ABSTRACT

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STUDIES ON THE PROTEOLYTIC HYDROLYSIS OF TISSUES FOR THE LIBERATION OF THE MUCOPOLYSACCHARIDES AND ON THE DETERMINATION OF THE CONSTITUENTS OF MUCOPOLYSACCHARIDES BY GLPC

A study was carried out to assess various isolation procedures for mucopolysaccharides. The isolation procedures consisted of several combinations, using papain or pepsin-trypsin for hydrolysis, and absolute ethanol or cetylpyridinium chloride (CPC) as the precipitants. It was found that both enzymatic hydrolysis worked equally well as did the precipitants, CPC and absolute ethanol. Sulphate loss during both hydrolyses was quite extensive. In several of the avian tissues, the predominate fatty acids (as determined by GLPC) were found to be C_{16}, C_{16}^{-}, C_{18}, C_{18}^{-} and $C_{18}^{=}$. The components of mucopolysaccharides were studied by GLPC. The best GLPC column was found to be a 5% SE-30 on chromosorb W-AW-DMCS. Programs were developed for the separation of neutral sugars, uronic acids and amino sugars. The optimal conditions for the release of the components of mucopolysaccharides from samples were found and this procedure was used for the GLPC study of several mucopolysaccharide samples previously isolated from avian skin.

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by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

Department of Agricultural Chemistry,
McGill University,
Montreal, Quebec.

December 1970

Suggested short title

ISOLATION AND GLPC STUDIES ON MUCOPOLYSACCHARIDES

ALBRIGHT

ACKNOWLEDGMENTS

The author wishes to express his most sincere thanks to Professor P.A. Anastassiadis for his untiring interest and guidance which he displayed during the progress of this work.

The author is also indebted to the following for their helpful collaboration:

Mr. Keith Bruce and Mr. Michael Channon, for their co-operation when a helping hand was needed.

Professor G.O. Henneberry, whose watchful eye managed to keep the gas-chromatograph in good working order.

The author also wishes to acknowledge the financial assistance provided by the grants from the National Research Council of Canada, and the Department of Agriculture of the Province of Quebec.

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GENERAL INTRODUCTION

Within the last twenty years, research on mucopolysaccharides and glycoproteins has greatly advanced and has come to the forefront as one of the most progressively expanding fields. Much of this interest has been generated due to the close association of mucopolysaccharides to inflammatory diseases of the joints, such as arthritis and rheumatism.

In recent years this department has been keenly interested in mucopolysaccharides and glycoproteins. Much work has been done on electrophoretic separation, characterization, and isolation of these protein-carbohydrate complexes. Most of the work has been confined to bovine, porcine, and avian species.

This thesis is a study designed to advance the knowledge of mucopolysaccharides from an isolation and characterization point of view. Two methods of isolation were studied in an attempt to determine the best method of obtaining mucopolysaccharides from biological tissue, and gas chromatographic techniques were also investigated in an attempt to develop a simple and rapid method of analyzing mucopolysaccharides and the carbohydrate part of the glycoproteins.

once they had been isolated from the tissue.

I. LITERATURE REVIEW

A. NOMENCLATURE AND CHEMICAL STRUCTURE

1. Nomenclature

At present there is no universally accepted terminology for the definition of mucosubstances (of which mucopolysaccharides and glycoproteins are a part) formed by the combination of proteins and carbohydrates. The nomenclatures proposed by different authors are not consistent and many have used the same terms to mean different things.

In 1908 (1) a definition was proposed by the Physiological and Biochemical Committees on Protein Nomenclature for the term glycoprotein. It was defined as "compounds of the protein molecule with a substance or substances containing a carbohydrate group other than nucleic acid."

Levene (2) in 1925 made the first classification based on the chemical composition of the carbohydrate moiety of mucosubstances. His classification soon became obsolete.

Meyer (3) in 1938 classified the carbohydrate-protein complexes based upon their carbohydrate moieties. He introduced two terms, "mucopolysaccharides" and "glycoprotein".

Stacey (4) in 1946 and Kent and Whitehouse (5) in 1955 enlarged upon Meyer's original classification.

Bettelheim-Jevons (6) divided the carbohydrate-protein complexes into mucopolysaccharides which contained a large amount of carbohydrate and mucoprotein which contained small amounts of carbohydrate.

For a time it was argued that these substances should be classified according to the type of linkage between the protein and the carbohydrate units. However, it later became evident that this was not an appropriate criterion for classification.

One of the more recent classifications has been proposed by Winzler (7), and is actually a modification of Meyer's (3) original classification. This classification appears as follows:

I. Mucopolysaccharides:

High-molecular weight polysaccharides containing hexosamines.

A. Neutral mucopolysaccharides: Polysaccharides containing hexosamine and neutral monosaccharides.

B. Acid mucopolysaccharides: Polysaccharides containing hexosamine, uronic acid and/or sulphuric acid.

II. Mucoprotein: Protein combined with acid mucopolysaccharides in polar and other easily split types of linkages.

III. Glycoprotein: Substances with the properties of protein and which contain 0.5% or more of hexosamine bound to protein.

A. Glycoids: Glycoprotein containing from 0.5 - 4.0% hexosamine.

B. Mucoids: Glycoprotein containing more than 4% hexosamine.

The most recent system of nomenclature has been presented by Jeanloz (8). In this system the use of the prefix "muco" has been discontinued and the term "glycosaminoglycan" has been used for the protein-carbohydrate complex. The following table illustrates his nomenclature:

Glycosaminoglycan - polysaccharides that contain amino sugars

Glycoaminolipid - lipids that contain amino sugars

Glycosaminoglycuron - polysaccharides that contain amino sugars and uronic acid moieties

Glucosaminoglycan - polysaccharides that contain glucosamine

Galactosaminoglycan - polysaccharides that contain galactosamine

For well-defined pure polysaccharides, Jeanloz applied a suffix "an", e.g.:

Keratosulfate - keratan sulfate;

Heparitin sulfate - heparan sulfate.

The old established names such as chitin, hyaluronic acid, chondroitin and heparin were left unchanged.

Upon suggestion by Dr. K. Meyer, the terms chondroitin sulphate, A, B and C were changed to chondroitin 4-sulfate, dermatan sulfate and chondroitin 6-sulfate, respectively.

Such is the state of the nomenclature, but still confusion exists. The terms that appear in this thesis will be the most commonly used terms.

2. Chemical Structure of Various Mucopolysaccharides

Recent years have seen a great increase in the number of papers and reviews concerning the structure of the various mucopolysaccharides (8-15). Also many reviews have been published on their biological functions (16) and their metabolism (17). Some of the aspects, both chemical and biological, of the most common mucopolysaccharides will be discussed very briefly.

a. Chondroitin Sulphates A-B-C

After first being isolated over a century ago (18), chondroitin sulphate A was thought to contain acetic acid, sulphuric acid, hexosamine and hexuronic acid in equimolar amounts (19,20). The identification of the hexosamine and hexuronic acid was the subject of much research for many years.

The hexosamine moiety of chondroitin sulphate A was first shown to be either 2-amino-2-deoxy-galactose, or 2-amino-2-deoxy-D-talose (2). Later, by synthesis, it was proven that it was 2-amino-2-deoxy-D-glucose.

Bray (21) and James (22) established the actual conformation of the monosaccharides of chondroitin sulphate A.

