschalk (1972)[14]. Perhaps the realization of the importance of the sialic acids to the physical, chemical and physiological properties of the vast array of glycoproteins was the major achievement of that period. (For discussion see Gottschalk[12,13,14], Schmidt[19]).

The general concepts of glycoprotein structure, of the types of glycoproteins, of the carbohydrate involvement, etc., were developed after this period by workers who examined specific molecules. The intensive examination of a series of glycoproteins from highly varied sources clearly supports the basic definition presented by Gottschalk in 1962[12].

The basic polypeptide backbone superficially resembles that of classical non-glycosylated proteins. The primary structure of a glycprotein appears unique to the molecule in question.

It does appear, however, that due to the nature of carbohydrate attachment to the glycoprotein polypeptide backbone, certain generalities concerning unique properties within the primary structure can now be made. As the carbohydrate content of the glycoprotein increases, so does the content of the linkage amino acid (to be elaborated upon with specific regards to the submaxillary mucin). Furthermore, because of the nature of attachment, i.e. carbohydrate to protein, glycosylated amino acids are a distinguishing

feature of the primary structure of a glycoprotein. This is also especially true for glycoproteins with a high degree of carbohydration. Glycosylation[18] appears to involve the 1 carbon of the most internal sugar of the oligosaccharide unit and some functional group of an amino acid within the polypeptide chain. Marshall[15] lists the following amino acids for which glycosylation during glycoprotein synthesis has been shown to exist; L-asparagine, L-serine, L-threonine, L-lysine, L-proline and L-cysteine.

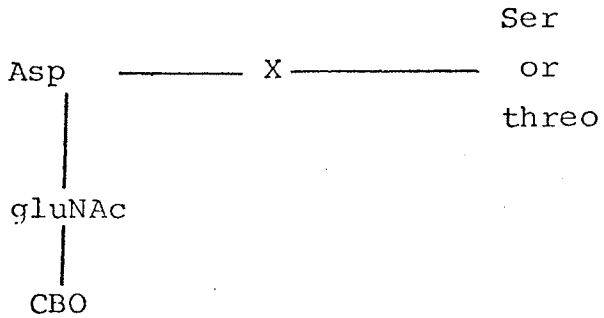
The earliest demonstrations[30,31] of a linkage amino acid involved the production of a glycosylated asparagine residue after extensive digestion of ovalbumin with a number of proteolytic enzymes. Gottschalk and co-workers[38-40] clearly identified serine and threonine as the linkage amino acids in ovine submaxillary mucin[37] and described the glycosidic nature of the linkage.

Until quite recently[42-45] linkage appeared to occur through one of two types:

A. The glycosylamine linkage which involves an N-glycosydic linkage to the amide group of asparagine. So far[41] only N-acetylglucosamine has been shown to be the carbohydrate partner in this type of linkage; and

B. The O-glycosydic linkage, in which the amino sugar is linked to serine and threonine residues. Interestingly enough, the linkage sugar appears to be N-acetylgalactosamine[14] in almost all cases for glycoproteins of mammalian origin.

Of particular interest, was the demonstration of the uniqueness of the amino acid sequence in the region of the glycosaminyl linkage[21-26]. (For literature listing see Neuberger et al.,[28] Table V, p. 466-467. Available amino acid data to date show no one preferred sequence to exist in the region of the linkage. The amino acids on the carboxy and amino terminal sides vary between proteins and even between glycosylation sites within the same molecule[20]. Therefore, no generalization can be made as to which amino acids might be expected to occur in the adjacent regions. However, it has been pointed out that certain amino acids do not appear in these positions. These include tryptophan, phenylalanine, tyrosine, cysteine, cystine and aspartic acid[15]. Early in the history of glycoprotein research, Eylar[27] pointed out the frequency with which threonine appeared in the vicinity of the glycosylated aspargine. The work of the last seven years has shown a much more specific sequence to occur. Almost invariably a serine or threonine residue occurs as the second amino acid away from the glycosylated asparagine. In almost every case to date the sequence in the region of the linkage is as follows:



Schmidt et al.[26], Neuberger et al.,[28] and Marshall[15] have extensively reviewed the literature regarding amino acid sequences about linkage regions, and especially comprehensive discussions can be found in the latter two articles. For example, of the five carbohydrate groups of the α-acid glycoprotein[26], all are attached via asparagine and all show the previously described sequence with the hydroxy amino acid being threonine in 4 out of the 5 cases. (For extensive discussions of the sequences of other N-linked glycoproteins see Table VI Neuberger et al.)[28] Several authors[49-51] have suggested this sequence might be a necessary but not a sufficient condition for glycosylation to occur. Neuberger and Marshall[49] hypothesize that the role of serine and threonine may be involved in hydrogen bonding or as a recognition signal for the sugar transferase. These authors and others (Neuberger et al.)[28] do, however, point out that the sequence -

$$x - asp - y - \underset{\text{(or threo)}}{\text{Ser}}$$

does occur in some proteins which have not been shown to be glycosylated[53-56]. It must therefore be concluded that other factors are also essential if glycosylation is to occur. Furthermore, it has yet to be shown whether the actual glycosylated amino acid is asparagine or aspartic acid prior to glycosylation. In contrast, Jett and Jamieson[26] in an interesting, albeit controversal paper, point out the survival value of this particular sequence on the basis of replacement mutations. Single point mutations for serine and threonine

generate asparagine codons and vice versa, whereas less probable two point mutations are required for conversion to other amino acid codons. These authors further suggest that, on the basis of their observations, and those of others[27,28], the primordial linkage amino acid was asparagine and that the others arose as single point mutations thereof. These authors[26] especially recognize the limited data available for such a complicated interpretation.

It was first suggested in 1959 from studies on the β-amylase from Aspergillis oryzae, that the carbohydrate may be linked through O-glycosidic linkage to some of its serine and threonine residues. Proof for such linkage was provided by several workers. Bhavanandan et al., (1964)[58] who unequivicably demonstrated that after an extensive alkali digestion of ovine submaxillary mucin, the amount of serine and threonine destroyed (40-50%) quantitatively agreed with the amount of N-acetylgalactosamine released into the medium. Tanaka et al.[59] have obtained similar results with bovine submaxillary mucin. Since it has been clearly shown[60,61] that alkali treatment of O-glycosidic linkages which are in β-position to a carboxyl group results in the release of the sugar and unsaturation of the aglycone, it was concluded that serine and threonine were the linkage amino acids and N-acetylglucosamine was in O-glycosidic linkage to these amino acids. (For a detailed account of the β-elimination reaction see (Neuberger et al., p. 474-475).

Anderson et al.,[62] Tanaka et al.,[63] and Harbon et al.,[64] have provided unambiguous proof for the O-glycosidic linkage in OSM and BSM. Other authors[65-67] have subsequently shown the presence of the O-glycosidic linkage for a wide variety of proteins including the human blood group substances[68,69]. There is little evidence to support an analogous situation in the primary structure around glycosidically involved serine and threonine to that which has been shown to exist for the glycosylamine linkage of asparagine. A few reports[81,82] of the sequence Pro-Pro located close to the linkage amino acid threonine have not been shown to occur in other molecules.

O-glycosidic linkages involving the hydroxyl of hydroxy-lysine and hydroxy-proline have been shown[70-77] recently to occur for a number of basement membrane and collagen proteins. An excellent review of these linkages can be found in Neuberger et al.[28].

THE CARBOHYDRATE GROUPS OF GLYCOPROTEINS

Several excellent reviews on the structure and function of the carbohydrate moieties of glycoprotein have been published in the last five years.[14,15,18-21,28,41] The extent and complexity of the literature makes it necessary to generalize about the oligosaccharide structure of glycoproteins. The oligosaccharide unit can vary[18] from a simple monosaccharide or disaccharide as found in ovine submaxillary mucin[83,84] to extremely complex heteropolysaccharide units[85-89]. Glycoproteins can have as few as one oligosaccharide unit per molecule e.g. ovalbumin[90,91] and Ribonuclease B[92] while e.g. OSM has in the region of 800 individual[83] units per molecule.

The sugar composition generally involves one, some or all of the following sugars; glucose, galactose, mannose, N-acetylglucosamine, N-acetylgalactosamine, N-acetylmannosamine, fucose and sialic acid. Furthermore, it appears[18] that the different sugars fulfill a positional "role" in the structure of the prosthetic group. That is, certain sugars can be found in certain positions with a high frequency in comparison to others. For example, the amino sugars, N-acetylglucosamine and N-acetylgalactosamine are invariably linkage sugars for serine, threonine and asparagine linked glycoproteins. Obviously then, these two sugars must occupy the most internal position on the oligosaccharide chain. Fucose and the sialic acids, when they occur, invariably have been shown to be terminal sugars, existing on the outside of the chain. For certain types of oligosaccharide structures, such as those

found in fetuin[99] and a number of other glycoproteins[100-103], a classical picture of glycoproteins of moderate carbohydrate content can be seen. The internal linkage sugar is N-acetylglucosamine[99,104] to which is attached a somewhat variable core region of several mannose and additional N-acetylglucosamines.[18,99,104] Externally attached to the core region is a trisaccharide, containing N-acetylneuraminic acid (NANA) galactose and N-acetylglucosamine (NAcGlu). The sequence, for fetuin, at least, involves an α-linkage between carbon two (2) of (NANA) and carbon three of galactose and a β-glycosidic bond between the one carbon of galactose and the four carbon of NAcGlu.[18,99,104] In other molecules showing similar sequences,[19] positional isomerism can occur in the trisaccharide region between NANA and galactose (involving linkages to almost every carbon of galactose except C_1). In addition, it has also been reported that in some, but not all, of the trisaccharide chains of a population, fucose can replace NANA in the terminal position.[18,105] Several authors have postulated a role for some of the mannose residues of the core region. For a number of molecules[85,100,104,106,107] it appears that these core mannose residues may act as branch points for the attachment of two of the outer trisaccharide chains. (Especially see reference 85 for partially complete structural models for the α-acid glycoprotein). Variations in this structure, even for molecules from the same source isolated at the same or different times are quite common,[18]

with the variations occurring in the content of both the mannose and N-acetylglucosamine residues. The number of mannose residues may vary upwards to twenty-five, depending on the molecule in question and the specific time of isolation.[18] This type of carbohydrate unit represents a complex type of structure for animal glycoproteins.

A tremendous number of simpler oligosaccharide units involving less than a pentasaccharide have also been reported. (See ref. 14,18,19,20 for listings and discussions) and the more relevant of these will be discussed later in the thesis.

In summary then, the complex type of carbohydrate prosthetic group shows a linkage region (one sugar), a core region of several sugars (extensively branched about one or more of the neutral sugars) and a short terminal oligosaccharide (most often containing either sialic acid or fucose or both).

The simpler type of prosthetic group contains only the linkage region and a short terminal oligosaccharide chain. Branches may be present but are not characteristic.

Of particular interest is the wide variability in oligosaccharide structures reported. This heterogeneity can be considered to be of two types. In one type minor variations can occur within the sugar sequences and/or the conformation of a single type of prosthetic group. For example, the substitution of fucose for sialic acid in some chains (of a population) of a molecule, or some molecules of the same species can have either two or three mannose residues.

Quite similarly, heterogeniety may be manifested in a more subtle manner by the appearance of several positional isomers at a particular linkage.

On a more gross structural level, heterogeniety is also manifested in the appearance, in some molecules,[18,71,81,107,108] of what appears to be, a second simpler type of carbohydrate prosthetic group in addition to a group of the complex type previously discussed. In some cases, two extra prosthetic groups, in addition to a major complex type have been reported,[18,107] linked to different amino acid residues.[18] The reason(s) for this type of heterogeniety, whether it is coded for genetically or whether it is the manifestation of a lack of specificity inherent in the glycosyl transferases remains yet to be determined.

GLYCOPROTEIN BIOSYNTHESIS

Although the work presented in this thesis does not directly deal with glycoprotein biosynthesis per se, it is thought that some information on this subject would further an understanding of the work presented.

The biosynthesis of glycoproteins has been the subject of many superb reviews in the past five years.[15,18,20,185-190] As a consequence of the extensive literature on this subject and the ready availability of these reviews, only the salient points of the biosynthesis of these molecules will be presented. For extensive discussions of the evidence for the

points made here, the reader will be referred as needs be. The biosynthesis of glycoproteins will be discussed to cover the following aspects; (i) biosynthesis of the peptide portion, (ii) biosynthesis of sugar precursors, (iii) attachment of the linkage sugar and (iv) elongation of the oligosaccharide chain.

The investigations of the biosynthesis of the protein component proceeded along lines determined by the available models for protein synthesis. It has now been concluded[15,18,185-190] that there is no reason to believe that the protein core is synthesized by any but the classical ribosomal model. In only one situation[20] has any evidence been presented to indicate any extraribosomal involvement in the production of the final polypeptide chain.

The case for the ribosomal model is supported by two lines of evidence. The well known effects of the puromycin inhibition of protein synthesis have been used by a large number of workers in an extensive variety of tissues to study the classical mechanism in glycoprotein production. The specific papers in this area are extensively reviewed by Clauser et al.[190] The overall conclusions derived from these papers indicates that puromycin inhibits both protein and glycoprotein biosynthesis more or less completely. Those tissues in which glycoprotein synthesis was inhibited less than protein synthesis generally show a large pool of previously synthesized peptide precursors. As a result, glycoprotein

synthesis (for e.g. labelled glucosamine incorporation) continued until this pool was exhausted. In some tissues this pool has been shown to be quite extensive. These data definitely support[190] the concept of a ribosomal mechanism for peptide synthesis not unlike that for ordinary proteins.

There is an extensive literature[190] demonstrating that the labelled amino acid uptake into glycoproteins occurs in the rough membranes of the endoplasmic reticulum, or more specifically, the ribosomes. These papers are thought by some[15,18,185-190] to provide the necessary evidence to directly support the classical ribosomal model as the process whereby the protein portion of glycoproteins is produced. Although other possible mechanisms for peptide bond production exist for shorter peptides only),Clauser et al.,[190] feel that the available data to date exclude the possibility that these mechanisms would be significant to glycoprotein biosynthesis. This conclusion has been supported by most other reviewers.[15,18, 185-190] Whether or not this conclusion is warranted, however, remains to be seen. This author eagerly awaits further work by Pigman et al.,[184-191] following their announcement (see ref. 18, for discussion) of the distinct possibility of a peptide polymerase activity in submaxillary glands of sheep and cattle. They postulate that only small peptides are formed at the ribosomes and that the linkage of these together occurs extraribosomally by a peptide polymerase located in the golgi membranes. In reviewing this literature,

Montgomery[18] concludes that this two stage process might very well represent a general model for glycoprotein synthesis not necessarily restricted to salivary glands. He reports evidence[191] indicating that this process also occurs in the liver. For a listing of all references to this development as well as their discussion, see this review.[18]

The source of sugar precursors for glycoprotein biosynthesis has long been established[190] as the nucleotide-sugar pool within the cell. That is, the substrates for the incorporation of the sugars into the glycoprotein are specific nucleotide derivatives of the sugars in question. It would be difficult to review, even superficially, the investigations of the last fifteen years and there are many excellent reviews available.[187,192-196] The pathways for the production of the UDP derivatives of gluNAc, galNAc and galactose; the GDP derivatives of mannose and fucose and the CMP derivatives of the sialic acids are shown in Fig. 1. For those interested in the discrete enzymology of these reactions, their controls, etc., Warren[195,196] and Davidson[187] should be consulted. It has long been established[190,195,196] that all the nucleotide-sugars can be produced from glucose. In addition, other sugars have been shown to be involved in nucleotide-sugar production. For example, exogenous fucose has been shown to be taken up into GDP-fucose directly (in some tissues only).[146] Direct uptake has similarly been shown for mannose, galactose and the sialic acids. (see Warren[196]) Glucosamine has been shown

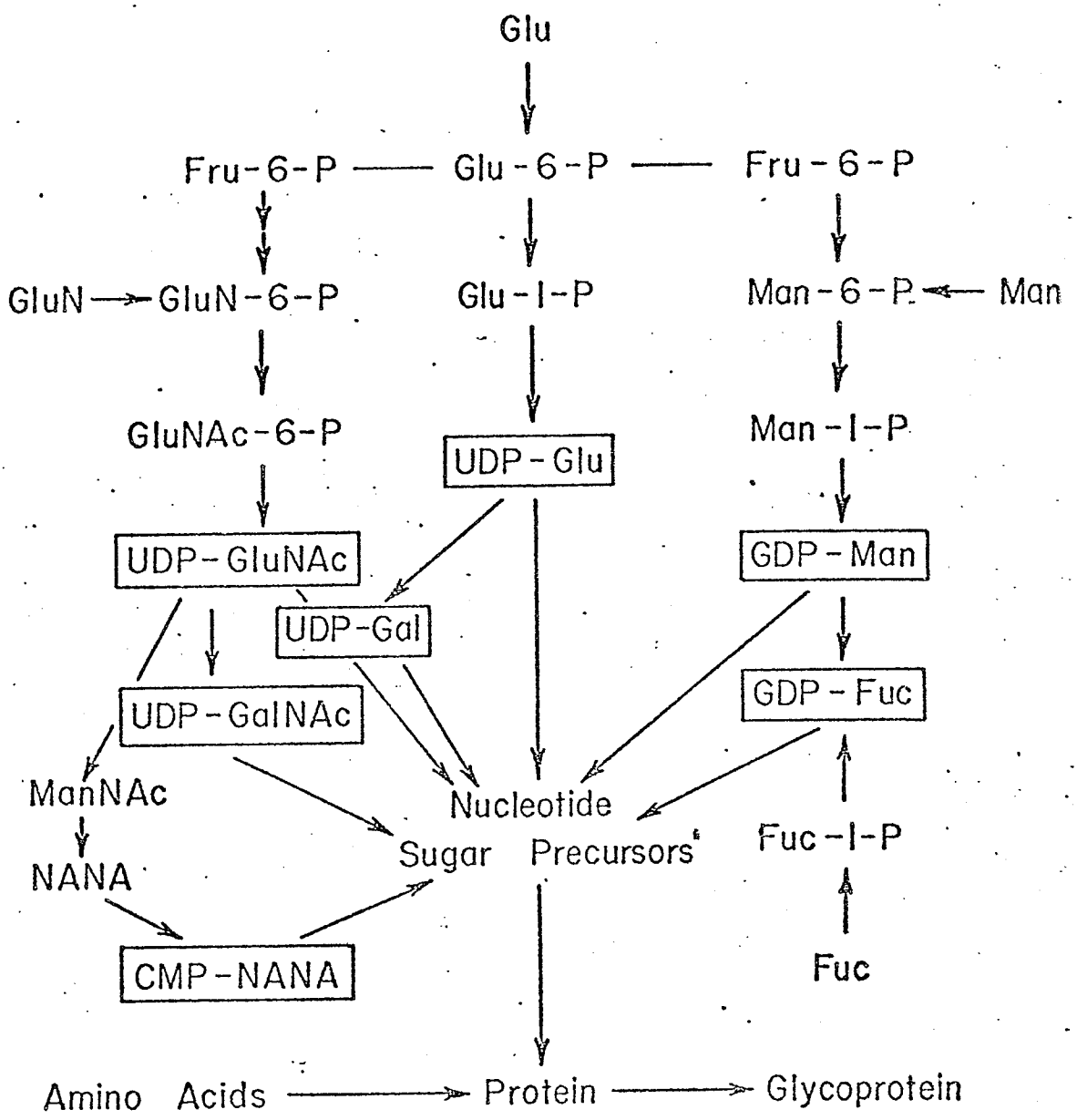


Fig. 1. Scheme showing intermediates involved in glycoprotein biosynthesis (modified from Winzler, 1968).[185] glu:glucose, fru; fructose, gluN: glucosamine, gluNAc: N-acetylglucosamine, Man: mannose, UDP: uridine diphosphate, gal: galactose, GDP: guanosine diphosphate, fuc: fucose, ManNAc: N-acetylmannosamine, NANA: N-acetylneuraminic acid, CMP: cytosine monophosphate, P: phosphate

to appear in several of the nucleotide sugars when used as carbon source by a large number of tissues. The biosynthesis of these sugar derivatives has been shown to occur by enzymes in the readily solubilized portions of the cell. The morphological organization of these enzymes within the cell in relation to the other machinery for the biosynthesis of glycoproteins is, at present, a highly debatable question.[185]

To date, the evidence[190] indicates that the mechanism of glycoprotein biosynthesis involves the sequential addition of the sugar monomers from the nucleotide-sugar precursors to a preformed protein or to a sugar residue already residing on a partially formed glycoprotein by a series of enzymes located within the membranes of the cell (most notably, the golgi membranes).[185,186,190]

The direct attachment of the linkage sugar to the polypeptide chain seems to imply a close spatial and temporal link between the classical mechanisms operating for protein synthesis and the reactions catalyzed by the glycosylating enzymes. The question of glycosylated amino acyl-tRNA's donating glycosylated amino acids to the growing polypeptide chain at the ribosome has been investigated[197] in the liver and no encouraging results were obtained. Direct evidence, however, has been obtained by a large number of investigators for glycosylating enzymes assumed to attach the first sugar to the polypeptide chain.[188,190] Many workers (see Clauser et al.,[190] for specific discussion) have shown that the initial

site of attachment lies within the smooth endoplasmic reticulum. It may be that, for some tissues, the linkage sugar may be first attached at some distance from the ribosomes, i.e. in the smooth membrane. A growing body of evidence, however, based on more sensitive techniques, has accumulated indicating that the initial site of attachment is in the ribosome-rich fractions of the rough endoplasmic reticulum. (See Clauser et al.,[190] or Roseman[186]). On the basis of this work it may be tentatively concluded that the rough membranes make a significant contribution to the incorporation of sugars into the glycoprotein, most probably being involved in the attachment of the linkage sugar.[190]

Several authors (see ref. 190 for listing and discussion) have demonstrated the uptake of labelled sugars into nascent proteins while still attached to the ribosomes. That is, direct glycosylation may be occurring at the ribosome while the polypeptide chain is still being formed. No theoretical objection exists against such a model[190] and a host of evidence has been accumulated in a wide variety of tissues to support it.

It would apppear then, that the attachment of the linkage sugars is accomplished as soon as possible, first occurring while the protein is still being formed, immediately after being released in the rough membranes and finally being completed in the smooth membranes.[185,190]

The presence of specific glycosyl transferases to accomplish this has been shown in a wide variety of tissues. Several reviews of this evidence are available.[186,188]

It has been proposed[192] that the unique sequence of sugars in the oligosaccharide chains of a glycoprotein is the product of the cooperative sequential specificity[186] of a multi-enzyme glycosyltransferase system present within the cell. The substrate of each enzyme is a specific nucleotide donor and a specific acceptor (sugar or amino acid of a partially completed glycoprotein). The specificity of these enzymes is determined by (i) the acceptor molecule, (ii) the penultimate sugar or amino acid sequence in the immediate vicinity and (iii) the linkage between the terminal and penultimate sugars.

As a result of this process, each acceptor molecule in the sequence is the product formed by the previous transferase reaction, (hence the term "cooperative sequential specificity"). The elongation of the oligosaccharide chains would be accomplished in this manner and the sugar sequence ensured by the specificities of the glycosyl transferases present. The characteristics of each enzyme, in turn, would be determined by the genotype of the cell according to the well known models for enzyme synthesis.

Recently, a number of workers[198-200] have presented evidence to confirm the involvement of lipid intermediates in the biosynthesis of some glycoprotein glycosylation reactions

(in the core region of serum glycoproteins). This requires much more work before any definitive pathways can be presented.

1. SUBMANDIBULAR GLAND CYTOMORPHOLOGY AND FINE STRUCTURE

Salivary glands show marked variation in structure which is species, age and sex-dependent. They do, however, show a number of common characteristics. They all appear to be complex tubulo-alveolar glands.[109] In simplest terms, each gland can be viewed as a series of secretory units, arranged in a linear sequence; the sequence being acini to intercolated ducts, to striated ducts, to excretory ducts. Shackleford and Wilborn[110] have classified acinar cells of salivary glands into two categories. They state that mucous acinar cells contain and secrete material rich in mucosubstance. Muco-substance may be taken to mean that the secretory droplets contain significant carbohydrate. Most probably the word "glycoprotein" should be substituted for, or at least be understood as the meaning of "mucosubstance" as used by these authors. The prefix "muco" has little meaning when applied in this way to salivary gland biochemistry. Serous[110] acinar cells are described as elaborating granules containing "a proteinaceous composition". This might more correctly be read that serous cells contain granules of a "more" protein-aceous composition than mucous acinar cells.

