Suggested short title:

Structural Aspects of Human Gastric Gel Mucin

ABSTRACT

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Thesis - Master of Science

The structure of human gastric gel mucin, a fucomucin of very high molecular weight, was studied by analyzing the effects of alkali treatment. The gel mucin was seen to undergo a β-elimination reaction as judged by the loss of both threonine and serine residues, the production of α-amino butyric acid and alanine residues in a reducing system, the formation of α-ketobutyric acid and pyruvic acid residues in a non-reducing system and the substantial decrease in molecular weight of the mucin after alkali treatment. It is concluded that gel mucin has polysaccharide side-chains of exceptionally large size, joined O-glycosidically to the protein core. As the existence of disulphide cross-links in the protein is not supported, it is proposed that the maintenance of the 'rigidity' of the gel structure is due to carbohydrate interactions between molecules.

STRUCTURAL ASPECTS OF HUMAN GASTRIC GEL MUCIN

by

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Thesis submitted in partial fulfillment of the requirements for the Degree of Master of Science

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July, 1971

To Judy.

PREFACE

In the preparation of this manuscript I received much assistance. I would like to thank each of the individuals who helped to make this report possible.

I thank Dr. S.C. Skoryna for the privilege of working in the Department of Surgical Research, McGill University, Montreal.

I am indebted to Dr. D. Waldron-Edward for her spirited advice and direction throughout the course of my work.

I sincerely appreciate the assistance and help of one sort and another that I received from Mr. P.L. Rojowski, Miss Barbara Meagan, Miss Miriam Weiser, Mr. Mark Yaffe and the other members of the Donner Building staff. In particular I would like to thank Mrs. V. Olenitsch and Mr. K. Christenson for the collection, purification and blood-group determination of the human gastric gel mucin.

I thank Dr. Rose Johnstone of the Department of Biochemistry, McGill University, for the generous offer of the use of the Amino Acid Analyzer, and Dr. Steve Potashner for his timely instruction on its use.

To Dr. B. Kopriwa of the Department of Histology, McGill University, and Mr. Wilkinson and Mr. R. Dixon of the Department of Radiology of the Royal Victoria Hospital, Montreal, my sincere thanks for their advice and technical assistance in the preparation of the autoradiograms.

I thank Mr. W..J. Latchem for the photography of the paper chromatograms.

The kindness of Mr. Gerry Rosebery of the Department of Chemistry, McGill University, in performing the infrared spectroscopy of gel mucin is much appreciated.

To Mrs. Iris Spooncer, without whose typing skills this manuscript would not have reached completion, my heartfelt thanks.

In carrying out this research I received the financial assistance of the Medical Research Council of Canada.

Finally I thank my father and mother for their efforts beyond the call of duty. Together with Christopher, Terri, Judy, and Timothy, they added much enjoyment to my work through their contagious sense of humour and goodwill.

This project, a study of the structure of human gastric gel mucin, belongs to the category of fundamental research. Fundamental research is justified through its ramifications and applications. The discovery reported herein, the nature of the protein-carbohydrate linkage, will find its first application in detailed comparative studies of gel mucin from healthy and diseased individuals. However, as other fundamental research has shown, the benefits to the populace in general will, most likely and regrettably, lag far behind the initial discovery.

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LIST OF ABBREVIATIONS

Apart from standard chemical formulae the symbols used in this manuscript are:

BA	bovine albumin
BSM	bovine submaxillary mucin
CMC	carboxy methyl cysteine
DNP	2,4, dinitrophenyl hydrazone (derivative)
DPN(H)	diphosphopyridine nucleotide (reduced form)
G	glycoside
GAL	galactose
GM	gel mucin (human gastric)
h	2,4, dinitrophenyl hydrazine; or as subscript hydrazone.
HGGM	human gastric gel mucin
HOG	hog gastric mucin (commercial preparation from mucosa)
ia	iodoacetamide
K	α-keto butyric acid
LDH	Lactic Acid Dehydrogenase
M	mucin (HGGM)
M_a	mucin hydrolyzed in acid without prior alkali treatment
M_D	mucin 'desialyzed' prior to treatment
N(Ac)GAL	N-Acetyl galactosamine
N(Ac)Glu	N-Acetyl glucosamine

LIST OF ABBREVIATIONS (Continued)

OA	ovalbumin
OD	optical density
OSM	ovine submaxillary mucin
P	pyruvic acid
PAS	periodic-acid Schiff
PSM	porcine submaxillary mucin
VV	void volume (non-retarded) fraction of HGGM on Sephadex G-200

I. INTRODUCTION

Historical

"Mucus, mucor, mucosus humor, mocositas, myxa, animal mucus, Blenna, animal mucilage, muqueux animal (F), Mosve, mucilage animal.

A substance analogous to vegetable mucilage, from which, however, it differs by affording subcarbonate of ammonia on distillation. Mucus exudes through the skin in a state of combination with a particular oily matter; and drying, forms the epidermis. It constitutes, in part, the different epidermeous productions, such as the hairs, nails, wool, and horn of animals, feathers of birds and scales of fish. It is found at the surface of the mucous membranes, and presents some difference in its composition and properties according to the particular mucous membranes from which it is obtained. It preserves the membranes moist; and in a state best fitted for the performance of their functions. The French give the term 'glaire' to the thick, sturgy mucus secreted by mucous membranes when in the state of disease."

- Definition of mucus from Dunglison R. 1833 Medical Lexicon. A new dictionary of medical science, 1st Edition, Philadelphia, p. 481[(1)].

Late in the seventeenth century, clinical literature referred to 'mucous secretions':

1690 A.C. Bentzius, De pituita vitrea insipida, 4° Aldorffi

The term 'mucus', denoting a slimy, viscid, tenacious material of animal origin, was freely used by the eighteenth century in both Latin and English medical texts:-

1734 C.H. Hoffbauer. De Ignobli Muco Ingrato Multorum Nobilium Hospite Halae, Magdeburg

1778 C. Darwin. Experiments Establishing a Criterion between Mucaginous and Purulent Matter, 8° Edinburgh, also (1780) London

1789 J.C.C. Lorleberg. Physiologia Muci Primarum Viarum, Wittenberg

1790 D. Henle. De Muco et Morbis a Muco Oriundis, Leiden

However, mucus was not characterized as a chemical substance distinct from albumen and gelatin until 1805. At that time, J. Bostock[2] published an article recording that in contrast to albumen, mucus was not coagulated "by exposing the fluid for some time to the heat of boiling water".

In 1835, Nicolas Theodore de Saussure introduced the name "muzin" or "mucin" to the clear, viscid secretion known as "Schleimstoff"[3]. In 1846 Scherer further characterized mucin by its property of being precipitated in the cold by acetic acid. In a paper, "Uber den Flussigen Schleimstoff des tierischen Korpers" he recorded the chemical analyses of mucia from a mediastinal cyst. The values for carbon and nitrogen were significantly lower than those corresponding to the class of substances called "protein"[4].

The fundamental discovery - that mucin contained a substance which reduced Fehling's solution, that is, carbohydrate - was made in 1865 by Eichwald[5] in Sherer's laboratory. Later Eichwald showed the wide distribution of mucins in the animal body: lymph glands, thymus, salivary glands, sarcoma.

An advance in characterizing the carbohydrate was made by Landwehr in 1882[6]. He isolated a gum-like polysaccharide from the alkali-treated mucin of Helix pomatia. This was the first proof that the carbohydrate was not a single monosaccharide. Crystalline carbohydrate material was prepared in 1884 from frog egg mucin by Wolfenden[7]. The rhomboidal crystals had reducing power and, furthermore, contained nitrogen. The observation of the presence of nitrogen was confirmed a year later by Hammersten[8] in two out of three samples of polysaccharide.

Still, on the basis of prejudice rather than evidence, the carbohydrate present in mucins was thought to be a single sugar type (for example, glucose). Further progress in identifying the carbohydrate had to await the discovery of the structures of the simple sugars by the chemists of 1884-1900, notably Emil Fischer. During this time mucin was regarded "as a conjugated single compound consisting of a moiety with all the properties of a genuine protein and a moiety released as carbohydrate"[5]. Eichwald referred to mucin as "glycoproteid" on several occasions.

In 1901 Müller[9] proved hexosamines to be a key component of mucins. Another important contribution to the knowledge of the structure of mucins was the description by Bywaters[10] in 1908 of the ease with which mild alkali treatment releases the carbohydrate in the form of a polysaccharide precipitable by alcohol. Scant work was reported on neutral glycoproteins during the next thirty years, with the exception of Rimington's[11][12] "The Carbohydrate Complex of the Serum Proteins" II Improved method for isolation and redetermination of structure. Isolation of glucoamino dimannose from proteins of ox blood",and of the French scientists' references to protein-bound sugar in the blood plasma[13][14][15].

Instead,much research was done on the acidic mucopolysaccharides of connective tissues such as chondroitin sulphate. Krukenberg[16] first isolated chondroitin sulphate in 1884 and in pure form in 1889 by Morner[17].

The name chondroitin sulphuric acid was conferred upon this material isolated from hyaline cartilage by Schmeideberg[18]. His paper "The Chemical Composition of Cartilage" showed that sulphuric acid was joined to "chondroitin" and that "chondrosin" was the most stable fragment of chondroitin sulphuric acid. The chondrosin was conceived as a reducing disaccharide consisting of glucuronic acid and hexosamine. This hexosamine thought to be

D-chitosancine was shown by Levene and LaForge[19] to be the isomer called "chondrosamine". These workers also isolated D-glucuronic acid from chondroitin sulphate, thus proving Schmiedeberg's suggestion.

Levene held that "the formulation of the mode of union between carbohydrate and protein is simple. Alkali too low to bring about a disruption of the protein molecule, or a disruption of the carbohydrate radical, was sufficient to sever the union between protein and carbohydrate. Thus the simplest assumption is that the union is in the nature of an ester linkage."[20]

Mucoproteins of the chondroprotein type were also found in tendons, aorta and sclera. It seemed to Levene that the carbohydrate of all mucoproteins was conjugated with sulphuric acid[20]. Further he assigned to the carbohydrate a tetrameric structure consisting of hexosamine, glucuronic acid, acetic acid and sulphuric acid in equimolar proportions[20]. Levene generalized that in mucoproteins not of the chondroprotein type the carbohydrate had an analogous structure in which chitosamine replaced chondrosamine. These mucoproteins were labelled 'mucosin' and the carbohydrate moiety 'mucoitin sulphuric acid'. These mucoitin sulfates were to be found in the secretions of mucous membranes, in umbilical cord, vitreous humour, cornea and the mucoids, seromucoid and ovomucoid[21].

Levene soon disproved his own concept of a universal carbohydrate structure for mucoproteins. In 1927 Fraenkel and Jellinek [22] had used, of necessity, strong alkali conditions to obtain a protein-free carbohydrate (10% $Ba(OH)_2$, 100°C, 7 hours). Levene and Mori [23] confirmed the view of these previous workers that there existed "an intimate union between protein and polysaccharide, one more intimate than that of ions of an ionizable salt".

The discovery in the medical world of the agglutinability of human red blood cells by virtue of an antigen mucosubstance (glycoprotein) embedded in the cell wall and an antigenic receptor (antibody) present in certain sera, once again diverted researchers away from acidic mucopolysaccharides.

Landsteiner was the first to discover the serological blood group characters (neutral glycoproteins) of human erythrocytes in 1925 [24][25][26]. However, only the recognition of the existence of group specific macromolecules in epithelial secretions such as saliva, gastric juice and ovarian cyst fluid, rendered it possible to elucidate the structure of the glycoprotein underlying the specificity[27].

Another major stimulus in the study of mucoproteins such as submaxillary mucin and neutral mucopolysaccharides was the discovery of influenza virus haemagglutination and its inhibition by mucins of epithelial origin[28][29][30][31].

As late as twenty years ago the knowledge of the basic structure of mucosubstances was scanty. Hampered by the lack of detailed structural data it is no wonder that nomenclature suffered particular difficulties.

Nomenclature

The term "mucus" as applied to mucopolysaccharides, mucoproteins, mucin, - these latter names themselves being widely applied by various authors - is of venerable origin meaning no more than a viscous fluid of biologic origin. Moreover, the mucosubstances have been classified and reclassified according to source and to sampling technique. The inevitable spawning of multitudes of names prompted Glass(32) to outline mucosubstance in a system as follows:

I. Aminopolysaccharides, Mucopolyuronides, Glycosaminoglycans

1. Chondroitin sulfates (N-acetyl-D-galactosamine, D-glucuronic or L-iduronic acids, sulfates.

2. Heparin (Alpha) (Glucosamine, glucuronic acid, sulfates)

3. Hyaluronic acid (N-Acetyl glucosamine, uronic acids)

II. Mucopolysaccharides (mucoids, glycoids)

1. Fucomucins (protein, L-fucose, N-acetyl glucosamine, N-acetyl galactosamine, D-galactose).

2. Sialomucins (protein, sialic acid, N-acetyl-galactosamine).

III. Mucoproteins (glycoproteins)

1. Serum proteins (α-globulins, γ-globulin)

2. Di-hexose-hexosamine type: (serum mucoprotein) (protein, mannose, galactose, hexosamine)

3. 'Glandular' mucoprotein (protein, uronic acid, hexosamine, sialic acid (?), sulfates).

General Structural Aspects of Muco-Substances

Mucosubstances are rather complex high molecular weight materials containing carbohydrate and protein, and in some instances lipid [33], held together by various types of linkages[34]. Glycoproteins pose, of course, all the problems which are encountered, both in the field of carbohydrates and in that of proteins. Moreover, the combination of carbohydrate and protein joined together by covalent bonds in the same macromolecule creates certain specific analytical problems to be met with specific treatments and careful interpretation.

The physical and biological properties of the macromolecules are conferred upon them by their chemical structures. Inter and intra-molecular interactions in biological macromolecules can be classified into hydrophobic, interionic and hydrogen bonding interactions. Hydrophobic bonding, being the association of non-polar chain groups, includes the attractive, orientation, polarization and dispersion forces and the important entropic contribution due to the decrease in the ordering of water molecules surrounding the associated non-polar groups. Such non-polar groups in glyco-

proteins include the non-polar hydrocarbon side-chains of amino-acids of the protein moiety: tryptophan, phenylalanine, proline, methionine, isoleucine, leucine, valine. Interionic interactions include site-site interactions due to charge fluctuations and cation-anion associations involving uni or multivalent external ions and/or charged side-groups of a biopolymer. Polyelectrolytes with a preponderance of anionic groups include the acidic glycosaminoglycans and the glycoproteins containing sialic acid. The presence of the sialic acid units in the carbohydrate complicates the interpretation of sedimentation, viscosity and electrophoresis experiments. The protein moiety of the glycoprotein contains amino acids which are capable of forming hydrophilic bonds through charged polar side-chains, namely aspartic acid, glutamic acid, lysine, arginine and histidine. Hydrogen bonding associations can be subdivided in proteins into interpeptide, peptide - side-chain, and inter side-chain interactions. Also, the numerous hydroxyl groups of the sugar residues will interact with the water molecules of the solvent, probably through hydrogen bond formation, giving the macromolecule a larger effective volume than might be indicated by the molecular weight.

It is well known that polypeptides may assume an α-helix, β-pleated or "random coil" formation and thus take on the secondary and tertiary structures of globular proteins. In the case of

synthetic polysaccharides, coiling also occurs[35], the large degree of flexibility of carbohydrates being limited in some polysaccharides by the presence of sulphated monosaccharide and hexosamine electrostatic interactions.

In some macromolecules that have a high proportion of carbohydrate the structure of the carbohydrate chains will impose an architecture on the molecule nullifying the polypeptide forces. Creeth and Knight[36] have recently studied the physical properties of the blood-group specific substances. From the model they proposed of a highly flexible macromolecule, in which the polypeptide does not retain any tertiary or extensive secondary structure, it is apparent that the carbohydrate dominates the character of this glycoprotein. Similar flexible models or random coil structures have also been proposed for bovine cervical glycoprotein[37][38].

Three-dimensional aggregate structures composed of many macromolecules are common in biology. Witness the polysaccharides cellulose and chitin. These substances have no cross-links but rather are held together by the interlacing of long filaments. The effectively rigid structures are maintained by non-covalent bonds[35]. In glycoproteins, the protein moiety is thought to form a backbone (non-polar) which allows the externally oriented hydrophilic, polar oligosaccharide chains to interdigitate with those of other macromolecules. Thus, the glycoproteins form

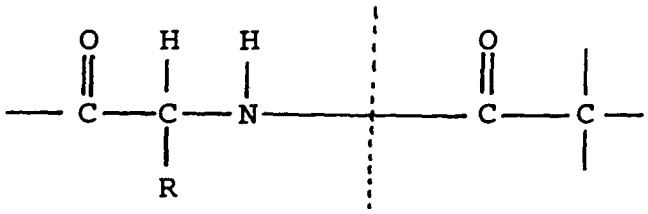
gums, slimes and gels variously noted as viscous, sticky, adhesive or cohesive. Indeed, in some parts of the world, surface mucus from fish has been used as a cement in the construction of large domes[39]. In proposing a structure for chondroitin sulfate - protein complex, Partridge[40] took into account the inter-molecular interactions which enforce aggregates.

An obvious display by glycoproteins of their viscous, slippery nature is the protection of animal surfaces (by lubrication). This is but one of the biological functions identified with glycoproteins. Amongst other activities attributed to the mucinous secretions of the gastric mucosa alone are those of an anti-coagulant, a lipid absorption promotion factor, a histamine binding factor and a lipotropic factor. The secretions are also active in the inhibition of viral haemagglutination and show blood-group antigenicity (A,B,H,Le^a)[32].

The structure of glycoproteins is not only determined by the various non-permanent forces described earlier, but also by the covalent bonds between protein - protein, carbohydrate - carbohydrate and protein - carbohydrate. These comprise the branch points and cross-links within a molecule: the monosaccharides may be arranged in branched as well as linear structures, two peptides may be joined together, or one doubled back on itself, (by disulphide bonds through cysteine residues, or by glutamyl-lysine cross-links[41]). The nature of the carbohydrate-protein linkage is of vital importance to the structure

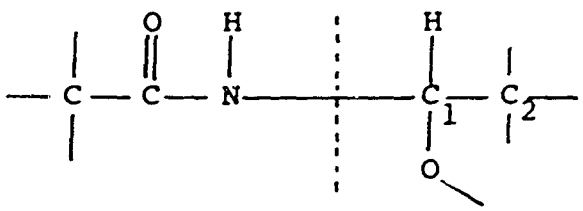
The type, number and position of these linkages in many glycoproteins have been the subject of much research and remain to be the subjects for much more. The protein - carbohydrate linkage may be one of several types[42].

1. <u>True Peptide Bonds</u> : such as exist between the carboxyl from muramic acid (an analogue of neuraminic acid)and the free amino group of N-terminal alanine in bacterial cell walls. This type of bonding has not yet been found in mammalian material.



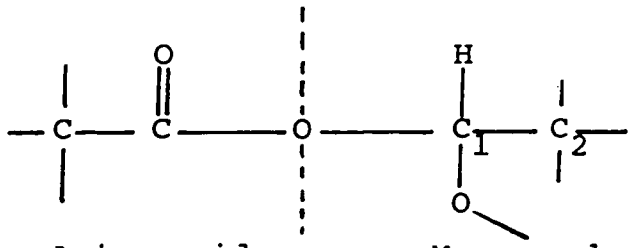
Amino Acid Muramic Acid

2. <u>N-acyl-glycosylamine bond</u>: (<u>N-glycosidic bond</u>): formed by the elimination of a molecule of water between the glycosidic hydroxy group (at C_1) of the initial amino-sugar (N-acetyl glucosamine) of the oligosaccharide chain and the amide group of the amidated free carboxyl of either aspartic or (more rarely) glutamic acid of the polypeptide material. This linkage occurs in α_1-acid glycoprotein[43], ribonuclease B, ovalbumin and some plant glycoproteins[44]. The linkage is stable to acid and alkali and the proteins concerned are usually globular.



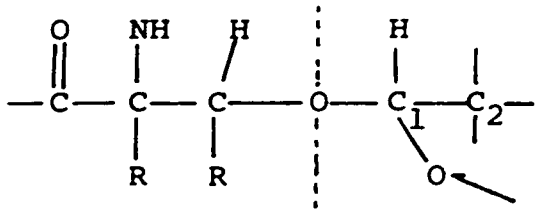
Amidated amino acid Monosaccharide

3. <u>True ester bonds</u> : formed by the esterification of the initial sugar residue (in this case C_1 hydroxyl from N-Acetyl galactosamine) by the terminal carboxyl group of the amino acid. This type of bond has been suggested for the submaxillary gland mucins of ox and sheep[44][45].



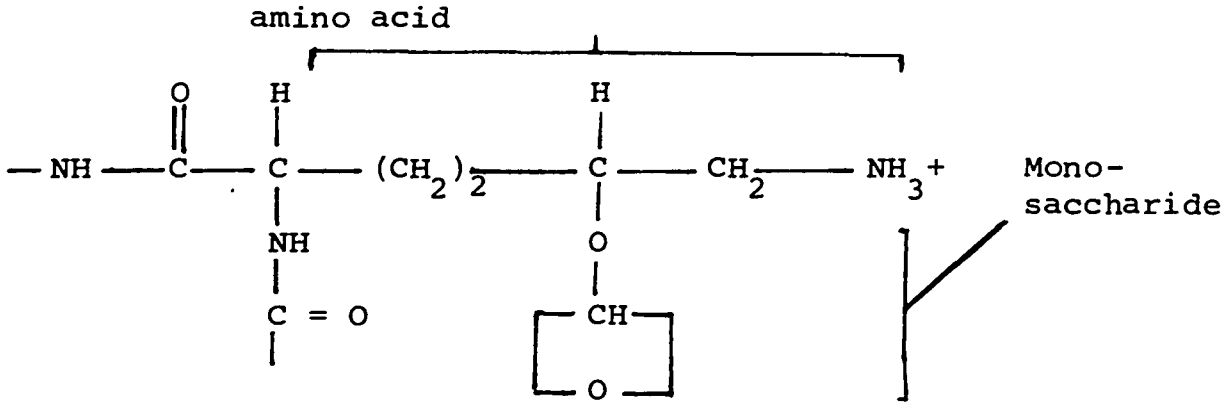
Amino acid Monosaccharide

4. <u>O-glycosidic bonds</u> : formed between the C_1 of the monosaccharide and the hydroxyl of serine or threonine amino acid residue. This type of bond has been found in acidic mucopolysaccharides, submaxillary gland glycoproteins, in the mucoid of colloid breast carcinoma and in the blood group specific substances[43][46][47][48][49][50][51] and in low sulfate heparin of the aorta. The linkage is labile to alkali.