Levene (23) made the wrong assumption that the disaccharide contained a (β-1-4)-hexosaminidic linkage. This was later corrected to be a (β-1-3)-hexuronidic linkage by Davidson (24).

Chondroitin sulfate B was first isolated from pigskin in 1941 (25). It was found to contain equivalent amounts of chrondrosamine, acetic acid, uronic acid and sulphate. The amounts obtained for uronic acid by the carbazole method gave figures much lower than for either chondroitin sulphate A or C. It was later established by Jeanloz and Stoffyn (26,27) that these low values were due to the existence of iduronic acid.

The structure of chrondroitin sulphate B was further investigated by methylation studies carried out by Jeanloz, Stoffyn, and Trenege (28). The results showed that the hexosamine residues were unsubstituted at C-6 and the sulphate group was located at C-4 position (of the hexosamine residue).

Recently it was shown by Hoffman et al. (29) that chrondroitin sulphate A and B were stereoisomers, and only differed at C-5 of the hexuronic acid moieties.

From this information it is possible to give chrondroitin sulphate B the structure of repeating units of (1-4)-0-L-idopyranosyluronic acid-(1-3)-2 acetamido-2-deoxy-4-0-sulpho-β-D-galactopyranose.

Chrondroitin sulphate C differs from chrondroitin sulphate A in the position of the inter-sulfate groups. In the chondroitin sulfate A C-4 of the hexosamine moiety is esterified, while in chrondroitin sulphate C, the C-6 of the hexosamine moiety is esterified.

This difference of the sulfate group was first established by Ori (30) who showed the differences in the infrared spectras of the two substances. These conclusions were confirmed in the latter studies of Jeanloz and Stoffyn (26) on chrondroitin sulphate B.

In conclusion, while chrondroitin sulphate A is composed of repeating units of (1-4)-0-β-D-glucopyranosyluronic acid-(1-3)-2 acetamido-2-deoxy-4-0-sulpho-β-D-galactopyranose, chrondroitin sulphate C is made up of the repeating units (1-4)-0-β-D-glucopyranosyluronic acid-(1-3)-2 acetamido-2-deoxy-6-0-sulpho-β-D-galactopyranose.

b. Hyaluronic Acid

Hyaluronic acid has been shown to be composed of 2-acetamido-2-deoxy-3-0-β-D-glucopyranosyluronic acid-D-glucose, linked by 1,4-β-glycosidic linkages. This structure has been reviewed by many workers and was revised by Meyer (31) and Jeanloz (32).

While various other studies (33-36) have provided further evidence of this chemical structure of hyaluronic acid, the physical characteristics of hyaluronic acid isolated from various tissues varY greatly. It is not known whether this difference in physical characteristics is due to a difference in the molecular structure or an artefact caused by isolation and purification procedures.

Davidson and Small (17) have suggested from studies of the incorporation of (^{14}C6) glucose into rabbit skin, that these different hyaluronic acid entities are due to the existence of more than one metabolic pool for the formation of this constituent. In other words, different fractions occur as a consequence of the mechanism by which hyaluronic acid is synthesized, and these small amounts of low molecular weight hyaluronic acid are firmly bound to insoluble structural tissue components, and it is these components which are used to form the large macro-molecules characteristically

present in the ground substance of the mucoid layer connective tissue.

c. Heparin

Heparin was first discovered by McLean (37) while studying the blood-coagulating properties of the phosphatide cephalin from the liver.

Heparin was first recognized to be of great importance when its anticoagulant and antilipaemic properties were fully realized.

The identity of the hexosamine component of heparin was first established in 1936 (38) by Jorpes and Bergstrom who isolated 2-amino-2-deoxy-D-glucose hydrochloride from an acetic hydrolysate of the polysaccharide.

Because of acid-induced degradation (39), the isolation and characterization of the uronic acid moiety has been a problem. D-glucuronic acid was conclusively shown to be present in heparin by Foster et al. (40) by hydrolysis of heparin by 2N sulphuric acid at 95-100^{o} for three hours.

Various other reports (41-43) have confirmed the presence in heparin of glucuronic acid. Also, the fact that no other hexoses can be detected suggests the presence of glucuronic acid as the principle acid component of heparin.

d. Heparitin Sulphate

Heparitin sulphate is a polysaccharide closely related to heparin. It is found in various mucopolysaccharides (44) and in large amounts in amyloid deposits (45).

It is a polymer that consists of equivalent amounts of 2-amino-2-deoxy-D-glucose, and D-glucuronic acid and one sulfate group per repeating unit. Indications are that half of the glycosamine units are O-sulfated and the other N-acetylated (46), and that the linkages are D-linkages, an indication based on the strong positive optical rotation of the substance.

There is an uncertainty which still remains about the structure of heparitin sulfate and its relation to heparin. Both share common features. One difference is the presence in the heparitin sulfate of N-acetyl groups accounting for half of the amino groups present. The other half, like heparin, is N-sulfated. Depending upon its source, heparitin sulfate may be fractionated into two fractions: one with a high N-acetyl content and a second with a high N-sulfate content (47). Also the presence of L-iduronic acid as a minor component has been established for both heparitin sulfate and heparin (48).

All this raises the inevitable question of whether heparitin sulfate is actually two simple polysaccharides not adequately resolved by the present techniques.

e. Keratosulfate

Keratosulfate was first isolated by Meyer et al. (49) from bovine cornea.

The structure of keratosulfate has been studied mainly by methylation techniques (50). A comparison of the monosaccharide products from methylated keratosulfate and methylated, disulphated keratosulfate has provided evidence of the positions of the sulphate ester grouping and the glycosidic linkages.

Keratosulfate contains D-galactose and 2-acetamido-D-glucose, as well as a sulfate ester grouping.

B. ISOLATION OF MUCOPOLYSACCHARIDES

Since 1860 when Rollett (51) extracted a mucopolysaccharide from skin with lime-water and precipitated it with acetic acid, many new methods of mucopolysaccharide isolation have been attempted.

Methods of mucopolysaccharide isolation in general fall into two categories, those employing alkaline hydrolysis of dried, defatted tissue and those employing proteolytic enzymes. Certain mucopolysaccharides, such as hyaluronic acid from umbilical cord (52) and chondromucoid can be extracted by water, but these may be considered as exceptional cases.

Most early methods for the isolation of mucopolysaccharides used very drastic methods. Most methods used hot acid or alkali, or 90% phenol (53). Many workers however, contested the severity of the procedures, as they felt that some of the mucopolysaccharide was being degraded (19).

Recent methods used for the isolation of mucopolysaccharides have used weaker isolation procedures, usually salt solutions or weak alkali.

Meyer and Smyth (54) developed the first procedure that extracted mucopolysaccharides with a salt solution. They treated tissues with 10 percent calcium chloride. After treating the extract with chloroform-amyl alcohol and Lloyd's reagent to remove protein, the mucopolysaccharides were precipitated with absolute ethanol.

Various concentrations of calcium chloride were used successfully by several workers (55,56,57,58).

Weak alkali extractions have been used successfully by several workers (59,60,61).

Pearce and Watson (62) combined weak alkali and salt extractions for the isolation of mucopolysaccharides from human skin.

Various salts have been used for extractions, but the most commonly used salt is calcium chloride.

Proteolytic enzymes are now considered to be the best

method to isolate mucopolysaccharides from biological tissues. For a long while commercial enzyme preparations contained high concentrations of a mucopolysaccharide and also mucoids, which inhibited the enzyme. Since then, the enzyme preparations have become more purified, and this is no longer a problem to the researcher.