They[110] further sub-classify serous cells, but this

classification is of little interest to this thesis.

Acinar cells, (be they mucous or serous types) are invariably arranged about an irregularly shaped lumen. The lumen is for the most part, centrally placed amidst the cells,[109] which are generally pyramidal with some microvilli on the luminal surfaces.

In the adult rat, the submandibular gland consists of the 5 previously mentioned zones, being; acini, intercolated ducts (i.c.) granular ducts (g.d.) striated ducts (s.d.) and secretory ducts (sec.d.), with the intralobular volume occupied by each division within the gland listed[111] as follows: acini 63; i.c. 4; g.d. 18; s.d. 1 and sec.d. 14. Acini, per se, demonstrate extreme heterogeneity in size and shape. The lumen of the acini is much smaller than the total luminal surface area of the cells present.[111] (approximately 4-6 cells/lumen). Hence, cells not juxtaposing directly onto the lumen itself are served by secretory canaliculi (luminal extensions or canals) which empty into the lumen. Tamarin and Sreebny[111] suggest that the lumen may be regarded simply as a junctional zone between secretory canaliculi and the lumen of the intercolated ducts. Intercolated duct cells abut rather abruptly with acinar cells, and the lumen is slightly larger than the lumen of the acinus. Myoepithelial cells[109,111] lie in apposition to both acini and intercolated duct cells. Since these stellate-shaped cells share similar pharamocologic[112] and immunological[113] properties to smooth muscle cells,

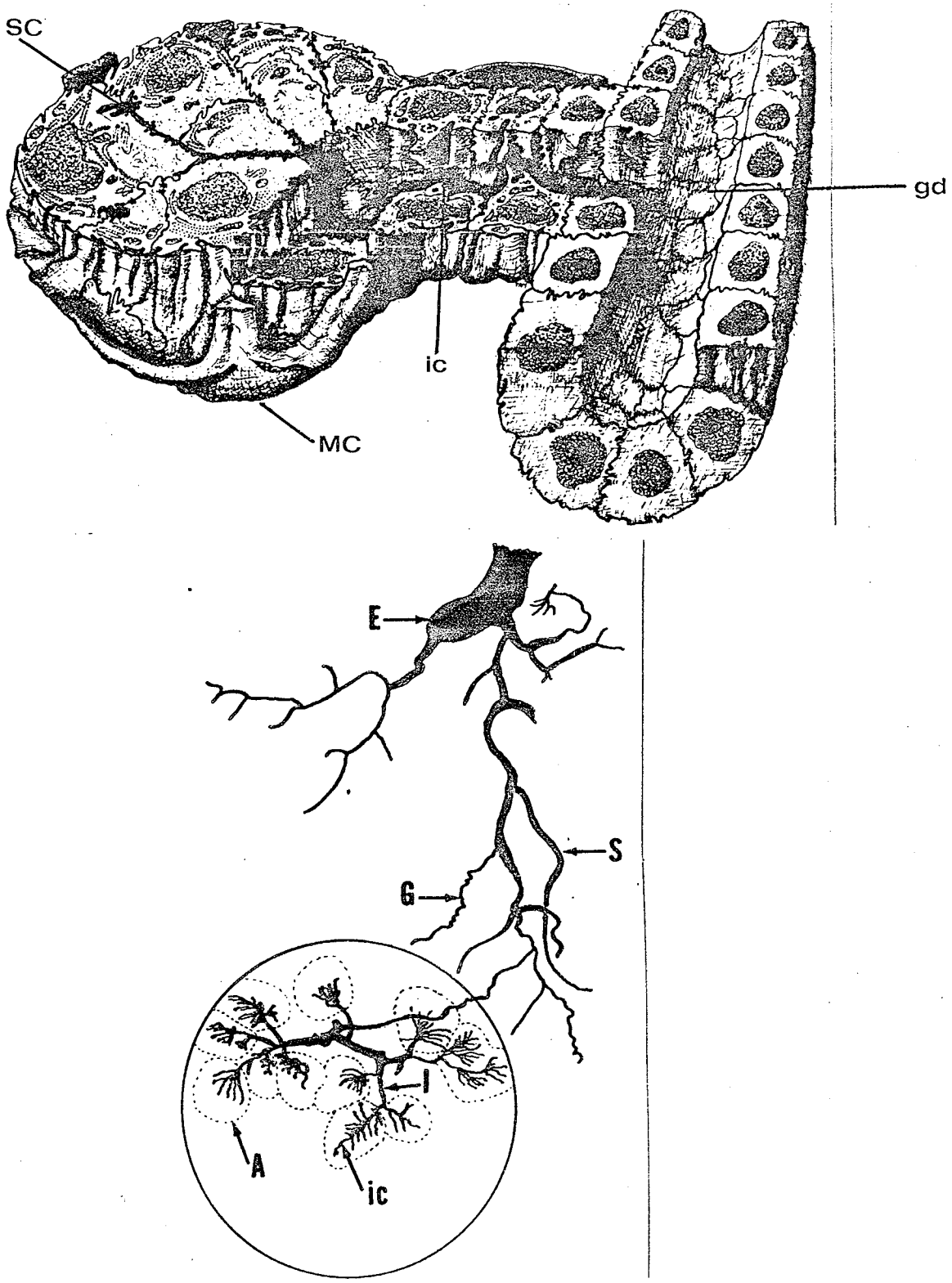


Fig. 2. Diagramatic representation of submandibular gland secretory unit cytomorphology.

(a) Three dimensional composite diagram of secretory unit
(b) Diagramatic relationship of ducts of the secretory unit.

A, acinus; MC, myoepethelial cells; SC, secretory canaliculi; ic, intercolated cells, gd, granular duct; S, secretory duct.

it is thought[109] that these cells can, given appropriate stimuli contract and force the contents of the acinar and ductal lumens out, causing saliva to come gushing into the mouth.

Immediately, but not obviously following the intercolated cells are cells showing discrete granules and which are termed granular duct cells.[111] Granular ducts merge after a very short distance with the striated ducts. Striated ducts have lumens of larger diameters than the other ductal tissues and the cells of this duct do not show any granules. Excretory ducts, following the striated ducts and travelling to the hilar region, merge to form the major excretory duct. The exit from the gland at the hilus in the loose fascia, along with the sublingual duct, nerves and blood vessels.

METHODS AND MATERIALS

Protein Assay

Total protein was assayed by the method of Lowry et al.[95]

Sialic Acid Assay

All sialic acid values except those for the final analysis of the component sugars of the mucin were determined with the Thiobarbituric acid procedure of Warren.[96]

Tissue Fractionation Experiment

Male, white Sprague Dawley rats, 4-5 months old, were used in all experiments unless otherwise indicated.

For this experiment, eight (8) rats were killed by exsanguination under light ether anesthesia. The submaxillary glands (combined submandibular and sublingual glands) were immediately removed and stored on ice. The adventitia was stripped away and the sublingual glands carefully dissected away and discarded. The remaining tissue was the submandibular gland (SMG or simply "glands"). The glands were homogenized with a tight fitting teflon pestle in a glass homogenizing tube after thorough mincing with dissecting scissors. The glands were homogenized with 10 firm strokes of the pestle in either (a) cold distilled water or (b) cold 0.154 M saline such that the final concentration was 100 mg/ml wet tissue weight (4 pairs of glands per group). The speed control setting on the homogenizer (Tri-R-Stir-R Model S63C, Tri-R Instruments, Inc.) was 6 (approximately 6000 rpm). This was termed the whole homogenate (W.H. see Fig. 3). The W.H. was decanted into

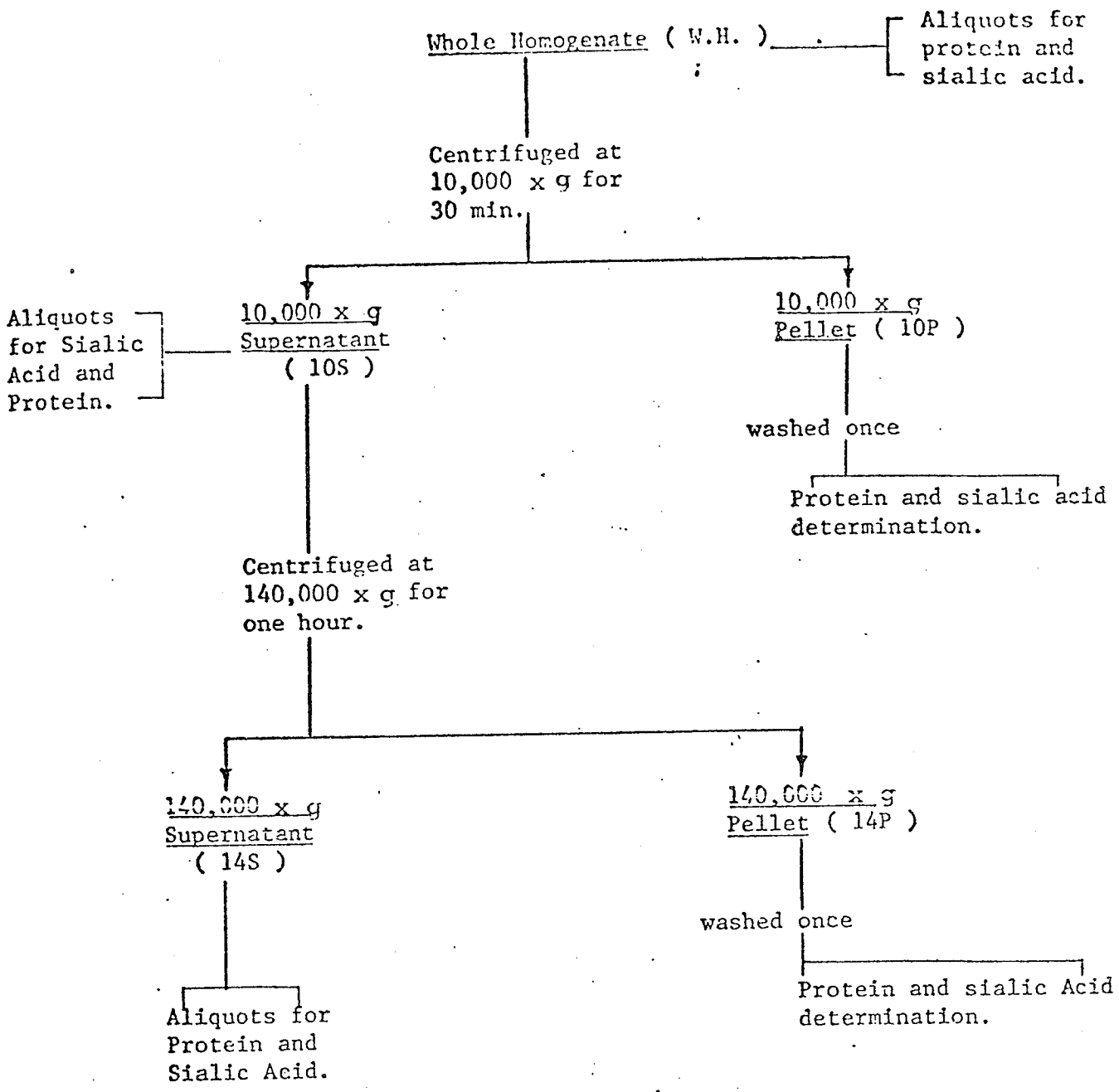


Fig. 3. Initial tissue preparation scheme for differential centrifugation of submandibular gland.

plastic centrifuge tubes and centrifuged in an International B-20 Centrifuge at 10000 xg for 30 min at 4°C. The supernatant was decanted into fresh plastic centrifuge tubes and the pellet washed once with 500 µl of either distilled water or saline. The washings were combined with the supernatant. The 10000 xg supernatant and pellet were designated the 10S and 10P fractions respectively. Suitable aliquots were taken for protein and sialic acid determination. The 10S fraction was then centrifuged at 140,000 xg in an International B-60 Centrifuge at 4°C under reduced pressure for 1 hour. The supernatant was decanted and the pellet was washed once with 200 µl of either distilled water or saline and the washings combined with the supernatants. The 140,000 xg supernatant and pellets were designated the 14S (HSS) fraction and the 14P fraction respectively. Suitable aliquots were taken for protein and sialic acid determination. The data from this experiment are presented in the results section under Tissue Fractionation Experiment. (Table 1 p. 49A)

Tissue Fractionation Scheme

As a result of the tissue fractionation experiment the following fractionation scheme was adopted. The whole homogenate was prepared in distilled water and centrifuged at 10,000 xg for 30 min. The pellet was discarded and the supernatant recentrifuged at 140,000 xg for 1 hour under reduced pressure at 4°C. The pellet from this centrifugation was also discarded and the supernatant (14S fraction or HSS) was stored

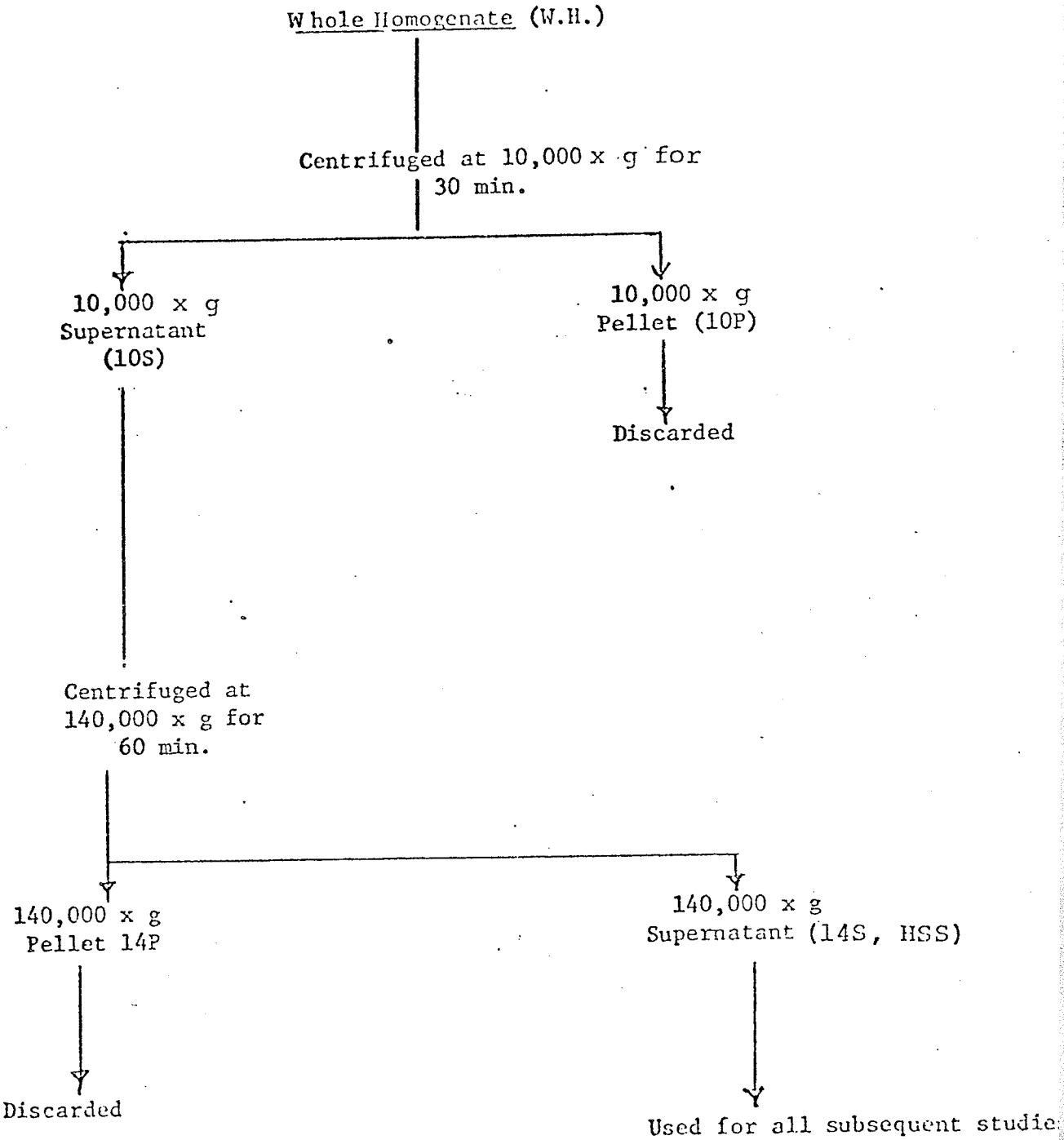


Fig. 4. Final tissue preparation pattern.

in the freezer at -60°C to be used for subsequent experiments (see Fig. 4).

Electrophoretic Studies

Extensive electrophoretic studies were carried out on the proteins found in the 14S fraction. Initially, our primary interest was to survey the number of sialoproteins found in this fraction. We necessarily had to survey the total glycoprotein composition as well. Several electrophoretic systems involving polyacrylamide gels were used. These are now listed and described. All electrophoresis was accomplished by methods essentially described by Davis (1964)[93].

System (A) 7% Anionic Acrylamide Gels (7% Anionic PAGE[94]).

The stock solutions for these gels are listed as follows: (as per Canalco literature 1965).

Solution A	Solution B
48.0 ml 1N HCl	48.0 ml in HCl
36.3 gm Tris	5.98 gm Tris
0.23 ml Temed	0.46 ml Temed
with the pH adjusted to 8.8-9.0	with the pH adjusted to 6.6-6.8

Solution C	Solution D
28.0 gm Acrylamide	10.0 gm Acrylamide
0.735 gm Bis.	2.5 gm Bis.

Solution E	Solution F
4.0 mg Riboflavin	80.0 mg Ammonium Persulfate

Solution G	Upper Electrode Buffer (Cathodal)
140.0 mg Ammonium Persulfate	5.16 gm Tris
	3.48 gm Glycine
	with the pH adjusted to 8.91

Lower Electrode Buffer (Anodal)

14.50 gm Tris

60.0 ml IN HCl with the pH adjusted to pH 8.07.

Solutions A to G were all made up to a final volume of 100 ml with distilled water. The cathodal and anodal buffers were all made up to a final volume of 1 litre with distilled water.

System (B) 3.5% Anionic Acrylamide Gels.

In order to increase the mobility of the larger molecules during the time of electrophoresis, the concentration of the acrylamide was halved.[97] This gives a markedly reduced frictional resistance to large molecular weight molecules ($MW > 8 \times 10^5$). The stock acrylamide solutions of system A were subsequently changed as follows:

Solution C	Solution D
14.0 gm Acrylamide	5.0 gm Acrylamide
0.735 gm Bis.	2.5 gm Bis.

As before, these solutions were made up to 100 ml with distilled water. All other stock solutions and buffers were unchanged.

System (C) 7.5% Anionic Urea Acrylamide Gels (Urea Gels).

The stock solutions for this system were derived from those recommended for use with the Shandon Preparative Electrophoresis Apparatus. They were subsequently modified and their final form appears as follows:

Solution A	Solution B
4.8 ml 10 N HCl	25.6 ml IN H_3PO_4
36.3 gm Tris	5.7 gm Tris
0.46 ml Temed	

Solution C	Solution D
30.0 gm Acrylamide	10.0 gm Acrylamide
0.8 gm Bis	2.5 gm Bis
0.015 gm KFeCN	

Solution E	Solution F
0.250 gm Ammonium Persulfate	0.008 gm Riboflavin

Solution G	Electrode Buffer for both Chambers
360.0 gm Urea	6.0 gm Tris
	28.8 gm Glycine with the pH adjusted to 8.45.

Solution G was made up to 1 litre with distilled water. The solvent phase for solutions A through F was solution G, and the final volume for these solutions was 100 ml . The electrode buffer was made up in distilled water to a final volume of 1 litre.

System (D) 3.75% Anionic Urea Acrylamide Gels (Large pore Urea Gels).

As before, the stock acrylamide solutions for these large pore gels were modified from those of System C as follows:

Solution C	Solution D
15.0 gm Acrylamide	5.0 gm Acrylamide
0.8 gm Bis	2.5 gm Bis

These solutions were made up to a final volume of 100 mls with Solution G. All other stock solutions and buffers remained unchanged from System C.

System (E) 7.5% Cationic Acrylamide Gels (Cationic Gels)

Cationic gels were prepared and run by methods very similar to those described by Nagai et al. (1964).[98] The following stock solutions were prepared accordingly and used:

Solution A	Solution B
6.8 ml IN KOH	10.0 gm Acrylamide
8.7 ml IN HAc	2.5 gm Bis
0.3 ml Temed	

with the pH adjusted to 6.2

Solution C	Solution D
4.0 mg Riboflavin	13.6 ml IN KOH
	26.5 ml IN HAc
	0.5 ml Temed

with pH adjusted to 5.3.

Solution E

30.0 gm Acrylamide

0.8 gm Bis

Solution F

0.15 gm Ammonium Persulfate

Solution G

35.6 gm B-Alamine

3.0 ml 1 N HAc

with pH adjusted to 5.5

Solution H

4.85 gm Tris

2.40 ml Concentrated HAc

with the pH adjusted to 5.5

Solution I

33.3 gm KAc

5.1 m Concentrated HAC

with the pH adjusted to 5.3.

Solutions A through F were suspended in and made up to a final volume of 100 ml with distilled water. Solutions G, H and I were suspended in amd made up to a final volume of 1 litre with distilled water.

Gel Casting and Electrophoresis Proper

The final model to which the gels were cast can be seen in Fig. 5. Gels were cast as described by Davis.[93] The apparatus used was a Buchler Polyanalyst with a Buchler power source. The ratios of stock solutions used in the preparation of the separating gels were as follows:

(i) Systems A and B (Anionic acrylamide)

Solutions A:C:G = 1:1:2 by volume

(ii) Systems C and D (Urea gels)

Solutions A:C:E:G = 1:2:4:1 by volume

and

(iii) System E (Cationic gels)

Solutions D:E:F:Water = 1:2:4:1 by volume.

Separating gel volumes were 1000 µl for systems A through D and 800 µl for the cationic system. The mixtures of stock solutions for the stacking gels were as follows:

(i) Systems A and B.

Solutions B:D:E:F = 1:1:1:1 by volume

(ii) Systems C and D.

Solutions B:D:F:G = 1:2:1:4 by volume

and

(iii) System E

Solutions A:B:C:Water = 1:2:1:4 by volume.

Stacking gel volumes were invariably 200 µl for all gel systems.

Sample gel preparation proceeded as follows. Sufficient stacking gel solution was added to the protein sample (which contained the amount of protein required for the run, be it 50, 100, 200, 400 µg, etc. of Lowry detectible protein in less than 100 µl total volume, to bring the final volume of the sample gel solution (protein and stacking gel) to a final volume of 100 µl. If more than one gel was to be run of the same sample (as was the case the large percentage of the time) then the sample gel mixture was made up to some integral multiple of 100 µl (e.g. 400 µl if 3 samples were required).

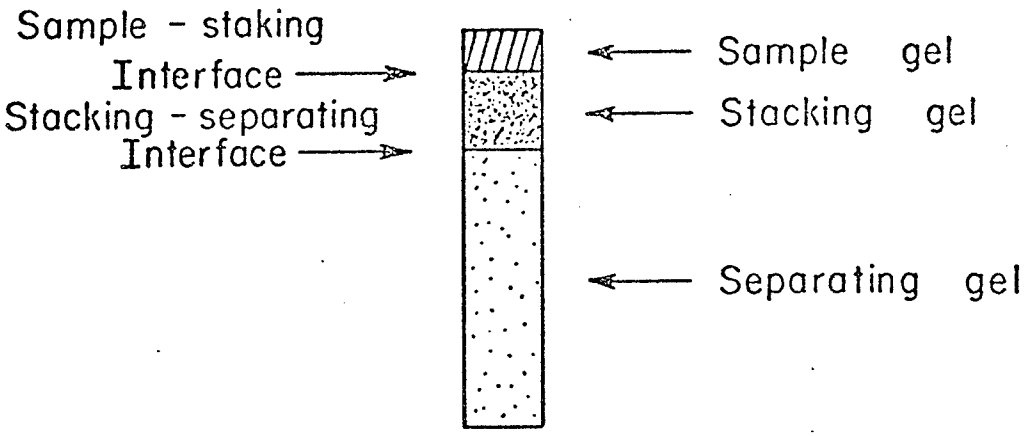


Fig. 5. Diagramatic representation of gel architecture.