Hydroxy amino acid Monosaccharide

5. <u>α-Hydroxy proline - carbohydrate linkage</u> : found in plant cell walls and collagen[44][52], or hydroxylysine-carbohydrate linkages. Clearly these are limited to those glycoproteins which have these unusual aminoacids.



A given glycoprotein is <u>not</u> limited to having a single type of linkage[53][54][55]. For example, an A-myeloma globulin (type K) from serum contains a glycopeptide fragment in which there is no serine or threonine but only aspartic acid. This strongly suggests an N-glycosidic (type 2) bond. A second glycopeptide, mainly serine and threonine, has been shown to have o-glycosidic (type 4) bonds[54].

The most common sugar residue to be linked with the amino acid of carbohydrate - protein linkages has been galactosamine (N-acetyl galactosamine)[53][54][55][56]. In some instances mannose[53], arabinose[52], and xylose[57] have been implicated. The question of which sugar residue is involved in the protein - carbohydrate linkage(s) in <u>human gastric gel mucin</u> has not to date been investigated. This important information could be obtained

by an analysis with gas liquid chromatography of the oligosaccharides produced by the action of enzymes or alkali on the linkage.

The first linkage to be definitely established, in which N-acetyl glucosamine forms a secondary amide with L-asparagine (type 2 N-glycosidic bond), was found in ovalbumin by Marshall and Neuberger[58]. In glycoproteins with this linkage the number of heterosaccharide units per total number of amino acid residues tends to be low. The macromolecules have in general the physical properties determined by the peptide moiety. As stated earlier, this bond is stable to acid and alkali.

From data derived from bovine and ovine submaxillary mucins[44][59][60] Gottschalk suggested that these were true ester bonds (type 3) joining aspartyl or glutamyl residues to the carbohydrate. He believed these ester bonds were responsible for up to 80 percent of all protein - carbohydrate linkages in the submaxillary mucins.

The first reports of an O-glycosidic linkage (type 4) involving the hydroxyl group of serine concerns a bacterial glycoprotein from Aspergillus orgazae[61][62]. In 1963 Blix[63] reported the O-glycosidic linkage to be present in submaxillary mucin. This report was confirmed a year later by Tanaka and Pigman[64][65]. The molecular weight of glycoproteins which have this type of linkage is usually of the order of several millions. The number of heterosaccharide residues per total

number of amino acid residues is very high and solutions of these proteins are very viscous. A characteristic property of this linkage is its considerable lability to alkali (greater lability than that of type 3), even in the cold[64][65]. The removal of the sugar residue in alkali is not a hydrolysis but a cleavage facilitated by a migration of electrons, the so-called β-elimination reaction. This reaction will be dealt with in greater detail on page 35 . Further evidence for the existence of this type of linkage has been obtained by enzymatic cleavage of the carbohydrate from the glycopeptides[66].

Finally it should be noted that the α-hydroxyl-lysine bond (type 5) in contrast to the O-glycosidic bond (type 4) is not expected to be alkali-labile as there is no carbonyl group in a β-position to facilitate a β-elimination reaction (page 35).

Partially Defined Mucosubstances (Glycoproteins)

Amongst the number of authoritative reviews that have been published concerning the structure and function of glycoproteins are:

R.G. Spiro (1963) Glycoproteins structure, metabolism and biology. New England Journal of Medicine 263, p.566

A. Gottshalk (ed.) (1966) Glycoproteins: Their Composition, Structure and Function. Elsevier Publishing Co. Amsterdam, London, New York

N. Sharon (1966) Polysaccharides (glycoproteins) Annual Review of Biochemistry, 35, p.507

The glycoproteins which have received the most attention are ovalbumin, α_1-acid glycoprotein, submaxillary gland mucins, blood-group specific substances, thyroglobulin and several immunoglobulins. For the purpose of comparison, the history and structure of the submaxillary mucins and of the blood-group specific substances will be discussed along with that of human gastric gel mucin.

1. Submaxillary Mucins

The submaxillary glands, in ruminants, are the main secretory organs of the viscous component of saliva. Purified submaxillary glycoproteins have been isolated from the glands of sheep (OSM), cattle (BSM) and pigs (PSM) using two principal methods[56]. That employed by Gottschalk and coworkers[67] (g)* consists of aqueous extraction of the minced glands, followed by pH and methanol fractionation. In the other, that of Pigman (p)* and coworkers[68][69][70], the glycoprotein is separated from aqueous extracts of the glands at pH 3.5 as a mucin clot or is precipitated by a cationic detergent. As a result of investigations in a number of laboratories, a concept of the structure of these glycoproteins was substantially agreed upon. As put forward by

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* (g) denotes isolation method of Gottschalk

(p) denotes isoltation method of Pigman

Gottschalk in 1952[29], the glycoproteins are best visualized as conjugated proteins consisting of a polypeptide chain (core, backbone) to which relatively small polysaccharides are linked as individual groups. In the case of submaxillary mucins, these small polysaccharide branches are known to be disaccharides of sialic acid joined to N-acetyl-galactosamine[56].

Comparatively little is known about the sequence of amino acids in the peptide moiety and there are divergent views about the nature of the disaccharide - peptide linkage. Purified OSM(g) was obtained as a viscous material sedimenting as a single component of molecular weight $1.0 \pm 0.2 \times 10^6$ daltons and consisting of about 42% (weight) carbohydrate and 58% (weight) peptide. When it was heated for twenty minutes at 100°C with very dilute acids, or merely in water, sialic acid residues (identified as N-acetyl-neuraminic acid) were liberated quantitatively and accounted for 25% (weight) of the whole composition of the glycoprotein. Trace amounts of N-glycollyl neuraminic acid were present in the hydrolysate. The sialic acids could be enzymatically cleaved from the structure by the action of neuraminidase from Vibrio cholerae or Clostridium perfringens. The principal structure of the macromolecule appears to be unaltered by the removal of the sialic acid residues, the terminal sugar units then being N-acetyl-galactosamine. These are susceptible to the action of N-acetyl-β-hexosaminidase.

Treatment of the intact OSM(g) with barium hydroxide (0.2%) at 80°C for ten minutes liberated a reducing dissacharide with the structure (∝-N-acetyl neuraminidyl)-(2 → 6)-N-acetyl-galactosamine) in yields which accounted for some 40% of the whole glycoprotein.

On this basis, a structure has been proposed for the glycoprotein in which 600-700 disaccharides are attached to the peptide chain, an average of one for every 800 m.w. units or one for every 6.4 amino acids of the peptide chain. In the early stages of the investigation Gottschalk[44][60][71] had suggested that the disaccharide linkage to the peptide was an ester linkage (Type 3 of page 3), whilst not excluding the existence of o-glycosidic linkages (type 4). The three lines of experimental evidence for this view were:

1. The carbohydrate - peptide bond was sensitive to alkali (88% "hydrolysis" by 2N NaOH at 100°C for ten minutes).
2. Hydroxylamine at pH 12 and 37°C brought about cleavage of the bonds with the formation of hydroxamates.
3. Lithium borohydride caused a reduction in the presence of tetrahydrofuran, with the conversion of the amino acids into the corresponding β and γ alcohols.

Results of other workers[68][69][70] investigating the structure of BSM(p) did not support this view. Hashimoto, Pigman and co-workers[69] isolated a glycopeptide in 16% yield from pronase (Streptomyces griseus) digests of BSM(p). The product contained serine and threonine in ratios of 0.56 and 0.58 to the sialic acid and galactosamine residues. The values for aspartic and glutamic acid were 0.05 and 0.18 respectively, and no other amino acid residues carrying functional groups were detected. The number of aspartic and glutamic acid residues was insufficient to provide the necessary linkages.

Tanaka and Pigman[64][65] later reported the reductive cleavage of mucin by alkaline sodium borohydride. The decrease in serine and threonine amino acid residues and the consequent increase of alanine amino acid residues and the presence of α-amino butyric acid, an amino acid residue normally absent, indicated that a β-elimination of the O-seryl and O-threonyl glycoside bonds had occurred.

Therefore it was apparent that in BSM(p) disaccharide chains are, to a considerable extent, attached as O-glycosides of serine and threonine.

Later work by Gottschalk and co-workers[72] confirmed the O-glycosidic (type 4) linkage for OSM(g). However, in the earlier work on OSM(g), the glands of four-month old lambs

(Australian) were used. Later the work was done with glands from three-year old sheep (German). Age dependent changes can be ruled out only after more investigations. It is also pertinent to note that differences exist in the fractionation of BSM(g) and BSM(p). Immunochemically BSM(p) has at least two distinguishable components while BSM(g) has at least three.[73]

The amino acid composition of BSM(p) and BSM(g) alike is characterized by a high content of serine, threonine, glycine and proline.

The presence of sialic acid residues in submaxillary mucins confers strongly anionic properties on the macromolecule. At physiological pH values these strong acids are completely ionized, so that ion repulsion contributes to the maintenance of an extended conformation.

A diagram of the linkage between the disaccharide and peptide appears in the appendix.

2. Blood-Group Specific Substances

The blood-group substances are soluble fucomucins in saliva, gastric juice, pancreatic and biliary secretion and in ovarian cyst fluids. They bear the ABO(H) antigenicity of the same specificity as the erythrocytes and other cell bodies of the individual. Probably the purest of the blood group

substances isolated are those from ovarian cyst fluid[74]. Analysis shows that such preparations of A, B, H and Le^a substances are glycoproteins of very similar qualitative composition. Each consists of about 15% peptide and 85% carbohydrate, the latter being comprised of D-galactose, L-fucose, N-acetyl glucosamine, N-acetyl galactosamine with some sialic acid.

TABLE I

Composition of Blood-Group Substances (%)[75] isolated from human ovarian cyst mucins

Blood-Group	A	B	H	Le^a
Nitrogen	4.9	4.8	5.0	4.9
Fucose	20	18	19	13
Hexosamine (as glucosamine after acid hydrolysis)	32	33	28	29
Reducing sugar (as glucose after acid hydrolysis)	51	50	50	56

The composition of the peptide portion of the blood-group substances is always closely similar, in that in all cases two-thirds is comprised of serine, threonine, proline and alanine.

TABLE II

Amino Acid Composition of Human Blood-Group Specific Substances isolated from Human Ovarian Cyst Fluids[76]

Amino acid	Blood Group of Specimen A (4 samples)	B (3 samples)	H (5 samples)	Le^a (3 samples)
Aspartic acid	3.2- 4.0*	3.7- 4.8*	2.6- 4.1*	2.7* 4.3
Threonine	24.7-26.4	25.9-28.0	28.8-31.0	27.0-28.5
Serine	16.7-17.6	15.6-17.6	17.3-20.1	14.7-17.0
Glutamic acid	4.3- 4.6	4.0- 4.3	3.3- 5.0	3.6- 5.6
Proline	13.1-13.9	14.2-14.6	11.3-14.4	12.6-14.3
Glycine	5.5- 6.6	5.8- 6.6	4.7- 5.5	5.8- 6.6
Alanine	8.5-10.4	10.0-10.9	8.1-10.3	8.8-10.6
Crystine(half)	-	0 - 0.7	0.5- 1.3	0 - 0.9
Valine	4.6- 4.8	4.3- 4.7	4.0- 4.5	4.6- 5.5
Isoleucine	2.1- 2.6	1.9- 2.0	1.8- 2.6	1.6- 2.4
Leucine	2.4- 3.2	1.9- 2.1	1.5- 2.7	2.3- 4.8
Tyrosine	0.3- 0.6	0 - 0.4	0 - 0.7	0 - 0.7
Phenylalanine	0.8- 1.4	0.7- 1.4	0.6- 1.0	0.7- 1.4
Lysine	1.4- 2.3	1.4- 1.9	1.4- 1.8	0.9- 2.1
Histidine	1.3- 4.8	2.1- 2.6	1.1- 2.3	2.1- 2.8
Arginine	2.9- 3.2	2.8- 3.0	2.3- 2.8	2.6- 3.1
TOTAL amino acids	13.15-15.65+	10.41 -16.35+	13.92 -26.45+	7.35 -16.36+

* expressed as μmoles of amino acid per 100 μmoles of total amino acid

\+ expressed as grams of amino acid per 100 grams of blood-group substances

The sialic acid content of isolated blood-group specific substances is variable (generally up to 2%) and the removal of sialic acid under mild conditions does not affect the blood-group reactivity.

The blood-group specificity lies within the terminal oligosaccharide chains. The structures determining the specificities have been discovered by using the four following techniques[50][77][78][79]:-

1. immunochemical inhibition studies
2. partial acid hydrolysis followed by identification of the resulting di, tri and oligosaccharides (by gas or paper chromatography of their derivatives).
3. reductive alkaline cleavage (β-elimination reaction) followed by a study of the amino acid and carbohydrate products.
4. selective enzymic degradation.

The oligosaccharide structures are shown below:

Specifity	Proposed Structures for Chain Determining Antigenicity
A	N(Ac)GAL - GAL - N(Ac) GLu - GAL - N(Ac). GAL - O - | Fucose
B	GAL - GAL - N(Ac) GLU - GAL - N(Ac) GAL - O - | Fucose
H	GAL - N(Ac) GLU - GAL - N(Ac) GAL - O - | Fucose
Le^a	GAL - N(Ac) GLU - GAL - N(Ac) GAL - O - | Fucose

N(Ac) GAL = N-Acetyl galactosamine
GAL = Galactose N-Ac GLU = N-acetyl glucosamine

The oligosaccharides are known to be joined to the protein by O-seryl and O-threonyl glycosidic linkages in molecules with a molecular size ranging from 300,000 to several million[78][79][80].

Recently Kochetov and co-workers[81] have presented evidence that the N-acetyl hexosamines of the peptide-carbohydrate linkage bear no branch-points. In the model they developed for the blood-group substances, the structure of the oligosaccharide chain, at residues further removed from the peptide backbone, is highly branched.

3. Gastric Mucosubstances

Following a suggestion of Webster and Komarov[82], it has been agreed to distinguish between visible mucins (that is, the glistening layer adhering to the mucosa) and the dissolved mucus of the stomach contents. The study of these materials was put on a firm basis by the work of Glass and Boyd (1949-1954)[83][84][85][86] but no single chemically and physically homogeneous gastric mucin could be claimed to have been isolated at that time[32][87][88].

There are several classes of mucosubstances in the gastric secretions. The largest percentage of gastric juice components is the group of fucomucins abundant in the periodic-acid Schiff (PAS) positive peak of the electrophoretic fraction (neutral) and the sulfated polysaccharides present in the fastest moving of the electrophoretic fractions upon paper electrophoresis[89].

The former material is presumed to originate from the breakdown of visible mucus[90]. The polypeptide moiety set free during this mucolytic process is thought to migrate as part of the peaks remaining near the origin of the paper electrophoretogram.[91][92] The gastric juice contains, besides visible mucus, other neutral glycoproteins (dissolved), some sulfated glycoprotein and enzymes.

The secretions of the gastric mucosa represent the combined activities of six histologically distinguishable cell types secreting more or less independently at varying secretory rates. The complexity of the macromolecular components of the gastro-intestinal tract has been considered by Horowitz[93]:

Overview : A comparison of proteins, glycoproteins, mucopoly-saccharides, polysaccharides, with respect to protein and carbohydrate content:

The relative position of mucin in the realm of these molecules is indicated in the following spectrum:

	% Carbohydrate Content *	
	.0	Hemoglobin, insulin
Glycoproteins	10	19 s γ-Globulins
	20	3 s α_2-Glycoproteins
	30	4 s α_2-β_1-Glycoproteins
	40	α_1- Acid Glycoprotein
Mucopoly-saccharides	50	Human gastric gel mucin
	60	Submaxillary mucins
		Neuramino-glycoprotein
		Protein - acid mucopolysaccharide complexes
	70	
	80	Blood-group substances
	90	
	100	Glycogen

---

* Adapted from K. Schmid in Biochemistry of Glycoproteins and Related Substances, ed. E. Rossi and E. Stall, 1968, Kaiger, Basel, N.Y., p.4

Description of Human Gastric Anatomy as Pertains to the Secretion of Mucus[94]

There are three histologically distinct areas of the stomach:

(i) cardia

(ii) fundus and corpus

(iii) pyloric

The mucosa is furrowed with bulging areas on which the gastric pits or foveolae open. The foveolae serve as ducts to the gastric glands. Two layers of cells are active in the production of mucous:

1. Surface epithelial cells which are columnar cells. Mucigen, the precursor to mucus, fills the cytoplasm above the level of the depressed nucleus.
2. Mucous neck cells of the cardiac pyloric and fundic glands which are rounded or wedge shaped. The cells are generally filled with mucin.

The histological techniques used to distinguish the secretory cells are:-

1. Periodic-acid Schiff Reaction (PAS) [95]

considered specific for the detection of neutral carbohydrates The reaction involves two steps:

(i) The oxidation of vicinal hydroxyl (glycol) groups by periodic-acid to the dialdehydes.

(ii) The conversion of the newly formed dialdehydes into a magenta (red-purple) colour upon addition of Schiff base (leucofuchsin).

2. Toluidine Blue : Metachromatic Staining Technique

This stain is considered specific for acid polysaccharide (polyanions). Metachromatic dyes are bound to negatively charged compounds forming electrostatic links between the polar groups of the substrate and the dye molecule[96].

3. Alcian Blue is a textile pigment (a copper phthalocyanin derivative) used to stain weakly acidic groups such as carboxyl groups. Although sialic acid is itself alcian blue positive, it has been shown that chrondroitin sulfates and heparin react only through the glucuronic acid carboxyl groups.[96] The dye is thought to bind through electrostatic linkages[97]. Quintarelli and co-workers[98] were able to demonstrate that the dye staining reaction was pH dependent. Since alcian blue has at least two, and up to four, cationic solubilizing groups, these groups would satisfy an equivalent number of negative charges in a tissue with but one dye(chromophore) molecule binding through the copper phthalocyanin and give rise to frequently obtained poor (light) staining.

The visible mucin, having accessible vic-hydroxyls in hexose residues, and mucus producing cells, give a positive periodic-acid Schiff stain. Thus the surface epithelial cells and the mucous neck cells stain red-purple. The deep foveolae cells and some neck cells are responsible for the more acidic mucins.[99][100]

General Biosynthesis of Glycoproteins

In 1888, Goblet cells (intestinal epithelium) were thought to be cells in which the nucleus had become one huge droplet of secretion[101]. Since then, it has been shown by Leblond and others that glycoproteins are synthesized in stages — protein first followed by the attachment of oligo- or polysaccharides before secretion. This synthesis involves the golgi complex[102][103][104]. There is some evidence to support a concept of "one enzyme joining one linkage" in the biosynthetic network[105].

A competition between enzymes which link the monosaccharides together and between enzymes which attach groups such as acetyl and sulfate groups, would account for the observed heterogeneity of the carbohydrate side-chains of many glycoproteins[106][107].

It has been difficult to isolate the granules of secretion (mucigen) before they leave the cell because the cells that elaborate it tend to change soon after death and fixatives tend to disrupt the droplets[108]

Much work has been carried out to determine the possible pathways for the biosyntheses of the blood-group substances and the genetic control of these pathways[77][78]. However, the prerequisite knowledge of the total structure (sequence and geometry) of these molecules and isolation of the enzymes involved in their construction have yet to be achieved.

Report of Isolation of a Single Glycoprotein Component from Gastric Juice

It is reasonable to assume that gastric juice contents will reflect the dynamic state of the mucosa, its health or ill-health. However, studies done by Hoskins and Zamcheck[109] in order to correlate the composition of the gastric juice with various diseased states were unsuccessful because the 'non-dialyzable' fraction investigated was not a single well-defined fraction. Furthermore, this 'fraction' represents a stage in the degradation of the mucosal layer of mucus and not the functional layer which is a three-dimensional insoluble gel.

In 1964, Waldron-Edward and Skoryna[110] reported the isolation and partial characterization of a macromolecular component from the ropey threads as well as the masses of gel-like "visible" mucus from gastric juice. The component was isolated from gastric juice, neutralized in situ by the instillation of phosphate buffer. In its native state the gel-like substance acted as a single entity on paper electrophoresis.

This human gastric gel mucin (HGGM) was a high molecular weight fucomucin (greater than 2×10^6 daltons) with high blood-group activity and having a composition of 30% polypeptide and 55% carbohy drate and 1% sialic acid. Human gastric gel mucin stained intensely PAS positive. It is likely that the material described by the authors as 'gel mucin' represented the main component of

'visible mucus'. It is not clear by what means the mucin layer on the mucosa becomes the closely related visible gel mucus, then to be degraded to smaller soluble fractions of the gastric juice.

Function of Gastric Gel Mucin

In the past decade there have been many efforts to establish a correlation (qualitative and quantitative) between variations in gastric mucous secretions and diseased states (or drug and chemical stimulation).[109][111][112]. An impetus to this level of research was the finding that people of the blood-group specificity "O", non-secretor, are significantly more susceptible to peptic ulcers or gastric cancer[113].

The concept that gastric mucus may protect the gastric membrane from injury (or from auto-digestion) dates from Claude Bernard's anology of the mucus lining of the stomach to a "vase imperméable", but it is still not known how the mucin makes the mucosa invulnerable. There appear to be several levels of defence:

1. It may well be that mucin functions intracellularly to protect the mucosa. Leblond[114] has shown that there is a rapid turnover of epithelial cells, and further, that the turnover within the cells is so large as to mask the disappearance of mucin into the lumen. In this way the mucosa would be kept separated from the stomach contents of enzymes and acid.

2. Heatley[115][116] suggested a more detailed view of how mucus could function as a "mucus barrier" (a term first applied by Hollander[117]) to diffusible ions. It is known that mucin, an almost neutral glycoprotein, has little or no capacity to neutralize or buffer H^+ (acid) of the gastric lumen. Thus the soluble or degraded mucus cannot neutralize the acid before it comes in contact with the gel surface. Nevertheless, the mucin must protect the membrane from the gastric acid. The three dimensional gel structure is important in this respect, for it holds the solution of ions like a sponge. Bicarbonate - containing solutions, possibly exuded from epithelial cells, would then form a concentration and pH gradient toward the lumen. This gradient would act as a chemical barrier to acid attack on the membrane itself.
3. The tenacious mucin layer on the mucosa is a physical wall to abrasion. Specifically the mucin is a three-dimensional gel structure of many hydrated macromolecules extending over the whole surface. The mucin gel is comparable to the cross-linked dextrans of gel filtration systems. Small ions such as protons (acid) and dyes (picric acid) penetrate the gel. Large molecules such as the proteolytic enzymes are excluded. A continual renewal of the gel mucin from the mucosa cells would result in a dynamic physical barrier to the onslaught of enzymes, bacteria and drugs[118].