Various enzymes have been used for proteolytic hydrolysis. Among those used are pepsin, trypsin, papain, pronase, ficin and pancreatin. Combinations of these enzymes have also been used. One of the first isolations involving proteolytic enzymes was carried out by Meyer and Chaffee (63), who used a combination of pepsin and trypsin. Meyer et al. (64) later modified this method, and it became the standard method used for isolation of mucopolysaccharides after hydrolysis of the tissue with pepsin and trypsin.

Following the work of Meyer et al. (64) many isolations were carried out using pepsin-trypsin combinations (65-67). One of the latest isolations involving pepsin-trypsin was carried out by Oppenheimer et al. (68). They were able to isolate mucopolysaccharides found in fibre sarcomas and Flexner-Jobling carcinomas.

In 1961 Bertelsen and Marcker (69) isolated the mucopolysaccharides from human aortas by the use of pancreatin.

They found hyaluronic acid and chondroitin sulfate.

The use of papain or a combination of papain with trypsin has increased greatly in the last few years. One of the first to use such a combination was Shiller et al. (70). First they digested acetone dehydrated rat skins with papain. At this point most tissues were in solution; however, if they were not, sodium hydroxide was added to a concentration of 0.5M, the mixture shaken, and dialyzed. The dialyzed sample was then digested with trypsin.

Fabianek et al. (71,72) followed the method of Shiller et al. (70) for digestion of their tissues, except that more papain and more trypsin was used per gram of tissue. After digestion, the mucopolysaccharides were precipitated with cetylpyridinium chloride.

Many workers have used papain alone for their digestions. It has a slightly wider specificity than most animal proteases, and is readily available in pure form. It has a few disadvantages. It must be activated by a reducing agent such as cystine, 2-3-dimercaptopropanol or potassium cyanide. The addition of EDTA maintains the activity.

Slack (73) has used papain to digest various tissues at a temperature of 45^{o}C and pH 6.0. The digestion was more complete than if a combination of pepsin-trypsin had been used.

Papain, however, is quite resistant to inactivation and can be used at temperatures of up to 65°C.

Searls (74) used papain to digest tissue from embryonic chicks that was not digestible with 0.5N sodium hydroxide. Following digestion with papain, the mucopolysaccharides were separated with Sephadex.

Antonopoulos et al. (75) extracted released glycoaminoglycans from fracture callus from rabbits after digestion with papain.

Prame et al. (76) used papain to digest eye tissue to release the mucopolysaccharides. Pearce and Mathieson (77) recently carried out an investigation as to the effectiveness of various methods. Those methods compared were those of Bollet et al. (78), Meyer et al. (64), Scott (79) and Schiller et al. (70). Grossly impure products were obtained for the procedures of Bollet et al. (78) and Scott (79). Shiller's (70) method gave a product of similar purity, while the method of Meyer et al. (64) gave a product of low purity, because of difficulties with removal of protein with chloroform-amyl alcohol and kaolin.

Other plant proteases such as ficin (80,81) have been used with some success to liberate the mucopolysaccharides from tissue.

After the tissue has been solubilized by whichever means available, the mucopolysaccharides must then be extracted from the solution. Often the solutions have been dialyzed to remove amino acids, peptides and small molecular weight by-products. This, however, is not often used, as frequently large losses of mucopolysaccharides may also occur. For example, heparin is easily lost through the membrane.

Perhaps the easiest way to obtain the mucopolysaccharides from the solution is by selective precipitation, either by ethanol in the presence of a high concentration of NaCl, $CaCl_2$, or sodium acetate, or by precipitation of the mucopolysaccharides by a quaternary ammonium salt, such as cetylpyridinium chloride (CPC), cetyltrimethyl-ammonia (CTA) or cetyltrimethylammonium bromide (CTAB).

In 1956 Meyer *et al.* (64) first outlined a fractionation scheme using ethanol to separate the mucopolysaccharides as their calcium salts. Below is their scheme.

Polysaccharide precipitated	Concentration (%) of alcohol
chondroitin sulphate B	18-25
hyaluronic acid	30
chondroitin sulphate A	30-40
chondroitin sulphate C	40-50
keratosulphate	50

Instead of precipitating the mucopolysaccharides as the calcium salts, Jorpes and Gardell (82) and Smith and Gallop

(83) precipitated the mucopolysaccharides by ethanol as the barium salts.

Hilborn (14) precipitated the mucopolysaccharides as their calcium salts, using ethanol. He claimed that a 1.2 volume ethanol concentration precipitated the mucopolysaccharides, while an ethanol concentration of 4.0 volumes formed a precipitate largely composed of glycoprotein material.

The precipitation reaction of quaternary ammonium salts was first observed by Kuhn in 1940 (84). Kuhn only applied the precipitation to proteins. Later the precipitation reaction was applied to polysaccharides (85-87).

Shiller et al. (70) after papain hydrolysis of rat skin, precipitated all the mucopolysaccharides by adding CPC until an excess had been added. Hyaluronic acid and chondroitin sulphuric acid were quantitatively extracted and separated, the principle involved was based upon the differential solubility of the mucopolysaccharide-CPC complex in NaCl.

Many workers have now presented many different methods using quaternary ammonium salts to isolate the mucopolysaccharides.

Pearce et al. (88) made a study of the fractionation of mucopolysaccharides on different columns (DEAE, ECTEOLA, and Dowex 1-X2) and different salt concentrations to fractionate

the CPC-mucopolysaccharide complex. They found that DEAE and ECTEOLA gave inferior results to Dowex 1-X2 resin, and that the elution profile depends to a greater extent on the support and condition of elution than on the properties of the glycosaminoglycan.

Kanamoto et al. (89), while determining acidic mucopolysaccharides in tissues with cetyltrimethyl ammonium bromide (CTAB), found that DNA interfered with the reaction, giving high values. DNAase was added to prevent this interference. It was found that RNA did not interfere.

Cetyltrimethyl ammonia was used by Harris and Fraser (90) to recover the mucopolysaccharides from small volumes of serum and cell cultures. Control of pH, ionic conditions and of reactants gave a degree of recovery of mucopolysaccharides and an inherent variability of assay which compares favorably with methods needing larger samples.

C. GAS-LIQUID-PHASE-CHROMATOGRAPH (GLPC) OF MUCOPOLYSACCHARIDES COMPONENTS

Gas-liquid-chromatographic studies on mucopolysaccharides essentially are studies of the gas-liquid-chromatographic separations of carbohydrates, or more specifically, the hexoses, pentoses, amino sugars, and uronic acids.

The development of methods for the separation of carbohydrates and other polyhydroxy compounds by gas-chromatography has been much slower than with other biological compounds such as amino acids, fatty acids or steroids, because of the inherent nature of the carbohydrates themselves. They are highly polar and nonvolatile. Most of these problems have now been solved by the use of methylation (91), acetate formation (92), acetal formation (93) or trimethylsilyl formation (93). All are volatile products.

1. O-methyl Ether Derivatives

The formation of methyl ethers of carbohydrates is actually the replacement of the primary or secondary alcoholic groups of a carbohydrate by methoxy groups. The first report of the separation of fully methylated sugars was described by McInnis (94). He used single anomers, which were easily separated on Apeizon-M grease. When McInnis tried anomeric pairs, the α and β structures were not separated. A later effort by Kircher (95), using butanediol-succinate polyester as the stationary phase, also failed to separate anomeric pairs of the fully methylated derivatives.