After the stacking gel had completely polymerized, the sample gel was layered on top of the stacking gel and left stand for 1 hour. Unfortunately, some inhibition of polymerization generally occurred for protein volumes greater than 25 µl of the total 100 µl of the sample gel mixture. The time of 1 hour was chosen to allow the gels to be run under fairly standard conditions. The buffers were then added to their respective chambers. In the case of the cationic gels (System E) the upper buffer (Anodal) was prepared by mixing 75 µl of solution G and 25 µl of solution H and brought up to a final volume of 1 litre. The buffer for the lower chamber was prepared by diluting 50 ml of solution I to a final volume of 1 litre with distilled water.

Electrophoresis Conditions

A tracking dye was added to the upper chamber in all cases as follows:

(i) for systems A & B 1.0 ml of 0.005% Bromophenol Blue

(ii) for systems C & D 2.5 ml of 0.005% Bromophenol Blue

(iii) for system E 20 µl of 0.5% Pyronin Y solution.

The electrophoresis conditions were as follows: initially the current setting was 1.25 mA per gel column until the indicator had reached the sample-stacking interface (see Fig. 5). At this time, the current was then increased to 2.5 mA per column and maintained there until the indicator had travelled to within 2-5 mm of the end of the separating gel (see Fig. 5). At this time electrophoresis was terminated. The gels were

removed as described by Davis.[93] Staining of the separated components is described in the following section.

Staining of Electrophoretic Gels

Protein Staining. In order to visualize protein containing bands, the gels were placed in 10 x 75 mm test tubes and covered with excess 0.1% Amido Shwartz in 7% Acetic Acid for 1 hour. Destaining (removal of excess stain), in most cases, was done by electrophoresis against 7% Acetic acid at 5 mA per column. In a few cases excess stain was removed by washing against several washes of 7% Acetic acid for 48 hours. Gels were stored in 7% Acetic acid for photography.

Carbohydrate (Glycoprotein) Staining

The periodic acid Shiffs procedure developed in this laboratory was used to detect glycoprotein-containing bands. (See Appendex A). The final staining procedure consisted of the following steps:

(i) immediately after electrophoresis the gels were placed in 10 x 75 mm test tubes and covered with excess 12.5% TCA for 1 hour.

(ii) after fixation (step (i)) the gels were placed in clean 10 x 75 mm test tubes and covered with excess 1% Periodic Acid for 2 hours.

(iii) the gels were then washed free of Periodic acid as described in Appendix A for 2 hours against 15% HAc.

(iv) the bands were then visualized by immersing the gels in Shiff's reagent at 4°C in the dark for 2 hours.

(v) removal of excess Shiff's reagent was accomplished by washing against 7% HAc for 24 hours in a covered beaker (completely covered with tinfoil to prevent excess exposure of bands to light).

(vi) gels were stored not more than 1 week in 7% Acetic acid in the dark before photography and scanning.

Column Preparation

Sephadex was prepared and columns packed as follows. The Sephadex was swollen in distilled water for 8 hours at 96°C in a water bath. It was then left to stand on the desk top overnight and defined (removal of small particles) thoroughly. Defining generally took 1-2 days of rinsing and decanting the fines (small particles) until reasonable visual homogeniety was achieved. The Sephadex was then placed in the cold room overnight. Immediately prior to packing, the Sephadex was thoroughly evacuated to remove trapped air bubbles. The column was then packed by gently pouring the Sephadex down the side of the column into a short water column (10% total column length). After the Sephadex had finished packing, the column was washed for 24-48 hours (48 hours for 2.5 x 100 cm. columns) to equilibrate the column to pressure and to further remove soluble high and low molecular weight contamination. The columns were then loaded

with the protein to be eluted. All columns were packed and run in the cold room.

Column packing was done under very rigidly standardized conditions. After the initial washing the quality of each column packed was determined by passing a dextran blue solution down the column. The volume of dextran blue applied to the column was exactly the same as the sample volume to be applied. Columns with any defects were repacked (usually only one out of every five or six columns was acceptable!) Void volume and flow rate determinations were made before and after each run.

Fractionation Experiment I 1.5 x 30 cm column.

Preliminary separation of HSS protein was accomplished on Sephadex G-200 coarse in a column 1.5 x 30 cm (Sephadex bed length 24 cm) prepared as described. Two (2) glands were obtained and HSS protein prepared as usual. The high speed supernatant was then frozen in the -60°C freezer and lyophylized to dryness. Just prior to chromatography the HSS powder was resuspended in 1.5 ml cold distilled water and 50 µl 10N NaOH was added to facilitate dissolution and suspension. One (1) ml of this solution (17.7 mg) was applied to the column and eluted with distilled water at a flow rate of 1 ml /hour and 1 ml volumes were collected by drop in an ISCO Golden Retriver fraction collector. Protein and sialic acid determination was done on each fraction in duplicate. Fractions were pooled, frozen and lyophylized as shown in Fig.12.

Electrophoretic analyses of fractions 14Sf 1-4 was done with system A and the gels were stained with Amido Shwarz and Alcian Blue. At the time of these experiments the PAS procedure had not been fully worked out therefore PAS stains were not obtainable.

Fractionation Experiment II 1.5 x 90 cm column.

A column 1.5 x 90 cm was packed with Sephadex G-200 superfine to a final bed length of 75 cm as described. HSS protein from the combined glands of ten (10) rats was prepared as previously described. The HSS was frozen and lyophylized and resuspended in 3.0 ml distilled water with 100 µl 10N NaOH. An aliquot (57.70 mg protein) was applied to the column and eluted at a flow rate of 1.5 ml /hour with distilled water in the cold room. 1.0 ml fractions were collected and analyzed for protein and sialic acid. The tubes were then pooled into 6 fractions (14Sf1-6) and assayed electrophoretically. Electrophoresis was done with system C and the gels were stained with Amido Shwartz and the PAS Procedure.

Preparative Fractionation Experiment III 2.5 x 100 cm column.

For preparation of the mucin in amounts suitable for chemical assay, HSS protein was repeatedly prepared and chromatographed on Sephadex G-200 in a column 2.5 x 100 cm (bed volume 90 cm). HSS protein (100-500 mg) was loaded onto the column after lyophylization and resuspension, and eluted with distilled water at a flow rate of 4.5 ml /hour. Four (4) ml fractions were collected by drop

and assayed for protein and sialic acid. The tubes were then pooled and assayed electrophoretically with system C to determine the fractions containing the mucin. Bands were stained with Amido Shwartz and the PAS stain.

Hydrolysis of Mucin and Preparation of Methyl-glycosides for Gas Chromatography.

Mucin to be processed for sugar analysis by the gas chromatography method of Chambers and Clamp[114] was treated as follows: The mucin fraction prepared from the preparative Sephadex G-200 column was lyophylized. One or two mg were weighed out with great care into drawn glass hydrolysis tubes and suspended in 500 µl dry methanol. One ml of 2.0 M methanolic HCl and 500 µl of internal standard (90 µg of D-mannitol in 500 µl dry methanol) was added and nitrogen gently bubbled through for 30 seconds. The tubes were then immediately sealed. The final concentration of HCl was one molar. The mucin solution and standards were hydrolized for 24 hours at 85°C.

Dry methanol[114] was prepared by refluxing methanol for one hour with magnesium turnings (2.5 gm /500 ml Methanol) and iodine crystals (100 mg/500 mls Methanol). After one hour, the methanol was distilled into a clean, dry glass-stoppered flask. Acidification was accomplished by bubbling dry HCl gas into a quantity of dry methanol until a final concentration of 2.0 M was reached.

Standard methyl glycosides were prepared by dissolving known amounts of the following sugars: fucose, N-acetylneuraminic acid, N-acetylglucosamine, N-acetylgalactosamine, N-acetylmannosamine, glucose, galactose, mannose, glucuronic acid and mannitol in dry methanol. Fixed amounts of each were pipetted into a single drawn glass hydrolysis tube and taken to dryness in a vacuum oven at 38°C over NaOH pellets. The sugars were re-dissolved in 2 ml of 1 M methanolic HCl and gently flushed with nitrogen for 30 seconds prior to sealing.

Hydrolysis of the mucin and preparation of the standards was done in the oven at 85°C for 24 hours. After hydrolysis, neutralization[114,115] of the acid was done by the addition of 450 mg silver carbonate (Ag_2CO_3). The pH was tested and more Ag_2CO_3 added if necessary. Since acid hydrolysis cleaves the N-Acetyl-groups from acetylated sugar amines and acids, the re-N-acetylation was done by the addition of 100 µl of acetic anhydride immediately after neutralization. The tubes were allowed to stand for at least 6 hours at room temperature for completion of this reaction.[114,115]

The methyl glycosides (from both mucin and standards) were obtained from the supernatant of the neutralized hydrolyzate after centrifugation. The pellet was washed three times with 500 µl dry methanol and the washings added to the supernatant. The methyl glycosides were then taken to dryness in a vacuum oven at 38°C over NaOH pellets and stored in a vacuum desicator overnight. Great care was taken to keep the samples free from water throughout the preparation steps.

Since the methyl glycosides were chromatographed as the trimethyl silyl derivatives, 50 or 100 µl of Sil-prep reagent (Applied Science Laboratory) was added to the samples and standards allowed to react for 30-40 minutes. They were then centrifuged in tightly stoppered, glassed stoppered centrifuge tubes and 1 or 2 µl aliquots of the supernatant were injected into the gas chromatograph.

The gas chromatograph used was a PYE series 104 chromatograph with a Honeywell Braun Electronik chart recorder. Samples were chromatographed on a 6 ft x 1/4 inch glass column packed with OV-1 on gas chrom Q (Applied Science Laboratory, State College, Pa.). The chromatograph was temperature programmed at $1^{o}C$/min from 100^{o}-$200^{o}C$ and maintained at $200^{o}C$ for 30 minutes, or until the N-acetylneuraminic acid peak emerged. The detector temperature was $210^{o}C$ with the injection port setting at 5-6. Nitrogen flow rate was 40 ml min^{-1}, hydrogen flow rate 40 ml min^{-1} and the air flow rate was 20 ml min^{-1}. The amplifier gain was set at 2×10^{3} and the recorder chart speed was 1/2" minute.

Quantitation of the sugars was done by triangulation and comparison to the internal standard. The calculations were done by the method described by Chambers and Clamp[114,115], and the molar relative response factors used were those supplied by these authors.[114,115]

1. RADIOSOTOPE INCORPORATION AND TISSUE INCUBATION

Rat submandibular gland slices were incubated with radioisotope - labelled sugar precursors in order to study the incorporation of these sugars into the glycoprotein fractions separated by column chromatography and electrophoresis.

Submandibular glands were removed from rats under light ether anesthesia and immediately placed in warm Krebs Ringer Phosphate (KRP) buffer[117] with 11 mM glucose. Shortly afterwards (approximately 10 min) the glands were weighed and sliced (approximately 1 mm thickness) and placed in KRP buffer with 11 mM glucose (1 mg tissue wet weight per ml) and placed in a 37°C shaking water bath. Ten or twenty microlitres of radioisotope-labelled sugar (Fucose-1-C^{14}, 50 µc/ml, (Calbiochem), D-glucosamine-1-C^{14}, 100 µc/ml. (New England Nuclear) or D-galactosamine-1-C^{14}, 100 µc/ml (New England Nuclear) were added to the slices and the tissue was incubated for 2-3 hours. At the end of the incubation the slices were washed with distilled water several times in a buchner funnel over gentle vacuum. The slices were then homogenized in distilled water as previously described (Tissue Fractionation Procedure) and the HSS fraction prepared. Initially, the protein was dialyzed against distilled water to remove unbound label, but this procedure caused irreversible precipitation of some of the protein of the HSS. For this reason decontamination was done by passing the protein through a column 1.5 x 25 cm long of Sephadex G-25 coarse (prepared and run as previously

described). The entire void volume protein was collected (the first peak) and the retarded fraction (second peak) (consisting of small peptides and very low molecular weight compounds) was discarded. The eluent (washed HSS) was lyophylized and resuspended in 1 ml distilled water and chromatographed on Sephadex G-200 column length 1.5 x 25 cm). The labelled protein was eluted as previously described, and 30 µl aliquots of each tube were taken for scintillation counting. The scintillation cocktail for the HSS and the column fractions consisted of 1 ml methanol and 8 ml of scintillation fluid (4% PPO in 0.05% POPOP in toluene). The tubes were then pooled into four fractions for electrophoretic examination and lyophylized.

2. ELECTROPHORESIS OF LABELLED SUBMANDIBULAR GLAND PROTEIN.

Labelled HSS and the column fractions to be electrophoresed were resuspended in distilled water. Aliquots of 200 or 300 µg protein (Lowry) were then electrophoresed in duplicate or triplicate on gels prepared from system C and stained with Amido Shwartz and the PAS stain. Stained gels were photographed and the bands sliced out by hand. The slices were hydrolyzed for 2-3 hours in 1 ml 60% H_2O_2 at 60°C in scintillation vials. The hydrolysate was then suspended in 15 ml of Aquasol. Initially a scintillation fluid of 20% BioSolv BBS-3 (Beckman) in 4% PPO in 0.5% POPOP in toluene was used. However, on cooling to 4°C, a high degree of

quenching was observed. The scintillation fluid was subsequently changed to Aquasol (New England Nuclear, Montreal). This showed significantly less quenching than any other solubilization media used. All experiments reported here were done with 15 ml Aquasol as the scintillation medium.

Unstained and unfixed protein was also prepared after electrophoresis for counting as follows. Immediately after electrophoresis, unstained and unfixed gels were sliced (continuously) in a Savant Gel Slicer. The gel particles were eluted with distilled water. The fractions were collected into scintillation vials and hydrolyzed with 1 ml of 60% H_2O_2 for 2-3 hours at 60°C and suspended for counting in 15 ml Aquasol (Beckman). Satisfactory suspension of the hydrolysates could only be achieved if the vials were left to stand at room temperature for 24 hours or so prior to counting. Presumably, this allowed more complete solubilization of the hydrolysate by the Aquasol to occur.

Gel scanning of the PAS stained gels was done with a Joyce-Loebl Chromoscan using a 520 nm filter.

Amino Acid Analysis

The mucin was hydrolyzed for amino acid analysis in 6N HCl (at a final concentration of 0.5% mucin) under vacuum for 22 hours at 110°C. The amino acid analysis was preformed on a Beckman model 120C Amino Acid Analyzer.

Aliquots of the molecule were subjected to 24,48 and 72 hours to determine the loss of amino acids due to degradation. Values presented in Table 3 represent the subsequently corrected amino acid determinations.

RESULTS AND DISCUSSIONS

The presence of sialic acid in the rat submandibular gland has been known for several years.[125-130] That much of the sialic acid may be found in glycoprotein material is not unexpected and has been suggested a number of times in the past.[125-130] A stricter examination of the nature of the sialic acid containing compounds seemed the next important step in examining the sialic acid metabolism of the gland. Phillips (1971)[131] had indicated a significant recovery of sialic acid in the 140,000 x g supernatant of a combined submandibular-sublingual preparation. Prior to this, many other workers had clearly shown the ability to isolate glycoprotein rich fractions from a number of sources by various types of aqueous extraction. To this end the following series of experiments were done.

Salivary glands were fractionated by differential centrifugation according to the tissue fractionation scheme (Fig. 3) and the fractions obtained analyzed for protein and sialic acid by the methods already described. The results of the differential centrifugation appear in Tables 1 and 2.

When submandibular glands were fractionated in distilled water, over 80% (83.8%) (Table 2) of the total sialic acid of the gland could be found still soluble after centrifugation at 140,000 x g for 1 hour. However, preparation of the glands in saline resulted in considerably less (43.4%, Table 2) sialic acid appearing in the high speed supernatant

Table 1. Distribution of the sialic acid and protein fractions of the rat submandibular gland after differential centrifugation.

Fraction	W.H.[1]	10S[2]	10P[3]	14S[4]	14P[5]
No. of Animals	4	4	4	4	4
Distilled Water					
Total Sialic Acid (nM)	739.01 ± 7.9*	764.02 ± 65.24	150.69 ± 29.30	619. 17 ± 43.65	85.58 ± 11.67
Sialic Acid per mg. Total Protein.	12.37 ± 1.51	16.19 ± 2.26	12.52 ± 1.39	17.71 ± 1.87	25.64 ± 7.44
Total Protein (mg.)	60.63 ± 8.4	48.17 ± 9.24	11.96 ± 1.51	35.17 ± 3.46	3.48 ± 0.66
Saline					
Total Sialic Acid (nM)	820.29 ± 17.45	469.25 ± 50.31	238.6 ± 58.6	355.37 ± 43.42	68.0 ± 43.75
Sialic Acid per mg. Total Protein.	14.29 ± 1.15	13.74 ± 2.22	16.63 ± 3.86	11.19 ± 1.85	45.41 ± 28.29
Total Protein (mg.)	57.79 ± 5.46	36.75 ± 5.84	14.33 ± 0.51	32.01 ± 2.34	1.50 ± 0.07

1. Whole homogenate, 2. 10000 x g supernatant, 3. 10000 x g pellet, 4. 140000 x g supernatant and 5. 140000 x g pellet.

* Mean ± standard diviation

Table 2. Mean per cent recoveries of protein and sialic acid from fractions derived by differential centrifugation of rat submandibular gland.

	Sialic Acid (nM)		Protein (mg.)	
Fraction	Distilled water	Saline	Distilled water	Saline
10S + 10P / W.H.*	123.7 ± 12.7	89.4 ± 12.1	99.3 ± 11.9	90.8 ± 5.8
$\frac{14S + 10P + 14P}{W.H.}$ *	115.7 ± 6.7	80.7 ± 4.5	83.8 ± 5.7	83.4 ± 5.0
10S / W.H. **	103.3 ± 8.9	60.4 ± 6.1	79.6 ± 11.5	63.4 ± 6.1
14S / W.H. **	83.8 ± 6.4	43.4 ± 6.0	58.3 ± 4.8	58.2 ± 6.0

* Represents total percent recoveries at both levels of fractionation.
** Repersents soluble percent recoveries at both levels of fractionation.

Symbols as per Table 1.

(HSS) despite the fact that the per cent total protein appearing in the HSS was almost identical (58% of total) for both preparations (Table 2). As a consequence, the sialic acid per unit protein of the high speed supernatant showed a 1.5 fold enrichment over the whole homogenate (from 12 to 17 nM/mg, Table 1), whereas that of the saline preparation showed a slightly decreased ratio (from 14 to 11 nM/mg, Table 1). The 10,000 x g pellet for the saline preparation showed an increased ratio of sialic acid to protein in comparison to the distilled water preparation (30% of total vs 20% of total respectively) accounting for the observed difference.

Several possible factors could affect these differences in sedimentation. The increased ionic strength of the saline preparation may sufficiently neutralize the zeta potential of the sialic acid containing compounds, such that their solubility and sedimentation properties are affected (salting out effect). Alternatively, the saline may protect the granule-bound sialic acid from osmotic lysis. As a consequence, the granules would tend to sediment with their contents intact. In the distilled water preparation the granules would have certainly ruptured. In addition, the zeta potential of the molecules would be maximized, and hence their resistence to sedimentation at high speeds would be high. The increased recovery of sialic acid in the high speed supernatant may, therefore, be a manifestation of both of these effects. Using a homogenization medium containing tris, sucrose, magnesium chloride and potassium chloride,

Phillips[131] has reported similar recoveries for a saline preparation. Unfortunately, this worker failed to remove the sublingual gland (personal communication) from the submandibular gland and consequently his sialic acid fractions were highly contaminated with sialic acid containing components from the sublingual gland. It is well established[127] that the sublingual gland (in comparison to the submandibular gland,) contains 6-7 times the sialic acid concentration on a wet weight basis and has almost twice the total gland content of sialic acid. The high degree of contamination thus incurred by Phillips[131] makes direct comparisons between his and this work all but impossible. Several attempts were made to determine the relative amount of sialic acid contained within the different possible sialic acid containing components of the HSS. On the theory that only the sialic acid of the glycoprotein fraction would be affected by bacterial neuraminidase (Vibro cholera), HSS was incubated with this enzyme and the rate and amounts of sialic acid release were recorded (Fig. 6). Almost all of the bound sialic acid (greater than 90%) could be released after 24 hrs incubation with V. cholera neuraminidase. Approximately 20% of the total sialic acid of the HSS was free at any given time (Fig. 6) Unfortunately, the interpretation of the data was obscured by significant release of sialic acid by processes inherent within the gland itself. Incubation of the HSS with V. cholera neuraminidase (Fig. 6) suggested at least two different pools

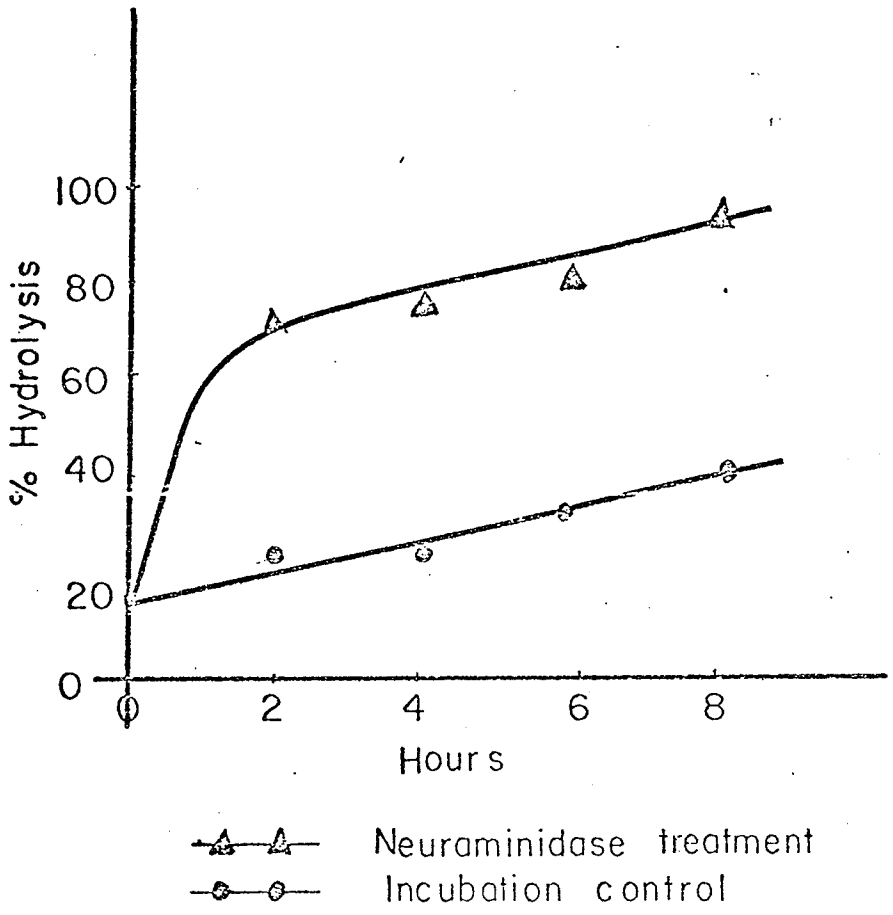


Fig. 6. Effect of incubation with neuraminidase (Vibrio cholera) on the rate of hydrolysis of bound sialic acid from rat submandibular gland HSS.

of sialic acid exist within the HSS. The linkages of only 60%-70% of the total bound sialic acid were susceptible to exogenous bacterial enzyme. The remainder of the linkages (representing approximately 30% of the total bound sialic acid), as shown by the incubation control, are susceptible only to some hydrolytic process originating with the tissue itself. It is a well known fact that the glycosidic linkages involving sialic acid in glycoproteins is extremely labile (this is in some contrast to the linkages involving the other sugars). Therefore, this release of sialic acid may simply represent the inherent instability of the sialic acid involved linkages. This is, by no means, the only explanation; nor the least likely. The presence of endogenous neuraminidase(s) has/have clearly been shown in rodent salivary glands[119] as well as human salivas.[121] Although the neuraminidase levels previously demonstrated[119] for the rat submandibular gland have been significantly less than those indicated by the hydrolysis rates of the incubation control (Fig. 6), our data may be more indicative of the true levels within the gland. The failure of these workers[119] to detect any neuraminidase activity in the different human salivas even though their presence has since been conclusively demonstrated by others[121] may very well indicate that these workers were significantly underestimating the neuraminidase activity of the gland.