A difference in chemical structure strategically located within the macromolecule would result in a breakdown in the protective capacity of the mucin.

Experimental Design

Before a working model of gastric gel mucin (HGGM) can be postulated, it is essential to know the sequence of amino acid residues, the sequence of carbohydrate residues, the size and number of polypeptide and carbohydrate chains, and the nature of the linkage(s) between the carbohydrate and protein. The major object of this research, a study of the structure of gel mucin, has been to ascertain the nature of the protein - carbohydrate linkage(s).

The β-elimination reaction is of diagnostic value in determining the type of linkage to be O-glycosidic (Type 4) (47)(48)(49)(64)(65)(72). It is possible to recognize that the β-elimination reaction has occurred by the following criteria:

1. An amino acid analysis of the acid hydrolysate of the reaction products shows a specific decrease in serine and/or threonine amino acid residues.
2. Chromatographic analysis or enzymatic assay of the acid hydrolysate demonstrates the presence of α-keto acids (pyruvic acid and α-keto butyric acid).
3. An amino acid analysis of the borohydride-reduced, acid hydrolysed, reaction products shows an increase in alanine

amino acid residues and the presence of the unusual amino acid residue α-amino butyric acid.

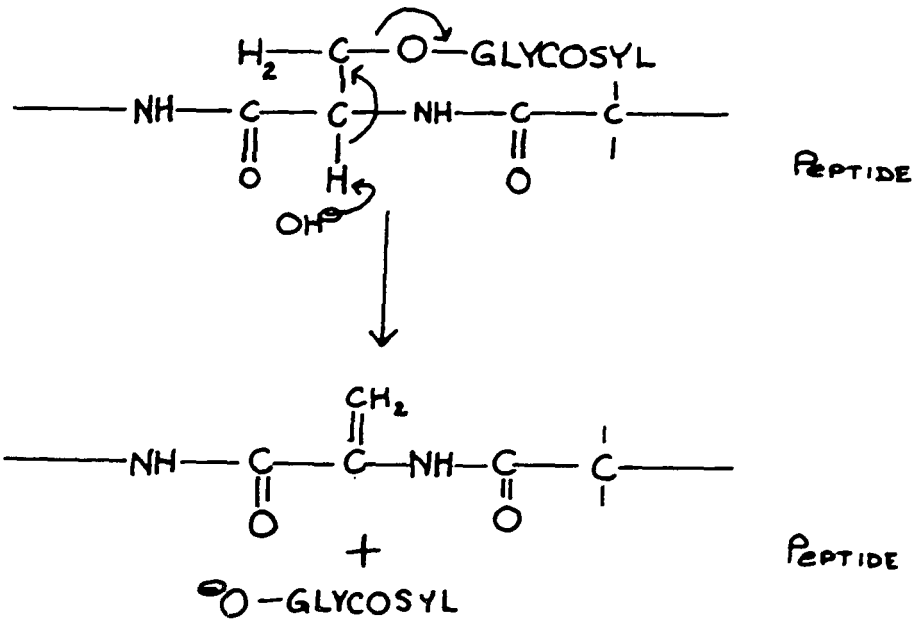
4. Gel filtration of the reaction products shows products which have a substantially smaller molecular weight than the original glycoprotein.

5. An increase in ultra-violet light absorbance at 241 and 231 mμ *.

The β-elimination Reaction (71)

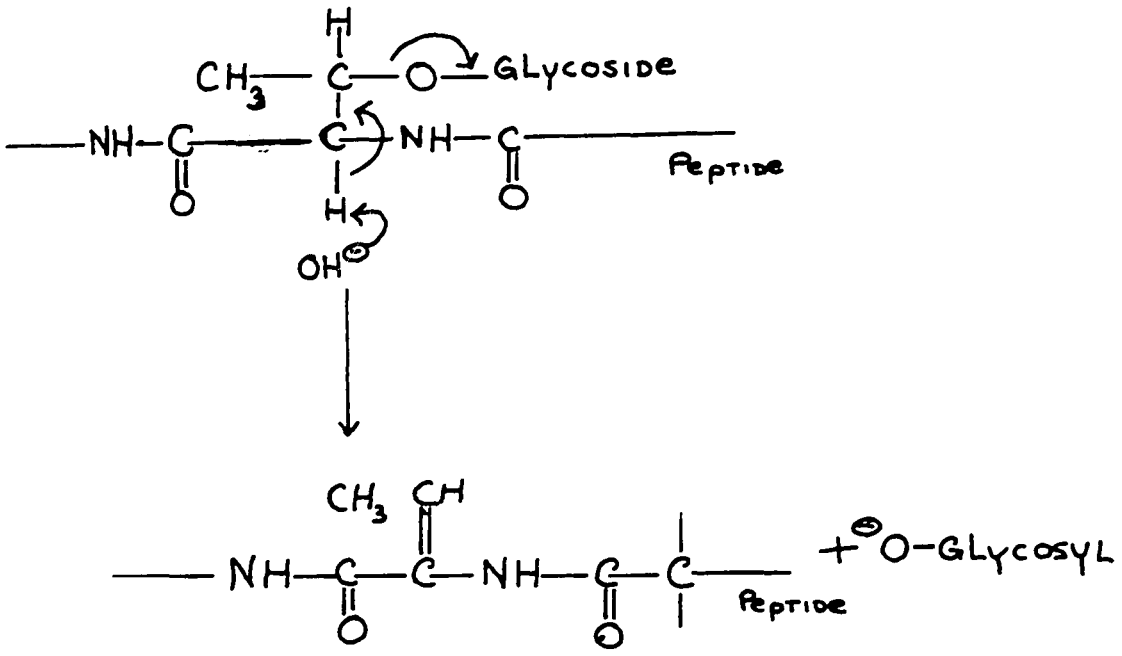
The β-elimination reaction is caused by aqueous alkali. A proton is released from the α-carbon and a nucleophile from the β-carbon forming an α-β double bond with the elimination of the O-glycoside. This reaction is diagrammed below:-

(a) O-seryl glycosidic linkage



* see discussion (page 79).

(b) O-threonyl glycosidic linkage



The development of the unsaturated bond of the newly-formed α-amino acrylic acid and α-amino crotonic acid residues from serine and threonine respectively can be monitored by the increase in absorbance at 241 and 231 mμ [72]. The N-substituted derivative of α-amino acrylic acid derived from serine has an extinction coefficient (ε) = 5300 at 241 mμ . That of the corresponding threonyl derivative has an extinction coefficient (ε) of 6250 at 231 mμ [119].

The β-elimination reaction is not a hydrolysis but a cleavage, facilitated by a migration of electrons. This electronic shift is greatly enhanced by the engagement of the neighbouring amino acid carboxyl groups of the serine and threonine residues

in peptide bonds. The reaction does not occur or occurs very slowly in model compounds in which the carboxyl or amino group is free[120].

The unsaturated amino acids resulting from the elimination of the glycoside are unstable to acid hydrolysis conditions yielding ninhydrin negative α-keto acids. This sequence of events is diagrammed below:

O-seryl glycoside $\xrightarrow{\text{alkali}}$ (α-NH_2 acrylic acid) (unsaturated) $\xrightarrow{\text{acid}}$ Pyruvic acid

O-threonyl glycoside $\xrightarrow{\text{alkali}}$ (α-NH_2 crotonic acid) (unsaturated) $\xrightarrow{\text{acid}}$ α-keto butyric acid

An amino acid analysis then shows the loss of serine and threonine residues resulting from the production of the α-keto acids. If, however, prior to acid hydrolysis the reaction products are reduced (with sodium borohydride) the newly formed amino acids alanine and α-amino butyric acid residues are produced.

Serine $\xrightarrow{\text{alkali}}$ α-NH_2 acrylic $\xrightarrow{\text{1. Reductant}}$ $\xrightarrow{\text{2. acid}}$ alanine

Threonine $\xrightarrow{\text{alkali}}$ α-NH_2 crotonic $\xrightarrow{\text{1. Reductant}}$ $\xrightarrow{\text{2. acid}}$ α-NH_2 butyric

II. MATERIALS AND METHODS

Human Gastric Gel Mucin (HGGM)

Freshly aspirated anacid gastric juice from human volunteers was immediately chilled. The soluble mucoproteins were removed by filtration on fritted glass filters at 4°C as described by Waldron-Edward and Skoryna[110]. The gel mucin was allowed to swell in distilled water and was then lyophilized. The blood-group specificity and secretor status were determined routinely on all donors.

For subsequent analysis the lyophilized gel mucin samples were dissolved in 8M urea for a period of 5 to 7 days at 4°C. At this stage after a short period of exposure to urea, the gel structure is re-established upon removal of the urea[121]. The dissolved mucin was centrifuged on an IEC centrifuge at 30,000 r.p.m. at 20°C for thirty minutes. The surface lipid layer or sedimented debris which was evident in a few cases was removed. The mucin solution thus prepared lacked any specific absorption due to nucleic acid derivatives in the region of 260-270 mμ. The mucin solution was exhaustively dialyzed against distilled water and lyophilized. In one instance mucin (blood-group A, secretor) was partially fractionated on a Sephadex G-200 column[121]. The void volume fraction was used for comparative amino acid analyses.

Human plasma - Fresh human plasma (mixed sample) was supplied to us through the courtesy of the Department of Biochemistry, Royal Victoria Hospital, Montreal, Quebec.

Hog gastric mucin (NNR) was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Labtrol protein standard (LT25YD) was obtained from Dade Reagents Inc., Miami, Florida.

Bovine albumen(twice recrystallized) was obtained from Calbiochem, Monsey, New York; Insulin (Zn-insulin, 40 units/ml) from Connaught Medical Research Laboratories, Toronto, Ontario;

Oxytocin (Sytocinon (L) 15 EO(E) S.1973) from Sandoz, Montreal, Quebec; and Ovalbumin from Worthington Biochemical Corporation through Winley-Morris Company Limited, Montreal, Quebec. (10 i.u./ml. suspension of oxytocin)

Reagents

Iodoacetamide (recrystallized twice prior to use); 5,5' dithiobis -(2-nitro benzoic acid)(Ellman's reagent); dithiothreitol (Cleland's reagent); N-acetyl-galactosamine and N-acetyl neuraminic acid (Type II) were purchased from Sigma Chemical Company, St. Louis, Missouri.

Radioactive C^{14}- 1 iodoacetamide with an activity of 0.05 millicurie (2.9 mg.) was purchased from New England Nuclear, Dorval, Quebec (Lot No. 252-152).

S-Carboxymethyl cysteine and DL (∝)-amino butyric acid were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

The chlorine gas (lecture bottle size) was obtained from Matheson Company Inc. Whitby, Ontario.

Hydrochloric acid (reagent grade) for protein hydrolysis was purchased from Mallinkrodt Chemical Works Limited, Pointe Claire, Quebec.

The Lactic Dehydrogenase enzyme assay kit for pyruvic acid was purchased from Sigma Chemical Company, St. Louis, Missouri.

All other chemicals were reagent grade and purchased from Fisher Scientific Company, Montreal, Quebec.

Sephadex G-200 was purchased from Pharmacia Fine Chemicals Inc. (Canada) Ltd. of Pharmacia, Uppsala, Sweden.

Chemical Assays

Nitrogen determination. The determination of nitrogen was carried out by the Kjeldahl method using a Markham Still (122). The mucin sample size varied from 1 to 3 milligrams. Sulphuric acid hydrolysis was carried out for four hours in the presence of selenium dioxide as catalyst. Titrations were done with either mixed methyl red - methyl blue or methyl red - bromocresol green as indicators. Labtrol was used as a standard.

Fucose (Methyl-Pentose) Determination

Fucose was determined by the Dische and Shettles[123][124] cysteine method. The aliquot size was 1 ml. Concurrent standard determinations were made using freshly prepared or freshly thawed fucose solutions.

Protein Determination

Protein was routinely monitored by the absorbance at 230 mu on a Beckman DU spectrophotometer. Occasionally protein was determined by the method of Lowry[125] using 0.8 ml. aliquots.

Sialic Acid Determination

N-acetyl neuraminic acid was determined by the Warren method[126][127] after mild acid hydrolysis with 0.1 N H_2 SO_4 one hour at 80°C.

Urea Recrystallization

The urea (U.S.P. grade) was recrystallized three times from 80 percent ethanol in order to remove optically absorbing contaminants.

Acid Hydrolysis

Glycoprotein samples were hydrolyzed under reflux conditions with 6N HCl at 107-111°C for twenty-four hours. As a precaution against humin formation, a common destructive reaction which occurs during glycoprotein hydrolysis, the glycoprotein was hydrolyzed at high dilution (at concentrations of less than 0.02 percent)[76]. The volume was reduced rapidly by rotary flash

evaporation (Buchler Instrument) at 35°C. The hydrolysate was taken to dryness from water three times. The final solution was lyophilized.

Samples of mucin which were acid hydrolyzed in preparation for amino acid analysis were taken to dryness twice more from small volumes of water at 65°C in order to achieve a better analytical separation.

Amino Acid Analysis

The soluble residues of acid hydrolyzed mucin were applied to a 75 cm. (Type C resin) column of a Techicon Auto Analyzer and the analysis carried out over a standard five hour run. The pH range of the eluting citrate buffer containing Brij detergent was 2.75 to 6.10. The flow rate was 57 ml. per hour. Norleucine was used as the internal standard.

Glucosamine, galactosamine and α-amino- butyric acid were run with a standard amino acid solution in order to calibrate the times of elution.

β-Elimination Reaction

Glycoprotein solutions of concentrations ranging from 0.04% to 0.6% (by weight) were treated with either 0.1N or 0.2N NaOH (pH = 11.5 - 12.4) in the cold (4°C) for 216 hours. The samples were treated with alkali either in the presence or in the absence of 0.3M $NaBH_4$, a reducing agent. After the 216 hours reaction time the solutions were neutralized with HCl (pH 7.0-7.5). When sodium borohydride was present the solutions

were acidified below pH 5.0 before neutralization. In one experiment the borohydride-reduced, alkali-treated, mucin was hydrogenated for one hour at room temperature in the presence of purified $PdCl_2$ (palladium chloride) catalyst, according to the method of Tanaka and Pigman[65].

Mucin treated with alkali with or without borohydride, was analyzed spectrophotometrically, by gel filtration, or subjected to acid hydrolysis before analysis by amino acid analyzer, paper chromatography, or enzymatic assay. Ovalbumin similarly treated, was analyzed spectrophotometrically or by paper chromatography.

Paper Chromatography

Ascending or circular ascending chromatography of the mucin or ovalbumin hydrolysates and of α-keto acid standards was carried out on Whatman No.1 Chromatographic paper which had previously been 'washed' by a 12 hour "dry run" in the respective solvent systems. The solvent systems used were:-

(i) n-butanol : H_2O : ethanol :: 3 : 2 : 2[128]

(ii) methanol : benzene : n-butanol : H_2O :: 5 : 2 : 2: 2 (128)

(iii) n-butanol (saturated with IN sodium bicarbonate)[129]

(iv) water saturated butanol : formic acid :: 95 : 5 (130)

The solvents were permitted to 'flow' up the paper for periods of 3 to 5 hours. The hydrolysate samples to be applied were taken to dryness by flash evaporation at 35°C until the pH was greater than 3.0 as preliminary chromatograms showed 'tailing' and a large variation in R_f values due to low pH.

Samples were applied with drawn-out pasteur pipettes being careful not to scrape the paper. The standard solutions of 1% α-keto butyric acid (by weight) and 1% pyruvic acid (by volume) were applied twice to the origin. In contrast, hydrolysates dissolved in 2 ml. water (at least 0.7% (by weight) of original glycoprotein)were applied five or six times to the chromatogram.

The chromatograms were visualized after thorough drying, by one of the following methods for free keto acid detection:

1. Exposure to ammonia fumes for 5 to 10 minutes followed by airing for at least thirty minutes or until the background is pale yellow upon dipping in Nessler's reagent[131].
2. Dipping through a mixture of 2% O-phenylene diamine hydrochloride, 2N sulphuric acid and ethanol (in a ratio of 1 : 1 : V/V). The spots are detected by their pale yellow colour and their greenish-white fluorescence under a Wood's ultraviolet lamp[132].

Preparation of 2, 4 dinitro-phenylhydrazone derivatives of α-keto acids

The α-keto acids of glycoprotein hydrolysates and standard solutions of pyruvic and α-keto butyric acids were used to prepare 2, 4 dinitro-phenylhydrazone derivations for paper chromatography. The method of preparation is adapted from that of Kvamme[133] and of Seligson[129].

The hydrolysate residue was taken up in 10 ml. of water. To this solution was added 0.5 ml. of 0.5% 2,4 dinitro-phenyl-hydrazine in 6 N HCl. The mixture was allowed to react at room temperature (30°C) for 45 minutes. The derivatives were then extracted in a separatory funnel with three 10 ml portions of chloroform-ethanol (4:1). The combined solvent layers were extracted with 10 ml of 1N Na_2CO_3 and the solvent discarded. The Na_2CO_3 solution of hydrazones was washed with 5 ml of chloroform-ethanol. The carbonate solution was acidified in the cold (4°C) with 3 ml. 6N HCl. The hydrazones were then extracted from the aqueous layer with three successive portions of chloroform-ethanol (5 ml., 3ml. and 3 ml) respectively. The combined extracts were concentrated under an air stream and applied immediately.

Absorbance Changes at 241 and 231 mμ

Mucin, bovine albumin, ovalbumin, insulin and oxytocin solutions in 8M urea* were treated with concentrated alkali (Na OH) and the absorbance changes were recorded. The concentrations of the solutions were low:

mucin 0.2 mg/ml, 0.5 mg/ml

bovine albumin 0.18 mg/ml to 0.25 mg/ml

insulin diluted eight or ten times

oxytocin diluted two or four times

The initial pH range was 7.8-8.3. The final pH after addition of alkali (up to 0.2 ml) was 11.2 to 12.5.

In some experiments the reversibility of the optical density changes was investigated by neutralizing or acidifying the solutions with concentrated HCl.

Gel Filtration

Alkali-treated (with and without borohydride) mucin solutions were fractionated on an Amicon XM-100 membrane. The non-diffusible supernatant was applied to a Sephadex G-200 column (2.5 cm. x 67 cm.) in the cold ($4^{\circ}C$). The sample was eluted with a phosphate buffer pH 5.8 I = 0.1 and collected in 5 ml. fractions. The flow rate was 2 ml. per hour. The absorbance at 230 mμ was measured and 1 ml. aliquots of each fraction were used to determine the fucose content.

* or in water

Enzymic Assay of Keto acids

Pyruvic acid was assayed with the enzyme Lactic Acid Dehydrogenase using the assembled kit made available commercially by Sigma Chemical Company, St. Louis, Mo. The keto acid, α-keto butyrate, contributes a positive error in this assay. The enzyme reaction is outlined below:-

$$\underset{(\text{high O.D.}_{340})}{\text{Pyruvic acid} + \beta\text{-DPNH}} \rightleftharpoons \underset{(\text{low O.D.}_{340})}{\text{L(+) Lactic acid} + \beta\text{-DPN}}$$

In the presence of an excess of β- DPNH the reaction is driven to the right and substantially all the pyruvic acid is converted to lactic acid. The amount of β-DPNH which is converted to β-DPN is measured spectrophotometrically (Beckman DU Spectrophotometer) at 340 mμ , and becomes a measure of the amount of pyruvic acid originally present[134]. The method described by Sigma was adhered to with the exception that twice the volume of enzyme was used.

Removal of Sialic Acid

Sialic acid was removed from some mucin samples, termed 'desialyzed' mucin, for purposes of comparison with whole gel mucin. The sialic acid was removed by mild acid hydrolysis (0.1 N $H_2 SO_4$) for one hour at 80°C, followed by exhaustive dialysis. The remaining sialic acid was determined by the thiobarbituric acid method of Warren[126].

Blood-Group Activity was determined at the time of gastric juice collection, by the method of Kabat[27].

Infrared Spectroscopy

The infrared spectrum of gel mucin (dried in vacuo over P_2O_5 for 14 days) was determined in a KBr disc at a concentration of 1% (by weight) courtesy of the Department of Chemistry, McGill University, on a Perkin-Elmer 257 instrument.

III. Observations and Results

Human gastric gel mucin (HGGM) has already been identified as a glycoprotein comprising two-thirds of the non-dialyzable gastric juice [110]. Gel filtration studies of the major component of HGGM give a molecular weight of greater than 2×10^6 daltons. Minor glycoprotein components associated with the very high molecular weight major component can be separated by free flow electrophoresis [121]. The HGGM failed to migrate on filter paper during electrophoresis at pH 9.0. It dissolved in 8M urea and in this form it sedimented in the ultracentrifuge as a single boundary with a coefficient $S^{25}_{8M\ urea} = 3.18 \times 10^{-13}$ [110].

HGGM upon standing in 8M urea for a period of over one week at 37°C became slowly converted into a breakdown product of faster electrophoretic mobility and smaller sedimentation coefficient. The original HGGM and the second component both stained intensely PAS positive indicating that the breakdown was not a cleavage of carbohydrate from the glycoprotein. Mucin dissolved in 1% K_2CO_3 or N/100 sodium hydroxide gave a composite pattern with mobilities of 0.4 and 0.52 respectively. The mobility of the urea breakdown product was 0.38. On further standing in alkali, further changes took place [110].

Mucin is also slowly degraded by acid alone under physiological conditions, that is, pH 1.0-2.5, 37°C, in the absence of pepsin. The carbohydrate residues, galactose, fucose, sialic acid and at least two peptides, were liberated[135].

Chemical Analysis

The chemical composition of gel mucin consists of 30% protein, 30% galactose, 9% fucose, 15% hexosamine and 1% sialic acid*. No glucose, mannose, xylose, uronic acids or tryptophan have been detected[110]. The nitrogen content of gel mucin was determined to range from 5.85 mg./100 mg. dried mucin to 7.15 mg./100 mg. dried mucin, with a mean value of 6.55 mg./100 mg. dried mucin (cf. 5.9 to 7.5 mg./100 mg. dried mucin, average value 6.61 mg./100 mg. mucin)[110].

An analysis of an 18 hour acid hydrolysate of mucin on a Beckman Automatic Amino Acid Analyzer indicated the following composition of amino acids and hexosamines (see Table III)[136].

---

* The sialic acid content of 'desialyzed' mucin described in "Materials and Methods" was undetectable.