Retention times of methylated glycosides are generally increased as the number of methyl groups decreases. Partially

methylated glycosides have been examined by Jones and Perry (96). An important factor in their work was the substitution of diatomaceous earth for coated glass beads. Jones and Perry were only able to methylate the hexoses. Neither the amino sugars nor the uronic acids were successively methylated.

2. O-acetyl Derivatives

Perry (97) was the first to report the successful separation of acetylated sugars, by the formation of acetylated alditols. Wells et al. (98), Berry (99) and Bishop (91,100) have published reviews of the O-acetyl esters of carbohydrates.

Perry (97) was the first to use mixtures of polar and non-polar liquid phases applied in a very thin layer. Using this method he had hoped to combine the advantages of both types of coatings, as well as reducing the retention times by thin coatings. This method gave a rapid separation of acetates C_4 to C_8, but isomeric alditol acetates were not separated. Fully acetylated methyl glycosides have been separated by gas-chromatography on mixed liquid phases; indications are, however, that the isomers are not resolved (101). Sawardeber et al. (102) quantitatively acetylated alditols with pyridine and acetic anhydride. Interference by borate ions, which are used to reduce the aldoses to their corresponding alditols,

was decreased by using minimum amounts of sodium borohydride and increasing the reaction time. Because of the excessive tailing of the pyridine, it was replaced by sodium acetate.

Crowell and Bennett (103) used the method of Sawardeber et al. (102) to acetylate alditols from wood pulp. Pyridine was evaporated and replaced by methylene chloride.

Kim et al. (104) for glycoprotein analysis, removed excess borate as the volatile trimethyl borate.

Glucosamine and galactosamine have been examined by gas-chromatography of their acetate derivatives by Jones et al. (105). It was shown, however, by Bishop (106) that glucosamine tetraacetate yields two volatile products by gas-chromatography, neither of which is the original compound. He suggested that these rearrangements may have been caused by: (1) the high temperatures of the columnar injection port; (2) the liquid phase used; (3) the solvent.

Since then Perry has modified his method (107) by first quantitative reduction of the glucosamine to the corresponding glycitol derivative, and then acetylation to form the 2-acetamido-1,3,4,5,6-penta-0-acetyl-2-deoxyhexitol. Using polar liquid phases, he obtained single peaks with no evidence of formation of artifacts during preparation or of decomposition during gas-chromatography.

3. Acetals

Kircher (95) first demonstrated that 4,6-O-ethylidene-D-glucose-1,2,3-O-triacetate could be chromatographed. An attempt was made to resolve a mixture of the 3-methyl, 3-ethyl, and 3-vinyl ethers of 1,2:5,6-di-O-isopropylidene-D-glucofuranose, but these three esters were not separable.

Jones et al. (105) made a thorough investigation of the gas chromatographic separation of acetal derivatives of amino sugars.

It has been shown that acetals can undergo rearrangements during gas chromatography (106) and for this reason they have not gained much acceptance. Prior substitution of all available hydroxy groups can stabilize the acetals but this is a long and involved procedure.

4. O-trimethylsilyl Ethers

The O-trimethylsilyl derivative, the most popular derivative, provides a practical and routine method for increasing the volatility of all compounds containing polar substitutes, such as carbohydrates.

Hedgley and Overend (108) first silyated sugars using an excess of trimethylchlorosilane in pyridine. The reaction took six hours at room temperature.

The first improvement in the silyation reaction was made by Makita and Wells (93), who used hexamethyldisilazane as well as trimethylchlorosilane in pyridine to silyate bile acids. The reaction was quantitative and rapid.

Bentley et al. (109) first applied this method of Makita and Wells (93) to carbohydrates, and found no isomerization, and that the reaction was quantitative and rapid.

Sweeley et al. (110) made an extensive investigation of the trimethylsilation reaction on 100 or more different carbohydrates. They found that all of the various carbohydrates and related substances which may occur as components of mucosubstances, such as glucose, galactose, hexuronic acids, hexosamines, and sialic acid, are subject to this method of analysis.

The reaction according to Sweeley (110) involved the suspension of the carbohydrate material in pyridine followed by the addition of hexamethyldisilazane and trimethylchlorosilane in the ratio of 1:0.2:0.1. The hexamethyldisilazane silyated the carbohydrate while the trimethylchlorosilane catalyzed the reaction. Sweeley, upon investigation of the silyation reaction found that the reaction was complete in a few minutes, and that relatively insoluble carbohydrates only required gentle warming. Usually a precipitate of NH_4Cl was

formed, but this had no effect upon the chromatography.

One disadvantage of the use of pyridine is the long, broad solvent peak that results. Perry and Hulyalkar (111) and Karbbainen et al. (117) eliminated this problem by substitution of pyridine by chloroform in the first case, and hexane in the second case.

Trifluroacetic acid has been used as a catalyst instead of trimethylchlorosilane. There are two advantages: (1) no precipitate is formed, and (2) quantitative silation occurs in samples containing up to 40 mg of water.

The success of O-trimethylsilyl ether derivatives has prompted research for new and more effective silyl donors.

Trimethylsilylacetamide has proved to be an effective silyl donor for D-glucose, and O-substituted D-glucose derivations (112).

The most promising new silyating reagent available is N-O-bis-trimethylacetamide (113,114). Analytical procedures using N-O-bis-trimethylacetamide have been devised for almost all classes of water-soluble substances of biological interest.

For example, sugar phosphates (115,116), hexosamines (117), hexosaminitols (118) and anhydroglycoses (119) are among the types of compounds which have been analyzed using N-O-bis-trimethylacetamide. The reactions are very rapid, as

the equilibrium for the reactions was far to the right.

5. Special Silyations for Amino Sugars

Many of the procedures for the silyations of amino sugars requires first that the N-acetyl grouping be formed (120). By this method, the N-acetyl-tetra-O-trimethylsilyl hexosamine give good quantitative and qualitative results.

Other reports on the other hand (110,121) have shown that the hexosamine-HCl may be directly silyated by Sweeley's method (110) producing anomeric penta-N,O-trimethylsilyl hexosamine derivatives.

Mass spectrometry has shown, however, that the amino group is not silyated by this method and the tetra-O-trimethyl-silyl hexosamines are actually formed.

A new method has now been described for the silyation of hexosamine-HCl (122). The reagents are a 20% solution of hexamethyldisilazane in dimethylformamide. The mixture is heated and then injected into the gas-chromatograph. The hydrochloride provides sufficient acidity to catalyse the reaction. Both anomeric forms of glucosamine-HCl and galactosamine-HCl are formed.

6. Release and Separation of Carbohydrate Components of the Carbohydrate-Protein Complexes

Mucopolysaccharides and glycoproteins are similar complex

polysaccharides and therefore before analysis they must be split into their components, namely the neutral, acidic and basic sugars.

Marshall and Kummeron (123) in 1962 isolated the carbohydrate components of serum β-lipoprotein, by hydrolysis of the samples with 2N hydrochloric acid for eight hours at 100°C. This was found to release optimum amounts of amino sugars. They separated the hexosamines and neutral sugars by chromatography of the hydrolysate on Dowex-50 (H^+). Sialic acid was released after hydrolysis by 0.1N sulphuric acid for one hour at 80°C. This hydrolysate was passed through Dowex-2 (HCO_3^-) and the released sialic acid separated. Sweeley and Walker (124) in 1964 hydrolyzed glycolipid material with 0.5N methanolic hydrochloric acid for 24 hours at 75-80°C. The carbohydrate and lipid fractions were isolated by various extraction techniques. The released components were chromatographed as their trimethylsilyl derivatives.