The somewhat fastidious substrate specificity of the different neuraminidases, especially that shown by the

V. cholera form has been the subject of much work and discussion. (For specific discussions see reviews by Gottschalk 1972, 1973 and Drozeniuk 1972, 1973).[118,120] In light of these articles, it may very well be, that at least two, and perhaps several, different types of sialic acid involved linkages exist in the rat submandibular gland HSS. In addition, it should be pointed out that V. cholera neuraminidase has not been shown to be especially active when submandibular mucins of other sources (bovine and horse) were used as substrate.[118] It seems a possibility, albiet how remote, that the V. cholera neuraminidase resistant fraction could contain the rat submandibular gland mucin under the conditions used.

The possibility of ganglioside and ceramide-bound sialic acid, in a gland so richly furnished with membranes, being a small component of this resistant pool also seems likely. In a recent publication, Nijjar and Pritchard[24] have shown low but significant sialic acid levels in their membrane preparations from the rat submandibular gland. Unfotunately, these workers attributed the entire sialic acid content of their membrane to glycoprotein, which seems entirely unlikely. In any case, the membrane (and lipid bound) sialic acid of the rat gland may very well be partly or wholely resistant to the V. cholera enzyme as has been the case for some gangliosides and ceramides of other sources.[122,123]

The analytical separation of the HSS fraction of the

rat submandibular gland into the different protein and glycoprotein components proceeded along two classical lines. The HSS was subjected to analytical column chromatography and polyacrylamide gel electrophoresis (PAGE). The individual fractions obtained by column chromatography were then subjected to examination by PAGE.

Initially, fractionation of the HSS was carried out by a salting out procedure (ammonium sulfate fractionation).[153] However, serious problems associated with the prolonged dialysis necessary to remove the ammonium sulphate, and consistently low recoveries forced us to abandon this approach as an initial separation technique and rely solely on the use of columns and PAGE.

The application of electrophoretic techniques utilizing various media (free boundary electrophoresis[140-142], paper[136-138,143,144,148-150], starch gel[133,135], cellulose acetate[133], and PAGE[132,134,139,147,151] of various forms) to the separation of the various salivary components goes back a number of years and the early literature has been reviewed by Mandel (1966)[152], (with special emphasis on the applicability to oral health problems). The vast majority of the papers reviewed deal with the various human salivas, especially parotid saliva. The enzymatic constitution of saliva (see ref. 152) was the object of many of these studies. Only a few studies[132,134] concerned themselves with the nature of the protein and glycoprotein components specifically. Dawes[138]

(1966) has presented a paper of immense practical significance to workers involved in the electrophoresis of salivary proteins in which he dramatically demonstrated the TCA solubility of many of the proteins and glycoproteins in saliva. This was disturbingly re-emphasized a few years later.[153] The ramifications of this work to the fixation and staining of salivary material of any source is only just begun to be appreciated,[153] (to be discussed later in the thesis) (see Appendix A ref. 153).

The use of stains of different specificities to demonstrate the chemical nature of the various components in salivary materials after PAGE is a technique which has been largely neglected by saliva workers. Even when these techniques were used[132,134] their applications have led to questionable results when re-examined by others later.[153] For example, the excellent work of Steiner and Keller[134] or even that of Caldwell and Pigman[132] is deficient. Their method of PAS staining[132] has now been shown to be of limited sensitivity and probably involved a significant loss of protein from the gel.[153] It was, however, the best method available at the time. (For discussion see appendix A, ref. 153).

There are few publications regarding the electrophoresis of rat saliva. The early papers of Sweeney et al.[148] and of Hall and Schneyer[149,150] using electrophoresis on paper strips to separate rat parotid and combined submandibular-sublingual saliva are of historical interest only. More recently, Rabinovitch et al.[149] (1969) and Abe et al.[139] (1970)

(the latter unfortunately being in Japanese) have presented PAGE analyses of rat whole, parotid and combined submandibular-sublingual salivas. Although the quality of the work, especially the latter paper, is excellent, the use of stains specific for proteins only is disappointing (this point will be discussed at length later in this thesis). Furthermore, since neither of these workers ligated the sublingual glands, the submandibular saliva was heavily contaminated with sublingual secretions. Therefore, the significance of these works to the present one is at best questionable and probably irrelevant. Abe et al.[139] did however report a very interesting observation. They found that some bands in rat serum electrophoresed with mobilities not unlike those of the secretions of the salivary glands examined. The significance of this observation for saliva remains obscure. To the best of our knowledge no PAGE examinations of the contents of rat glands have been done to date.

Electrophoretic analysis of the HSS in a number of gel systems with the three stains described in the previous section indicated that the HSS was, in fact, a highly complicated glycoprotein mixture that was predominantly anionic in nature. The results of these studies are presented in Figs. 7 through 11, inclusive. Electrophoresis of 200 or 300 µg (Lowry) protein in system A (anionic 7.5%) indicated approximately 10 protein bands (Fig. 7(a)) of a wide variety of molecular weight (as evidenced by the distribution of the bands

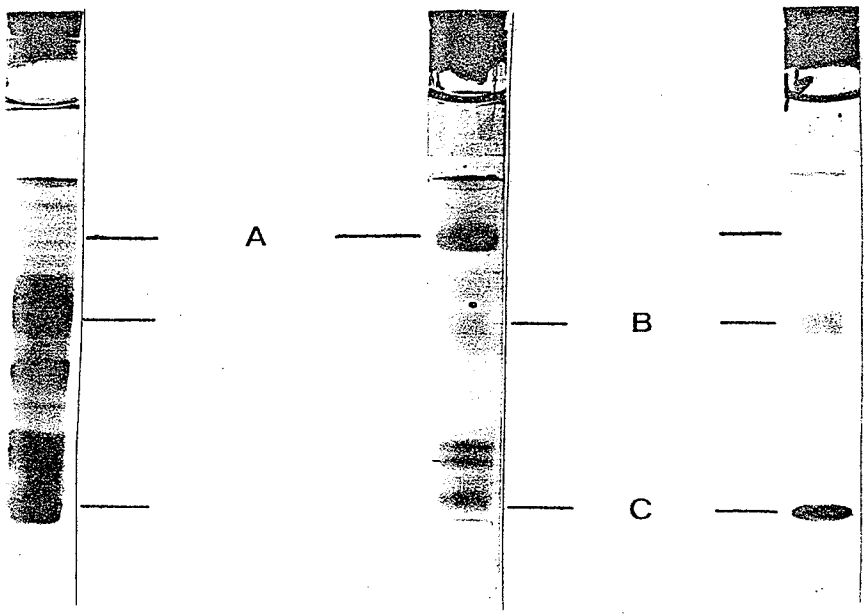
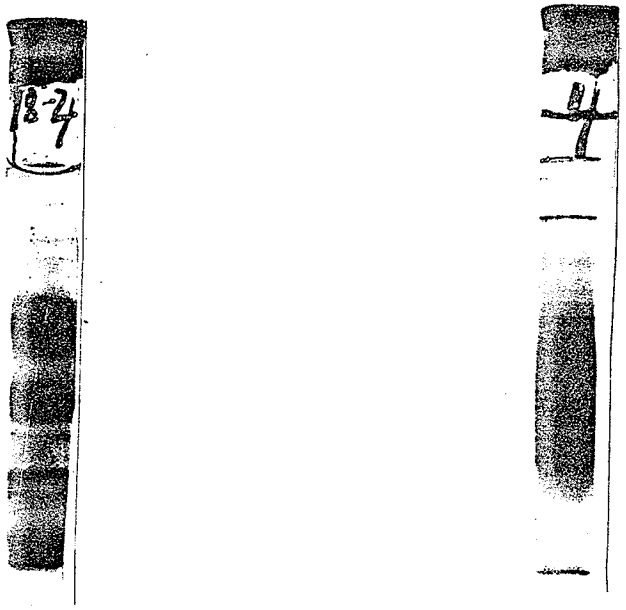


Fig. 7. PAGE separation (System A) of 300 µg HSS protein. Gels are stained with (a) Amido Shwarz, (b) PAS, and (c) Alcian blue.

throughout the body of the separating gel. Surprisingly, PAS staining (Fig. 7(b)) indicated that almost all of the proteins of the HSS that ran in this system were glycoprotein in nature (or ran with glycoproteins). Furthermore, the PAS gels indicated an intensely staining PAS positive band of low mobility which did not show any appreciable representation on the protein stained duplicate gel. (Area A). Significant Alcian blue (AB) staining (Fig. 7(c)) appeared for two bands (Areas B & C) with faint representation appearing for two or three others.

Since a number of the bands in the Amido Schwarz (AS) stained gel (Fig. 7(a)) showed the characteristic fuzziness indicative of aggregation (Area B especially),a urea gel system (system C) was devised to decrease aggregation and increase resolution.[155]

Anionic electrophoresis in the presence of urea (Fig. 8,9(a)) increased the band number to 14 and 15 (concentration dependent) and markedly increased individual band resolution as evidenced by increased band number and increased band compactness (Fig. 8). For the same reasons we also concluded there was an increase in band homogeniety. PAS staining was qualitatively the same as before (Fig. 9(b)) with the expected concomitant increase in band number and resolution. The PAS staining obviously indicates that almost all the proteins are glycoproteins. The band previously (Fig. 7(b)) found in Area A was also present, and as before, there was



(a) (b)

Fig. 8. Comparison of PAGE separation of 300 µg HSS protein on gels of (a) System A and (b) System C. The stain is Amido Shwarz.

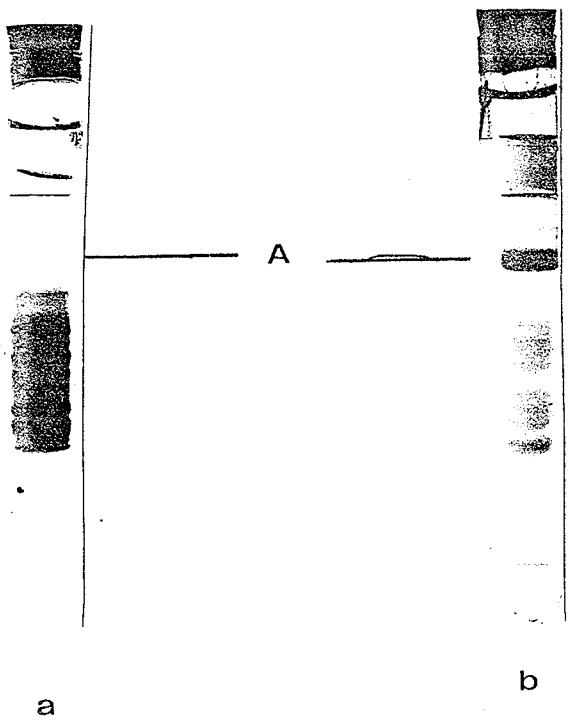


Fig. 9. PAGE separation (System C) of 300 µg HSS protein. Gels were stained with (a) Amido Shwarz and (b) PAS.

extremely poor protein representation for this band (Fig. 9(a) and (b)). This property of intense PAS staining and poor protein staining as well as its position in the gel region (indicated[97] to contain molecules of approximately 10^6 m.w.) led us to believe that this was the rat submandibular gland mucin. This hypothesis was supported by two papers published shortly thereafter by Holden et al.,[156,157] who demonstrated similar staining characteristics for purified salivary mucins of other species. We have termed this phenomenon the "inverse staining relationship[158]" for the following reasons. Since the salivary mucins of other sources have had protein contents ranging from 30-60% depending on the source, species and worker; these molecules should give a protein stain with Amido Shwarz of similar staining intensity to the PAS stain, but they do not.[156-158] Hence, some effect of the carbohydrate, most likely steric hindrance, is interfering with the access to or the free interaction of Amido Schwarz with the protein core. It should be noted that this effect is not merely specific for Amido Shwarz, but is maintained for coamassie blue and fast green also (personal communication by E.J.Zebrowski) We have subsequently identified this phenomenon as the inverse staining relationship to denote the significant and unexpected reduction in staining intensity presumably caused by carbohydrate.

Unfortunately, due to the properties of the urea - polyacrylamide gel complex, irreversible uptake of the Alcian

blue stain by the gel occurred. This produced a sufficiently high background stain, such that the bands could not be visualized in any way. Data, therefore, on Alcian blue staining for the urea gels could not be obtained.

Despite this problem, the urea gels were the gels of choice for resolving the different proteins and glycoproteins, with no area showing the patterns characteristic of aggregation of proteins.

In order to check the completeness of electrophoresis of HSS protein, it was thought that an increase in pore size[97] would allow larger molecules (m.w. in excess of 1.5×10^6) to enter the separating gel. New bands would then imply incomplete electrophoresis. To this end, large pore (3.75% Acrylamide) gels were prepared in urea solution (system D). According to the theory of Ornstein,[97] this should double the pore size and increase the size of the molecule that can enter the separating gel up to about 2.5×10^6 M.W. When HSS protein was run under these conditions (Fig. 10(a,b)) no new bands of any significant quantity (not detectible by visual inspection, therefore less than 0.5 µg protein) appeared in either the AS or PAS stained gels. As others[132] have reported, the 3.75% gels did not give the same quality of separation as 7.0 or 7.5% gels for salivary protein (which is not surprising since this is exactly what the theory predicts[97]). The AS and PAS relationships were largely unchanged by the change in pore size, i.e. almost all the bands staining for protein also

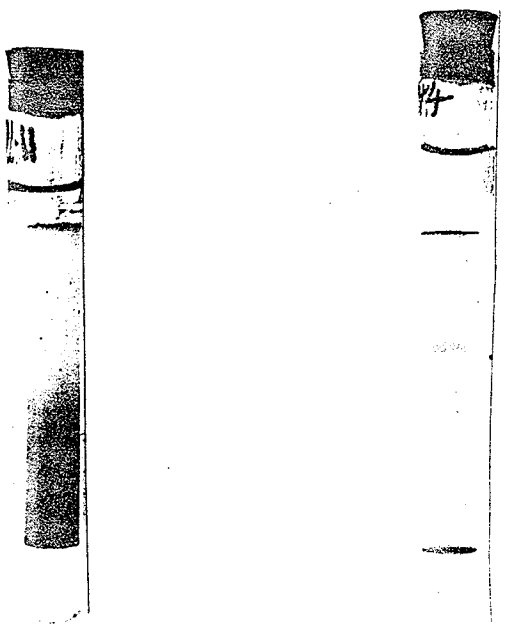


Fig. 10. PAGE separation (System D) of 200 µg HSS protein. Gels were stained (a) AS and (b) PAS.

stained for carbohydrate, and the inverse staining relationship was maintained for the mucin.

Previously, some salivas have been shown to contain many positively charged (cationic) components[147]. Cationic gels were therefore prepared (System E), and the HSS electrophoresed and stained with the AS, PAS and AB stains. The results are shown in Fig. 11. AS staining indicated (Fig. 11(a)) that there are only one or two proteins of cationic nature of any significant quantity. Both bands showed weak PAS positivity (Fig. 11(b)), indicating that they were glycoproteins. As expected, no detectible uptake of Alcian blue was observed. Previously, Steiner and Keller,[134] has reported Alcian blue positivity for cationic components of human parotid saliva. These workers have accepted the claims asserted by Pearse[159] that Alcian blue in 3% Acetic acid stains acidic components (a claim similarly accepted by Caldwell and Pigman[132]). However, it would seem that there is little reason to believe acidic components would appear anywhere in cationic gels, having migrated in the other direction. In a paper fraught with technical problems (by their own admission), the specificity of their staining technique is open to considerable discussion.

In summary, then, the preliminary examination by PAGE indicated that the HSS supernatant contained approximately 17 glycoproteins of which at least two were basic in nature. Tentatively, the alcianophilia of at least 2 and perhaps 4 of

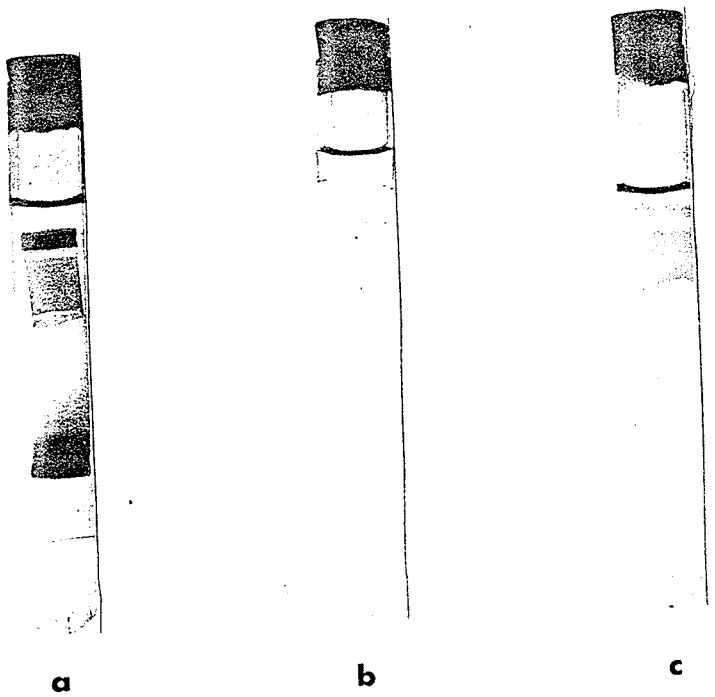


Fig. 11. PAGE separation (System E) of 200 µg HSS protein. Gels were stained with (a) Amido Shwarz, (b) PAS and (c) Alcian blue.

the anionic components represents some acid carbohydrate residues (most likely sialic acids), since the Alcian blue stain in Area B and approximately half of the stain in Area C is sensitive to loss of bound sialic acid from the HSS material (see Appendix 2). The HSS supernatant also appears to contain the rat submandibular mucin.

The application of chromatography on Sephadex to the separation of proteins is standard laboratory procedure. Use of this technique for the examination or preparation of salivary proteins and glycoproteins is also well established[38,131,160,161] and has been recommended as the standard method for the preparation of salivary mucins.[164]

Preliminary experiments with columns of Sephadex G-50 and G-100 indicated that most of the proteins within the gland were of sufficiently high molecular weight that they appeared in the void volume as a single peak or were poorly resolved from void volume material.

Chromatography of the HSS on Sephadex G-200 (column 1.5 x 25 cm) indicated several molecular weight fractions within the HSS. The results of a typical experiment can be seen in Fig. 12. Four (4) protein peaks were observed and these were separated into five (5) fractions (f1-f5) for electrophoresis. The f1 fraction was particularly interesting, having come off with the void volume (exclusion limit for Sephadex G-200 approximately 800,000 m.w.) and showed significant sialic acid. In addition, all the other protein

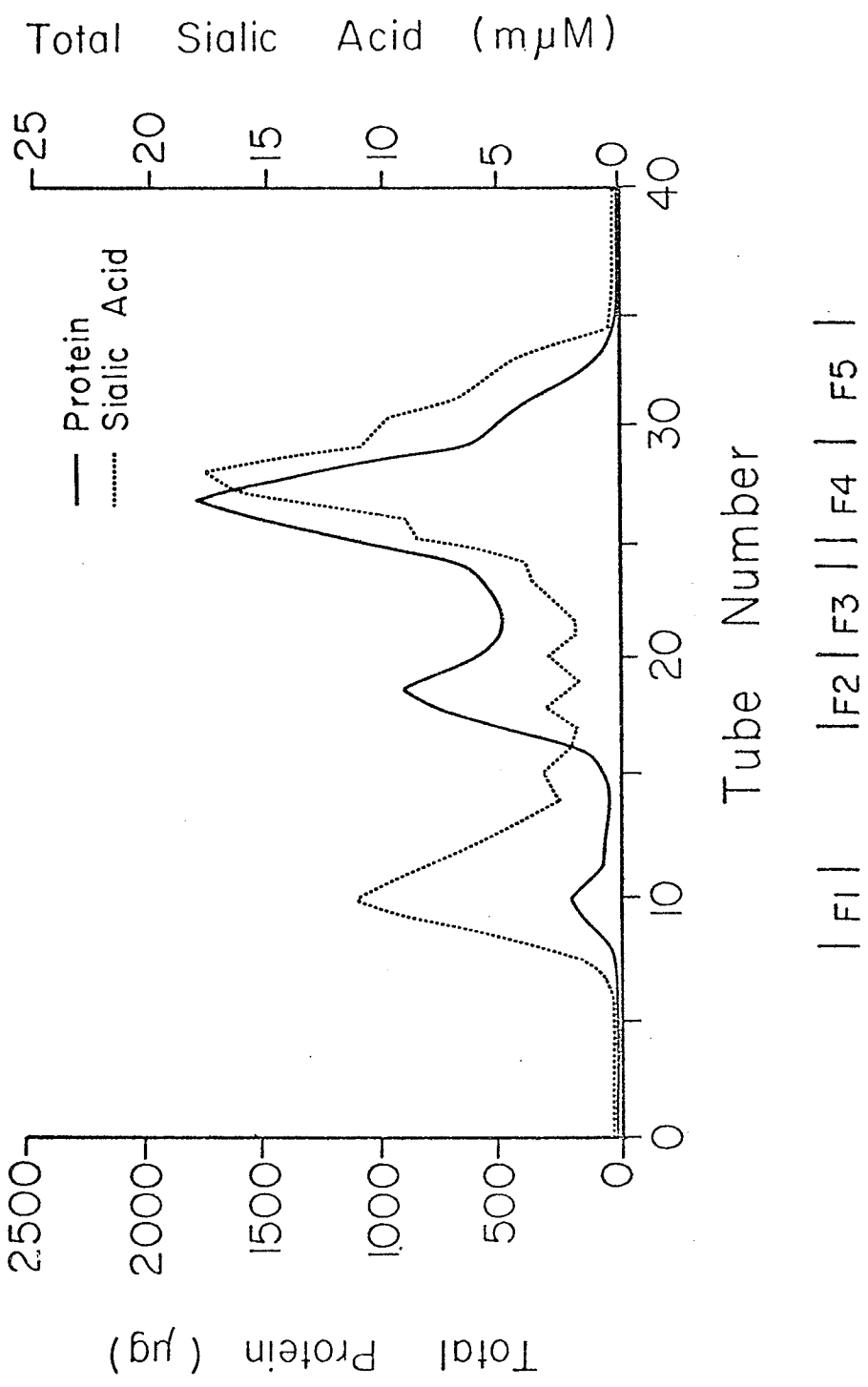


Fig. 12. Typical elution profile obtained from the chromatography of the HSS on columns of Sephadex G-200 superfine (1.5 x 25 cm). Flow rates were 1.0 ml/hour.

peaks showed the presence of variable quantities of sialic acid. The peaks contained within fractions f4 and f5 contained the largest part of the sialic acid of the HSS. Furthermore, it appeared that most of the sialic acid chromatographed with the very low molecular weight components. Presumably then, this region contained many small glycoproteins, glycopeptides, as well as the free sialic acid. When the contents of f5 was analyzed by electrophoresis in System A, (Fig. 13(e)) it showed little stainable protein, and those bands present were located in the regions of the separating gel thought to contain low molecular weight components.[97] It might also be noted that in the gel of the HSS (Fig. 7(c)), this same area shows a strong alcianophilia (Area C). Unfortunately, due to the small amounts of material obtained from these columns, the PAS and AB stains could not be done and only Amido Shwarz stains appear.

The appearance of f1 as a distinct peak of such high molecular weight with no demonstrable Amido Shwarz staining suggested that this fraction contained the rat submandibular gland mucin.

The absence of a band provides an excellent demonstration of the seriousness to analytical electrophoresis of phenomena such as the inverse staining relationship. The proteins fractionated on the column in much the same sequence as they fractionated in the acrylamide gel, which indicated that comparison of molucular weights could be made on the basis of the position in the gel for the proteins of the HSS.