TABLE III

Analysis of Gel Mucin[a] after an 18 hour acid hydrolysis[136]

Amino acid	Gel Mucin	
	M amino acid/ 100 mg.mucin[b]	Ratio of residues of amino acid to residues of threonine
Aspartic acid	5.16	0.264
Threonine	19.54	1.000
Serine	11.96	0.612
Glutamic acid	6.35	0.325
Proline	9.29	0.475
Glycine	7.50	0.384
Alanine	8.85	0.453
Cystine (half)	0.00	0
Valine	4.67	0.238
Methionine	0.59	0.030
Isoleucine	2.86	0.146
Leucine	5.96	0.308
Tyrosine	1.38	0.706
Phenylalanine	1.93	0.988
Lysine	2.12	0.185
Histidine	1.49	0.075
Arginine	3.29	0.168
Glucosamine	34.3	1.760
Galactosamine	17.0	0.882

a. Mucin (blood-group A, secretor, partially fractionated on Sephadex G-200, void volume (excluded) fraction.

b. Values not corrected for water content or loss on hydrolysis

The preliminary amino acid analysis showed gel mucin to have a high percentage (30%) of hydroxy amino acids (serine and threonine). This fact, coupled with the high blood-group activity of gel mucin[110], suggests that an O-glycosidic linkage between the protein chain(s) and carbohydrate prosthetic groups exists in HGGM as in the blood-group specific substances.

Detection of β-elimination Reaction in Alkali-Treated Mucin

A. Amino Acid Analysis of 24-hour Acid Hydrolysates

The retention times of galactosamine, glucosamine and α-amino butyric acid were determined prior to analysis of mucin samples. The peaks of glucosamine and galactosamine overlapped those of half-cystine and methionine respectively, thus precluding quantitative analysis of these components.

The HGGM samples analyzed were characterized as:

(i) whole gel mucin (blood-group B, non-secretor)

(ii) whole gel mucin (mixed sample of blood-group A and B, both secretor)

(iii) void volume fraction of fractionated gel mucin by Sephadex G-200 filtration (blood-group A, secretor)

The Table IV of the amino acid composition of mucin before and after treatment with various solutions of alkali (subjection to various alkaline conditions) appears on Page 54.

The alkaline conditions used to effect a β-elimination reaction were specifically:

Sample (i)

1. 0.6% solution of mucin in 0.1N NaOH, for 216 hours at 4°C.

Sample (ii)

1. 0.2% solution of mucin in 0.1N NaOH, for 24 hours at 4°C.
2. 0.2% solution of mucin in 0.1N NaOH 0.3M $NaBH_4$,, for 216 hours at 4°C.
3. 0.2% solution of mucin in 0.1 N NaOH, 0.3M $NaBH_4$ for 216 hours at 4°C, followed by hydrogenation with $NaBH_4/PdCl_2$ for 1 hour at room temperature (25°C).

Sample (iii)

1. 0.56% solution of mucin in 0.1N NaOH, 0.3M $NaBH_4$ for 216 hours at 4°C, followed by $NaBH_4$/ $PdCl_2$ hydrogenation for 1 hour at room temperature (25°C).

TABLE IV

Amino Acid Analysis of Alkali Treated Human Gastric Gel Mucin[a]

Amino acid	Sample and treatment moles amino acid/100 moles amino acid					
	(i) whole HGGM		(ii) whole HGGM			
	No alkali	1.Alkali 216 hrs.	No alkali	1.Alkali 24 hrs.	2.Alkali 216 hrs. $NaBH_4$	3.Alkali 216 hrs. $NaBH_4$ $PdCl_2$
Aspartic acid	9.3	8.6	7.81	9.3	8.22	7.3
Threonine	13.0	11.7	14.02	14.7	10.64	10.3
Serine	8.8	7.4	10.11	9.6	7.22	8.5
Glutamic acid	8.8	8.7	8.85	9.6	9.34	8.9
Proline	11.0	9.8	9.05	10.7	10.6	11.5
Glycine	9.2	9.5	9.90	9.0	9.64	12.4
Alanine	7.4	9.3	8.60	9.0	10.64	11.0
α-amino butyric	0	0	0	0	1.37	2.1
Valine	8.0	6.3	7.31	5.7	6.81	6.1
Half-cystine	b.	b.	b.	b.	b.	b.
Methionine	b.	b.	b.	b.	b.	b.
Isoleucine	2.7	3.0	2.50	2.98	2.8	3.7
Leucine	5.2	6.2	7.25	5.7	7.45	6.5
Tyrosine	2.7	4.4	2.10	2.98	3.20	1.9
Phenylalanine	2.2	3.3	2.51	2.48	3.52	2.2
Lysine	4.9	3.3	3.07	2.71	2.76	2.8
Histidine	2.1	2.2	2.30	2.0	1.82	1.7
Arginine	6.5	4.7	5.35	4.7	4.94	3.6

Amino acid	Sample and treatment moles amino acid/100 moles amino acid (iii) void volume mucin	
	No alkali	1. Alkali, 216 hrs. Reductant - $PdCl$
Aspartic acid	6.90	7.20
Threonine	14.21	11.52
Serine	8.75	7.40
Glutamic acid	8.75	9.00
Proline	9.21	11.51
Glycine	10.50	10.00
Alanine	10.50	12.12
α-amino butyric	0	1.08

See page 54A

TABLE IV (Continued)

Amino acid	Sample and treatment moles amino acid/100 moles amino acid (iii) void volume mucin	
	No alkali	1. Alkali, 216 hrs. Reductant - $PdCl_2$
Valine	6.95	7.48
Half-cystine	b	b
Methionine	b	b
Isoleucine	3.42	
Leucine	6.86	7.00
Tyrosine	1.76	b
Phenylalanine	2.64	2.46
Lysine	3.72	3.18
Histidine	1.77	1.95
Arginine	5.28	4.65

[a] Values not corrected for water content or losses on hydrolysis

[b] Not determined

The changes in the amino acid composition of HGGM after prolonged solution in mild alkali, notably the loss of serine and threonine residues and the gain of alanine and ∝-amino butyric acid residues, is summarized in Table V.

TABLE V

Changes in Amino Acid Composition due to Treatment of Gel Mucin with Alkali at 4°C

Sample and Treatment	Amino acid residue: moles amino acid/100 moles amino acid				
	Threonine	Serine	Glycine	Alanine	∝-amino butyric acid
Sample (i) No alkali	13.0	8.8	9.2	7.4	0
1. Alkali 216 hrs. No reductant	11.7	7.4	9.5	9.3	0
Loss	1.3	1.1	-	-	-
Gain	-	-	-	1.9	-
% loss or gain	10	12.5	-	25.7	-
Sample (ii) No alkali	14.02	10.11	9.90	8.60	0
1. Alkali 24 hrs. No reductant	14.7	9.6	9.6	9.0	0
Loss	-	-	-	-	-
Gain	-	-	-	-	-
% loss or gain	-	-	-	-	-

See page 56

TABLE V (continued)

Sample and Treatment	Amino acid residue: moles amino acid/ 100 moles amino acid				
	Threonine	Serine	Glycine	Alanine	α-amino butyric acid
Sample (ii)					
2. Alkali 216 hrs. reductant	10.64	7.22	9.57	10.64	1.37
Loss	3.38	2.89	-	-	-
Gain	-	-	-	2.04	1.37
% loss or gain	24.0	28.6	-	24.2	137
Sample (ii) 3. Alkali 216 hrs. Reductant $PdCl_2$	10.3	8.5	12.4	11.0	2.1
Loss	3.72	1.61	-	-	-
Gain	-	-	2.50	2.40	2.1
% loss or gain	26.5	15.9	25.2	27.9	210
Sample (iii) No alkali	14.21	8.75	10.50	10.50	0
1. Alkali 216 hrs. Reductant $PdCl_2$	11.52	7.40	10.00	12.12	1.08
Loss	2.69	1.35	-	-	-
Gain	-	-	-	1.62	1.08
% loss or gain	19	15.4	-	15.4	108

Detection of β-Elimination Reaction in Alkali-Treated Mucin

B. Detection of α-keto Acids in Acid Hydrolysate of Alkali-Treated Mucins

1. Paper chromatography of the free keto acids

The chromatograms were developed as described in "Materials and Methods" for periods varying from 3 to 5 hours. Samples were applied to the filter paper at pH values over 3.0. The pyruvic acid standard was applied at pH 6.5, the α-keto butyric acid standard at pH 5.0. Lowering the pH of the standards resulted in some variation of Rf values and increased "tailing". The chromatograms appear as figures 1, 2 and 3. The following designations are used to indicate the samples applied:-

M = Mucin of blood-group AB, secretor, incubated as a 2% solution in 0.1 N NaOH for 216 hours at 4°C (20 mg. total weight)

M_D = Mucin of blood-group AB secretor, desialyzed prior to alkali treatment, incubated as a 0.15% solution in 0.1 N NaOH, 216 hours at 4°C (15 mg. total weight)

M_a = Mucin of blood-group A secretor, not treated with alkali (18 mg. total weight)

OA = Ovalbumin incubated as a 0.1% solution in 0.1 N NaOH for 216 hours at 4°C.

No attempt was made to quantify the α-keto acids of the chromatograms.

FIGURE I (page 59)

The chromatogram was developed with sodium bicarbonate saturated n-butanol and visualized with Nessler's Reagent. The α-keto acids were recognizable as yellow-orange spots which faded with time, on a pale yellow background. The Rf values were calculated from the midpoint of the spots.

Rf values:

M	= 0.63
M_D	= 0.67
K	= 0.65
P (not shown)	= 0.05
M_A	no colour

FIGURE 1

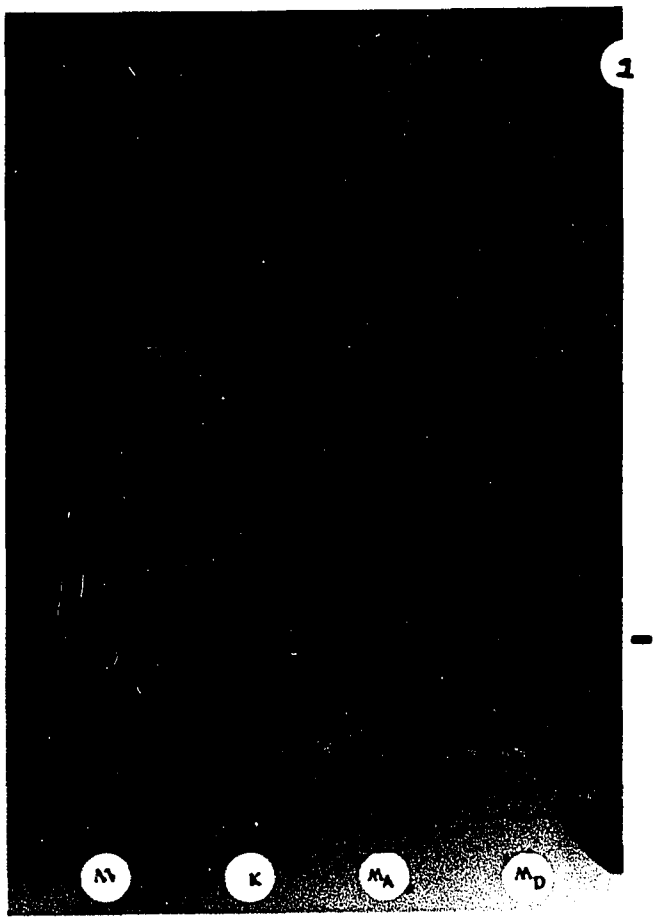


FIGURE 1

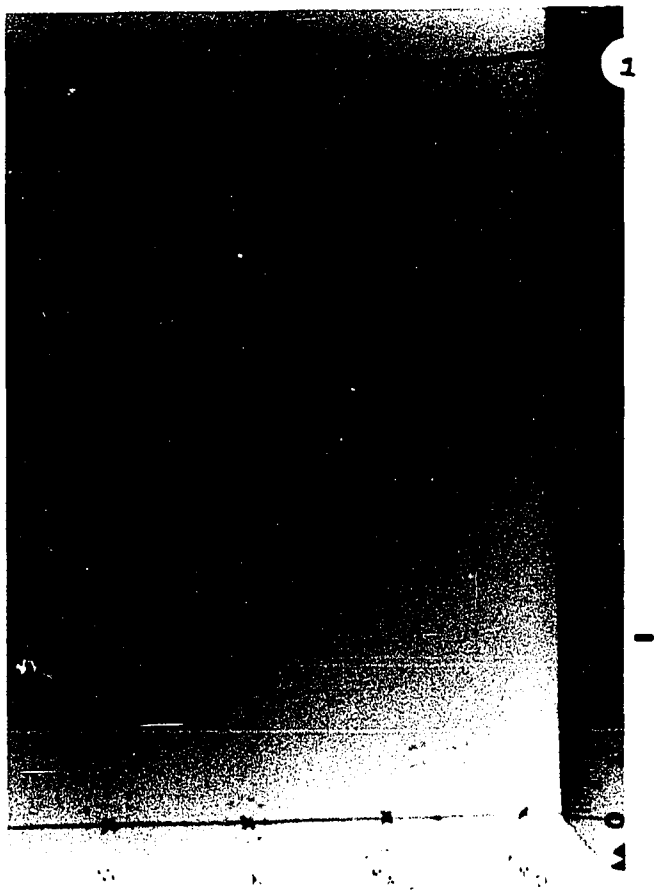


FIGURE 2 (page 61)

Chromatogram of alkali-treated mucin and α-keto acids developed with methanol-benzene-n-butanol-water (5:2:2:2 V/V) and visualized with O-phenylenediamine. The α-keto acids were visible as pale yellow spots which fluoresced green-white under a Wood's ultraviolet lamp.

R_f values:

M 0.77

M_D 0.68

K 0.75

P 0.62

M 1/40 mg. alkali-treated mucin (reduced with sodium borohydride) (not shown) faint fluorescence 0.73

HOG (100 mg. alkali-treated hog gastric mucin) (not shown) blue-white fluorescence 0.63

OA (not shown) no fluorescence

FIGURE 2

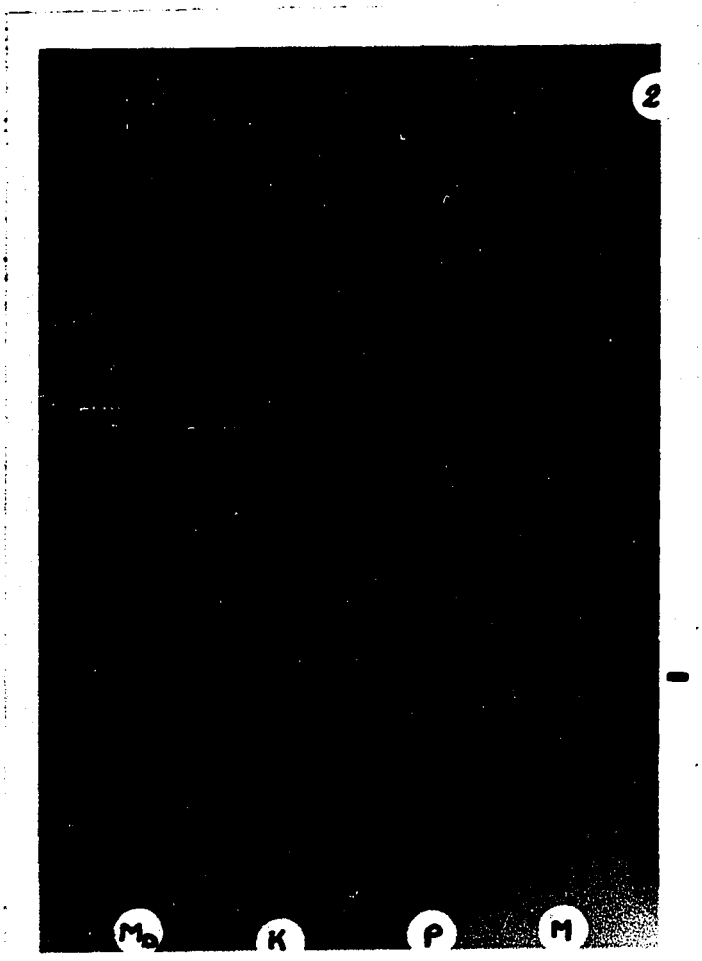


FIGURE 2

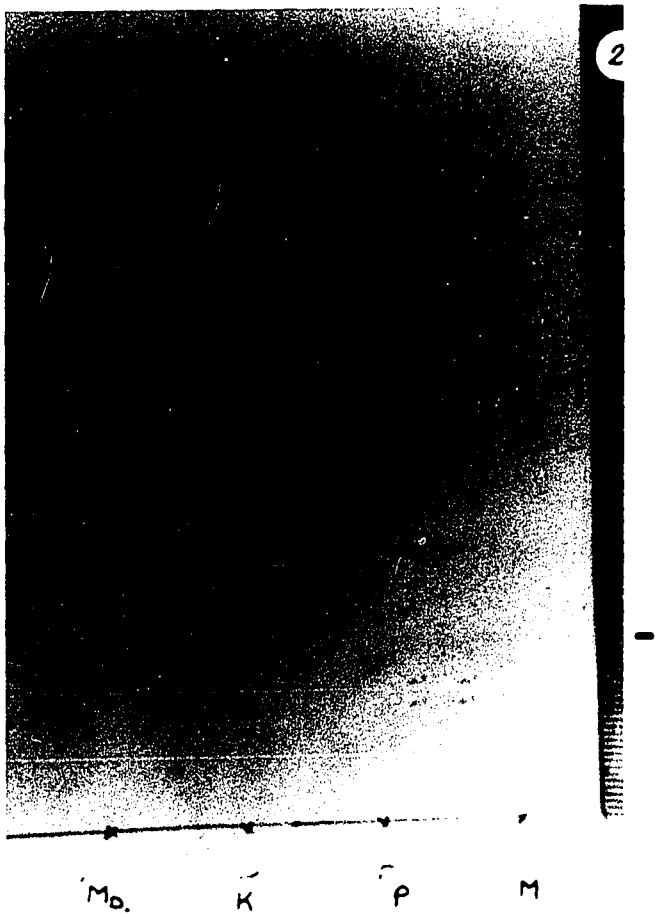


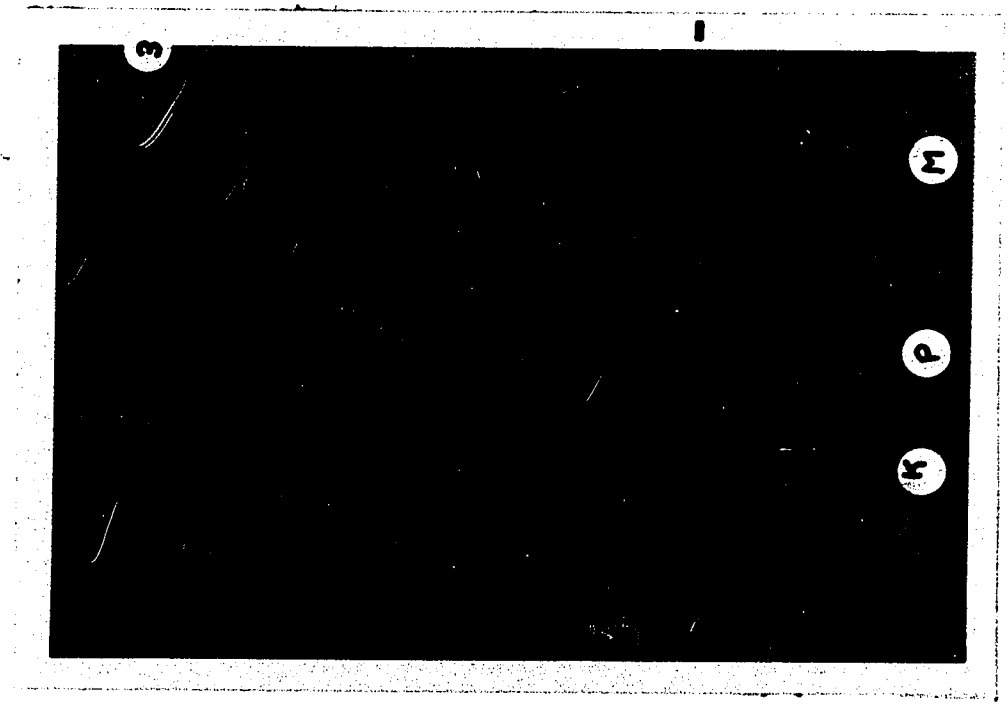
FIGURE 3 (page 63)

Chromatogram developed with water saturated n-butanol - formic acid (95:5 V/V) and visualized with Nessler's Reagent.