Karbbainen et al. (125,126) analyzed the carbohydrate components of mucopolysaccharides by gas-chromatography by hydrolyzing the sample in 2N hydrochloric acid at 103°C for seven hours. Prior to chromatography, the separation of the components was carried out on Dowex-50.

Oates and Schrager (127) hydrolyzed their mucopolysaccharide

samples with a suspension of Dowex-50 (X12, 200-400 mesh) in 0.25N hydrochloric acid, and heated it for 16 hours at 100°C. This hydrolysate was passed through Dowex-2 (200-400 mesh) and the eluate analyzed for neutral sugars.

Lehtanen et al. (128) first separated the mucopolysaccharides using cellulose acetate electrophoresis. The fractions were then hydrolyzed by 1.5N hydrochloric acid and chromatographed by Dowex-50. The components were then silyated and separated by gas-chromatography in an SE-30 column.

Oates and Schrager (129) first deacidified the hydrolysates before passing the hydrolysates through Dowex-50 columns. Using this technique, the amino sugars were N-carboethorylated, but the neutral sugars passed through unchanged.

Most recent procedures (104,130,131) first hydrolyze the sample with acid and then separate the hydrolysate into its carbohydrate components prior to gas-chromatography, by first passing the hydrolysate through Dowex-50 (H^+) and then through Dowex-1 or Dowex-2 both in their (HCO_3^-) forms.

Perhaps one of the most interesting procedures for the isolation and separation of the carbohydrate components of mucopolysaccharides prior to gas-chromatography was presented by Karkkainen et al. (121). They used a combination of acid hydrolysis and thin layer chromatography to separate them

II. ANALYTICAL METHODS

A. COLLECTION AND TREATMENT OF AVIAN TISSUE

Mature, laying Rhode Island Red hens were killed, exsanguinated, and the feathers removed. The hens were skinned, and as much subcutaneous fat as possible was scraped away from the skin. The skin was washed with water, and stored in acetone in the cold. After one week of dehydration, the acetone was replaced with a 2:1 chloroform:methanol mixture to ensure complete defatting. The skin was cut into small pieces and air-dried prior to proteolytic hydrolysis.

B. PROTEOLYTIC HYDROLYSIS OF SKIN TISSUE

1. Pepsin-Trypsin Hydrolysis

The air-dried skin was weighed and homogenized in a Waring Blendor with ten times its weight of water. After homogenization, the pH of the homogenate was adjusted to 1.5 with 8N hydrochloric acid, and two grams of pepsin (Nutritional Biochemicals, 1-20,000) was added for every liter of homogenate. The homogenate was layered with toluene and incubated at 37°C for 72 hours. The pH was adjusted to 7.8 to

8.0 after 72 hours, by the addition of 8N sodium hydroxide. Three grams of trypsin (Nutritional Biochemicals, 1-300) was added per liter of mixture.

The digestion was carried out for a further 24 hours. The hydrolyzed tissue was filtered through Celite Filter-Aid. The clear filtrate was processed as in Section C.

2. Papain Hydrolysis

This hydrolysis procedure was essentially the one used by Pearce and Mathieson (77). Air-dried skin was suspended in ten times its weight in 0.1M acetate buffer (pH 5.5, 0.005M with respect to both N,N'-ethylenediaminetetraacetate (EDTA) and L-cysteine HCl) and homogenized in the Waring Blendor. Concurrently, a 0.1% (w/v) suspension of crystalline papain in the same buffer was activated at 60°C for 30 minutes. After maintaining the sample at 60°C for approximately 30 minutes, the activated papain suspension was added to the skin suspension in a ratio of 1.0 ml to 1.0 gm of skin. The homogenate was digested for 16 hours at 60°C. If the sample was incompletely digested after 16 hours, more papain was added and the digestion continued for a further 12 hours. The digest was filtered through Celite Filter-Aid, and the clear filtrate processed as in Section C.

C. ISOLATION PROCEDURE FOR MUCOPOLYSACCHARIDES FROM ENZYMATIC HYDROLYSIS

1. From Skin Tissue

The hydrolysate from either the papain or the pepsin-trypsin hydrolysis was taken and divided into two equal parts "A" and "B". Calcium acetate monohydrate (22.7 gm/l) and acetic acid (to a final concentration of 0.25N) were added to "A". This first precipitation, called "A_1", was then formed by the addition of 1.2 volumes of absolute ethanol. The precipitate was collected and weighed. A portion (80% of the total weight) of "A_1" was redissolved in a 0.03M solution of sodium sulphate, while the remaining 20% was used for analysis (i.e., hexosamine, hexose and uronic acid).

The redissolved "A_1" solution was heated on a magnetic stirrer/heater to a temperature of 37°C. A 2% cetylpyridinium chloride (CPC) (1-Hexadecylpyridinium chloride, Matheson, Coleman & Bell) solution, made 0.03M with respect to sodium sulphate, was added dropwise with continuous stirring until a flocculent precipitate had formed.

An excess of CPC solution, as evidenced by abundant foaming, was added to ensure complete precipitation of the mucopolysaccharides. The sample was covered and left overnight at 37°C. The precipitate, called "A_2", was collected,

lyophilized, weighed, and analyzed. The supernatant was also lyophilized, weighed, and analyzed. It was labelled "A_3".

Next, the supernatant from the "A_1" precipitation was taken and to it was added 2.8 volumes of absolute ethanol to bring the alcohol concentration to 4.0 volumes. The precipitate formed was called "A_4". Again a portion was saved for analysis and the rest (80%) was redissolved in a 0.003M solution of sodium sulphate. CPC solution was added to precipitate "A_5" which was weighed and analyzed. The supernatant from "A_5" was dried and analyzed and labelled "A_6", as was the supernatant from "A_4" which became "A_7".

The "B" portion was made 0.003M with respect to sodium sulphate, and CPC solution was added to form a precipitate labelled "B_1". This was analyzed and a portion redissolved and precipitated by 1.2 volumes of absolute ethanol to form precipitate "B_2". To the supernatant was added absolute alcohol to make the alcohol concentration to 4.0 volumes, to form precipitate "B_3" and a supernatant "B_4".

To the "B_1" supernatant was added 1.2 volumes of absolute alcohol and then 4.0 volumes of absolute alcohol to form precipitates "B_5" and "B_6" respectively, leaving "B_7" as the supernatant after these precipitations. A schematic outline

of this separation can be seen in Figure I, page 34.

Tables were then set up to compare the various corresponding precipitates to assess the effectiveness of the two hydrolysis systems and the two methods of precipitation of mucopolysaccharides (see Figure I).

2. Isolation Procedure to Determine Loss of Sulphate in Enzymatically Treated Standard Mucopolysaccharides

Solutions of chrondroitin sulphate (2.5%) and heparin (1%) were made up and subjected to pepsin-trypsin hydrolysis or papain hydrolysis, as per Section B.

Each hydrolysate was divided into two equal parts called "A" and "B". To the "A" part, 1.2 volumes of alcohol as per Section C.1. was added to precipitate "A_1". Part "B" was precipitated using CPC and called "B_1". A schematic outline can be seen in Figure II. Each precipitate was analyzed for sulphate, hexosamine, uronic acid and hexose.