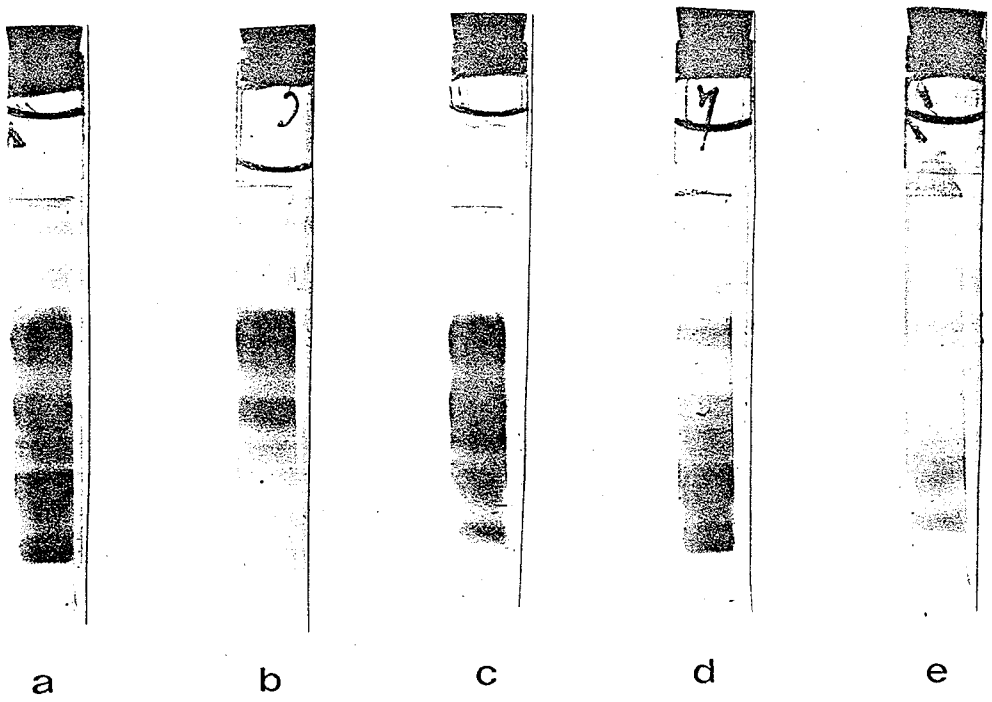


Fig. 13. PAGE separation (System A) of HSS and the following from the 1.5 x 25 cm column.
(a) HSS, (b) f2, (c) f3, (d) f4, (e) f5 fractions. Fraction f1 consistantly showed no bands therefore no photographs were made. Gels were stained with Amido Shwarz.

On the basis of the high quality of the chromatographic separations on such small columns, columns of longer bed length (1.5 x 75 cm) were prepared with Sephadex G-200 SF and the experiments repeated. The results of a typical experiment are presented in Fig. 14 and 15. The chromatographic results are qualitatively identical to the previous experiments. The elution profiles demonstrate four clearly resolved peaks and two partly resolved peaks. These were divided up into six fractions (f1-f6) for electrophoresis in urea gels of System C. The mucin fraction (f1) showed the same sialic acid peak and ran with the void volume as before. The results of the electrophoresis of the f1 fraction can be seen in Fig. 15(a,b). This fraction appears highly homogeneous showing marked PAS staining (Fig. 14(b)), but no significant staining with Amido Schwarz. The banding patterns for the fractions f2-f6 indicate, as before, that the proteins of the HSS were electrophoresing predominantly according to their molecular weight characteristics. The separation of f6 from f5 indicates the majority of the sialic acid is contained with the smaller glycoproteins (f5) and not in the glycopeptide- or free sialic acid fraction (f6) as was suggested, to some extent, by the smaller columns. The longer columns separated the sialic acid containing smaller molecules into two distinct peaks, the one in the f6 fraction probably representing the glycopeptides and free sialic acid.

These two chromatographic separations clearly indicate

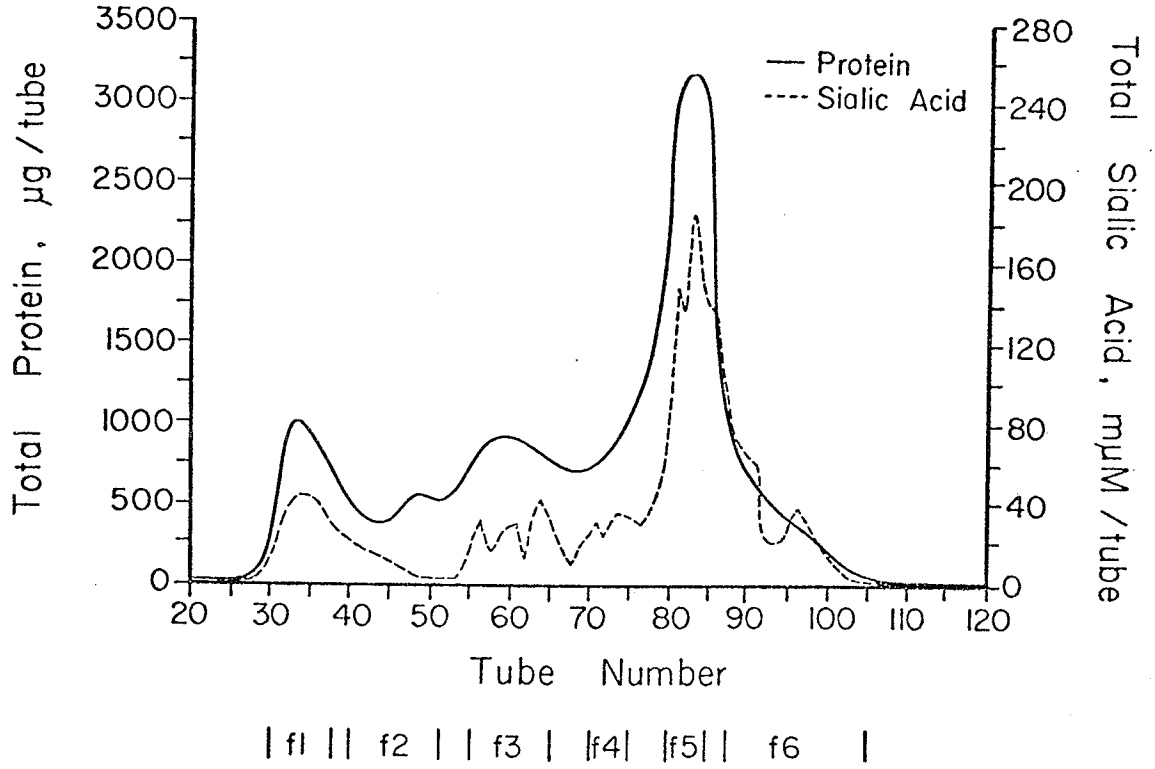


Fig. 14. Typical elution profile obtained from chromatography of HSS protein on columns of Sephadex G-200 superfine (1.5 x 75 cm). Flow rates were 1.0 to 1.5 mls/hour.

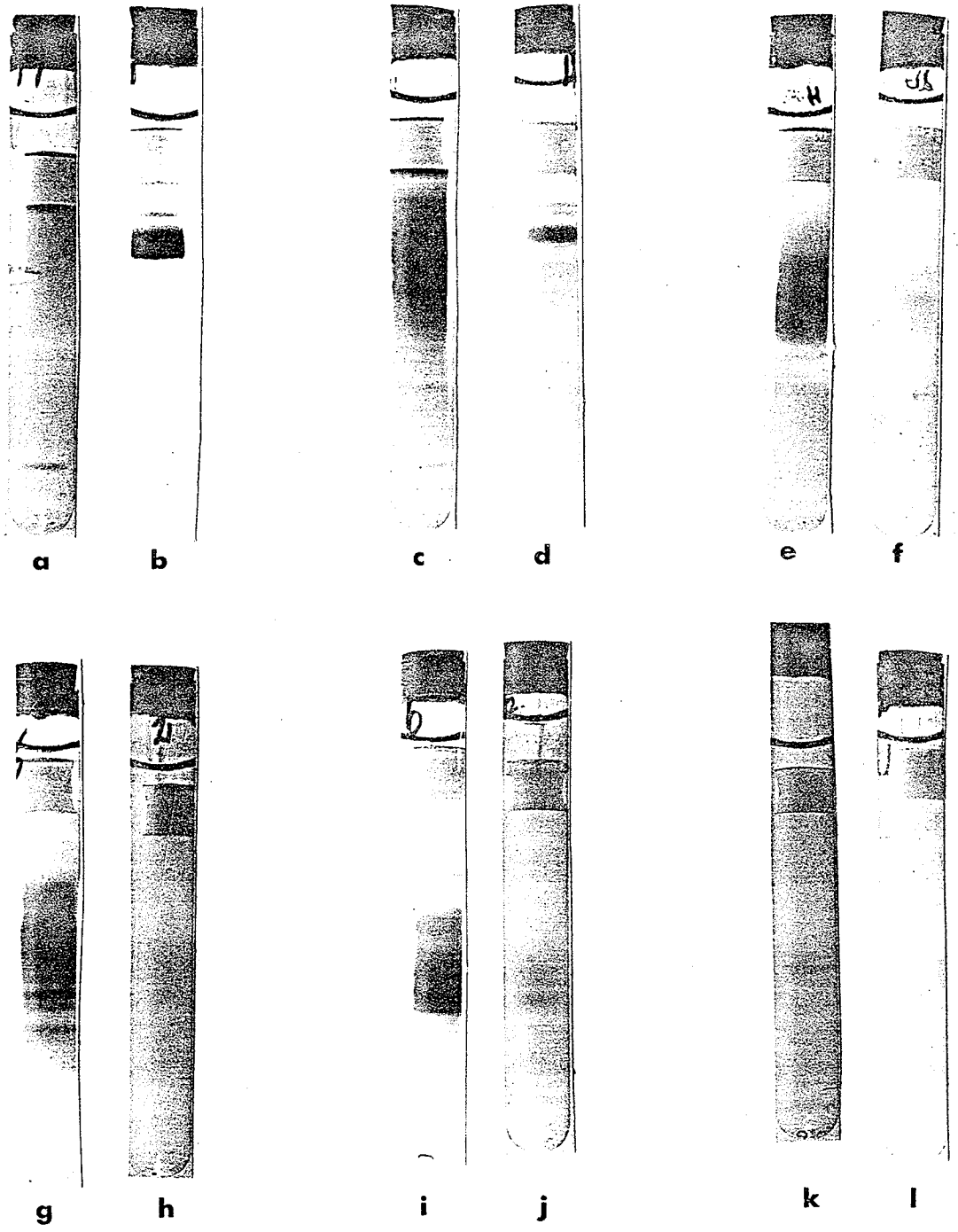


Fig. 15. PAGE separation (System C) of fractions from 1.5 x 75 cm column.
(a,b) f1; (c,d) f2; (e,f,) f3; (g,h) f4; (i,j) f5; (k,l) f6. Gels in a,c,e,g,i and k were stained with Amido Shwarz. Gels in b,d,f,h,j and l were stained with PAS.

that the HSS contains a large number of glycoproteins of wide range in molecular weights many of which contain sialic acid (at least 6). The RSM (fl) appeared to be relatively homogeneous and was located in the first peak which came off with the void volume. In addition, (Fig. 15 (a) and (b)) demonstrate the inverse staining relationship. All of the bands of the HSS (Figs. 7,8 and 15) appear in the column elution profiles in the same sequence in which they are electrophoresed, with the exception of the two bands in Area D on the urea gels which ran slower (lower negative charge) than their molecular weight would suggest. This was not observed for the non-urea gels (Fig. 13(g,i,k)). Recoveries from all columns were essentially 100% for both protein and sialic acid.

The use of Sephadex chromatography to prepare and purify submaxillary mucins is almost standard procedure.[160,162,164] However, a number of other methods are used almost as frequently and often in conjunction with column chromatography. These mostly involve precipitation from an aqueous extract of some sort with a variety of agents,[40,160,163,164,173-176] most often, but not always, followed by purification by some other technique, for e.g. Sephadex,[160,162,166] ion-exchange chromatography,[166] or zonal electrophoresis.[169] The use of columns of Sephadex G-200 immediately after the preparation of the whole aqueous extract for the isolation of the ovine submaxillary mucin (OSM) has not been recommended by Gottschalk et al.[177] because of the small amounts of material

that can be prepared by this method, however, they did point out that highly purified preparations could be achieved by this procedure. Lombart and Winzler,[166] using columns of very large diameter (9.7 cm) on the whole aqueous extract of dog glands have recently overcome this problem quite effectively. Fractionation of the HSS on Sephadex columns (2.5 x 90 cm) for the preparation of mucin material from the rat submandibular gland proved almost ideal; since (1) the small size of the glands (200-350 mg); (2) the recovery of mucin from the glands (approximately 1% by wet weight); and (3) the limitations on the numbers of animals that could be handled at a time (approximately 15) placed the amounts of HSS material prepared per day within the column capabilities available.

A typical elution profile for the preparative column (Sephadex G-200) is presented in Fig. 16. Preparation of the mucin was done with the coarse grade of Sephadex instead of the super-fine grade previously used in order to decrease the elution time as much as possible. As a consequence, peak resolution for the other fractions was markedly reduced. Similarly, due to the greater dilution, the smaller sialic acid peaks could not be assayed accurately. Since we were only interested in the mucin containing peak, the quality of the resolution of the other peaks was considered unimportant. As before, the mucin fraction (14Sf1, Fig. 16) demonstrated a significant sialic acid content which when in this position on the elution profile is now considered indicative of the presence of the mucin.[166,169,170]

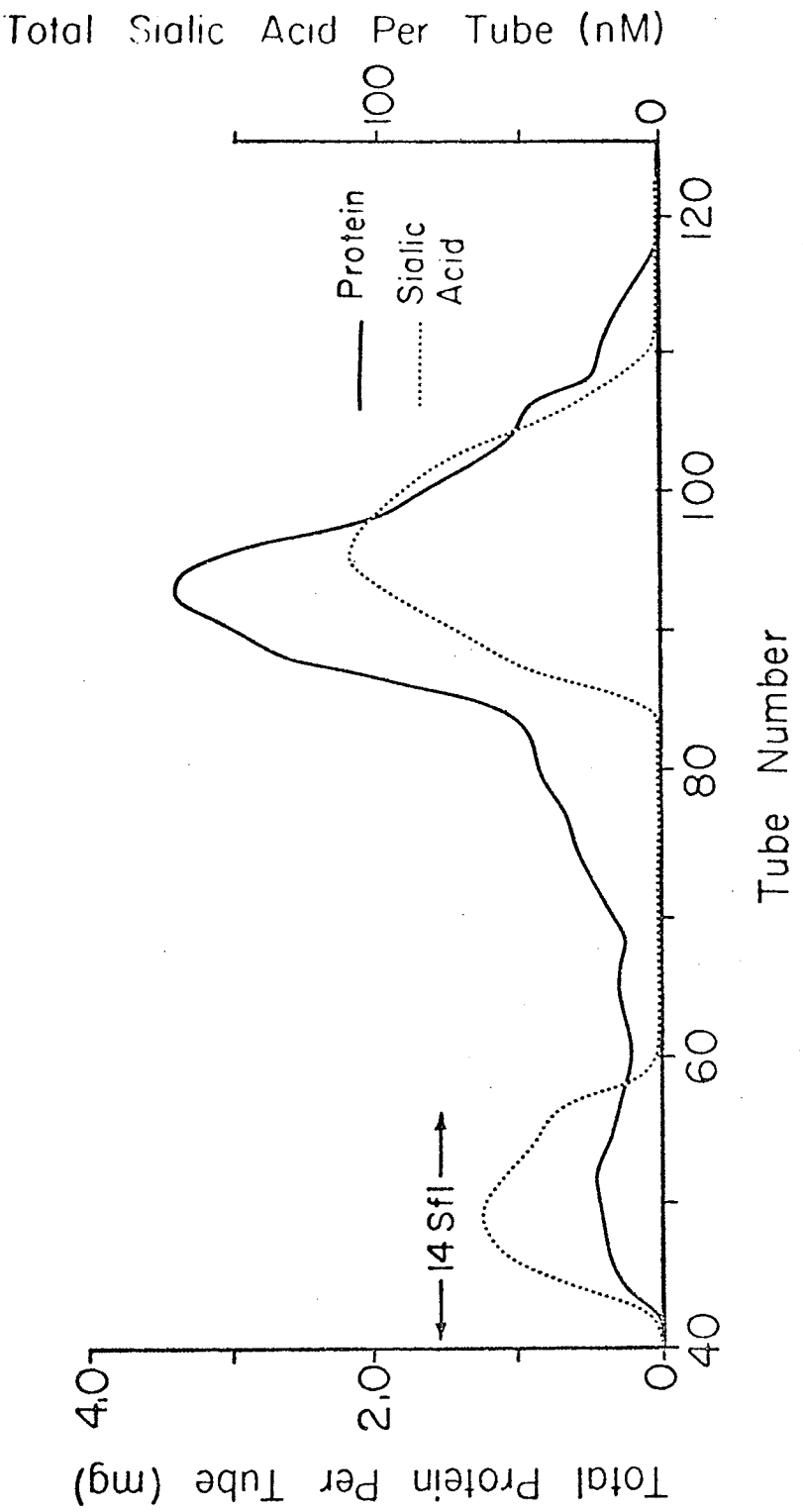


Fig. 16. Typical elution profile obtained from chromatography of HSS on columns of Sephadex G-200 coarse (2.5 x 90 cm). Flow rates were 4-5 mls/hour.

Amido Shwarz staining is considered to be an excellent irreversible fixative for a protein.[93,97] However, evidence of resistance to stable fixation by the mucin can be seen in Figs. 17(b) and 18(b). When the mucin containing gel was stained with AS, electrophoretically destained, and then subjected to the PAS procedure (Fig. 18(c)) it can be seen that (i) the protein (mucin) moved down the body of the gel, and (ii) due to a "smearing" effect became diluted, i.e. the band became broader and hence less concentrated, thereby significantly reducing the AS and PAS staining intensity. In addition to the reduction in staining caused by the "inverse staining relationship", this irreversible fixation would further reduce whatever AS stain did appear. The combination of these two problems appeared to effectively prevent AS staining for the mucin. These two effects appear to explain the problems others[156,157] have described in their attempt to detect mucins after electrophoresis with protein stains. To extrapolate farther, it does not seem unreasonable, especially in the light of previous work[153] on these types of glycoproteins, that the very broad, diffuse bands observed after electrophoresis by Holden et al.[156,157] with their PAS staining procedure for mucins from other sources might be the result of diffusion of the mucins after fixation during the very long and complicated procedures necessary for the PAS stain. We put great effort into minimizing the time required for washing, oxidation and staining, in our methods;[153] and yet,

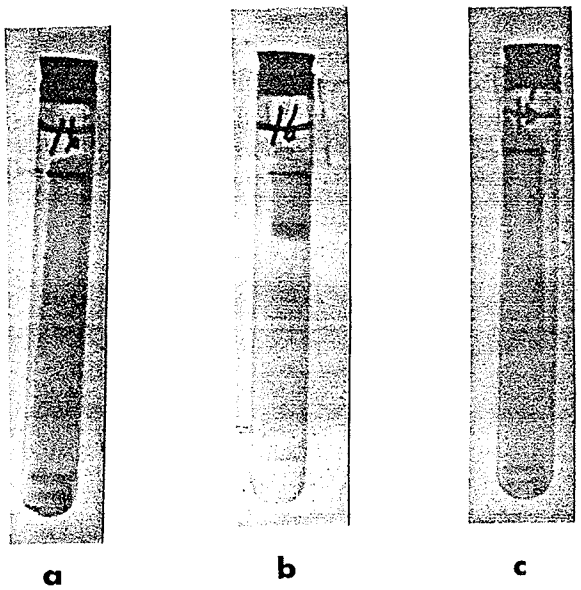


Fig. 17. PAGE separation (System A) of 100 µg 14sf1 protein. Gels were stained with (a) Amido Shwarz, (b) PAS and (c) Amido Shwarz followed by PAS stain.

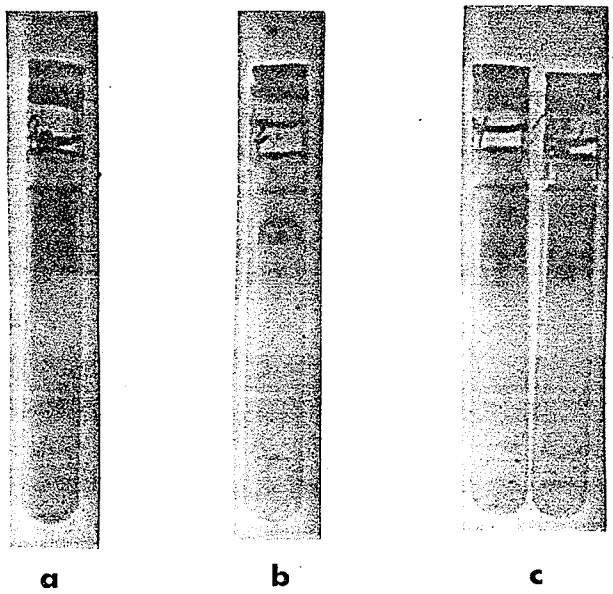


Fig. 18. PAGE separation (System C) of 100 µg 14sfl protein. Gels were stained with (a) Amido Shwarz, (b) PAS and (c) Amido Shwarz followed by PAS stain.

on the basis of the appearance of the bands in Figs. 17(c) and Fig. 18(c), it would seem that some diffusion and loss of material probably occurred. It would also appear from a comparison of Figs. 17 and 18 that electrophoresis in the presence of urea, in addition to providing increased resolution, might also increase the "fixability" of the mucin.

That the mucin preparation was reasonably pure was evidenced by the gel photographs (Figs. 17 and 18). Only one other band of any detectable quantity was present. Since salivary mucins have been shown to demonstrate molecular weights that are concentration dependent,[160,166] it would seem possible to conclude that this slower migrating band might represent an aggregate of the mucin.[160,166] This characteristic of concentration dependent aggregation makes molecular weight and homogeneity estimations of salivary mucins by most techniques quite suspect. In addition, the high carbohydrate content of mucins decreases the specific gravity proportional to concentration. The estimation from the Sephadex-column experiments of about 800,000 would seem to represent a reasonable lower limit for the molecular weight of RSM. Since the molecule enters the 7.0% acrylamide gels, its molecular weight must be less than 1.3×10^6, the approximate exclusion limit for these gels. Therefore, the molecular weight of the mucin is probably somewhere between 8×10^5 and 1.3×10^6, (note: the carbohydrate content would lower this estimate, since these estimates are made on the basis of the specific gravity of protein and therefore, a value closer to the lower limit would not be unexpected).

Due to difficulties inherent in the electrophoresis of this molecule, an accurate determination of the degree of purity or heterogeneity cannot be given. The small quantities of the molecule that can be prepared do not lend themselves to more traditional and extensive measures of homogeneity.

The extent of analysis of a glycoprotein, or of any compound for that matter, is defined and limited by the techniques available at the time. Since the mucin is a glycoprotein, the analysis of its structure was directed to determine (i) the amino acid composition and (ii) the carbohydrate composition. No attempt was made to determine the sequence of either the peptide core or the carbohydrate prosthetic groups.