R_f values:

M	0.77	
M_D	0.72	
OA	0. faint yellow	
K	0.76	cf. 0.76 (130)
P	0.51	cf. 0.64 (130)

FIGURE 3



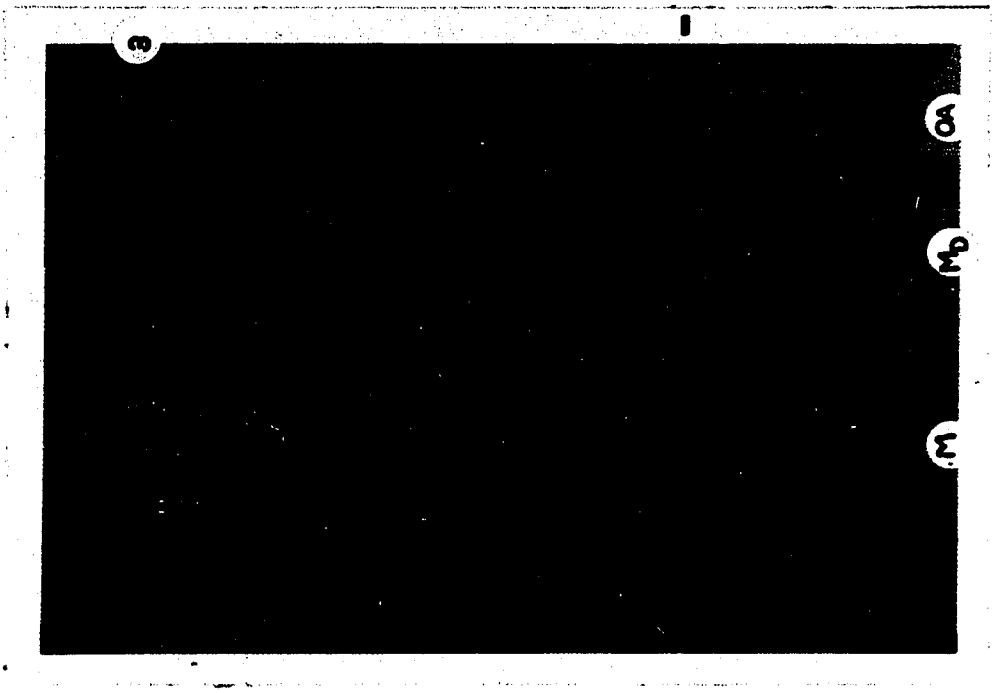
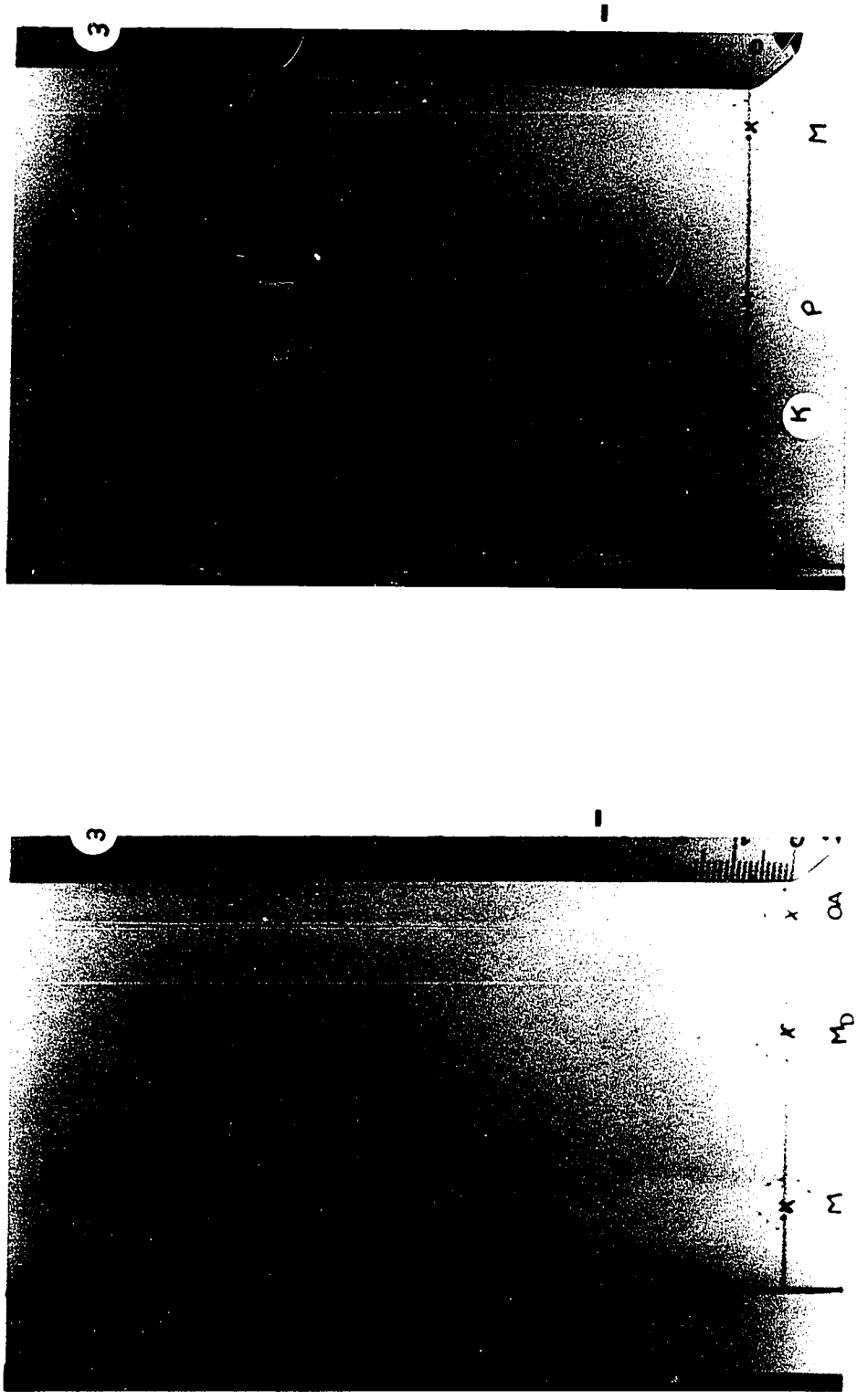


FIGURE 3



2. Paper Chromatography of 2,4 dinitrophenyl hydrazone derivatives of α-keto acids

The 2,4 dinitrophenyl hydrazone derivatives (DNP) of both α-keto butyric acid and pyruvic acid were separable into two distinct spots in ethanol-n-butanol-water (3:2:2 V/V) and in bicarbonate-saturated n-butanol. However, preparation of the DNP derivatives from a mixture of the two keto acids altered the R_f values of the fastest and slowest moving components and the derivatives resolved into three not four spots.

The appearance of DNP-isomers has been reported in the literature in the solvent systems alcohol-ethanol-water (5:1:4 V/V) t-amyl alcohol-n-propanol-ammonium hydroxide (13:1:6 V/V) and 0.5% sodium carbonate[137][138].

The DNP derivatives were located by their yellow colour prior to dipping the papers in ethanolic KOH, or by the green-yellow colour of the puruvic acid derivatives and the salmon coloured and brown coloured α-keto butyric acid derivatives after exposure to ethanolic-KOH.

The hydrazine reagent alone resulted in a slow-moving spot upon chromatography.

The chromatograms of DNP-keto acids are shown in Figures 4 and 5.

FIGURE 4 (page 66)

The chromatogram was developed with n-butanol-ethanol-water (3:2:2 V/V). The 2,4 dinitro-phenylhydrazones present themselves as yellow spots.

R_f values:

M	0.58 , 0.51
M_D	0.45
K	0.69 , 0.62 (cf. 0.63 in solvents (5:4:1)[128])
P	0.55, 0.49 (cf. 0.5 in solvents (5:4:1)[128])
P + K	0.68 , 0.59 , 0.55

FIGURE 4

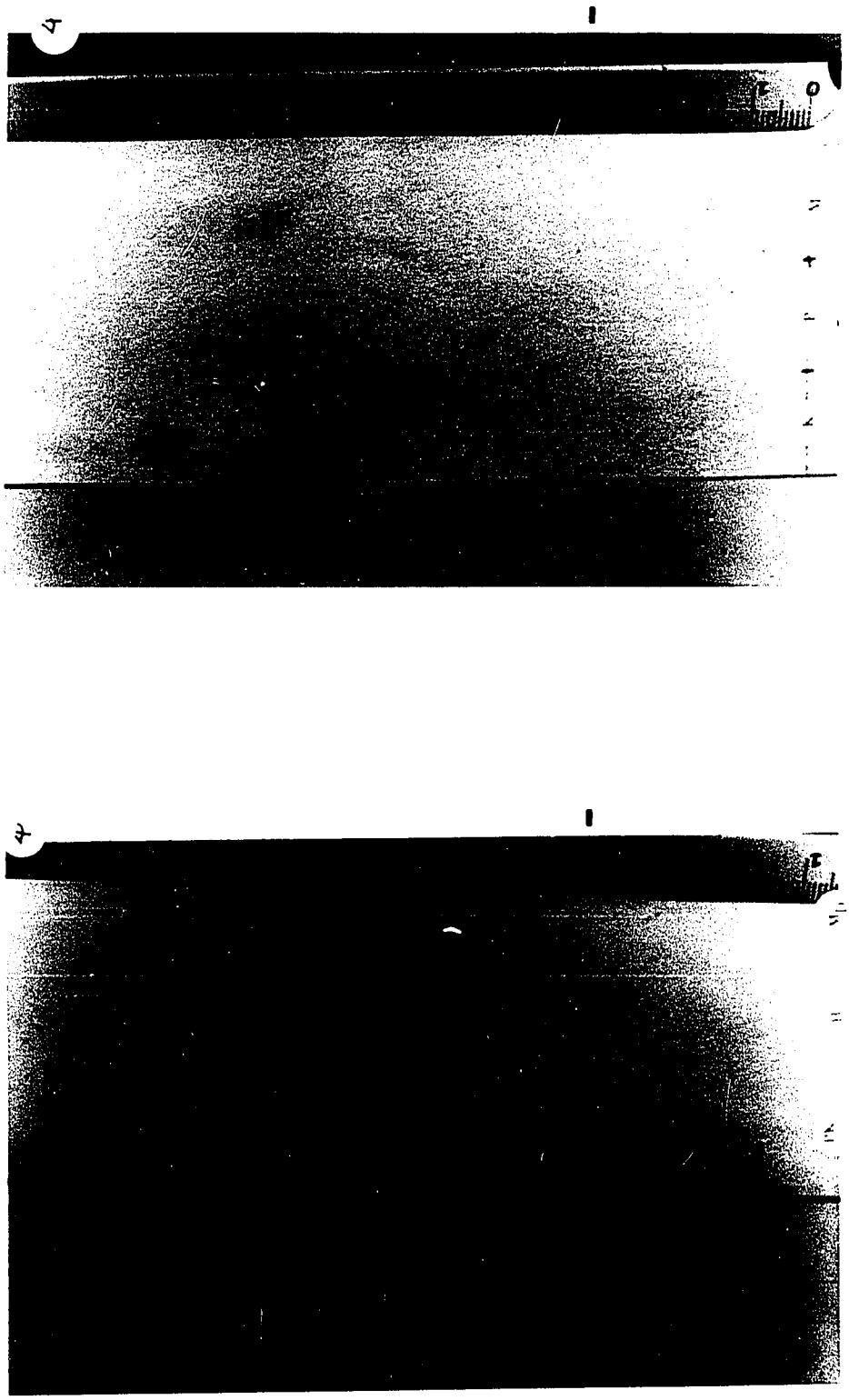


FIGURE 5 (page 68)

The chromatogram was developed in bicarbonate saturated n-butanol and treated with ethaolic KOH after drying.

M	0.83 , 0.25
M_D	0.74 , 0.24
K	0.39 , 0.30
P.	0.93 , 0.82 (cf. 0.80, 0.62 in solvent n-butanol - 1N Na HCO_3 (1:2 V/V)[129]

FIGURE 5

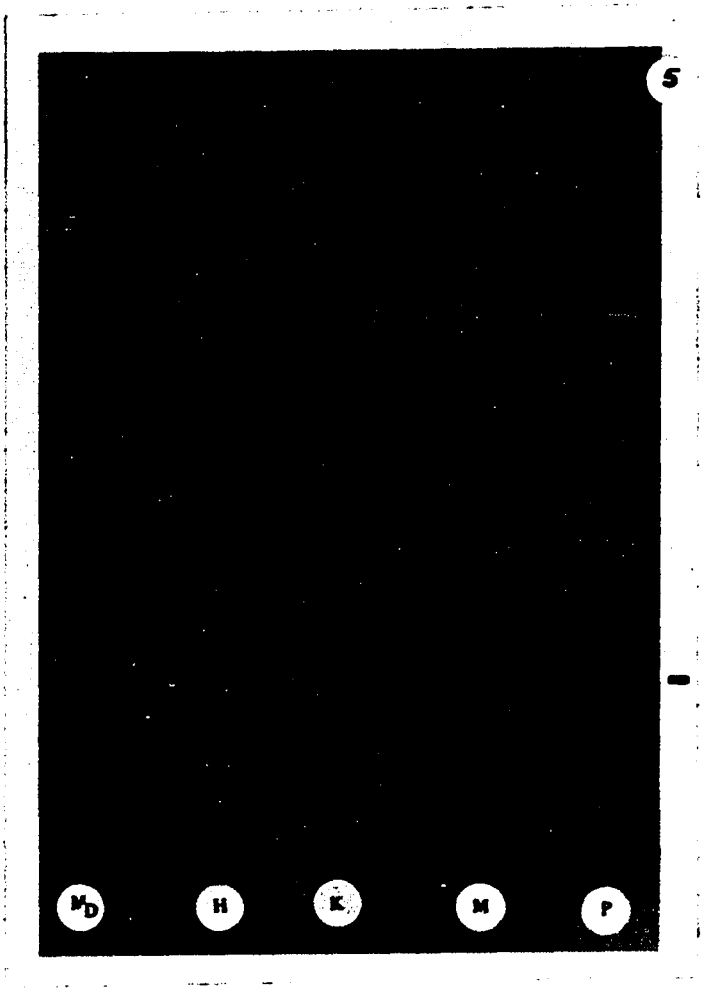
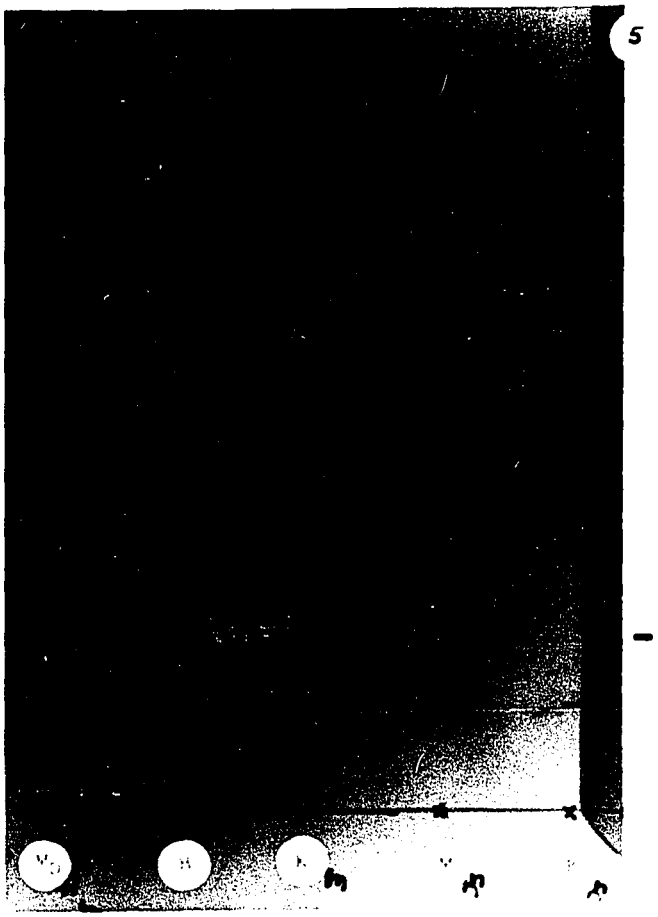


FIGURE 5



3. Enzymatic Determination of α-Keto Acids by Lactic Acid Dehydrogenase Assay of Alkali-Treated Mucin

A calibration curve determined for pyruvic acid in the range of 0.1 micromoles to 0.5 micromoles was found to be linear with respect to pyruvic acid concentration.

The pH of the reaction mixture used in the assay exceeded 9.5 in all cases. The effects of a high salt concentration and of the α-keto acid, α-keto butyrate on the assay were investigated confirming that α-keto butyrate contributes a 30% positive error to the spectrophotometric reading. The effect of a high salt concentration (1.6 N Na Cl) was judged to be within the variation of the samples of hydrolysate tested. The kinetics of the α-keto butyrate reduction by DPNH vary from those of the pyruvic acid reduction such that readings taken within three minutes from the addition of enzyme include a contribution due to butyrate of less than 10%.

From the Table VI below, it is evident that some component of the hydrolysate inhibits the enzyme reaction or drives the equilibrium to favour the reduced cofactor DPNH. No change in absorbance occurred in the absence of enzyme.

The mucin samples to be assayed were treated with alkali as listed below before acid hydrolysis.

1. Mucin of blood-group A, secretor, no alkali 37 mg. total weight)
2. Mucin of blood-group A, secretor, no alkali, (10 mg. total weight)
3. Mucin of blood-group A, secretor, in a 0.6% solution of 0.1N Na OH for 216 hours at $4^{\circ}C$ (6.8 mg. total weight)
4. Mucin of blood-group A, secretor, in a 0.4% solution of 0.3 N NaOH for 216 hours at $4^{\circ}C$ (20 mg. total weight)
5. Mucin of blood-group A, secretor, in a 0.4% solution of 0.3 N Na OH for 216 hours at $4^{\circ}C$ (20 mg. total weight)
6. Mucin of blood-group A, secretor, in a 0.4% solution 'desialyzed' prior to treatment with 0.3 N Na OH for 216 hr. at $4^{\circ}C$ (40 mg. total weight)
7. Ovalbumin in 0.15% solution of 0.1 N Na OH 216 hr. at $4^{\circ}C$

The expected values of $\Delta\ OD_{340}$ were calculated using the formula

$$\Delta\ OD_{340} = \frac{6.22 \times \text{moles of pyruvic acid in cuvette}}{3}$$

for a path length of 1 cm.[134] and basing the expectations on the losses of serine residues due to treatment of the mucin by alkali, as revealed by amino acid analysis.

The average loss of 1.6 micromoles serine per 100 mg. mucin shown by amino acid analysis generates, according to the mechanism of β-elimination, 1.6 micromoles pyruvic acid per 100 mg. mucin. The Δ OD_{340} resulting from this amount of pyruvic acid is calculated to be a decrease of 3.31 units per 100 mg. mucin.

TABLE VI

Lactic Acid Dehydragenase Assay of Pyruvic Acid and Hydrolysates of Alkali-Treated Mucin*

Description of sample (see details in text)	Amount of pyruvic acid added to medium	OD (340mμ)		
		Expected	Observed	Discrepancy
Standard	0.004 mg.	0.100	0.097±3%	-
1. Mucin (no alkali)(37 mg)	-	-	0.005±1%	0.004
2. Mucin (no alkali)(10 mg)	0.004 mg.	0.100	0.089±28%	0.011
3. Mucin (alkali) (6.8 mg.)	-	0.225	0.120±2%	0.105
4. Mucin (alkali) (20 mg.)	-	0.66	0.29 ±4%	0.37
5. Mucin (alkali) (20 mg.)	-	0.66	0.33 ±3%	0.33
6. Mucin (alkali) (40 mg.)	-	1.32	0.41 ±1%	0.91
7. Ovalbumin (15 mg.)	-	0	0.007±2%	0.007

* Average values of two determinations

Detection of β -Elimination Reaction in Alkali-Treated Mucin

C. Gel Filtration Studies of the Size of the Breakdown Products of Alkali-Treated Mucin

A major fractionation of the breakdown products of the neutralized alkali-treated gel mucin was effected by ultra filtration with an Amicon XM-100 membrane which allows molecules of under 100,000 molecular weight to diffuse through the membrane. The concentrated non-diffusible supernatant fraction was applied to the Sephadex G-200 column. The sample elution was monitored spectrophotometrically at 230 mμ and colorimetrically by fucose determinations on 1 ml. aliquots. The major characteristics of the elution profiles (figures 6, 7 and 8) are the four fractions labelled:

1. Void Volume: A narrow peak excluded from the Sephadex Gel with a molecular weight greater than 2×10^6 g , having a higher ratio of protein with respect to fucose than any other fraction.
2. Peak 12 : A fraction of smaller and more disperse molecular weights.
3. Peak 17 : A broad peak of still lower molecular weight
4. Peak 23 : A polydispersed fraction of molecular weight near 100,000 g.

FIGURE 6 (page 74)

Elution profile from chromatography on Sephadex G-200 of a 2 ml sample derived from 17 mg. of whole mucin of blood-group A, secretor, which had been incubated as a 0.4% solution in 0.2N NaOH for 216 hours at 4°C without sodium borohydride.

The solid bar indicates the void volume (V/V) fraction as determined by Blue Dextran 2000, open bar indicates the fraction described as peak 12 in the text.

Fucose X --------X

OD_{230} 0 ——————— 0

V_e = bed elution volume

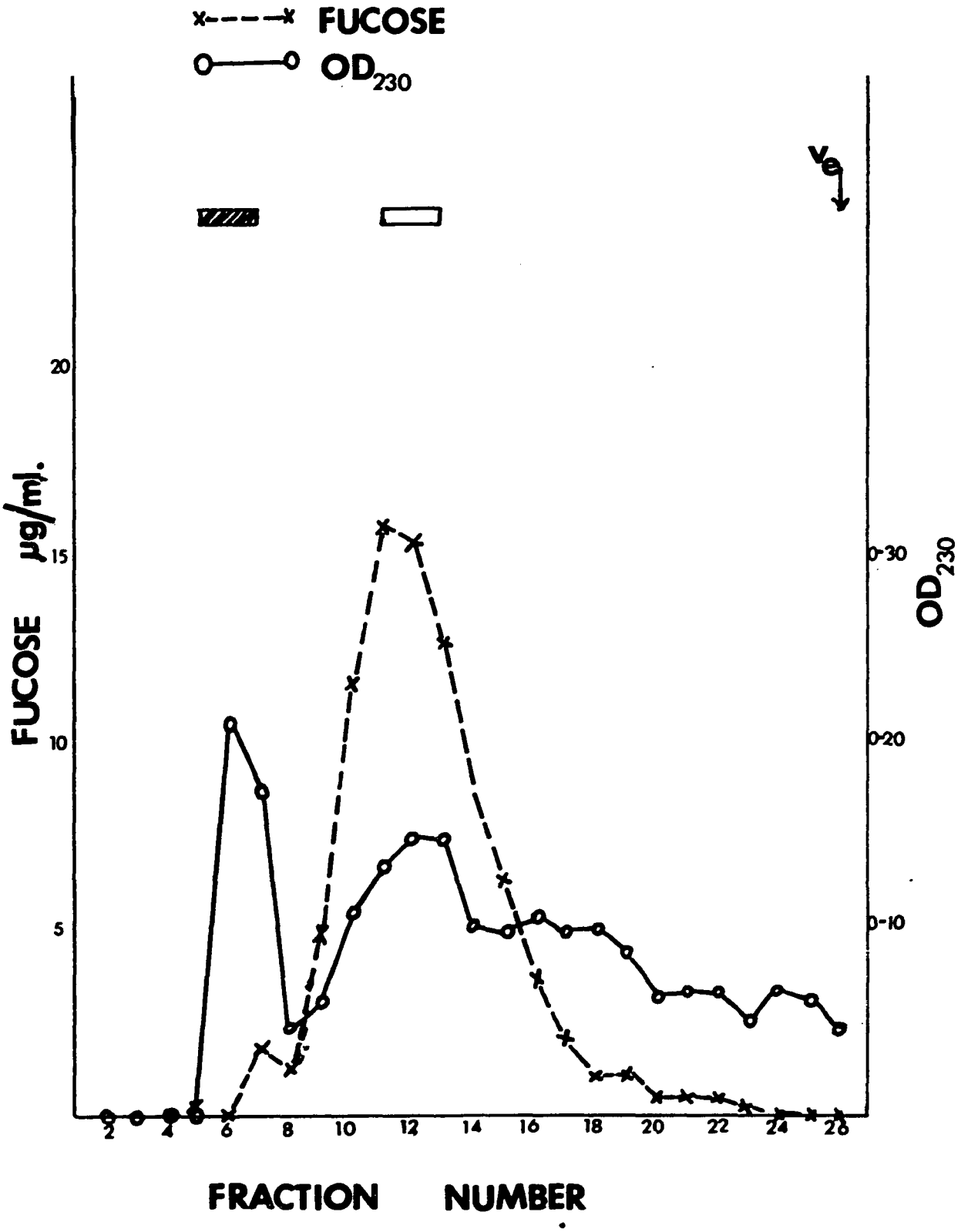

FUCOSE
OD230
Ve
FUCOSE µg/ml.
OD230
20
15
10
5
0·30
0·20
0·10
2
4
6
8
10
12
14
16
18
20
22
24
26
FRACTION NUMBER

FIGURE 7 (page 76)

Elution profile from chromatography on Sephadex G-200 of a 2 ml. mucin sample derived from 40 mg. of whole mucin of blood-group A, secretor, which had been incubated as a 0.4% solution in 0.2N NaOH, 0.3M, $NaBH_4$ for 216 hours at $4^{o}C$. Designations are identical to those of Figure 6 except that the open bars indicate fractions described as peak 17 and 23 in the text.