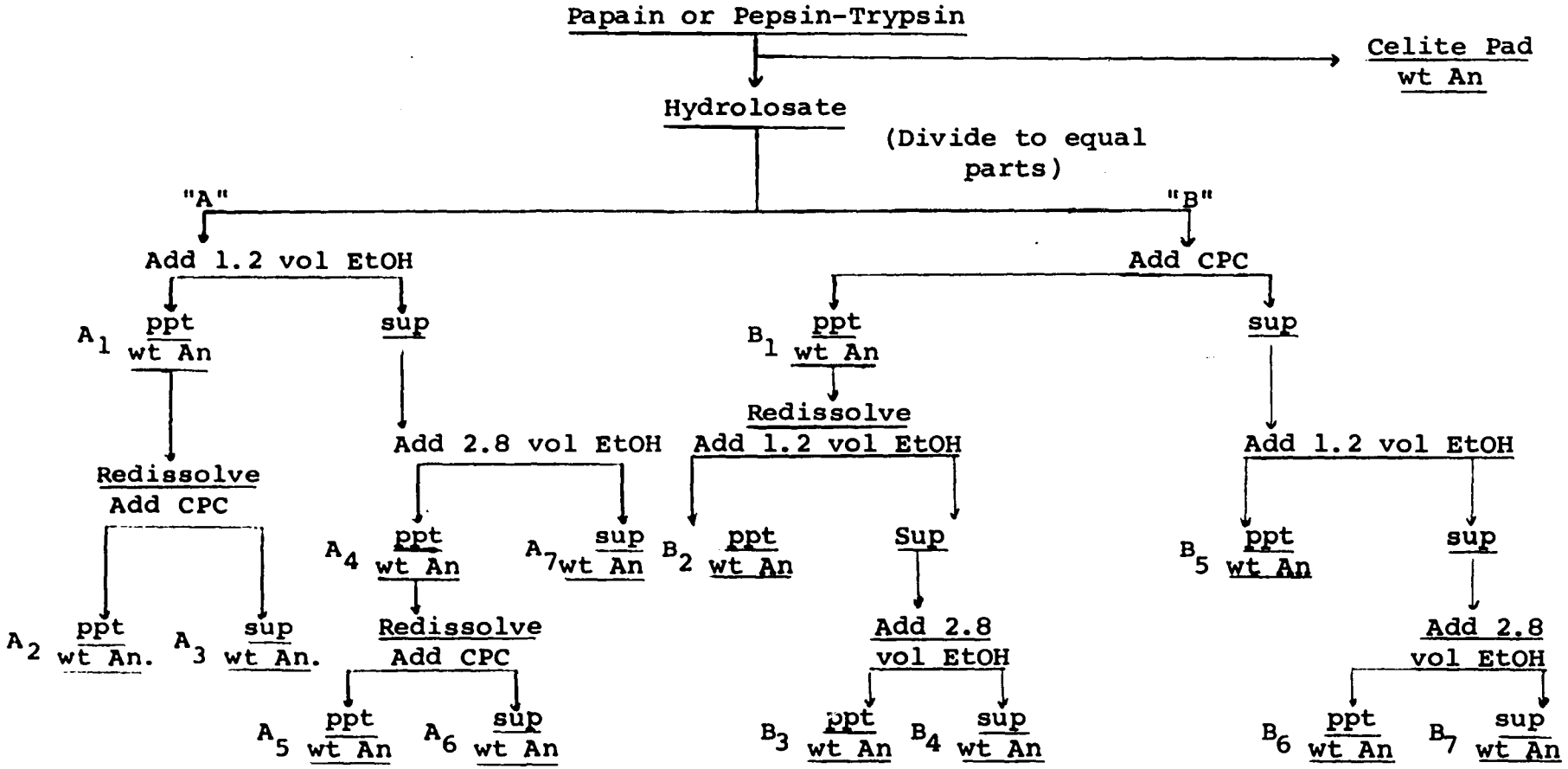
D. RESIN PREPARATION

1. Preparation of Dowex 50-X8 Cation Exchange Resin 200-400 Mesh Used for Hydrolysis and Fractionation of the Hydrolysates

The resin (Bio-Rad) was placed in a large sintered

Figure I

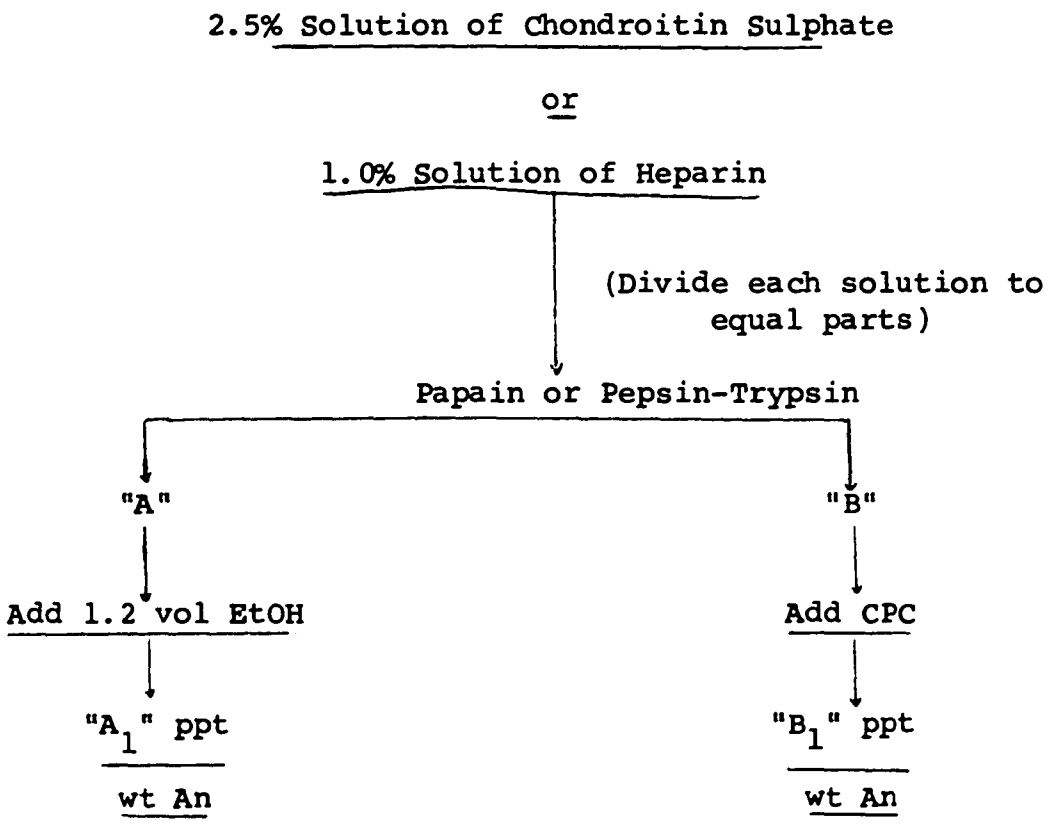
Schematic outline of the procedure for the isolation of mucopolysaccharides from enzymatic hydrolysis



wt - weight, An - analyse, ppt - precipitate, sup - supernatant, EtOH - ethanol, vol - volume

Figure II

Schematic outline of the isolation procedure to determine loss of sulphate in enzymatically treated standard muco-polysaccharides



wt - weight
An - analyse
ppt - precipitate
sup - supernatant
EtOH - ethanol
vol - volume

glass funnel and washed with 4.0 liters of 2N NaOH. The resin was then washed with distilled water until the washings were neutral to red litmus. Two liters of 2N HCl were passed through the resin and then distilled water until the washings were chloride-free. This process was repeated a second time. The resin was then washed twice with portions of 0.075N HCl and drained dry. The drained resin was weighed and suspended in 0.075N HCl (1:2 w/v) for use in resin hydrolysis and fractionation of the hydrolysate.