The amino acid analyses were performed by Dr. F. C. Stevens, (Department of Biochemistry, Medical College, University of Manitoba), as described in the methods section. The results are presented in Table 3. The mucin was approximately 48-50% protein by weight. Tryptophan and cysteine values are not presented since they were not determined by this method. The amino acid composition was rather atypical with respect to mucins of other sources for which amino acid compositions have been determined. The sum of the serine and threonine values (15%) was decidedly low, generally being in the region of 20-30% of the total amino acids. The serine and threonine to glutamate ratio was, however, normal. The serine, threonine glycine, proline, alanine and glutamate content was also lower than normal (41% instead of 70%). In

Table 3. Amino acid analysis : Rat submandibular mucin (RSM)

	gm/100 gm dry weight	mM/100 gm	mole ratio*
Lysine	3.7	23.31	3.2
Histidine	1.2	7.71	1.0
Arginine	3.2	18.36	2.5
Aspartate	4.6	31.79	4.3
Threonine	3.1	26.02	3.5
Serine	2.8	26.65	3.6
Glutamate	5.9	40.40	5.5
Proline	2.7	23.45	3.2
Glycine	2.5	33.30	4.5
Alanine	2.7	30.31	4.1
Valine	3.1	26.46	3.6
Methionine	1.1	7.37	1.0
Isoleucine	2.7	20.58	2.3
Leucine	5.0	38.12	5.2
Tyrosine	2.3	12.69	1.7
Phenylalanine	2.7	16.34	2.2

Total % amino acids by weight = 48.91%

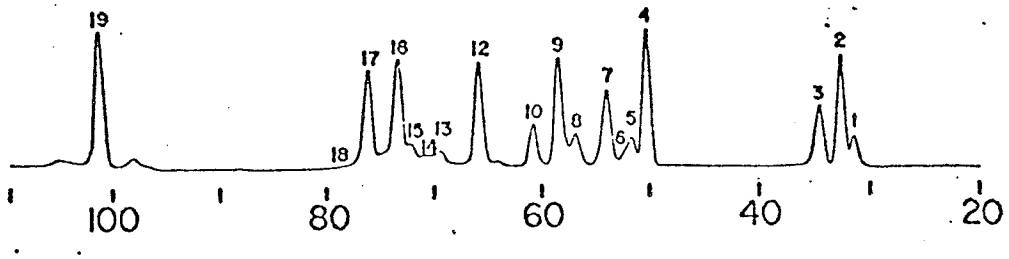
* mole ratio = $\frac{\text{mM/100 gm amino acid}}{\text{mM/100 gm methionine}}$

addition, the amount of valine, isoleucine, leucine and lysine was considerably higher (23%) than is usually reported. The linkage sugars were possibly serine and threonine as with other salivary mucins although direct evidence for such was not obtained. There was a good correlation between possible linkage sites (sum of serine and threonine was 52 mM/100 gms) and the number of prosthetic groups (determined by the concentration of the sugar in lowest amount (gluNAc) assuming 40 to 50% glycosylation[58,59] as has been shown for other mucins.

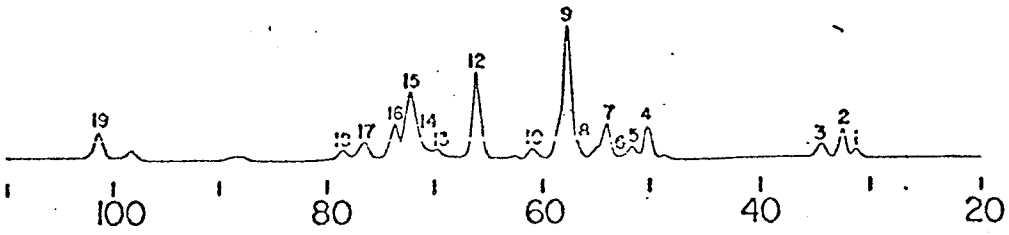
Gas chromatography was chosen as the means of analysis of the carbohydrate portion for a number of reasons, not the least of which was the ability to separate, identify and estimate in a single procedure all of the monosaccharides normally expected to occur in glycoproteins. To this end, all of the standard monosaccharides were prepared for gas chromatography by the methanolysis procedure of Chambers and Clamp.[114,178-180] They were then chromatographed individually, in groups (e.g. all acetamido-hexoses, all neutral sugars, etc.) and all together (Fig. 19(a)) at various concentrations to completely standardize the procedure and identify clearly all possible peaks. Our chromatograms compared very favorably to those reported by Chambers and Clamp[114,178] in spite of a much shorter column and poorer temperature programming. The typical chromatogram obtained from the hydrolyzate of the mucin can be seen in Fig. 19(b) and the quantitation of the individual sugars in Table 4.

Methanolysis produces several peaks (from 2 to 4) of varying areas but of fixed ratios for each sugar (see ref. 114, 178). The identification of a particular sugar by the retention time alone in biological prepparations is particularly unwise;[114] contamination being a problem almost impossible to eliminate.[178] Identification therefore, by multiple peak position and ratio of areas increases the confidence in the results. Furthermore, if the major peak is obscured or very asymetrical because of contamination, then quantitation can still be done on one of the minor peaks with confidence. Since biological preparations contain significant amounts of amino acids, organic buffers and lipids that can chromatograph with similar (in some cases almost identical) retention times[178] as the monosaccharides found in glycoproteins, the necessity of more complex identification parameters than a single retention time is very important. (For an extensive review on this subject see ref. 178). For these reasons methanolysis was chosen over the other methods available in the literature.[178,181-183]

The complexity of sugars identified in the preparation was surprising (Fig. 18, Table 4). Almost all the standard sugars found in glycoproteins were present in significant quantities (see Fig. 19, Table 4). Fucose and NANA were found in equimolar quantities and represented 5% and 12% of the total molecular weight respectively. Both of the usual acetamidohexoses were present with molar ratios; galNAc to gluNAc of 3:1, and constituted 14.6% and 4.5% of the total molecular weight respectively.



(a)



(b)

Fig. 19(a). Chromatogram of standard methyl glycosides prepared as described. Peaks were identified as below.

(b). Chromatogram obtained from the major rat submandibular gland glycoprotein (submandibular gland mucin). Peaks were identified as fucose, 1,2,3; mannose, 4,6; galactose, 5,7,8; glucose, 9,10; mannitol, 12; N-acetylglucosamine, 13,15,17,18; N-acetylgalactosamine 14,16; N-acetylneuraminic acid, 19.

Table 4. Carbohydrate analysis : Rat submandibular mucin (RSM

	gm/100 gm	moles/100 gm	mole ratio*	
N-Acetylneuraminic Acid	12.14**	0.040	2	(2.0)
N-Acetylglucosamine	4.52	0.020	1	
N-Acetylgalactosamine	14.66	0.066	3	(3.3)
Glucose	3.71	0.021	1	(1.05)
Galactose	6.76	0.038	2	(1.9)
Mannose	3.86	0.022	1	(1.1)
Fucose	5.44	0.034	2	(1.7)

Total % CBQ by weight = 51.1%

* Ratio = $\frac{\text{total moles/100 gm}}{\text{moles/100 gm NAc Glucosamine}}$

** Data representative of 5 determinations.
All determinations were within 5% of each other.

Two of the neutral sugars (galactose and mannose) were present with molar ratios 2:1. Both NANA and fucose were essentially equimolar to galactose. By subtraction, the mucin appeared to be 51% carbohydrate by weight. On the assumption that the glucose in the preparation is a part of the mucin, the glucose content would be about 3.7% of the weight. However, this may not be a reasonable assumption. Close inspection of peak 9 and 10 (Fig. 19(a)) clearly indicates that these two peaks have a ratio and retention time exactly characteristic of glucose.[114] In the mucin analysis, however, (Fig. 19(b)) it appears that although the retention time is almost exactly identical to glucose, the peak proportions were wrong. Peak 9 (Fig. 19(b)) then, appeared to be a composite of three peaks. Peak 8 (a minor peak of galactose) appeared as a shoulder. Peak 9 (major peak of glucose) appeared as a shoulder on the other side. A large peak due to an unidentified contaminant appeared almost exactly over peak 9. Originally it was thought tha this peak may be due to glucose.[170] However, it is most definitely, largely due to a contaminant (most probably a fatty acid.[178]) The identification of a small amount of glucose in the preparation is positive, however, no attempt was made to quantitate it directly. Assuming 100% recovery, a value for glucose of 3.71 gm/100 gm of mucin was arrived at. Often,[178] when glycoproteins are prepared by chromatography on Sephadex or cellulose, glucose appears as a contaminant. Glucose oxidase assays for free glucose in the mucin preparation were negative. Analyses for dextrans were not done. Most probably

Table 5. CARBOHYDRATE COMPOSITION OF SELECTED SALIVARY MUCINS (mMoles/100 gms)

	OSM[1]	OSM (major)[2]	OSM (minor)[2]	BSM (major)[2]	BSM (minor)[2]	PSMI[3]	PSM II[3]	PSMI (major)[4]	PSM (minor)[4]	CSM[5]	RSM[6]
N-Acetyl Galactosamine	99	103	78	38		81	111	117	104	69	66
N-Acetyl Glucosamine				76	82	0.9	1.6			57	20
N-Acetyl Neuraminic Acid	98	99	59							28	40
N-Glycoyl-Neuraminic Acid						38	48	50	51		
N-O-DiAcetyl Neuraminic Acid				97	56						
Galactose		2.4	17.0	8	28	52	109	58	61	81	38
Mannose						2.2					22
Glucose											21
Fucose		4.1	5.0	7.5	5.4	.38	31	45	46	57	34
TOTAL % CARBOHYDRATE	54.0	63.0	42.2		47.7	45.5	64.1	59.2	57.3	61.3	51.1
TOTAL % PROTEIN	46.0	37.0	57.8		52.3	55.4	40.0	35.8	35.1	36.5	48.9

1. Gottschalk, A., Schauer, H. and Uhlenbruck, G. (1971) [162]
2. Tettamanti, G., Pigman, W. (1968) [163]
3. Katzman, R.L and Eylar, E.H. (1966)[164]
4. de Salequi, M. and Plonska, H. (1969)[165]
5. Lombart, C. and Winzler, R.J. (1972)[166]
6. Kapitany, R.A. and Zebrowski, E.J. (1973)[170]

OSM - Ovine Submaxillary Mucin
BSM - Bovine " "
PSM - Porcine " "
CSM - Canine " "
RSM - Rat Submandibular "

the glucose appeared as a contaminant in the form of dextrans released from the column. It can be seen from Table 5, that, for the mucins represented there, glucose has not been shown to occur in salivary mucins to date (with one exception, Lombart and Winzler[166] showed glucose to be present in their CSM preparation and were faced with the same problems of interpretation as us). The presence of mannose was unusual although not unreported (see Table 5). The simultaneous presence of both sialic acid and fucose is fairly common (Table 5) and indicated a branched oligosaccharide chain. It does not seem unlikely that at least one of the galNAc residues is also terminal on one of the branches of the prosthetic group. The high recovery (97-100%) suggest that the carbohydrate analysis was representative of the composition of the glycoprotein.

On the basis of these results, a tentative but reasonable arrangement of the sugars has been presented (Fig. 20). GalNAc is the linkage sugar and is O-glycosidically linked to serine and threonine. There is an internal region of neutral hexoses and acetamidohexoses. (Mannose to galactose to gluNAc to galNAc 1:2:1:1). The sugars in this region are probably arranged in at least two (possibly three) branches with mannose or galactose most probably serving as the branch point. NANA, fucose and probably one galNAc terminate the chains. If glucose is present in the mucin then it would occur in the internal region.

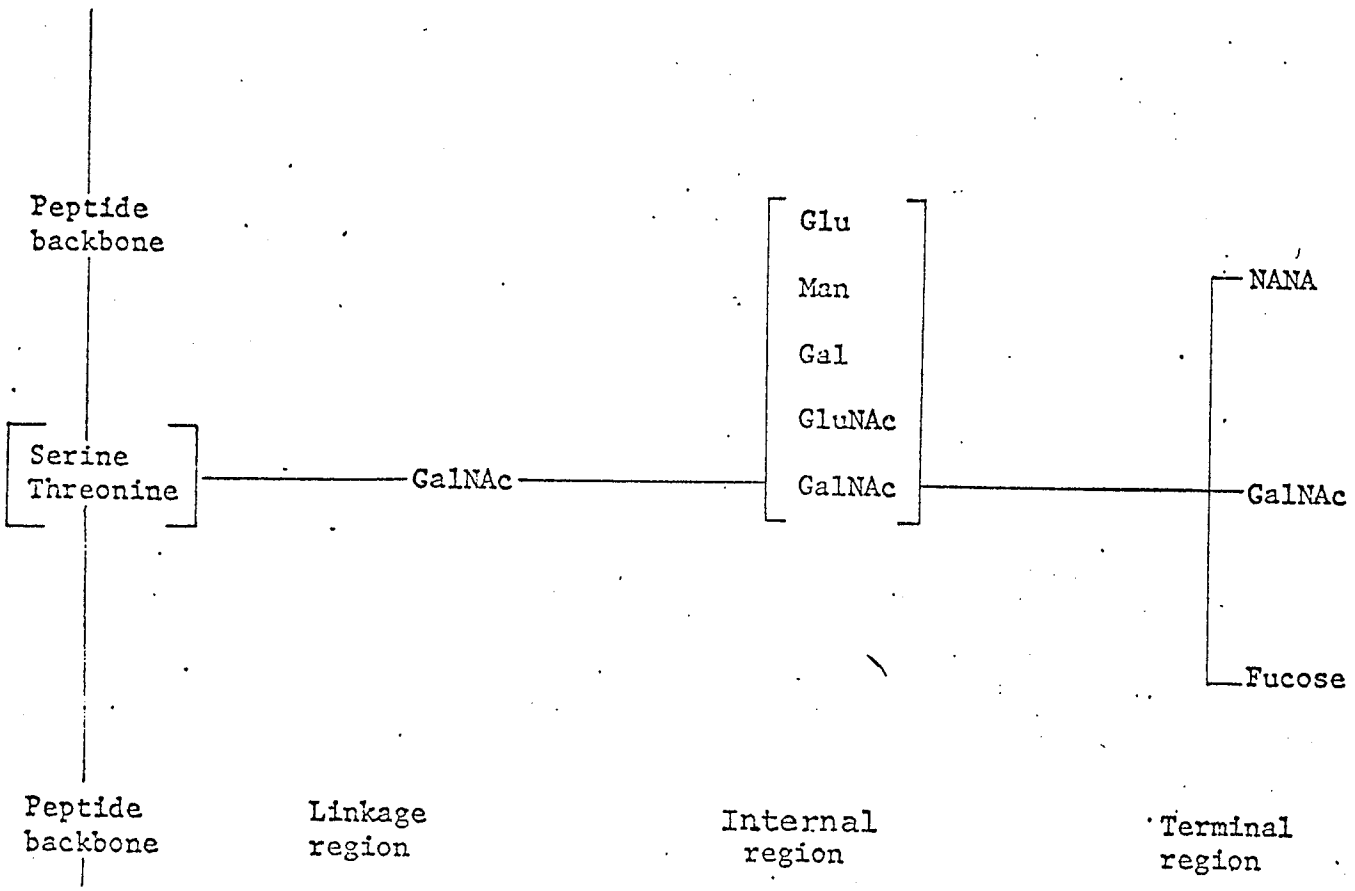


Fig. 20. Postulated partial structure of the major oligosaccharide group of RSM.

Pigman and co-workers[171,172,184] have suggested the possibility that the submaxillary mucins may be composed of a repeating series of small glycopeptides of approximately 20 to 28 amino acids (BSM[172]). Assuming that each glycopeptide contains all of the constituent amino acids to the same degree as the whole molecule, then the following analysis of the structure of the RSM can be done. Each glycopeptide would contain 52 amino acids. The glycopeptide would contain a total of 7 serines and threonines, of which 3 would be substituted by oligosaccharide chains, i.e. there would be 3 oligosaccharide prosthetic groups per glycopeptide, (Note: the ratio of methionine to gluNAc is 1:3). Since it has already been shown,[58,59] for other mucins that 40 to 50% of all the available acceptors are glycosylated, then the occurrence of 3/7 (42%) of the acceptors being glycosylated in the glycopeptide is in excellent agreement with the model. With the molecular weight of each oligosaccharide chain being approximately 2372, then the molecular weight of the total carbohydrate of the glycopeptide would be approximately 7200. The molecular weight of the peptide portion would also be in this region (7000) giving a molecular weight for the glycopeptide of approximately 14200. If we assume that the molecular weight is 800,000 for the whole molecule, then the mucin would be composed of 60 glycopeptides linked together. This would then mean that each mucin molecule contained approximately 180 oligosaccharide chains.

Several other preparations from rat submaxillary glands have appeared in the literature. The earliest preparation (Hashimoto et al., 1961)[173], which was a crude mucin clot from combined submaxillary glands, showed the presence of hexosamine, hexoses, fucose, glucuronic acid and sialic acid (as N, O, diacetyl-neuraminic acid). The total carbohydrate content was 70.4% by weight. The presence of glucuronic acid clearly indicates contamination by mucopolysaccharide. The identification of the sialic acid as the N,O,-diacetyl form is very unusual and no other worker has since shown its presence in the rodent gland. No attempt was made on the part of these workers to determine the degree of homogeneity of their preparation and it is quite reasonable to conclude that is was highly heterogeneous.

Shortly thereafter, Draus (1964)[175] described a mucin preparation isolated from the chloroform precipitate of combined submaxillary glands. This preparation was 42% carbohydrate and showed hexosamine (11.9%), sialic acid (9.4%), hexoses (12.9%) and fucose (3.1%). It did not show glucose and an assay for mannose was not done. The sialic acid was determined colorimetrically with N-acetylneuraminic acid as standard and was not identified as NANA. This preparation showed two bands on zonal electrophoresis which could not be separated by the techniques available to that author. Although superficially similar to our molecule the NANA/fucose ratio was higher (1:7 compared to 1). The NANA to hexosamine

and NANA to hexose relationships were quite similar. The relationship of this preparation to the present one is not clear. The heterogeneity of the preparation and uncertain tissue source seriously obscures any comparison of results.

The most recent isolation of mucin was that of Keryer et al., (1972)[169] which was made from submaxillary glands. These authors colorimetrically determined the presence of sialic acid, (measured as NANA), glucNAc, galNAc (ratio 3/2), galactose and fucose. Their preparation was approximately 50% carbohydrate and showed only a single peak of high molecular weight in the ultracentrifuge. Their amino acid composition appears very typical for a submaxillary mucin. Their gluNAc/galNAc ratio was 3/2 whereas ours was 1/3. They did not show the presence of mannose or glucose. Their sialic acid to galactose ratio was quite similar to our values and their sialic acid to hexosamine ratio was slightly higher.

Unfortunately, because of the uncertainty of the tissue source, the exact relationship of these molecule to ours is uncertain.

Several workers[131,161] have shown the uptake of glucosamine and galactose into glycoproteins in rat submaxillary glands. Ericson[161] demonstrated that radioactive glucosamine was taken up into most fractions when extracts of pilocarpine depleted slices were separated on columns of

Sephadex G-200. Philips[131] did extensive work on galactose uptake into glycoproteins prepared in much the same manner as Ericson's. It subsequently became of interest to see if radioactive isotope labelled sugar uptake into any of the glycoproteins of rat submandibular gland could be demonstrated after column chromatography and electrophoresis. Slices of submandibular gland were incubated for three hours as described. Initially, a two hour incubation was used, however, we found that the label uptake could be significantly increased by increasing the incubation time to three hours. The HSS prepared from the slices was subjected to electrophoresis in urea gels (system C) and the results presented in Fig. 21. Glucosamine-C^{14} uptake could be seen in all regions of the densitometry scan. No uptake of label could be detected for fucose-C^{14} and galactosamine-C^{14}. The mucin fraction was then prepared from the HSS by chromatography as before and then electrophoresed and fractionated as described. The results appear in Fig. 22. Uptake of both glucosamine-C^{14} and galactosamine-C^{14} into the mucin could be detected by these methods. No uptake of fucuse-C^{14} could be detected.

From these results it can be concluded that a combination of column chromatography and electrophoresis techniques as described and used herein could facilitate the study of the biosynthesis of the glycoproteins of the rat submandibular gland and other tissues. Larger preparations

would allow for separation and quantitation of the label uptake into both the carbohydrate and protein portions of a glycoprotein. The specific activity of the SMG preparations could be increased by pilocarpine or nor-epinephrine depletion prior to labelling. In addition, use of preparative gas chromatography would allow the separation, identification, isolation and quantitation of the component sugars as well as the determination of the specific activity in a single step.

CONCLUSION

1. The rat submandibular gland contains a surprisingly large collection of glycoproteins in significant concentration. Using the electrophoretic methods described we were able to detect 15-20 different glycoproteins. Fractionation of the HSS by column chromatography with Sephadex definitely supports these observations. The HSS contained the rat submandibular mucin (RSM).

2. Several of these glycoproteins (at least 6) contained sialic acid in significant quantities. RSM was shown to be a sialoprotein and the sialic acid was identified as N-acetylneuraminic acid by gas liquid chromatography.

3. RSM was 49% protein and 48-51% carbohydrate by weight although the linkage amino acids for other mucins have been serine and threonine. No evidence for this being the case with RSM was obtained. The amino acid content was atypical for that of a submaxillary mucin. The serine and threonine content was slightly low and the content of hydrophic amino acids was slightly high. The carbohydrate prosthetic group was complex and contained NANA, gluNAc, galNAc, galactose, mannose and fucose in the molar ratios of 2:1:3:2:1:2, respectively. The preparation showed detectible amounts of glucose which may be a contaminant or it may be part of the molecule.

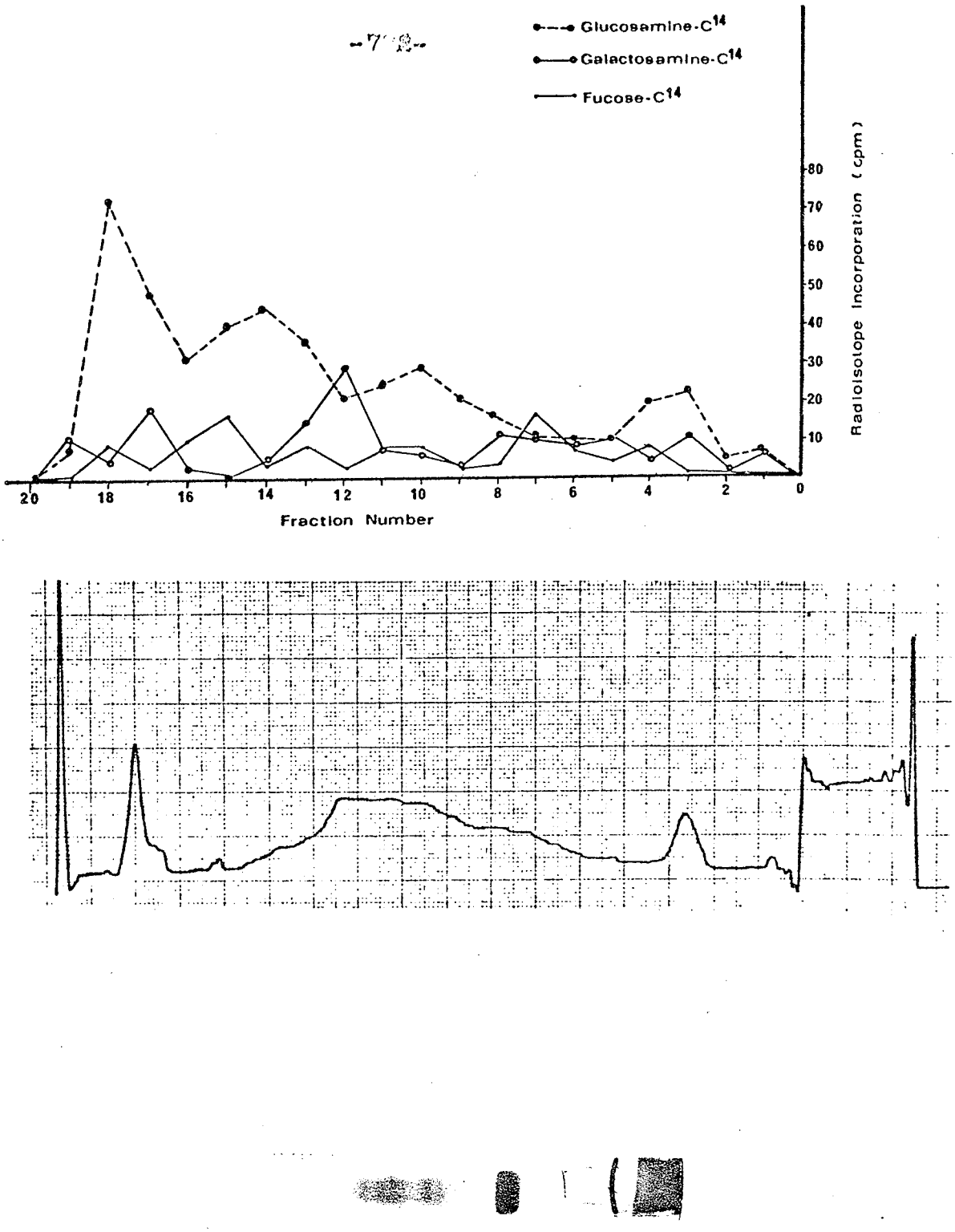
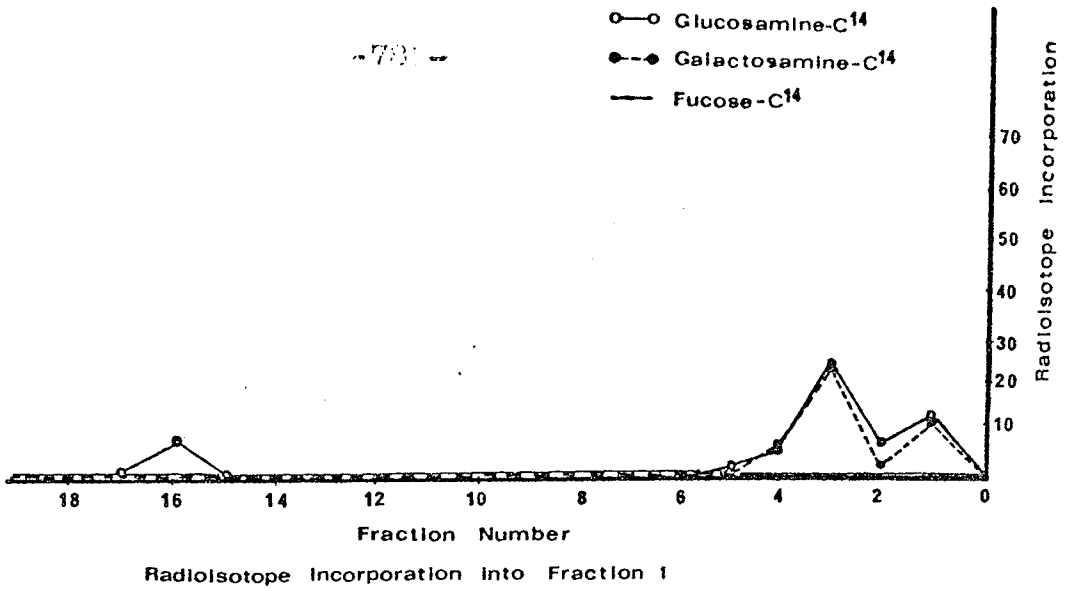


Fig. 21. Radioisotope incorporation into HSS.