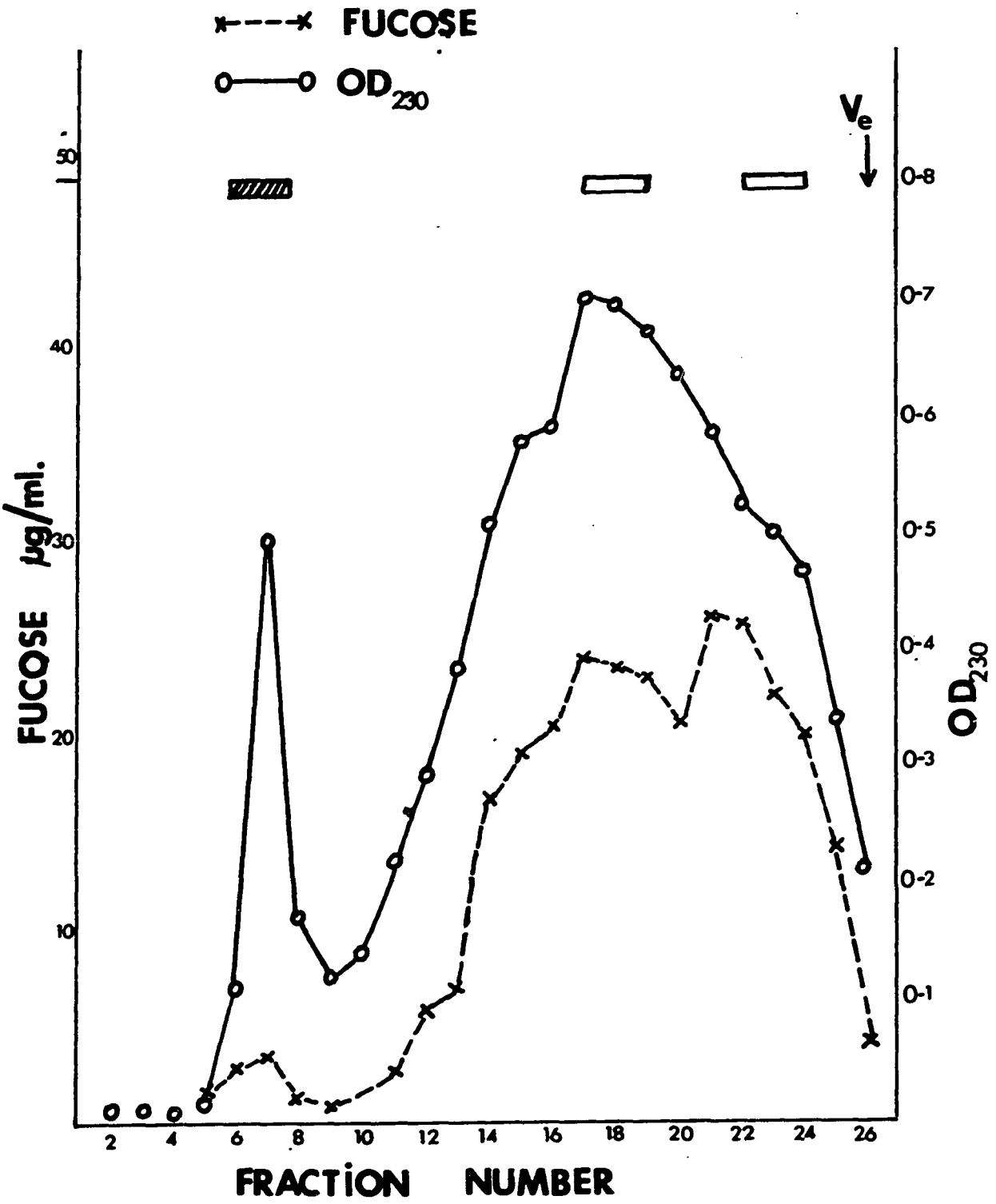
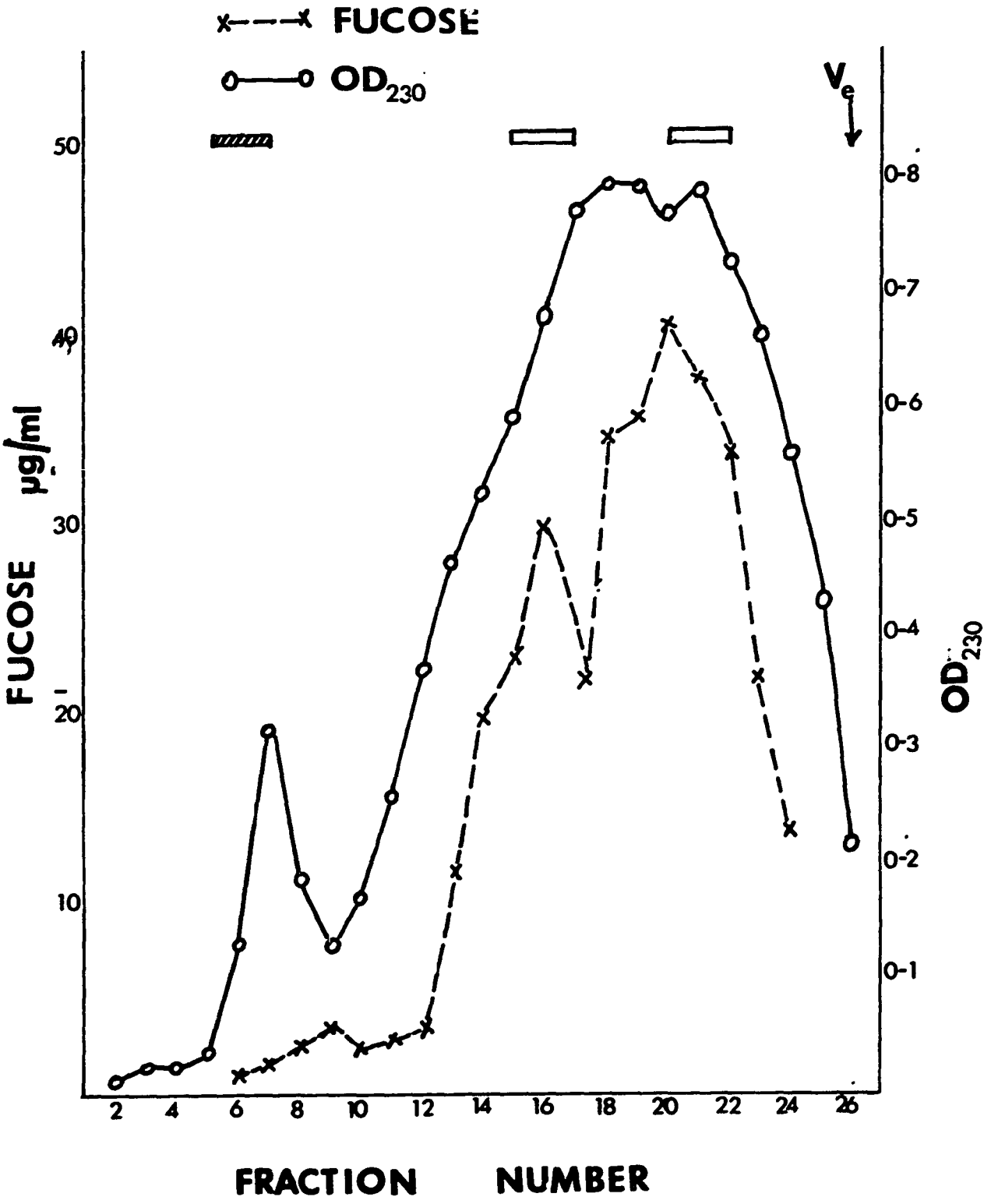

FUCOSE
OD230
Ve
FUCOSE µg/ml.
OD230
FRACTION NUMBER
50
40
30
20
10
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
2
4
6
8
10
12
14
16
18
20
22
24
26

FIGURE 8 (page 78)

Elution profile from chromatography on Sephadex G-200 of a 2 ml. sample derived from 40 mg. mucin of blood-group O secretor which had been treated with alkaline borohydride as in Figure 7. The neutralized mucin solution was reduced in volume by lyophilization prior to minimal concentration through an Amicon XM-100. The increased size of peak 23 reflects the less ardent membrane filtration. The designations used are identical to those of Figure 7.


FUCOSE
OD230
Ve
FUCOSE µg/ml
OD230
50
40
30
20
10
0-8
0-7
0-6
0-5
0-4
0-3
0-2
0-1
2
4
6
8
10
12
14
16
18
20
22
24
26
FRACTION NUMBER

Detection of β-elimination Reaction in Alkali-Treated Mucin

D. Spectrophotometric Evidence Derived from ΔOD_{241} and ΔOD_{231} of Mucin Solutions at pH 12.0

To reiterate, the β-elimination reaction mechanism, as outlined in the "Introduction", results in the formation of double bonds within the glycoprotein which absorb ultraviolet light at the wavelengths 241 and 231. Monitoring ΔOD_{241} and ΔOD_{231} from pH 7 to pH 12 has been used as presumptive evidence for the occurrence of the β-elimination reaction.

Solutions of oxytocin (an octa-peptide),* insulin,* ovalbumin, bovine albumin* and mucin (HGGM) in urea were studied. Recordings of OD_{241} and OD_{231} against water or 8M urea (at the sample pH) were made at neutral and alkaline pH at two minute intervals. At pH 12.0 all the solutions - peptides, proteins and glycoproteins - showed an immediate increase in absorbance at OD_{241} and OD_{231}. These changes are illustrated as bar graphs of ΔOD, with time due to the addition of alkali in Figures 10 and 11. All values are the averages of four determinations.

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* These proteins were studied as aqueous solutions

FIGURE 10 (page 81)

The change in absorbance at 241 mμ and 231 mμ of aqueous protein solutions of oxytocin, insulin and bovine albumin.

The graphs represent the following specific solutions:

10a oxytocin prepared by a four-fold dilution of the commercial preparation. The pH was adjusted to 11.2 after the initial recordings of OD_{241} and OD_{231}.

10b insulin prepared by a ten-fold dilution of the commercial preparation. The pH after initial readings was adjusted to pH 12.1.

10c bovine albumin in a solution of 0.25 mg/ml (wt/v). The pH of the solution, after initial readings, was adjusted to 12.4

The upper graphs portray ΔOD_{241}, the lower graphs ΔOD_{231}

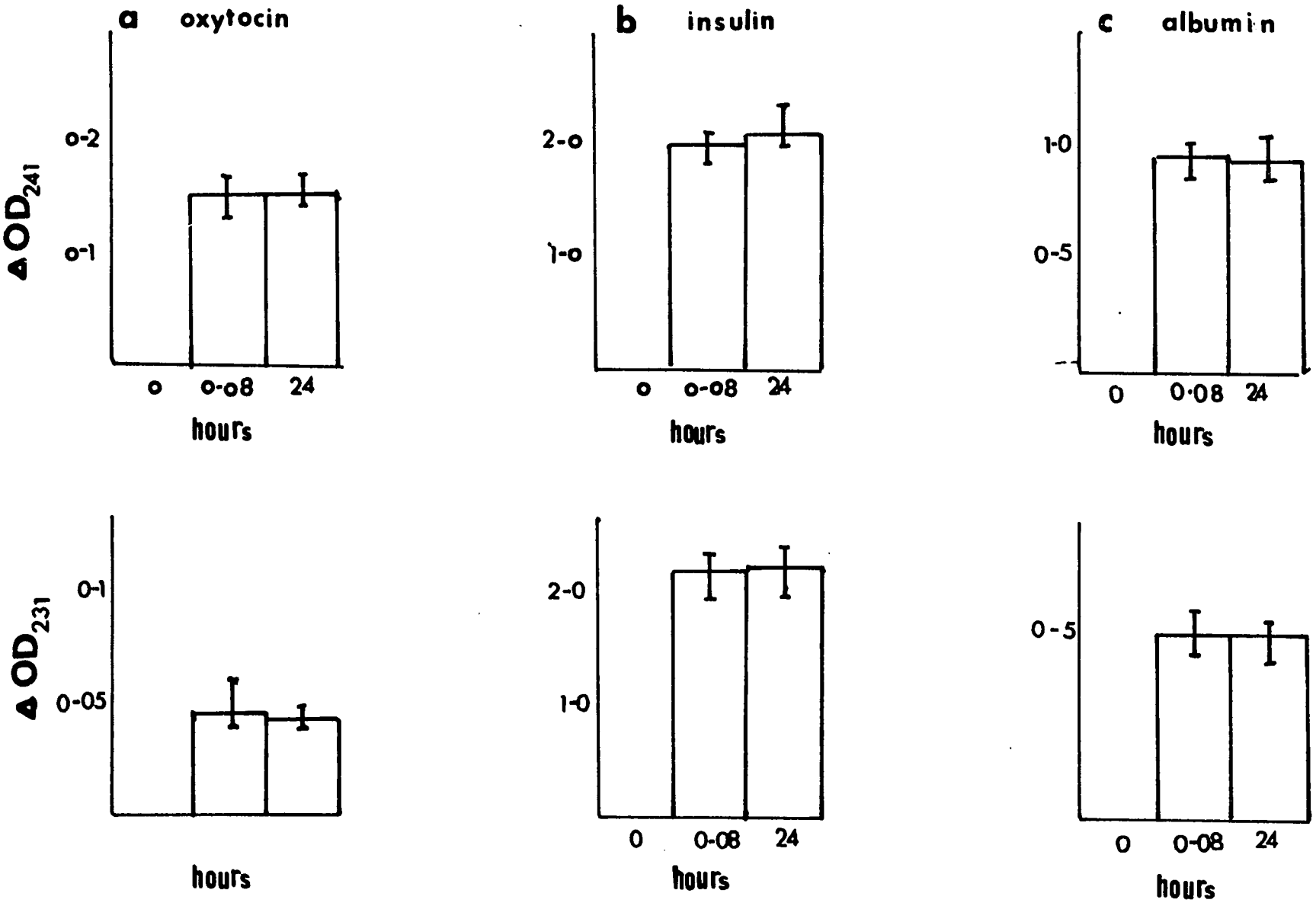

a oxytocin
b insulin
c albumin
ΔOD241
ΔOD231
0-2
0-1
0-05
2-0
1-0
0-5
0
0-08
24
hours

FIGURE 11 (page 83)

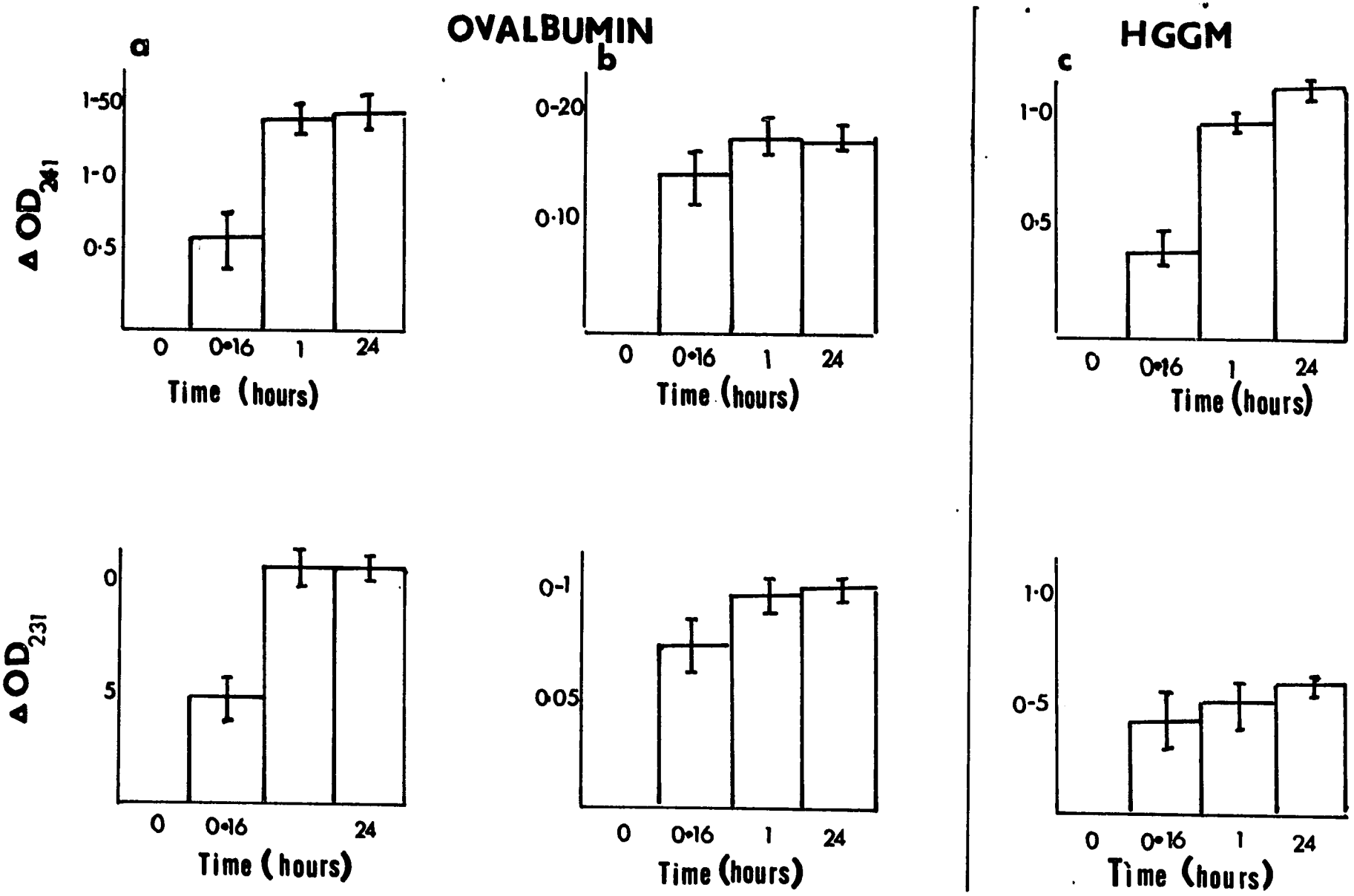
The change in absorbance at 241 mμ and 231 mμ of 8M urea solutions of the glycoproteins ovalbumin and gel mucin. The solutions were of the following concentrations:

11a ovalbumin 0.5 mg/ml, the pH, after initial readings, was adjusted to 12.5

11b ovalbumin 0.15 mg/ml, the pH after initial readings, was adjusted to pH 12.5

11c gel mucin of blood-group A secretor, 0.9 mg/ml (OD_{280} = 0.780), the pH, after initial readings, was adjusted to 12.2.

The upper graphs portray ΔOD_{241}, the lower graphs ΔOD_{231}.


OVALBUMIN
HGGM
a
b
c
ΔOD241
ΔOD231
1·50
1·0
0·5
0·20
0·10
0·5
0
5
0·1
0·05
0·5
0·16
1
24
Time (hours)

Clearly the time course of the optical density changes due to alkali for protein solutions and glycoprotein solutions differ.

Upon acidification of all the alkaline solutions examined, the changes in absorbance are reversed exactly (making corrections for the dilution due to added alkali and acid). Renewal of the alkaline state once again increases the absorbance.

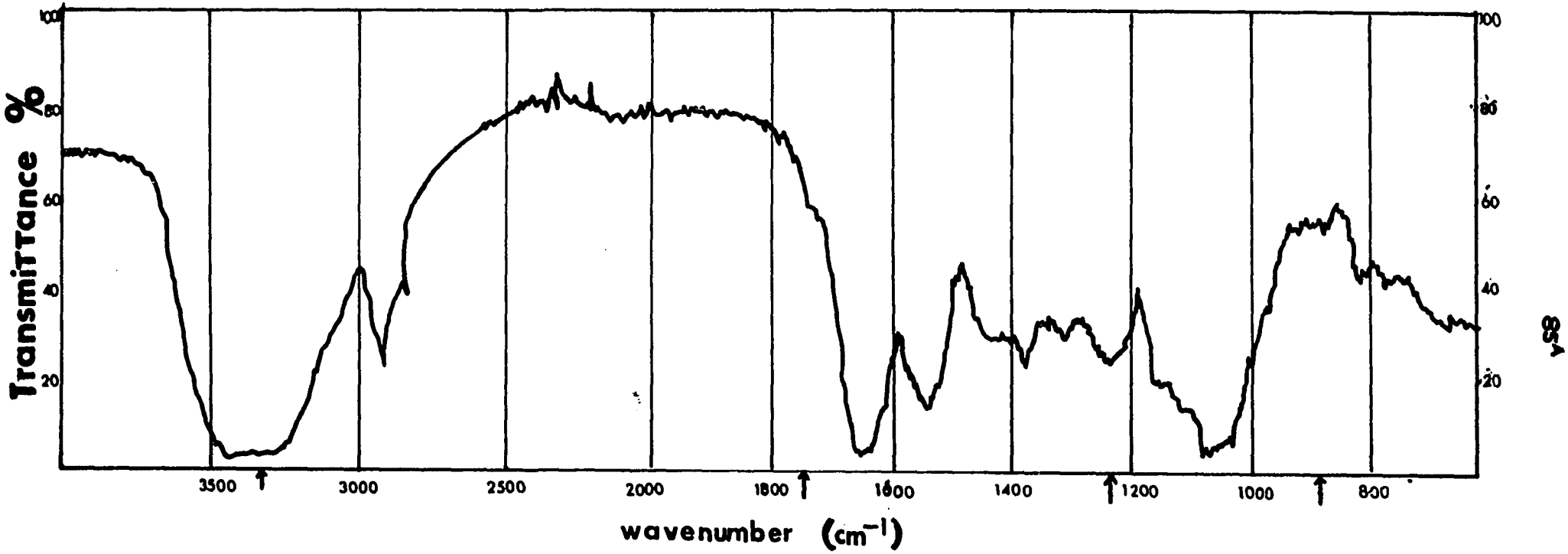
Infrared Absorption Spectrum

The infrared spectrum of dried mucin appears in Figure 12. The absorption peaks at 1240 $cm.^{-1}$, 1650 $cm.^{-1}$ and 3250-3480 $cm.^{-1}$ and lack of peaks at 890 $cm.^{-1}$ and 1750 $cm.^{-1}$ is supporting evidence for the existence of an O-glycosidic linkage within the glycoprotein HGGM.