2. Preparation of Dowex 50-X8 Cation Exchange Resin 200-400 Mesh for G.L.C. Analysis

The resin was first refluxed with methanol for three hours prior to preparation to remove interfering substances. After this initial step, the resin was prepared exactly as in Section D.1.

3. Preparation of Dowex 1-X8 Anion Exchange Resin 200-400 Mesh for G.L.C. Analysis

Before preparation, the resin was refluxed with methanol for three hours to remove any interfering substances. The resin was prepared as described by Simkin et al. (132). The resin was suspended in 2 liters of 6N NaOH for four hours with stirring. After removal of the NaOH by filtration, the

resin was washed with water until the washings were neutral to red litmus. The resin was then suspended in 2 liters of 2N sodium formate for two hours. The sodium formate was drained and the resin was washed with another 2 liters of 2N sodium formate. It was then washed with distilled water until the washings were neutral to red litmus. The resin was suspended in distilled water before use.

E. METHODS OF HYDROLYSIS USED TO LIBERATE CHARACTERISTIC COMPONENTS OF MUCOPOLYSACCHARIDES

1. Hydrolysis with Dowex 50-X8 for the Release of the Characteristic Sugar Units of Mucopolysaccharides

Ten to fifteen mg of each sample were weighed into pyrex test tubes (13 x 100 mm) and 5 ml of the Dowex 50-X8 suspension was added. The tubes were sealed and placed in an oven provided with a rotating apparatus at 100°C for 20 hours as first used by Anastassiadis and Common (133). After 20 hours, the tubes were cooled, opened, and chromatographed as in Section F.1., for colorimetric analysis, or used for G.L.C. analysis as in Section F.2.

2. Hydrolysis by 1N HCl to Release Characteristic Components of Mucopolysaccharides

Samples ranging from 10 to 15 mg were placed in 13 x 100 mm test tubes. Three ml of 1N HCl was added and the tubes were sealed. The hydrolysis was carried out for three hours at 100°C in the oven provided with a rotating apparatus. The tubes were opened and chromatographed as in Section F.1.

3. Hydrolysis by 1N HCl to Release Sulphate from Mucopolysaccharides

Samples ranging from 10-15 mg were sealed in 13 x 100 mm test tubes with 5.0 ml of HCl, and hydrolyzed for two hours at 100°C. The tubes were opened and the hydrolysate evaporated to dryness over NaOH.

F. CHROMATOGRAPHY OF PRODUCTS OF RESIN HYDROLYSIS

1. Chromatography of Products for Colorimetric Analysis

The contents of the resin hydrolysis tubes (see Section D.1.) were transferred to a glass column (1 x 30 mm) with a fritted disc, with the aid of 10 ml of distilled water.

This water eluate was collected in a 50 ml beaker. The water eluate beaker was removed and replaced with another 50 ml beaker. Elution was continued with 10 ml of 2N HCl, and then 5 ml of distilled water, all of which was collected in

the HCl eluate beaker. Chromatography was continued by passing the water eluate through the column again and collecting the effluent in another beaker. The column was washed with 11 ml of distilled water. The water eluate beaker was replaced with the HCl eluate beaker and 10 ml of 2N HCl was passed through the column. The water eluate and the HCl eluate were dried over NaOH. After drying, 5 ml of water was added to each beaker and these solutions were analyzed. The hexosamines were determined in the HCl eluate and hexoses and uronic acids in the water eluate.

2. Chromatography of the Products for G.L.C.

Initially the products of the resin hydrolysis were treated in the same way as those for colorimetric analysis. The HCl eluate from Dowex 50-X8 was used for hexosamine analysis after drying over NaOH. The water eluate was passed through a column of Dowex 1-X8 in the formate form. The neutral sugars were eluted with 20 ml of distilled water in four portions. The receiver was replaced with another 50 ml beaker into which the uronic acids were eluted with 15 ml of 2N HCl. The water eluate from Dowex 1-X8 was used for neutral sugar analysis while the 2N HCl eluate from Dowex 1-X8 was used for uronic acid analysis.

G. DETERMINATION OF CHARACTERISTIC COMPONENTS OF MUCOPOLYSACCHARIDES

1. Hexosamine

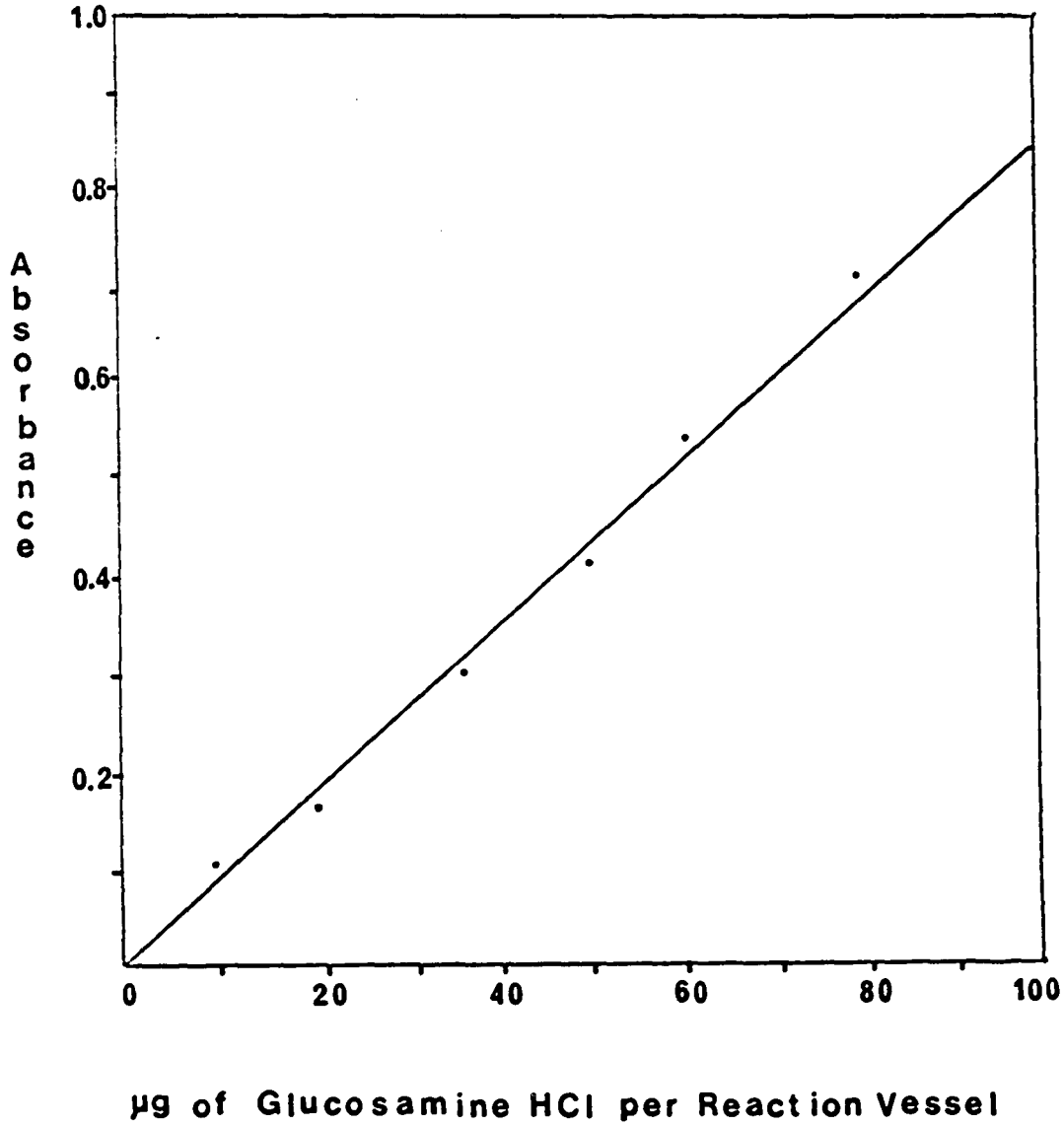
Hexosamine was determined by the modified Elson and Morgan reaction of Anastassiadis, Maw, and Common (134). This method was modified to fit 13 x 100 mm teflon screw cap matched test tubes. An aliquot of 0.5 ml of the hydrolysate was pipetted into the test tube. To this was added 1.0 ml of freshly prepared solution of 4% acetylacetone in 0.75M sodium carbonate. The tubes were shaken and heated at 100°C in boiling water for one hour. To the cooled tubes was added 2.5 ml of absolute ethanol. The tubes were shaken and then 1.0 ml of Ehrlich's reagent (0.8 gm p-dimethylaminobenzaldehyde was dissolved in 30 ml absolute ethanol, and 30 ml of concentrated HCl was added with shaking). The absorbance of each test tube was read at a wavelength of 530 mμ. A standard curve was prepared as in Figure III.