(a) Typical PAGE separation (System C) of 300 µg HSS protein stained with PAS.
(b) Densitometric tracing of gel.
(c) Radioisotope incorporation profile into HSS.



Radioisotope Incorporation into Fraction 1

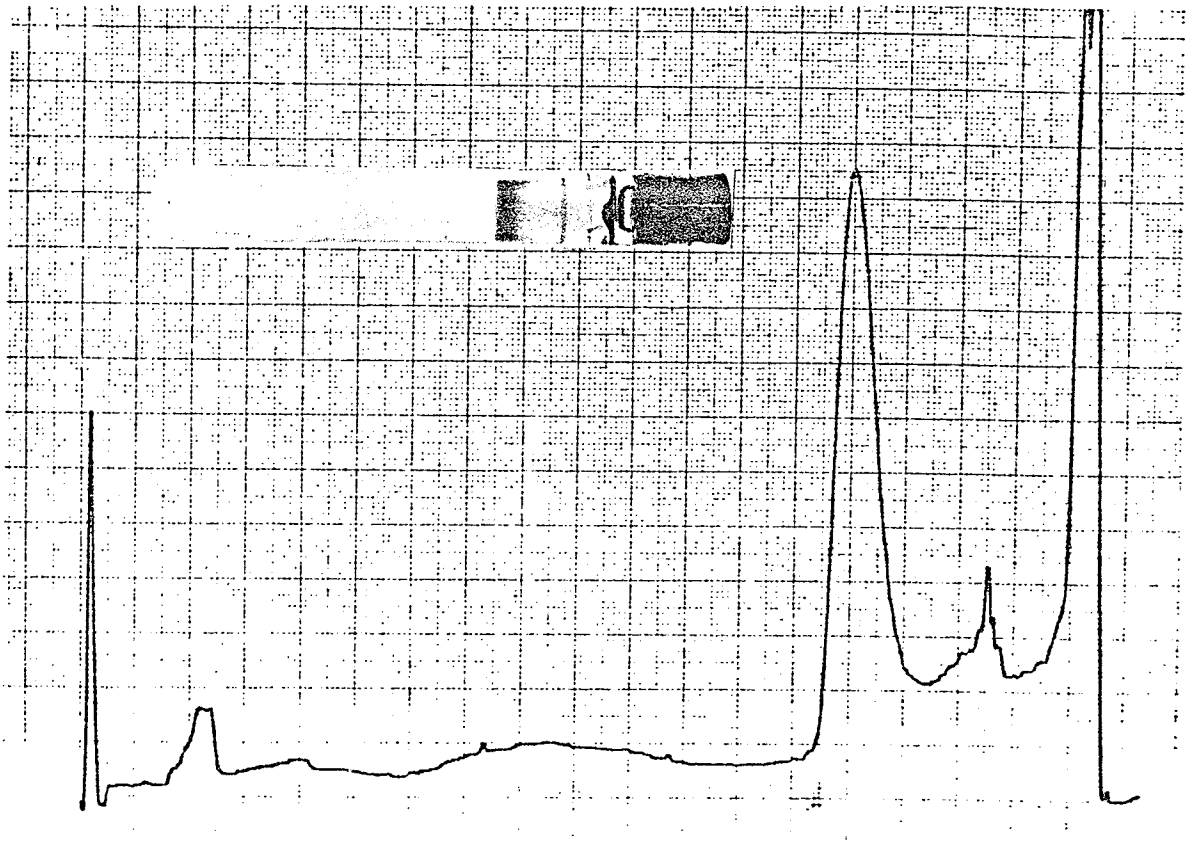


Fig. 22. Radioisotope incorporation into crude RSM fraction.

(a) Typical PAGE separation (System C) of 100 µg crude RSM fraction protein stained with PAS.
(b) Densitometric tracing of gel.
(c) Radioisotope incorporation profile into crude RSM preparation.

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APPENDIX A

ANALYTICAL BIOCHEMISTRY 56, 361 369 (1973)

A High Resolution PAS Stain for Polyacrylamide Gel Electrophoresis

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An improved PAS method for the detection of glycoproteins after electrophoresis on acrylamide or urea–acrylamide gels is described. Stronger oxidizing conditions and a more controlled washing for the removal of periodic acid resulted in both increased staining intensity and band resolution. The method will not stain protein and it appears that a 2–3 μg sample of bound or precipitable carbohydrate would easily be detected. Mucopolysaccharides were not detectable by this method.

The detection of macromolecular carbohydrate material, especially glycoproteins, on electrophoretic media is usually accomplished by some modification of the PAS stain (1–7). Since Koiw and Gronwall's (1) successful adaptation of the PAS method of Hotchkiss (8) to detect serum glycoprotein electrophoresed on paper, modifications for different tissue preparations have appeared throughout the literature. In principle, all of the methods involve an initial fixation stage, generally acidic or ethanolic, followed by oxidation of bound carbohydrate by periodic acid. The oxidation involves the cleavage of C—C bonds between adjacent α-glycols and their subsequent conversion into dialdehydes. The oxidation products are then reacted with Schiff's reagent to form a colored aldehyde addition product which appears a more or less intense red. Periodic acid is the preferable oxidizing agent in that its effect stops at the aldehyde stage, thus limiting overoxidation problems. If the procedure employed is successful, bands appear red on a white translucent background.

Zacharius and co-workers (7) investigated the earlier method of Keyser (3) and reported little success in adapting the procedure for use in acrylamide. These workers subsequently modified the method of Clarke (4) to stain plant and animal glycoproteins on acrylamide gels, however, they have presented no evidence to support their claims of a high degree of sensitivity. Our studies with rat submandibular gland glyco-

protein preparations have shown the Zacharius and Zell procedure to be less sensitive than the method of Caldwell and Pigman (5). On the basis of the work of Dawes (9) on human salivary proteins and the studies of Van Neerbos and De Vries Lequin (3) on PAS staining conditions, we felt the method of Caldwell and Pigman was also of limited sensitivity and was underestimating the number of glycoproteins present. The result of an extensive stepwise examination and subsequent modification of the procedure of Caldwell and Pigman form the subject of this report.

MATERIALS AND METHODS

Source of sample proteins. Aliquots of the 140,000*g* supernatant (HSS fraction) of rat submandibular gland (SMG), homogenized in distilled water (150 mg/ml) were used as the source of protein for most of the electrophoretic studies described. These electrophoretic observations were verified by comparison to those obtained with α-amylase (Worthington Biochemicals), (horse) transferrin (Nutritional Biochemicals), and (calf serum) fetuin (Calbiochem), trypsin and chymotrypsin (Worthington Biochemicals).

Protein determination. Protein was determined by the Lowry *et al.* procedure (10).

Determination of protein solubilized from gels. Following electrophoresis, the gels were immediately fixed in 12.5% trichloroacetic acid (TCA) for 1 hr and then transferred to 15% acetic acid in 10 × 75 mm test tubes. Aliquots of acid were removed at various time intervals up to 12 hr for protein determination (11). Gels identically prepared and run, but with no protein added, served as controls. Duplicate gels run at the same time were stained for total protein with 0.5% Amido Schwarz in 7% acetic acid for comparative purposes immediately following electrophoresis.

Electrophoresis

a. Nonurea gels. Seven percent acrylamide gels were cast from stock solutions prepared as described by Davis (14) with the following modifications: the stacking (large pore) gel was polymerized with the addition of an 80 mg% ammonium persulfate solution as well as the riboflavin solution recommended by Davis. Furthermore, the sample preparation involved the addition of stacking gel solution to the protein sample such that the final volume of the protein sample and gel solution was 100 μliters.

The ratio of stock solutions used in the preparation of the separating gel was A:C:G = 1:1:2 and for the stacking gel, B:D:E:F = 1:1:1:1.

The solutions referred to here have been put in the letter system used by Davis with solution F being the 80 mg% ammonium persulfate solution.

The electrophoresis buffer in the upper electrode (cathodal) chamber was 0.043 M Tris in 0.046 M glycine at pH 8.91 and in the lower (anodal) chamber was 0.02 M Tris in 0.06 N HCl at pH 8.07.

The gel columns were prepared using a 1000-μliter separating gel, a 200-μliter, stacking gel, and a 100-μliter sample gel, and electrophoresed at 1.25 mA per column (fixed current) until the indicator band (bromophenal blue) had entered the stacking gel. At this time, the current setting was increased to 2.5 mA per column and maintained there for the duration of the run. The electrophoresis apparatus used was a Buchler polyanalyst with a Buchler power source.

b. Urea gel. The 7.5% urea–acrylamide gels were prepared according to the method of Davis (14), with the following modifications: a 6 M urea solution was used as the solvent phase for all of the stock solutions. The buffer for the stacking gel was 0.256 N H_3PO_4 and 0.047 M Tris. The electrophoresis buffer for both chambers was 0.05 M Tris in 0.38 M glycine at pH 8.5. Electrophoresis conditions were the same as those already described for the nonurea gels.

Staining and Destaining Procedures

Amido Schwarz. Immediately following electrophoresis, the gels were immersed in 0.5% Amido Schwarz in 7% acetic acid in 10 × 75 mm test tubes for 1 hr and then destained electrophoretically at 5 mA per gel column.

Alcian Blue 8GX. Immediately following electrophoresis the gels were immersed in 1% Alcian Blue in 7% acetic acid for 2 hr. The gels were then destained by washing against 7% acetic acid as outlined below.

PAS. Immediately following electrophoresis, the gels were fixed for 1 hr in 12.5% TCA in 10 × 75 mm test tubes. The gels were then transferred into clean tubes and subjected to sample oxidation with 1% periodic acid for 2 hr and then washed as described below for 2 hr against 15% acetic acid. These steps were all carried out at room temperature. The gels were then placed in clean 10 × 75 mm test tubes containing Schiff's Reagent (Fisher Scientific) and stored in a refrigerator (in the dark) for 2 hr. The gels were destained as described below.

Gel destaining. For destaining and washing the gels were placed in highly perforated 1.2 cm × 11 cm polyethylene tubes. The tubes were individually numbered and stoppered at either end with No. 1 regular length corks. These numbered destaining tubes with gels contained were then placed into either 2 or 4 liter polyethylene beakers on a Lab-Line Multi-Magnestir with no more than 12 destaining tubes (free floating)

per liter of solution (7 or 15% acetic acid). Vigorous stirring provided the best results. For normal destaining the 7% acetic acid was changed three times over a 24-hr period. During the final removal of periodic acid, the 15% acetic acid was changed two to four times in the 2-hr destaining period. The destaining beakers were covered at all times with aluminum foil to prevent excessive exposure of the gels to light.

Densitometry. Gel scanning was done with a Joyce-Loebl Chromoscan using a 520 nm filter.

RESULTS

Protein Solubilization from Acrylamide Gels

The effect of overnight washing (against 15% acetic acid) on the protein pattern of TCA fixed, but unstained gels is shown in Fig. 1. Protein solubilization was indicated by a marked reduction in both Amido Schwarz staining intensity and band resolution and supported by protein assay of the wash media after 2 and 12 hrs, respectively (Table 1). Conditions for the protein assay were complicated not only by the low pH, but by interfering substances from the gels themselves as evidenced by the high optical densities obtained with control gels which contained no protein. The optical density values obtained were therefore statistically

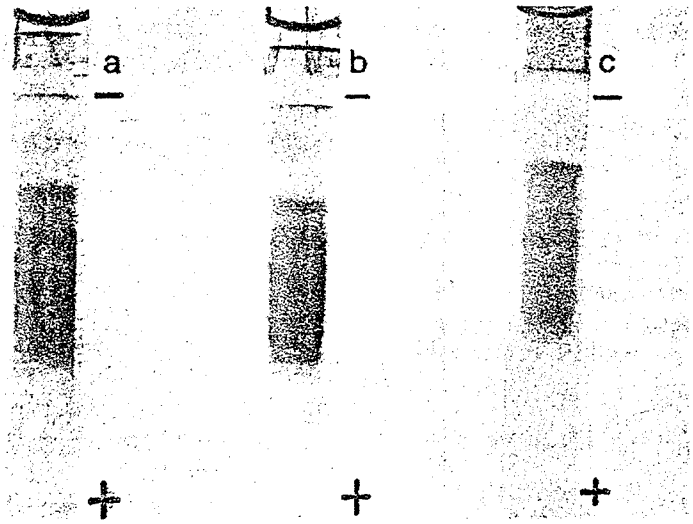

FIG. 1. Effect of TCA fixation and overnight washing on protein profiles of 200 μg HSS protein on 7.5% acrylamide gels (a) stained with 0.5% Amido Schwarz in 7% acetic acid for 1 hr immediately following electrophoresis, (b) 1 hr in 12.5% TCA and then stained as above, and (c) 1 hr in 12.5% TCA, washed overnight against 15% HAc (12 hr) and then stained.

TABLE I
Effect of Duration of Washing on Protein Solubilization from Acrylamide Gels

	OD (660 nm) of washes	
Control[a]	2-hr Wash	Overnight (12 hr) wash
123.9 (6)[b]	133.4 (6)	153.6 (6) $P < .01$

[a] Control gels contained no protein, but were treated identically.
[b] ( ) Number of gels dialyzed.

compared and were not converted to protein concentrations. In preliminary studies, we observed that only 10% as much HSS protein could be detected under these acidic conditions as was determined at neutral pH. It would therefore appear that a significant amount of the protein placed on the gel, perhaps as much as 30–50% could be solubilized and lost from the gel during washing and destaining, even after TCA fixation.

Oxidation Conditions

The increase of oxidation conditions from 0.2 to 1% periodic acid contributed significantly to the increased resolving power of the PAS procedure (Fig. 2a) reported here.

Comparison of Initial and Modified Procedures

The increased staining intensity and band resolution achieved by more controlled washing, stronger oxidizing conditions, and more rigorous

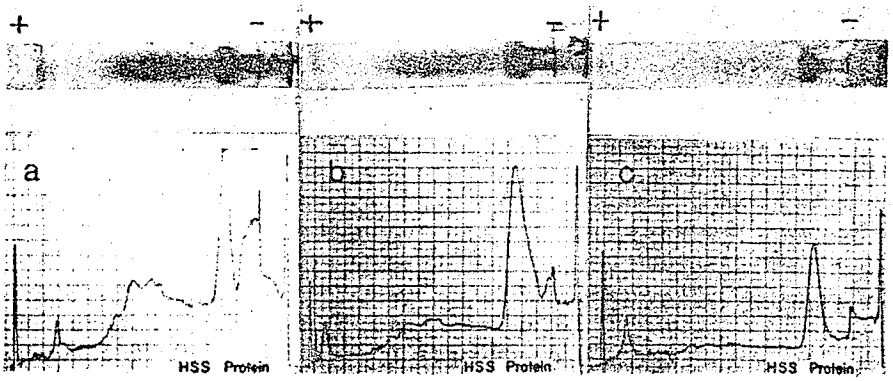

FIG. 2. Comparison of PAS staining patterns and corresponding densitometric scans obtained for 300 μg HSS protein on 7.5% Urea Acrylamide Gels by (a) using the PAS procedure described, (b) using the PAS procedure described with only 0.2% periodic acid for the oxidation step, and (c) using the procedure of Caldwell and Pigman.

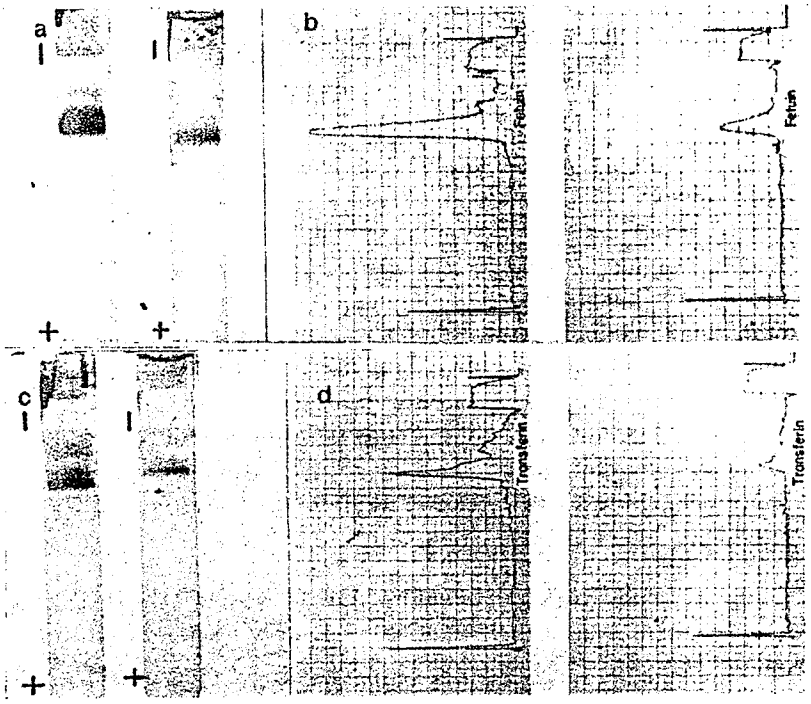

FIG. 3. PAS staining patterns and densitometric scans for 100 μg each of (a,b) fetuin and (c,d) transferrin and stained (a,c) with the PAS procedure reported and (b,d) the PAS procedure of Caldwell and Pigman on 7.5% urea acrylamide gels.

fixation is illustrated by the comparisons (Figs. 2 and 3) of the procedure presented here and that essentially described by Caldwell and Pigman. Increased band intensity and resolution improvement is especially obvious for the glycoproteins fetuin and transferrin (Fig. 3).

Specificity and Sensitivity of the Modified Procedure

The proteins α-amylase, trypsin, and chymotrypsin, which have not been shown to contain carbohydrate, did not stain with PAS (Fig. 4). The sensitivity and usefulness of the stain is shown by the detection of 14 distinct PAS positive bands in rat submandibular gland HSS (Fig. 5a and b). The distinct stain obtained with 50 μg transferrin established the lower limit of the stain sensitivity to be in the region of 2–3 μg of bound carbohydrate (Fig. 5c).

DISCUSSION

A major difficulty in standardizing the reported modification centered in optimizing the gel wash and oxidation conditions to ensure as com-

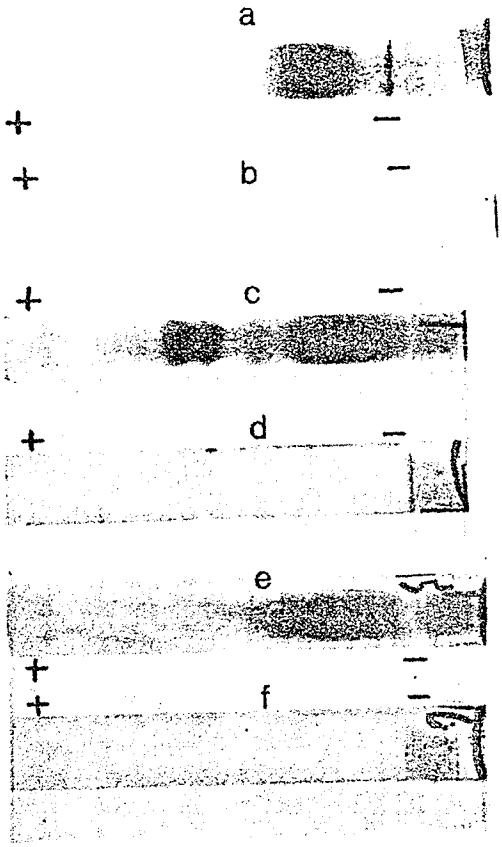

FIG. 4. Electrophoresis of (a,b) 100 μg α-amylase (c,d) chymotrypsin, and (e,f) trypsin used as carbohydrate-free protein standards on 7.5% urea acrylamide gels. Gels were stained with Amido Schwarz (a,c,e) and PAS (b,d,f) as described.

pletely as possible the removal of periodic acid from the acrylamide gels, but at the same time preventing any significant loss of PAS staining material through sample solubilization. Our observations on sample solubilization do not agree with the early report of Zacharius *et al.* (7) which suggested that sample fixation with TCA was effectively irreversible, even to an overnight washing against distilled water. It is possible that the relatively high protein concentration (500–900 μg) used by these workers made the comparative detection of sample loss difficult. Sample loss through solubilization is, however, not completely unexpected and has previously been demonstrated for both human salivary proteins (Dawes, 1963) serum glycoproteins (Appfel and Peters, 1969) as well as for relatively insoluble membrane proteins (Ray and Marinetti, 1971).

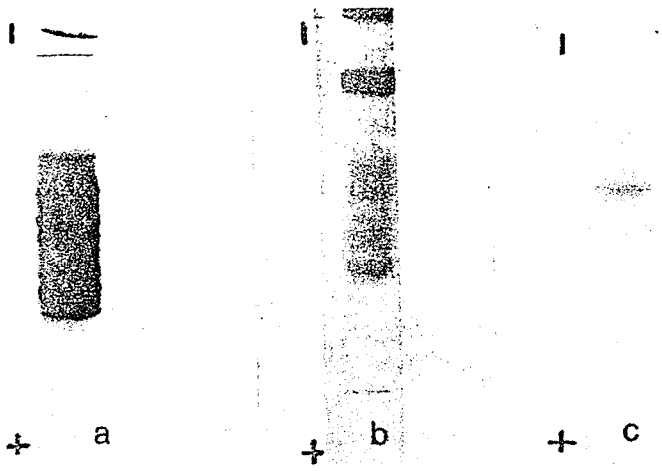

FIG. 5. (a) Amido Schwarz and (b) PAS staining of 300 μg of HSS protein using the modified procedure and (c) PAS staining of 50 μg of transferrin representing approximately 2.8 μg bound carbohydrate.

The results presented here (Fig. 1, Table 1) for rat submandibular protein clearly demonstrate that protein material can solubilize off the gel matrix during the stages following fixation, but prior to staining. The HSS protein as well as transferrin and fetuin all showed reduced staining intensity with Amido Schwarz following acid washes for periods as short as 6 hr. These results and those of others described above strongly suggest that this reversible fixation is a characteristic of glycoprotein in general and can be a major variable in PAS staining techniques.

The importance of completely removing periodic acid is well established (7) and only reemphasized in our study. Standardization of the gel washing and destaining conditions eliminated the inconsistent staining patterns we had routinely obtained using the method of Caldwell and Pigman (5). Although the washing and destaining conditions described herein were optimal for our gels, it should be emphasized that with changes in gel volumes, it may be necessary to reevaluate the wash time to ensure adequate destaining.