The indications of the absorption peaks are:

1. Absence of β-linked glycosides as characterized by a peak at 890 $cm.^{-1}$ (140)
2. Presence of sulphate as shown by S=O stretching vibration peak at 1240 $cm.^{-1}$. (141)
3. Presence of a carbonyl group (amide) absorbing at 1650 $cm.^{-1}$. (142)
4. Absence of ester group absorption at 1750 $cm.^{-1}$. (143)
5. The multiple stretching vibrations of inter-molecular hydrogen bonded O-H typical of polymeric associations characterized by the strong broad absorption at 3480 - 3250 $cm.^{-1}$. (142)

Infra-red Spectrum of HGGM



IV. DISCUSSION

It is tempting to speculate upon the inter-relation of glycoproteins which have in common a high blood-group activity and a high proportion of hydroxyamino acids. Three such glycoproteins are gastric gel mucin (HGGM) with a molecular weight greater than 2×10^6 g.[121], a gastric soluble glycoprotein of molecular weight 1.5×10^6 g.[141], and ovarian cyst fluid blood-group substances from 300,000 up to 1×10^6 g.[78]. Because of the chemical similarities borne by these molecules it is conceivable that each represents a stage of fragmentation of a large precursor macromolecule. As isolated however, these molecules display some striking differences. The inability of ovarian cyst fluid blood-group substances to interact with the substance Concavalin A contrasts with the ease of interaction of the gastric blood-group substances and illustrates the structural dissimilarities[142].

The comparable ratios of amino acid residues to threonine residues for each of these glycoproteins are shown in Table VII.

TABLE VII

Ratio of Amino Acid Residues to Threonine Residues

	HGGM[a]	HGGM[b]	Blood-Group substance A[c]	Gastric soluble glycoprotein[d]
Aspartic acid	0.485	0.56	0.15	0.09
Threonine	1	1	1	1
Serine	0.616	0.715	0.69	0.51
Glutamic acid	0.616	0.632	0.17	0.15
Proline	0.619	0.645	0.55	[e]
Glycine	0.738	0.706	0.23	0.21
Alanine	0.738	0.615	0.34	0.37
Valine	0.489	0.515	0.19	0.10
Isoleucine	0.251	0.179	0.11	0.07
Leucine	0.483	0.518	0.13	0.14
Tyrosine	0.129	0.15	0.02	0.03
Phenylalanine	0.186	0.18	0.05	0.07
Lysine	0.262	0.22	0.93	0.05
Histidine	0.125	0.16	0.19	0.09
Arginine	0.372	0.38	0.13	0.09

[a] HGGM of blood-group A, secretor, non retarded fraction on Sephadex G-200 chromatography (VV).

[b] HGGM (whole) of blood-group A and B, secretor

[c] Ovarian cyst fluid blood-group substance A reported by Pusztai and Morgan[76]

[d] Reported by Schrager (1969)[141]

[e] Not reported

The ovarian cyst fluid blood-group substances, bovine submaxillary mucin and pseudomyxomatous mucin have each been shown to have O-glycosidic bonds (Type 4 of page 13) As detailed in the Introduction, proof of the existence of this linkage lies in the demonstration of the occurrence of the β-elimination reaction.

My results show that HGGM also undergoes the β-elimination reaction. The first evidence that this had occurred was that the change in the amino acid composition of mucin treated with alkali was of the order and direction expected from theoretical considerations. That is, there was a loss of serine and threonine amino acid residues which was reflected by an increase in alanine and α-amino butyric acid residues. See Table VIII. The α-amino butyric acid was not detected when the alkali treatment had been carried out in the absence of sodium borohydride. Furthermore, the recovery of the reduced amino acid residues (alanine, α-amino butyric acid) was successfully increased from 33-46% to 55-110% by hydrogenation with $PdCl_2$. Thus the non-quantitative recovery of reduced amino acids is in part attributable to an incomplete reduction of the unsaturated amino acids[65]. In part the incomplete recovery can be attributed to another mechanism of β-elimination (see discussion page 86) or to alkali induced side reactions. One side reaction

TABLE VIII

Stoichiometry of Chemical Changes in Human Gastric Gel Mucin Resulting from Treatment with Alkaline Borohydride (0.1 N NaoH) for 216 hours at 4°C

	Amino acid residue		Change (μmoles/100 mg. mucin)	% Change
HGGM (whole) sample (ii)	Threonine	loss	36.6	26.7
	α-amino butyric acid	gain	12.8	128
Alkaline borohydride	Serine	loss	26.1	26.8
	Alanine	gain	17.6	21.8
	Glycine	gain	14.1	15.2
HGGM (whole) sample (ii)	Threonine	loss	36.9	26.9
	α-amino butyric acid	gain	19.7	197
Alkaline borohydride with $PdCl_2$	Serine	loss	14.0	14.9
	Alanine	gain	22.5	27.8
	Glycine	gain	23.5	25.1
HGGM (VV) sample (iii)	Threonine	loss	24.6	18.9
	α-amino butyric acid	gain	09.9	99
Alkaline borohydride with $PdCl_2$	Serine	loss	12.4	15.5
	Alanine	gain	14.8	15.4
	Glycine	loss	4.7	0.48
BSM Alkaline borohydride	Threonine	loss	16.6	33.7
	α-amino butyric acid	gain	2.1	210
	Serine	loss	31.2	55
	Alanine	gain	32.4	80
	Glycine	gain	0.27	0.35
BSM Alkaline borohydride with $PdCl_2$	Threonine	loss	17.0	34.5
	α-amino butyric acid	gain	14.7	147
	Serine	loss	31.7	56
	Alanine	gain	31.1	76
	Glycine	loss	0.19	0.30

See page 90

TABLE VIII (Continued)

Stoichiometry of Chemical Changes in Pseudomyxomatous Mucin Resulting from Treatment with Alkali (0.09 N LiOH) at 100°C for 1 hour[b]

	Amino acid residue	Change	μmoles/100 mg. mucin	% Change
PM-T	Threonine	loss	0.29	69
	α-amino butyric acid		c	c
	Serine	loss	0.07	43.7
	Alanine		d	d
	Glycine		0.08	80

[a] Reported by Tanaka and Pigman (1965)[65]

[b] Reported by Adams (1965)[49] for Trypsin isolated pseudomyxomatous mucin (PM-T)

[c] Not detected

[d] Not reported

which has been proposed is a peptide bond reduction that produces the amino alcohols, serinol and threoninol as it cleaves the peptide[143].

A destruction of serine and threonine that is not related to the β-elimination reaction can also arise from hydrolysis of the protein in the presence of carbohydrate (humin formation)[144]. However, the high dilution at which the glycoprotein hydrolysis was carried out kept these losses to a minimum[76] equal to that of the hydrolysate of mucin not treated with alkali. The control mucin and alkali treated mucin were hydrolysed in the presence of equal amounts of carbohydrate as none of the carbohydrate released by alkali was removed from the reaction medium before hydrolysis.

Supporting evidence of the occurrence of a β-elimination reaction in HGGM was the demonstration by paper chromatography of the formation of α-keto acids. The R_f values of the α-keto acids derived from HGGM after alkali treatment and those of the authentic α-keto acids co-chromatographed were similar. Evidently under the experimental conditions used the pyruvic acid and α-keto butyric acid dinitrophenyl hydrazone (DNP) derivatives existed in the two separable isomers. A failure to resolve the four isomers from a mixture of the two derivatives on paper chromatography might be remedied by using thin-layer chromatography. There has been a report of an artefact of DNP derivatives, namely:

1 hydroxy 6 - nitro - 1:2:3 benzotriazole. This artefact is characterized as slower moving than the slower of the pyruvic acid DNP derivatives and, as retaining its yellow colour when sprayed with alkali[145]. Although my results showed no separable artefact at this position, the pyruvic acid DNP derivatives turned a yellow-brown colour not the deep brown expected, upon dipping in alkali.

Quantitation of the ∝-keto acids (pyruvic acid) by enzymatic assay met with difficulty. The concentration of pyruvate as determined by the assay was 30 to 55 percent of the expected value. The colour reaction was affected by both the ∝-keto acids in the hydrolysate[134][146] and the presence of the hydrolysate itself. However, since the effect of the other ∝-keto acids (∝-keto butyric acid) increased with time[146], its influence (a positive error) was minimized by making readings within three minutes. The presence of the hydrolysate (2 ml. aliquot) in the reaction medium decreased the colour change although the pH remained the same[1]. A high salt concentration, a possible factor, did not alter the colour reaction.

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[1]Lactic dehydrogenase from beef heart remains stable at this pH of 9.5-10.0 for 1 hour at 30°C.[147]

In considering evidence for the occurrence of β-elimination the evidence offered by the losses of serine and threonine residues must be supported by the evidence of an accompanying substantial decrease in the molecular weight of the glycoprotein and vice versa.[148] HGGM was irreversibly degraded by alkali in the cold and was degraded more extensively by alkaline borohydride in the cold. This degradation was displayed by the assorted fragments which were separable upon gel filtration. The fragments ranged from dialysable carbohydrate, through carbohydrate chains of up to 100,000 g. and peptide - carbohydrate chains of perhaps 200,000 g. to a large mainly protein molecule of 2×10^6g. At this stage it is to be remembered that a demonstration of this two-way condition does not necessarily implicate a β-elimination reaction without the additional finding of the reduced amino acids or α-keto acids by an analysis of the reaction products. It is the simultaneous satisfaction of all these criteria which proves the occurrence of a β-elimination reaction. Thus HGGM has O-glycosidic linkages.

The presence of the large-sized fragments implies that fewer protein - carbohydrate linkages are involved than is the case for blood-group substances[(81)] or bovine submaxillary mucin[(64)].

The small loss of hydroxyamino acids in alkali-treated HGGM supports this hypothesis. In contrast to HGGM, bovine submaxillary mucin releases disaccharides upon alkali treatment and shows a large loss of hydroxyamino acids. Other carbohydrate-protein complexes with large carbohydrate chains have been found in connective tissue muco-polysaccharides[106].

Some consideration must be given to the fact that O-seryl glucosidic linkages which are generally susceptible to alkali may be located either internally or terminally along the peptide chain. A terminal O-seryl glycosidic linkage is less susceptible to alkali[71]. Thus losses of serine from mucin molecules with terminally located O-glycosidic bonds would be lower. This decreased lability would be especially evident under mild alkali conditions.

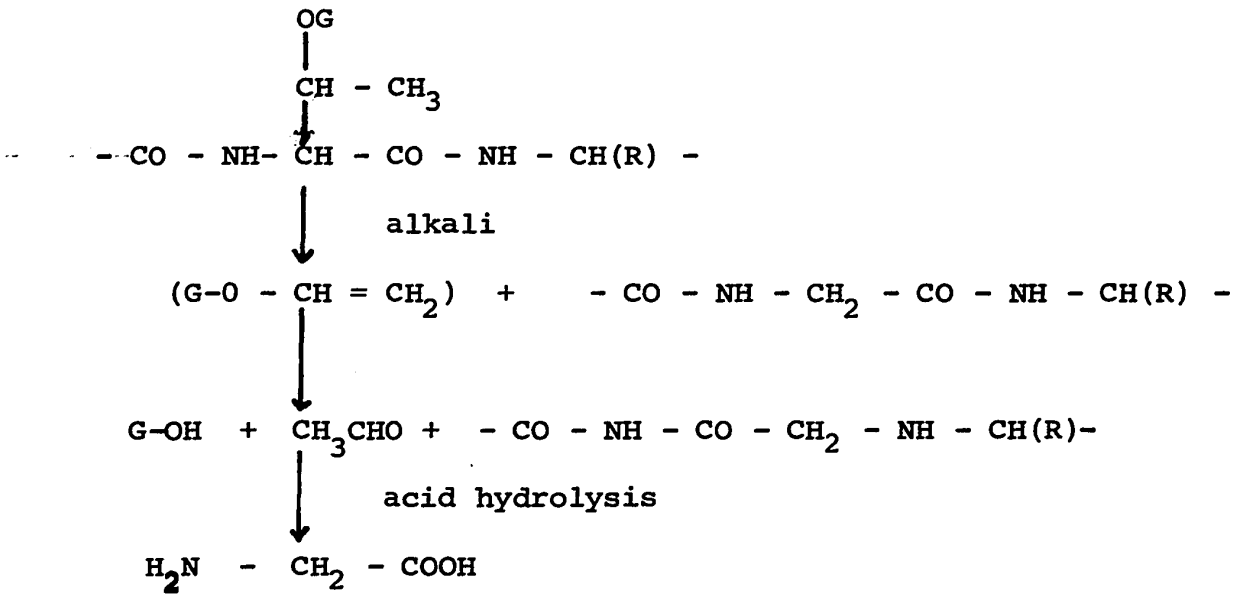
The temperature, sodium borohydride concentration, alkali concentration and length of time of reaction, are all critical factors in determining both the extent of the β-elimination reaction and which competing reactions may occur. Studies by Mayo and Carlson[149] of the effect of different conditions of alkali treatment on the rate of cleavage of N-acetyl chondrosine (O- β -gluopyranosyluronic acid-(1 $\longrightarrow$ 3)-2-acetamido-2-deoxy-D-galacto pyranose) have shown that when this compound is treated with 0.05 M sodium borohydride at 37°C the disaccharide

is completely cleaved within 30 minutes, but that no detectable destruction of the compound occurred for 60 minutes when the incubation mixture was maintained between 0°C and 4°C. Increasing the temperature to 37°C resulted in rapid cleavage.

It is known that HGGM treated with alkali at 37°C instead of 4°C is broken down to totally dialyzable units[150]. In comparing changes resulting from alkali-treatment of glycoproteins, one must be wary of the differences introduced by the reaction conditions.

Because different reaction products result from different conditions of alkali treatment, it is thought that several alkali-generated reactions are in operation. To explain the observed increase of glycine residues after alkali treatment of pseudomyxomatous mucin, Adams[48] invokes an alternate pathway of β-elimination (Process I). This process can operate only in threonyl-O-glycoside linkages and results in the hydrolysis of an internal threonine linkage after removal of the side-chain with glycoside from the peptide:

Process I (G = carbohydrate prosthetic group)



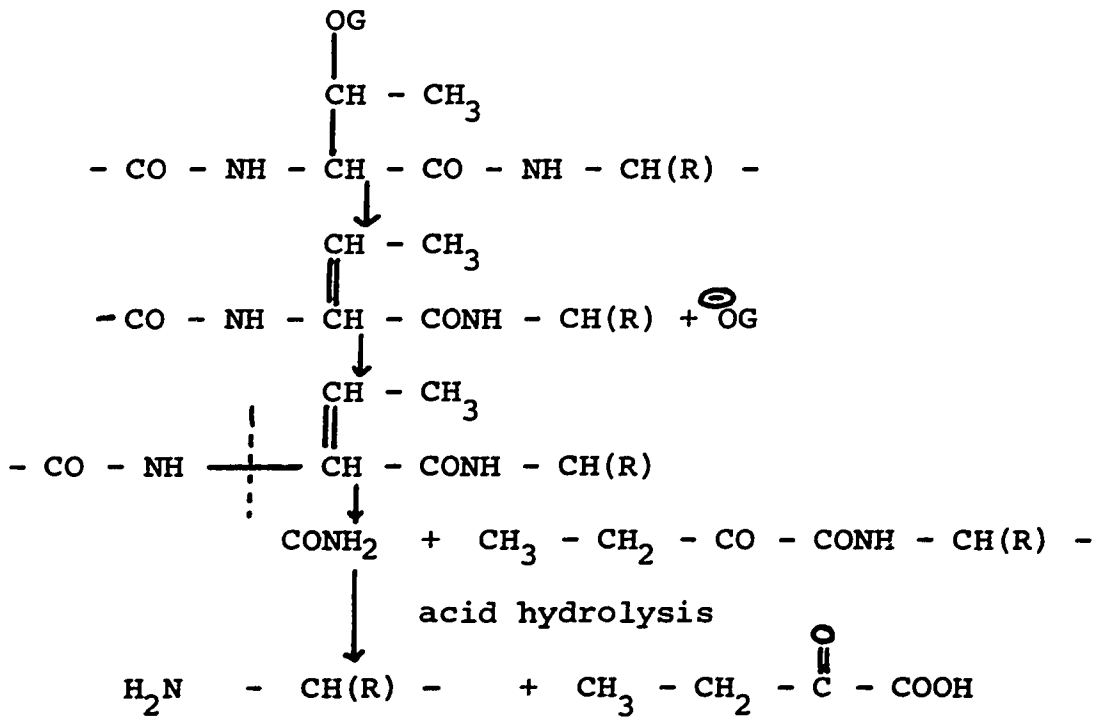
The comparatively small increases of glycine observed from alkali-treated HGGM suggest that this mechanism is less favoured than others under the experimental conditions[1].

By the alternate pathway, Process II devised by Adams, the dehydropeptide formed by elimination of the O-glycoside undergoes amide formation with the consequent rupture of the peptide chain. This results in ∝-keto butyric acid formation from O-threonyl glycosyl linkages. However the ∝-keto butyric acid found in hydrolysates of alkali-treated mucins may also have developed from the acid hydrolysis of the dehydropeptide, as the unsaturated amino acids are notoriously unstable to acid[(150)].

---

[1] An argument against this implied specificity of the glycine increase is the fact that amino acids can suffer a small number of internal bond breakages under acid hydrolysis conditions such as between C_2 and C_3 of serine which convert the amino acid residues to glycine residues. It is not known if bound carbohydrate protects the protein from this cleavage.

The difference between the products obtained by Process II and those obtained by the classical mechanism of the β-elimination reaction, (see 'Introduction', page 35), is that peptide cleavage has occurred before acid hydrolysis in Process II and thus the protein core size will appear to be smaller.



Notably there is some protein associated with the peaks representing the smaller fragments of alkali-treated mucin. The void volume fraction represents the peptide core protein (high molecular weight).[1] This fact is inconsistent with the dogma

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[1] The protein was measured both by the absorbance at 230 mμ and by the Lowry protein method.

presently held of a single large polypeptide backbone within a glycoprotein molecule, unless one invokes the mechanism for peptide cleavage during alkali treatment. The three processes by which the peptide cleavage can occur are:

1. Adam's proposed Process II of β-elimination reaction.
2. A non-β-elimination side reaction involving the reduction of hydroxyamino acids thereby splitting the peptide bond.
3. Base hydrolysis of the peptide bonds. However, this last reaction requires strenuous conditions such as refluxing of the glycoproteins with strong base and prolonged heat[151].

There are other alkali generated reactions which, if they occur, affect the carbohydrate prosthetic group. The polysaccharide which is cleaved initially as a unit from the peptide chain by alkali may suffer further degradation as the result of a 'peeling' or 'erosion' process from the reducing end of the carbohydrate chain[152]. Particularly susceptible to this reaction are the (1⟶3) linkages between sugar residues. Monosaccharides joined by (1⟶4) linkages, or having a high degree of substitution (as at branch points) stop the 'peeling' reaction. The ease with which a polysaccharide is degraded, therefore, yields information

about its internal structure. However, reduction reactions competing with 'peeling' reactions can reduce the unsaturated bonds required for 'peeling' and stop the reaction. Therefore, although (1 ⟶ 3) linkages may be predominant, certain conditions of treatment would give rise to oligosaccharides not monosaccharides. The influence of varied sodium borohydride concentration is not fully known.

The range of product possibilities due to the several β-elimination reaction mechanisms and several side reactions which can operate under alkaline conditions is not narrowed by the large size of the gel mucin molecule with its inherent variety of chemical components and types of linkages. An eloquent study of simpler glycoproteins has recently been published by DeVries and co-workers[153][154]. The results leave only one interpretation open to them. The glycoproteins studied were the curious freezing-point depressing glycoproteins of some Antarctic fishes. The ability of these glycoproteins to depress the freezing point is a direct result of their structure which therefore invited study. There are only five components which make up these molecules: threonine, alanine, N-acetyl galactosamine, galactose and sometimes proline. A β-elimination reaction was carried out in 0.5 N NaOH at 20°C for six hours[154].

The reaction was demonstrated by the loss of threonine, quantitative conversion of threonine to α-amino butyric acid and the quantitative release of reducing groups. The reaction was monitored at 241 mμ . The presence of an O-glycosidic linkage was thus unequivocably proven.

Precautions must be taken in the monitoring of the β-elimination reaction at 241 mμ . A cursory inspection of the results of alkaline pH on proteins and glycoproteins alike casts a grave doubt on the usefulness of this 'indication' of the reaction. Bovine albumin, ovalbumin, insulin and oxytocin are incapable of undergoing a β-elimination reaction, yet an increase at 241 mμ and 231 mμ is observed for each of these.

The absorbance change for the proteins and ovalbumin is immediate and reversible, implying the ionization of side groups or a change in conformation . That of HGGM is slower, requiring sixty minutes for completion but is also reversible.

The side chains of the amino acids cystine, histidine, methionine, phenylalanine, tryptophan and tyrosine all have chromophores which absorb in the middle ultraviolet range of 220 - 250 mμ . A change in the environment of these amino acids such as one perpetrated by a change in pH, will bring about a change in the absorbance (wavelength and intensity)

of these chromophores. Some ionizations of the tyrosine residues will occur at high pH (pH 13) with or without concomitant denaturation of the protein[1].

The greatest contribution to the chromophore absorbance is that of the multiple peptide bonds. These absorbances are measured at 230 mμ and rapidly reflect such conformations as random coils or α-helix[157][158].

Solvents such as urea and aqueous inorganic salt solutions are known to confer such spectral changes[159].

In glycoprotein solutions a marked discrepancy exists between the time required for a β-elimination reaction as monitored at 241 mμ and as detectable by changes of the amino acid composition. Whereas the increase in absorbance is complete after one hour at 4°C[72], no loss of serine or threonine residues can be detected even after 24 hours incubation. Furthermore, the losses of hydroxyamino acid residues calculated from the change in absorbance at 231 mμ and 241 mμ for gastric gel mucin exceed the number of such residues in the protein. There is thus insufficient evidence to support the use of a monitoring of the absorbance change at 241 mμ as an indicator of O-glycosidic linkage for glycoproteins in general. Such an

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[1] For example bovine albumin[155] need not be denatured while ovalbumin[156] need be.

interpretation may, however, be warranted if the glycoproteins involved have no aromatic or sulphur-containing amino acids and show upon treatment with alkali an irreversible time-dependent change in absorbance.

The results of this study of human gastric gel mucin by the β-elimination reaction are consistent with a model of HGGM as a huge fucomucin molecule with a large peptide core to which oligo or polysaccharide chains are joined by a small number of O-glycosidic linkages. The existence of O-glycosidic bonds does not preclude the existence of other linkages such as N-asparty-N-acetyl-glucosylamine bonds. This possibility deserves future attention[1].

The infrared absorbance spectrum of HGGM offers corroborative evidence for this structural model.

Attempts to detect thiol groups in reduced HGGM have been unsuccessful even with a sensitive radioactive technique (see Appendix 12). Evidently then the HGGM relies largely on its carbohydrate prosthetic groups for maintenance of its coherent gel structure as well as for its blood-group specific reactivity. The carbohydrate chains of one molecule would interdigitate with those of another to form the meshwork for which this mucin is named.

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[1]The application of N-aspartyl-N-acetyl-glucosaminidase in this regard might be successful[160]. The search for ester linkages might be carried out by hydroxamate formation [161][162].

The importance of the carbohydrate in determining the biologic function of mucin through a determination of the mucin structure leads one to ask various questions of the nature of the carbohydrate. How large are the carbohydrate chains? Which monosaccharide(s) is the partner to serine and threonine amino acid residues in the carbohydrate - protein linkage? Are the residues which determine the blood-group specificity in HGGM the same as in ovarian cyst fluid blood-group substances? Are the polysaccharide chains branched? Do any of the β-eliminated fragments carry blood-group reactivity. In what order, and in what orientation*, ((1⟶3) or (1⟶4)), are the monosaccharides attached?(81)

Studies of glycoprotein polysaccharide structure have been hampered by the presence of the protein. A controlled β-elimination reaction would be useful in releasing the carbohydrate for further study as would specific enzymes such as β-N-Acetyl-hexosaminidase(163). The understanding of the β-elimination reaction and its intricacies could profit from a thorough investigation using tritiated Na BH^3_4.(55)

The β-elimination reaction can also be applied to remove the protein from the presence of carbohydrate —long found detrimental to protein studies. In the absence of the

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* α or β linkage

carbohydrate, the end-group amino acids and the sequence of the peptide may be determined. The question of the size of the core peptide might be answered by rechromatography of the Sephadex G-200 void volume fraction on gels of higher fractionation range (Sepharose 2B, 4B)[1]. The size may also be determined by sedimentation equilibrium centrifugation studies of multiple pooled Sephadex G-200 void volume fractions.

Of critical importance to the development of a model accounting for the biological functioning of mucus is the spacing of the carbohydrate chains along the peptide core - the presence of these bulky chains-hinders pepsin digestion.

Determination of other facets of gel mucin such as a more closely approximated gel size must await the development of techniques which can handle these complex and somewhat polydisperse molecules[2].

The structural studies of HGGM will find their final application in the biological and medical studies of the physiological function and disfunction of gel mucin.

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[1] These gels, available from Pharmacia, Uppsala, Sweden, have a fractionation range between them of 300,000 to 30,000,000 as calibrated for proteins.

[2] Gel permeation chromatography of chemical derivatives of whole mucin thus made soluble in organic solvents is a possible method. Otherwise chromatography on aqueous gels of high fractionation range might be successful should enough well-defined glycoproteins be available to calibrate the elution volumes according to molecular weight (size and shape)

V. SUMMARY AND CONCLUSIONS

A preliminary chemical analysis of human gastric gel mucin showed that the hydroxyamino acids made up a high proportion of the total amino acid composition. This suggested the presence of O-glycosidic linkages between the protein and carbohydrate moieties. To test this hypothesis and to further investigate other aspects of the structure of gel mucin, the effect of alkali on mucin was studied.

The results of alkali treatment, namely those of a β-elimination reaction: the specific losses of threonine and serine residues, the formation (in the presence of reductant) of alanine and α-amino butyric acid residues, and the formation (in the absence of reductant) of pyruvic acid and α-keto butyric acid, in conjunction with the release of carbohydrate as smaller molecular weight fragments substantiated this hypothesis.

The presence of O-glycosidic linkages* in gel mucin is consistent with the infrared spectrum of gel mucin.

The gastric gel mucin is visualized as a fucomucin of very high molecular weight, consisting of a protein core to which are joined exceptionally large polysaccharide chains by O-glycosidic and possibly other linkages. The gel structure is maintained by the interaction between molecules of the poly-

---

* α linkages ?

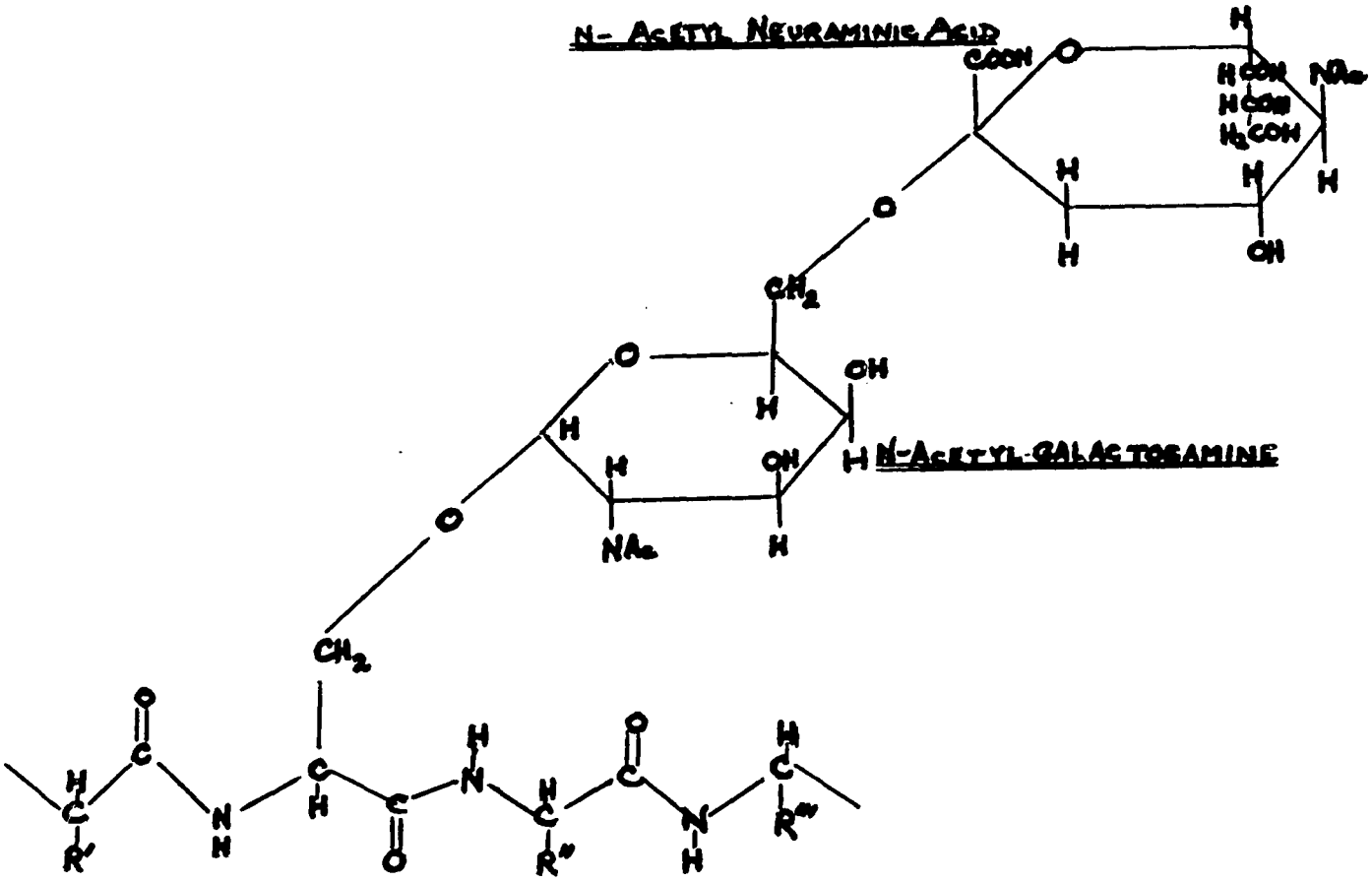
saccharide chains thus enabling the mucin to form a protective meshwork over the mucosa. It is not known at what intervals the polysaccharide chains are attached to the protein. With this information the geometry of the molecule could be defined well enough to propose a working model of how mucin protects the gastric mucosa.

APPENDIX VI

TABLE OF CONTENTS

VI. APPENDIX

The proposed structure of the O-glycosidic linkage in ovine submaxillary mucin



OVINE SUBMAXILLARY MUCIN

Investigation of Thiol Group(s) in Human Gastric Gel Mucin

The importance of disulphide cross-links in maintaining macromolecular structures as in hair keratin and insulin is not disputed. The continuous search for these cross-links or the sulphur amino acids of these linkages (cysteine, cystine) has encouraged the development of different methods of detection. These methods vary according to the degree of applicability and senitivity.

The amino acid analysis of HGGM showed occasional traces of half-cystine*. Therefore an attempt was made to detect the thiol group by two other techniques:-

1. Titration of thiol groups with Ellman's reagent[166]
2. Radioactive aklylation of thiol groups, the radioactivity of the reacted components being traced by autoradiography[167]

The source of materials appears earlier in 'Materials and Methods', page 41

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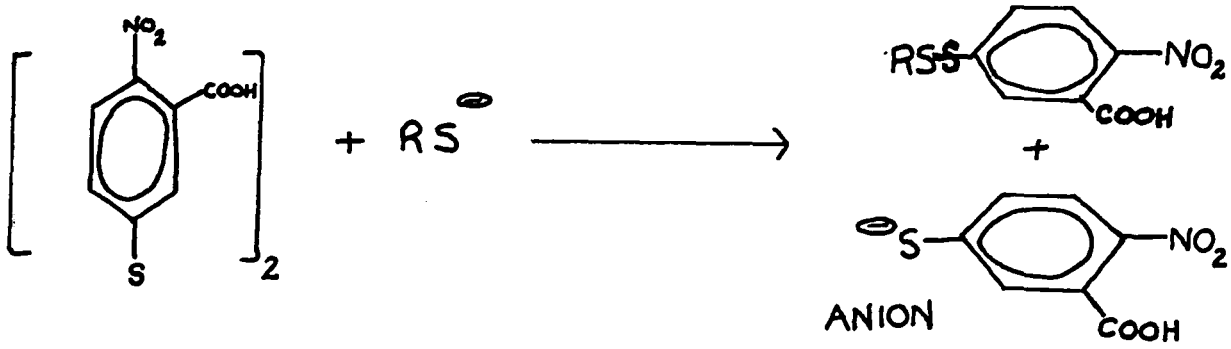
* The hydrolysates analyzed did not undergo oxidation with performic acid prior to acid hydrolysis. The major purpose of the amino acid analyses was a quantitation of the hydroxyamino acids not of cysteic acid[165]

Titration of Thiol Groups with Ellman's Reagent

Ellman's Reagent, 5, 5′ di thio-bis (2-nitro benzoic acid) undergoes the following reaction with free thiol groups:



The anion absorbs at the wavelength 412 mμ (ε = 13,600). The concentration of anion is a direct measure of the thiol groups which were accessible to the reagent. Ellman's[166] method was followed faithfully with the exception that twice as much Ellman's Reagent (at a concentration of 10 μM and a pH of 7.0) was added to the reaction mixture. A standard curve which was found to follow Beer's Law was calibrated with fresh solutions of N-acetyl cysteine. Because a variation in the development of colour with small changes of pH in aqueous solutions was noted, all experiments were done with 8M urea solutions (including the standard curve) in which no such variations occurred. Measurements of colour development (using the protein solutions as blanks) at pH 8.0 were made after twenty minutes reaction time on a Beckman DU Spectrophotometer.

The substances examined were bovine albumin, human citrated plasma and human gastric gel mucin. A table of the results appears on page 114.

Radioactive Carboxymethylation of Reduced HGGM

It has been reported that sulphydryl reagents vary in the extent of their reaction with thiols. This variation is due largely to differences in the size of the reagent molecules. As the size of iodacetamide is smaller than that of 5,5'-dithio-bis (2-nitro benzoic acid), an attempt to detect alkylated cysteine (carboxymethyl cysteine) was made with radioactive C^{14} iodacetamide.

The HGGM was reduced with dithiothreitol (Cleland's Reagent) prior to alkylation. The reduction and alkylation were carried out by the method of Hashimoto and Ludowieg[167]. The two samples of mucin (blood-group A, secretor; whole and the non-retarded Sephadex G-200 fraction) (50 mg.) were each reacted with 1.5 mg. C^{14}-1-iodoacetamide as described. The carboxymethylated mucin was dialyzed exhaustively and subsequently hydrolyzed in vacuo in 2 ml. 6N HCl. The final hydrolysate preparation, thrice died on a rotary evaporator, was taken up in 0.5 ml. water and centrifuged, and applied to Whatman No.3 MM paper electrophoresis strips. Electrophoresis was carried out at constant low voltage for three hours on a standard Beckman-Spinco vertical apparatus. The buffer employed was 0.05 M ammonium formate pH 3.5-3.6. After electrophoresis

the strips were visualized either by ninhydrin or by the starch-iodide technique of Rydon and Smith[169]. This latter method was relatively selective for iodoacetamide. With a longer exposure period (15 minutes) in chlorine gas the hydrolysate amino acids sometimes showed faintly.

The radioactive bands of the hydrolysate thus prepared were developed as autoradiograms by contact with films as follows:

1. Kodak medical no-screen X-ray film,
   3 weeks exposure time
2. Kodak medical no-screen X-ray film,
   5 weeks exposure time
3. Kodak screen film, 6 weeks exposure time
4. Anscofilm panchromatic camera film, 1 week
5. Anscofilm panchromatic camera film, 12 days

A diagram of the electrophoretograms and a discussion of exposures appear on pages 116-118.

RESULTS

Reactivity of Ellman's Reagent

Titration of Fresh Mixed Human Blood Plasma in 8M Urea

Volume of sample		Absorbance at 412 mμ	Concentration m moles SH/l[1]
Sample 1.	0.2 ml	1.80	6.5
	0.2 ml	1.83	6.6
Sample 2.	0.2 ml	1.73	6.2
	0.2 ml	1.79	6.45
Average	0.2 ml	1.76	6.29

Titration of Bovine Albumin in 8M Urea

Concentration of protein (m.w.65,000 g)		Absorbance at 412 mμ observed	Concentration moles SH/mole protein[2]	
			Expected	Observed
6.0×10^{-5}M (4 mg/ml)	1.	0.48	0.66	0.50
	2.	0.51	0.66	0.53
	3.	0.54	0.66	0.62
	4.	0.52	0.66	0.54
Average		0.51	0.66	0.55

[1] Calculated by the formula:

$$C_o = \frac{A * D'}{\varepsilon}$$

where C_o = concentration
A = absorbance at 412 m
D' = dilution factor
ε = extinction coefficient 13,600/M/cm and D' = 3.6

[2] The molecular weight of bovine albumin of 65,000 gm. The theoretical concentration of 0.66 moles SH/moles protein is reported by Cunningham L.W., Naenke B.J., Strayhon W.D. J. Biol. Chem. 228, 835 (1957)

Titration of HGGM in 8M Urea

Substance		Absorbance at 412 mμ
N-Acetyl cysteine 0.10 mg		0.80 ± 0.03*
HGGM (whole) Blood-group A, secretor		
	1.	0.002
8 mg/ml	2.	0.005
	3.	0.000
	4.	0.006

* The average value of eight determinations

Carboxymethylation of HGGM

All but two of the exposures to the β-particles of the carboxymethylated mucin were negative. In the two instances of 3 and 5 week exposure of Medical No-Screen film to the carbon-14, two slight bands of darkening occurred at a position corresponding to the carboxymethyl cysteine band on the electrophoretogram. Both the mucin hydrolysates were equally ineffective. The camera (visible light) films which have a thinner, and therefore more sensitive emulsion layer, failed to reveal any radioactivity.

Figure 13. on page 118 represents the electrophotograms.

DISCUSSION

The unsuccessful attempt to conclusively show the presence of sulphur groups in HGGM may indicate the absence of any sulphydryl amino acids in mucin, or alternatively, the insensitivity of the methods employed. Two other methods might meet with more success:

1. The radioactive alkylation might be repeated with more isotope and a subsequent automatic amino acid analysis in conjunction with a scintillation counter.
2. A micromethod for the detection of thiol groups in proteins employing atomic absorption spectrophotometry has been recently developed. This method depends on the ability of the mercurial p-hydroxy mercuribenzoate to interact with the thiol[170]

FIGURE 13

Electrophoretograms of Reduced and Alkylated Glycoproteins

To all the samples an amount of carboxymethyl cysteine was added in order to locate the position of this amino acid.

A. HYDROLYSATE OF OVALBUMIN. ELECTROPHORETOGRAM VISUALIZED WITH NINHYDRIN.

B. HYDROLYSATE OF WHOLE HGGM (BLOOD-GROUP A, SECRETOR). ELECTROPHORETOGRAM VISUALIZED WITH STARCH-IODIDE.

C. HYDROLYSATE OF HGGM (BLOOD-GROUP A, SECRETOR) (NON-RETARDED SEPHADEX G-200 FRACTION). ELECTROPHORETOGRAM VISUALIZED WITH NINHYDRIN.

BAND DESIGNATIONS

CMC = carboxymethyl cysteine

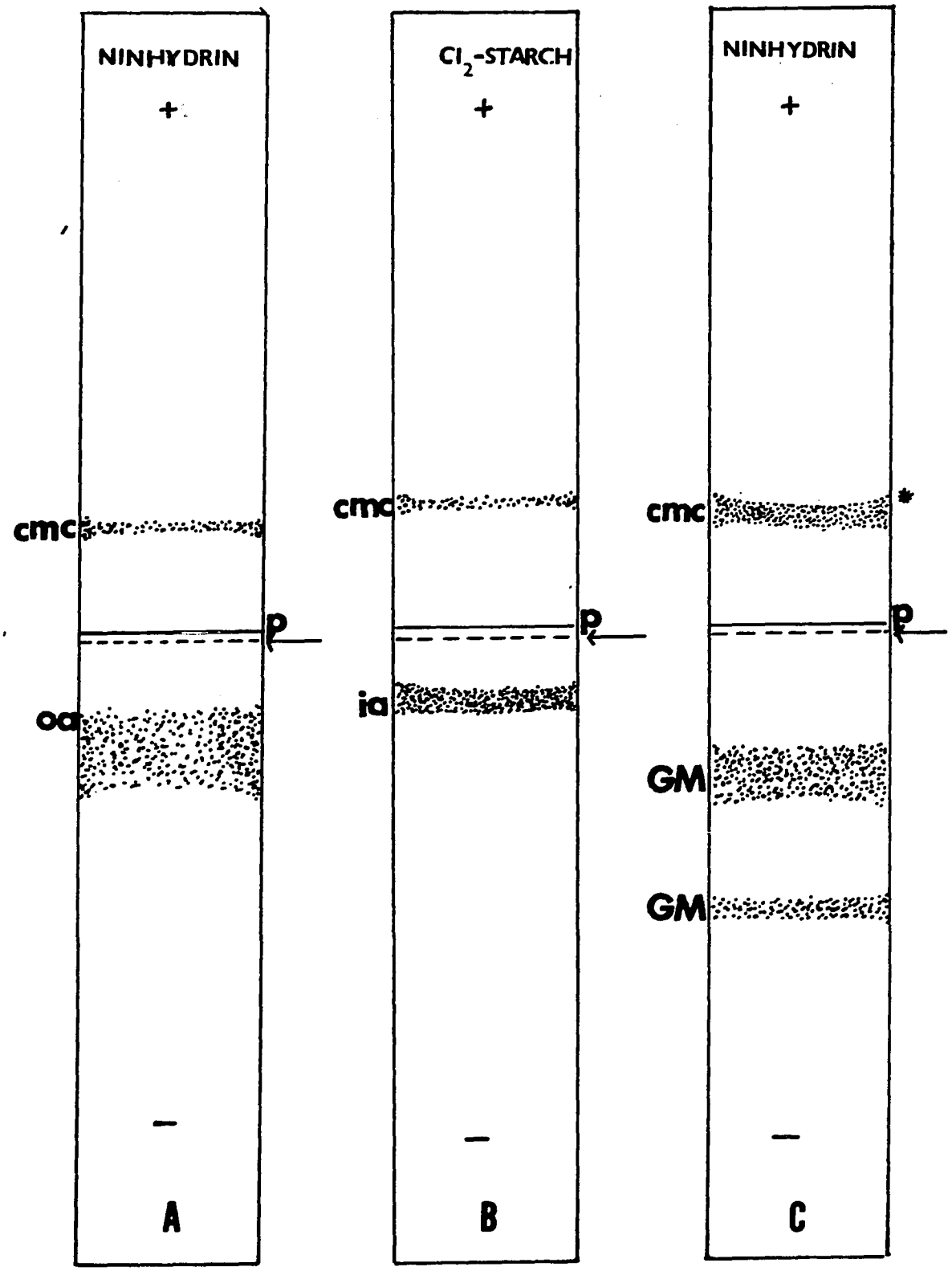
P = electroendosmosis marker

oa = ovalbumin

GM = human gastric gel mucin

The arrow indicates the origin of sample application.

* indicates suspected radioactivity


NINHYDRIN
+
cmc
P
oa
−
A
Cl2-STARCH
+
cmc
P
ia
−
B
NINHYDRIN
+
cmc
*
P
GM
GM
−
C

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Description of Pocket Contents

APPENDIX: Carboxymethylation of HGGM

The actual autoradiograms.

The paper strip number "4" on the X-ray film corresponds to paper strip "C" described on page 117