Without the added benefit of more rigorous washing and destaining conditions, an increase in the oxidation conditions alone produced a more marked staining intensity than was obtainable using the procedure of Caldwell and Pigman (5). Van Neerbos and De Vries Lequin (3) have investigated the oxidation conditions necessary for detecting glycoproteins on cellulose acetate and found that maximal staining intensity was obtained with 1.5% periodic acid after 50 min. We have extended the duration of oxidation to 2 hr in our study to ensure more complete

sample oxidation. The possibility of overoxidation occurring under these conditions, such as the oxidation of amino acids (e.g., tyrosine, serine) and the formation of PAS sensitive end products does not appear likely. Amylase, trypsin, and chymotrypsin which have not been reported to be glycoproteins, when run at a relatively high concentration (100 μg) using the procedure described here, remained PAS negative.

The sensitivity of this procedure is demonstrated not only by the detection of 14 distinct PAS positive components in a 300-μg sample of rat HSS protein, but in addition, the detection of 50 μg transferrin [2.8 μg bound carbohydrate (13)] suggests that 2–3 μg of bound carbohydrate could be easily detected using the procedure we have reported here.

Mucopolysaccharides (chondroitin sulfates) run in our system were PAS negative, possibly due to their solubility in TCA. They were, however, readily detectable with 1% Alcian Blue in 7% acetic acid.

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APPENDIX B

SOME EXPERIMENTS IN THE SPECIFICITY AND USEFULNESS OF ALCIAN BLUE AS A STAIN FOLLOWING PAGE.

Introduction

The presence of sialic acid as an important functional group of some of the glycoproteins in rat submandibular glands has already been demonstrated in this thesis. For this reason a stain localizing bound sialic acid with good certainty and reproducibility would have facilitated the electrophoretic studies of the glycoproteins present in the gland. Therefore, we attempted to determine the staining specificities and optional conditions for the use of Alcian blue in detecting bound sialic acid.

Since its introduction by Steedman in 1950, Alcian blue (A.B.) has become one of the most widely used cationic stains appearing in the histochemical literature.[2] Despite its popularity, a few reports of its application to electrophoresis preparations have been made. Caldwell and Pigman[3] have used Alcian blue in 3% acetic acid (HAc) to tentatively identify bound sialic acid in human saliva after PAGE. Hilborn and Anastassiadis[4] and others[12,13] have reported good Alcian blue stains for acidic mucopolysaccharides by the same methods. Recently, Wardi et al.,[12,13] have published an interesting paper describing the application of Alcian blue (in 2.5% HAc), after oxidation and bisulfite addition of the carbohydrate of glycoproteins as a substitute for the PAS stain. They claim

to be able to differentiate between acidic mucopolysaccharides (MPS) and glycoproteins by this method. Since their only criteria was the ability to stain mucopolysaccharides with Alcian blue in acetic acid without prior oxidation, etc., this procedure is highly disputible on the basis of the work of Caldwell and Pigman[3] and Kapitany et al.[15,16] who have shown glycoprotein staining under the same conditions. This work does show, however, that Alcian blue uptake can be increased by increasing the number of strongly acidic groups in the molecule.

The specificity and the mechanism of action of this stain has been the subject of much work in the past.[3-8] However, Anderson et al.[5] have pointed out problems with the assumption that Alcian blue stains sialic acid. Similarly, they have questioned the role of the salt linkage mechanism as the means of Alcian blue staining as suggested by Scott et al.[6] and Quintarelli et al.[7,8] The salt-linkage mechanism particularly, lacks direct support in electrophoretic preparations, since both workers[3,4] used Alcian blue in 3% HAc, the acidity of which is far below the pK of the carboxyl group of sialic acid.

Our experiments into the specificity and use of Alcian blue following the PAGE of rat submandibular gland HSS are presented here.

Methods and Materials

i) The HSS was prepared as described (Methods section).

ii) All electrophoresis was done with gels of System A, as described in the Methods section.

iii) Neuraminidase Incubation: Submandibular gland (HSS) was incubated with Vibro cholera neuraminidase for 18 to 24 hours. Incubation controls were incubated with the same quantity of distilled water under identical conditions. Aliquots were taken at regular intervals for free and total sialic acid determination and for electrophoresis. One duplicate set of acrylamide gels representing each time interval were stained with 1% Alcian blue in 7% HAc and another pair with PAS (see Methods section).

iv) For studies of the conditions for Alcian blue staining, the following Alcian blue preparations were used: 1% solutions of Alcian blue 8GX (Allied Chemicals) were prepared in (i) 3% HAc, (ii) 7% HAc, (iii) 15% HAc, (iv) 7% HAc with 0.5% Calcium chloride ($CaCl_2$), and (v) 7% HAc with 0.5% Sodium chloride (NaCl). Similarly, 1% Alcian blue solutions in distilled water (pH when used 3.5) were prepared with (i) NaCl and (ii) 0.5% $CaCl_2$.

v) The photography for (Fig. 5) was done under ultraviolet light against a piece of black velvet as background.

Results, Discussion and Conclusions

The results obtained by incubating HSS with neuraminidase are shown in Fig. 1. After 2 hours incubation all traces of the alcianophilia in region B were lost. The control sample had also lost its staining potential even though the sialic acid release (see Fig. 6) (in Results and Discussions section) from the control during that time was low. This band would therefore seem to represent the most readily hydrolyzable sialic acid linkages. This assumption is based on an extension of the conclusions reached by histochemists for the same types of experiments.[14] Others[5] have questioned this assumption for various reasons, most notably, for the difficulty in establishing adequate controls and the apparent variable susceptibility of sialic acid involved glycosidic linkages to neuraminidases.[14] The band in region C (Fig. 1) still maintained its alcianophilia quite indistinguishably from the controls at 2 hours. However, by 8 hours, the neuraminidase treatment severely reduced the staining in this region. This band, however, did not seem to lose its staining entirely. By 8 hours all of the hydrolyzable sialic acid had been removed and yet this alcianophilia persisted up to 18 hours. It may be due to the remaining bound sialic acid, a problem similarly encountered by the histochemists[9] many years ago. Since Alcian blue, in histochemical preparations at least, is not especially reactive with sulfate groups,[15] the presence of sulfated glycopeptides was unlikely. It seems most likely

that the residual stain was due to the uronic acids of the low molecular weight acidic mucopolysaccharides since these molecules are present in rodent salivary glands (chondroitin sulfates A & C) and have been shown to migrate in this region of the gel.[155]

The PAS stains of the duplicate gels at each time period appeared largely unchanged (Fig. 2). This appears to indicate that endogenous lysosomal glycosidase activity was not significantly altering the molecules in any undetermined way.[15]

It certainly seems, from these data, that, at least the alcianophilia in some areas of the gel (Area B especially) can be shown (indirectly) to be due to the presence of sialic acid. If that was true, then why did not all sialo-glycoproteins stain with Alcian blue. This problem was first noticed with the rat SMG mucin, which in both a highly homogeneous state and in the HSS failed to give a consistantly good Alcian blue stain. At best, it was only weakly Alcian blue positive. To investigate this problem further, commercially obtained fetuin[17] was electrophoresed and staining with Alcian blue was attempted. Consistantly, negative results were obtained.

The basic mechanism hypothesized for the uptake of Alcian blue by acidic groups involved a salt-linkage between the positive Alcian blue monomer and the dissociated carboxyl group of sialic acid[6-8]. This hypothesis has been criticised

a number of times in the past.[5,15] Under the acid conditions used by others,[15] and ourselves, the pH was sufficiently less than the pK of the carboxyl group of sialic acid (pK approximately 2.6) such that the carboxyl group would be largely associated. Increasing the acid conditions from 3 to 15% HAc had no effect on the intensity or specificity of the stain (Fig. 3). However, once the pH was increased past the pK of sialic acid (pH = 3.5), the staining pattern changed dramatically (Fig. 4,5). Alcian blue in water at pH = 3.5 caused a large change in the specificity of the stain. It seemed inconceivable that the loss of the acetate ion might be an important variable, but some support for this suggestion exists.[11] If the staining mechanism did involve a salt-linkage, then the increase in pH should have markedly increased the staining intensity for existing bands and not changed the specificity entirely (Fig. 4,5).

Putt,[10] who has advised the use of Alcian blue in calcium chloride as a routine method for staining mucins, found increased staining intensity in the presence of this divalent ion. When the HSS in this study was stained with Alcian blue in acid, with either 0.5% sodium chloride or 0.5% Calcium chloride, no effect could be visually detected (Fig. 6). These ions did, however, slightly increase the staining intensity when used with Alcian blue in water (Fig. 7). Since both ions produced much the same effect, it would seem to be a function of increased ionic strength and not a specific

ion effect. Theoretically, at least, an increase in ionic strength should decrease salt-linkage interactions by competing with the cationic stain for ligand binding sites.

We agree with Curran[15] that an amide linkage, involving orientation with the associated carboxyl group, to be the most reasonable hypothesis. It would also partly explain the earlier data which favored the salt-linkage mechanism.[6-8]

Furthermore, the failure of this dye to give good stains for the mucin[15,16] and fetuin,[17] both of which show the presence of significant sialic acid, raises more doubts as to the reproducable specificity of this dye when used by the procedures discussed for electrophoretically separated molecules. The sole consistant use for the dye in PAGE that we have been able to determine was in the identification of acidic mucopolysaccharides. These compounds give good Alcian blue stains[4,17] and do not stain with the PAS procedure,[17] whereas a glycoprotein invariably gives a PAS stain. Until these problems can be definitively solved, use of this dye as a detection method for sialoproteins following PAGE leaves a great deal to be desired.

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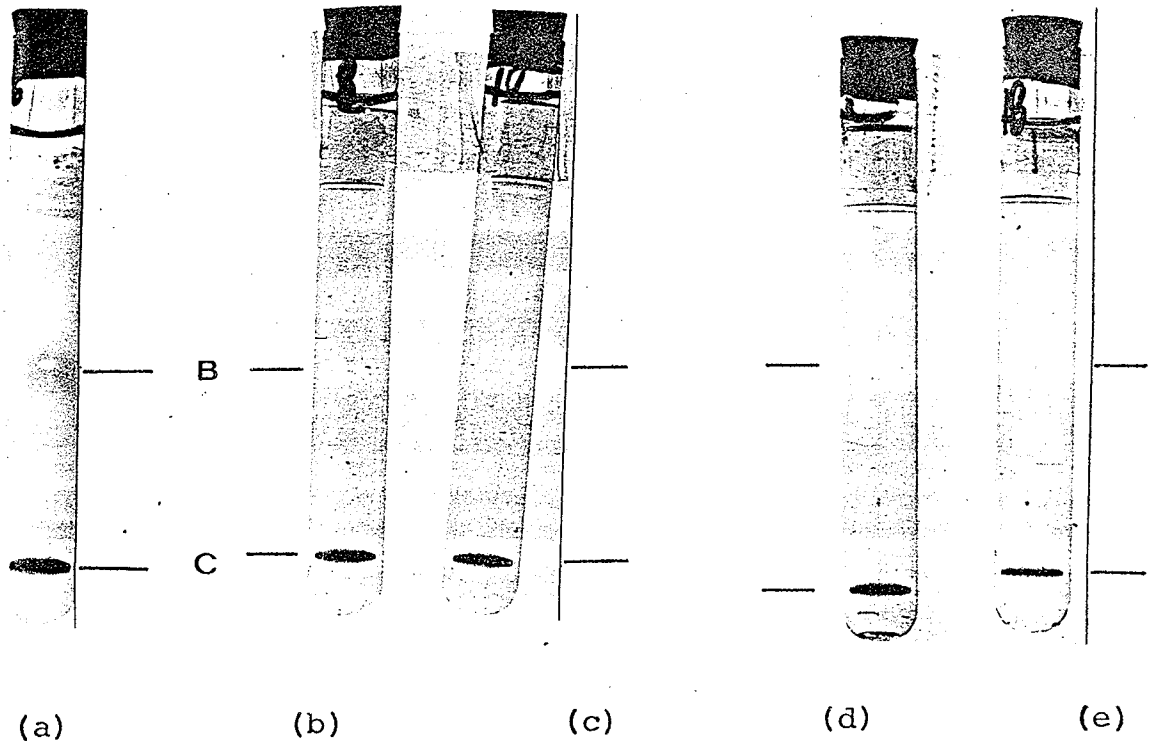
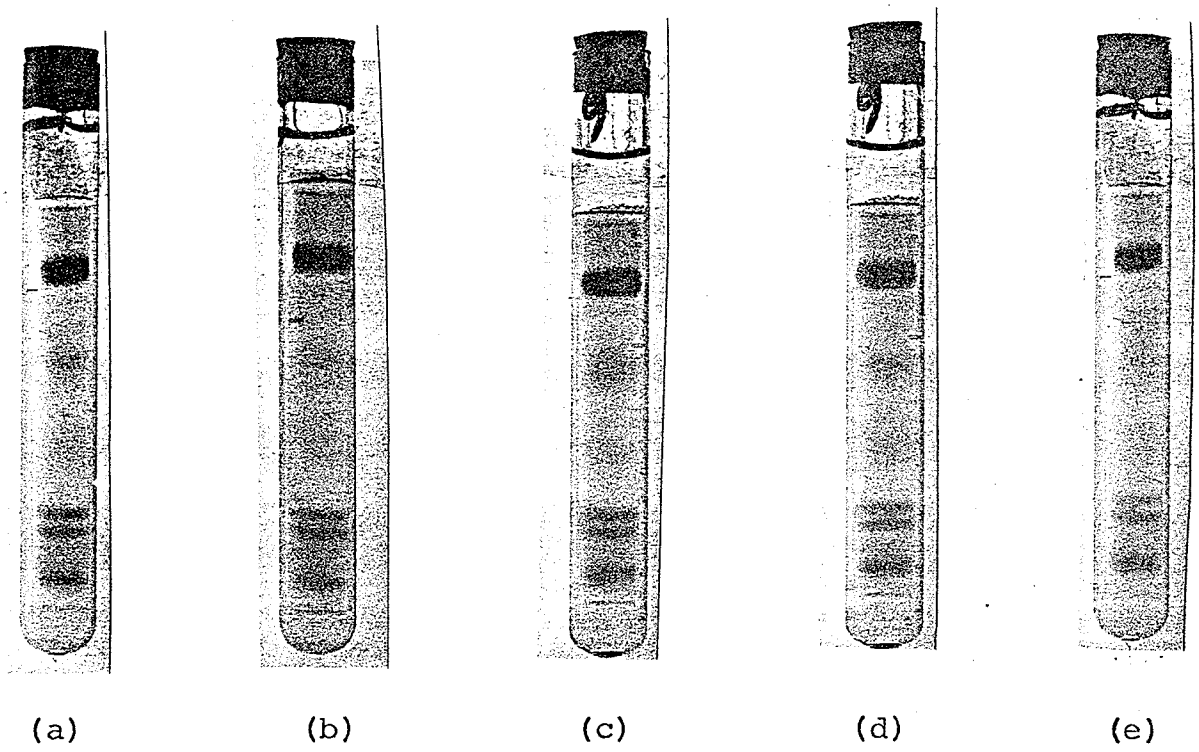


Fig. 1. Alcian blue staining of HSS after incubation with V. cholera neuraminidase. (PAGE, System A).

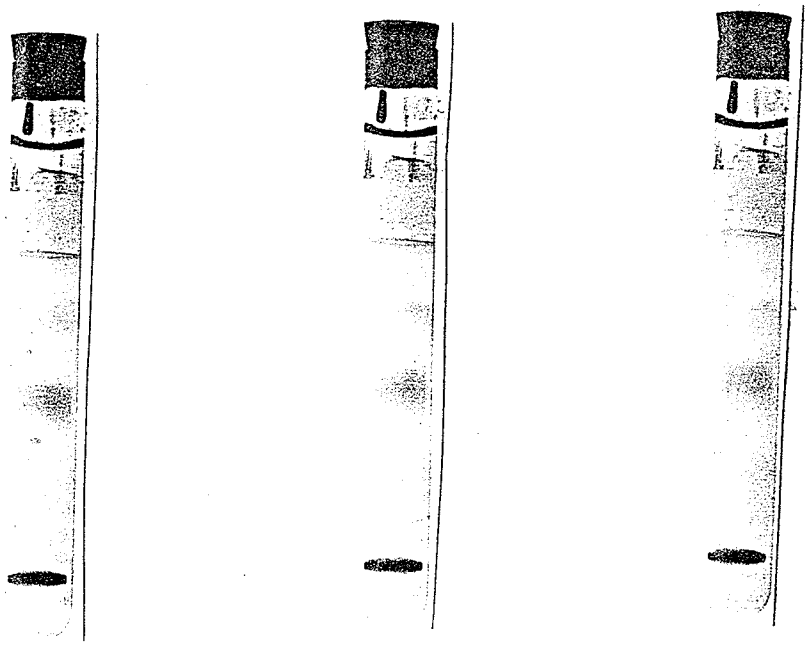
(a) incubation control at 0 time
(b) incubation control after 2 hrs
(c) neuraminidase treatment after 2 hrs
(d) incubation control after 8 hrs
(e) neuraminidase treatment after 8 hrs.



(a) (b) (c) (d) (e)

Fig. 2. PAS staining of both incubation control and neuraminidase treatment after 0, 2 and 8 hrs incubation.

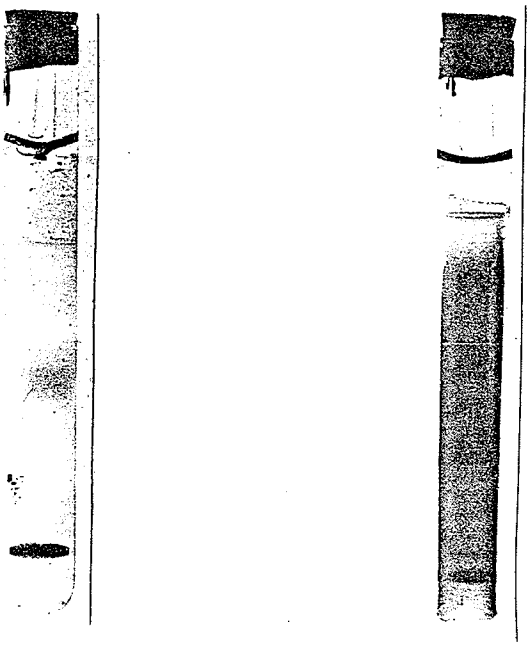
(a) 0 hrs
(b) 2 hrs incubation control
(c) 2 hrs neuraminidase treatment
(d) 8 hrs incubation control
(e) 8 hrs neuraminidase treatment.



(a) (b) (c)

Fig. 3. Alcian blue staining of 200 µg HSS with:

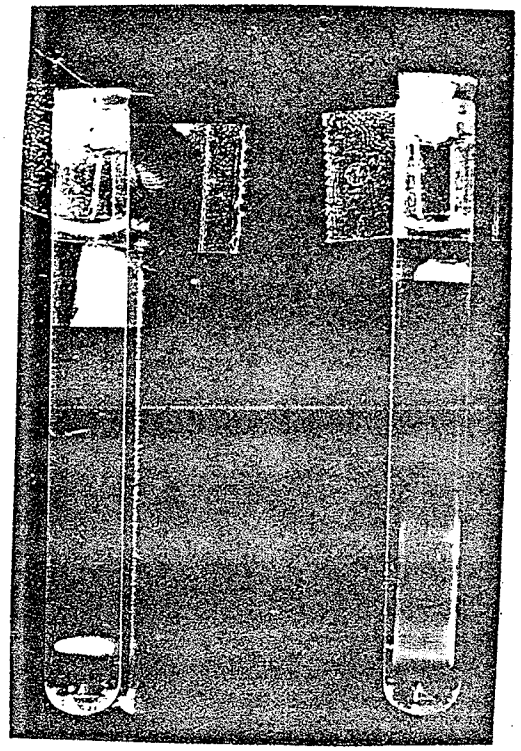
(a) 1% Alcian blue in 3% acetic acid
(b) 1% Alcian blue in 7% acetic acid
(c) 1% Alcian blue in 15% acetic acid.



(a) (b)

Fig. 4. Comparison of Alcian blue staining of 200 µg HSS in 3% acetic acid and water.

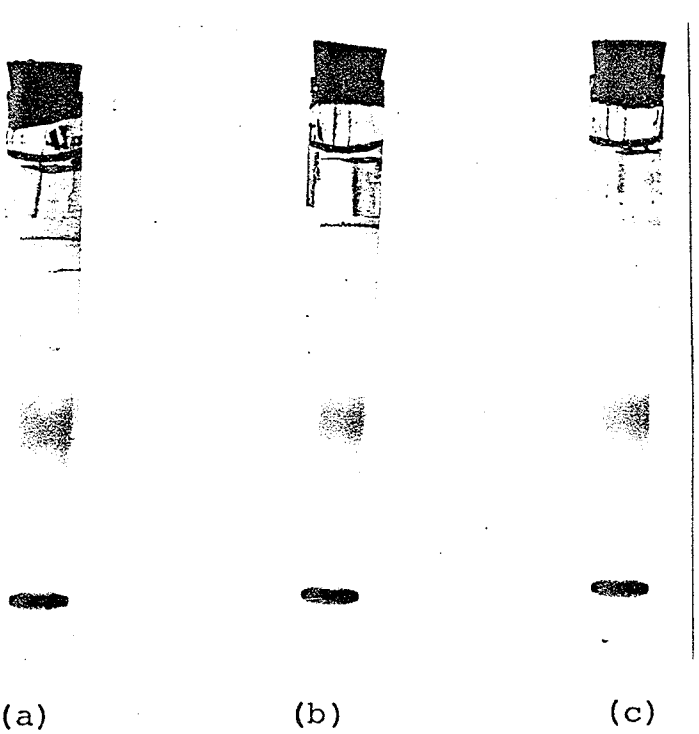
(a) 1% Alcian blue in 3% acetic acid
(b) 1% Alcian blue in H_2O at pH = 3.45.



(a) (b)

Fig. 5. Gels from Fig. 4 photographed under ultraviolet light against black velvet background.

(a) 1% Alcian blue in 3% acetic acid
(b) 1% Alcian blue in H_2O at pH = 3.5.



(a) (b) (c)

Fig. 6. Alcian blue staining of 200 µg HSS stained with:

(a) 1% Alcian blue in 7% acetic acid
(b) 1% Alcian blue in 7% acetic acid with 0.5% NaCl
(c) 1% Alcian blue in 7% acetic acid with 0.5% $CaCl_2$.

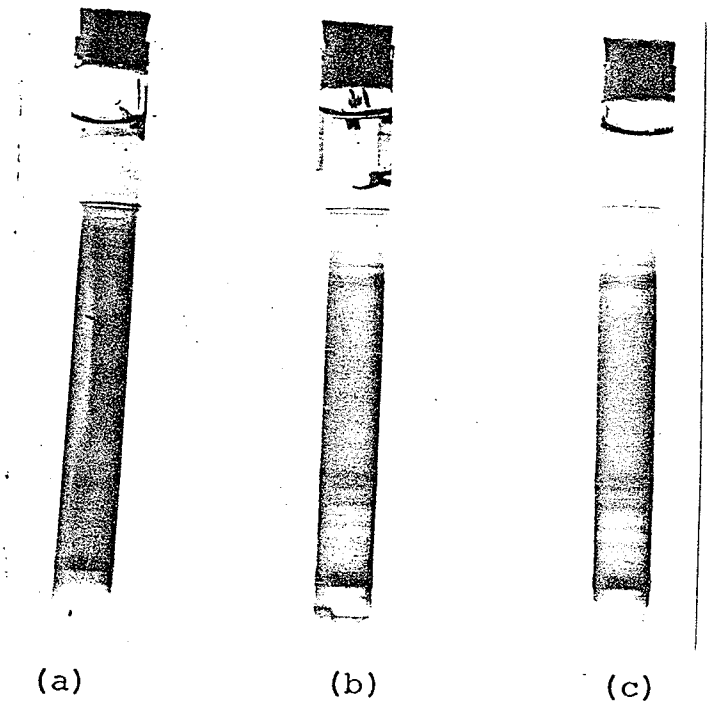


Fig. 7. Alcian blue staining of 200 µg HSS stained with:

(a) 1% Alcian blue in H_2O at pH = 3.45

(b) 1% Alcian blue in H_2O at pH = 3.45 with 0.5% NaCl

(c) 1% Alcian blue in H_2O at pH = 3.45 with 0.5% $CaCl_2$.