2. Uronic Acid

Uronic acid was determined by the method of Dische (135). This method was modified to fit 13 x 100 mm teflon screw-capped test tubes. A 0.5 ml aliquot of the test solution was added to the test tubes. The tubes were cooled in

Figure III

Standard curve for the colorimetric analysis of hexosamine.



an ice bath and 3 ml of concentrated sulphuric acid was added. The tubes were shaken, and heated in boiling water for 20 minutes. The tubes were cooled and 0.1 ml of a 0.1% solution of carbazole in absolute ethanol was added to each tube. The test tubes were shaken and allowed to stand for two hours in order for the solutions to reach maximum color development. The absorbances were read at 530 mμ. A standard curve was prepared as in Figure IV.

3. Hexose

Hexose was determined by the method of Fairbairn (136). This method was modified to fit 13 x 100 mm teflon screw-capped test tubes. A 1.0 ml aliquot of the test solution was pipetted into the test tubes. Five ml of anthrone reagent (1.0 gm of anthrone dissolved in a sulphuric acid solution of 760 ml of sulphuric acid made up to 1,000 ml with distilled water) was added to each tube and the tubes were heated at 100°C for 12 minutes. The tubes were cooled and read at 620 mμ. A standard curve was prepared as in Figure V.

4. Sulphate

Sulphate was determined by the method of Alicino

Figure IV

Standard curve for the colorimetric analysis of glucuronic acid.

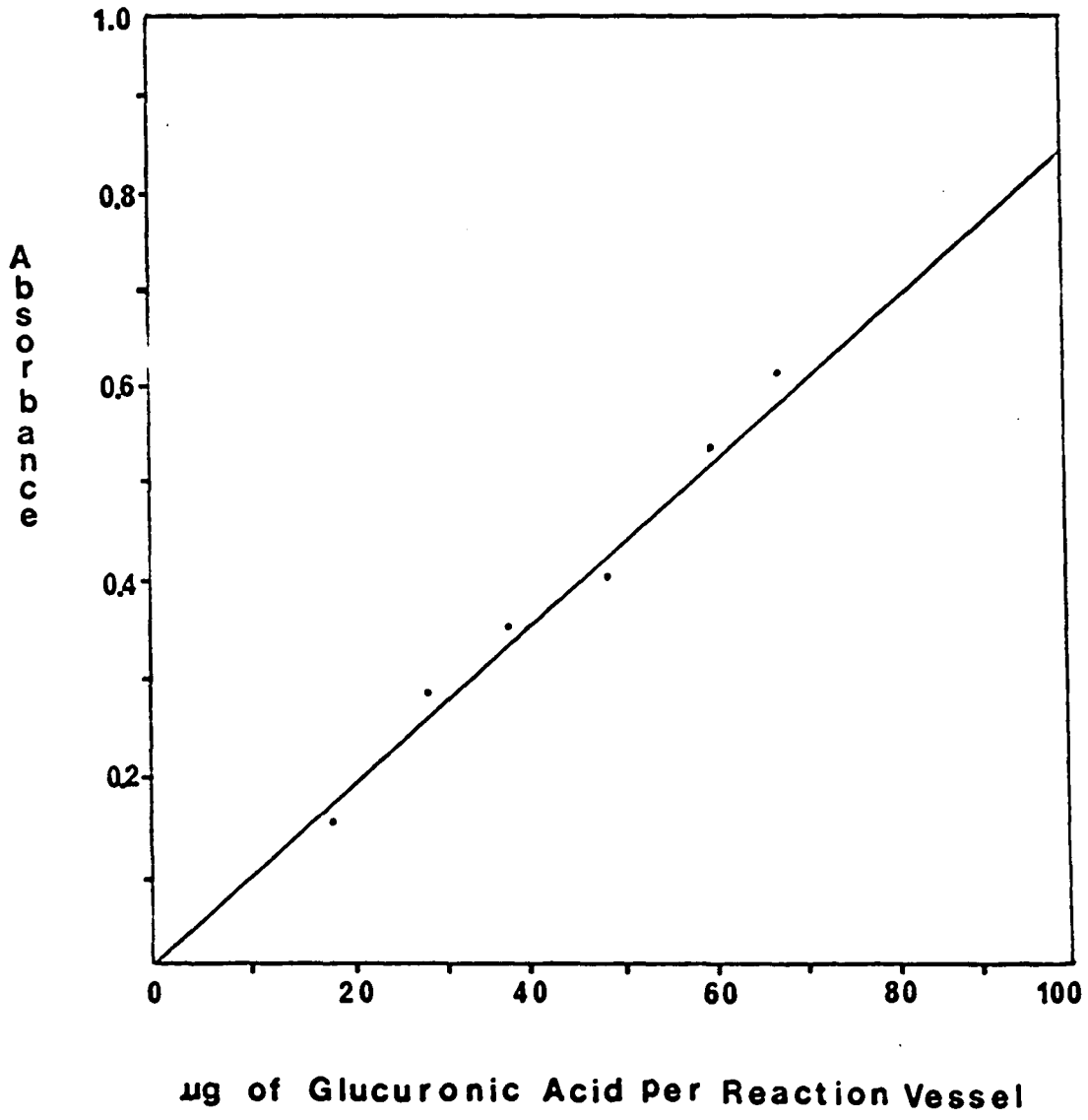
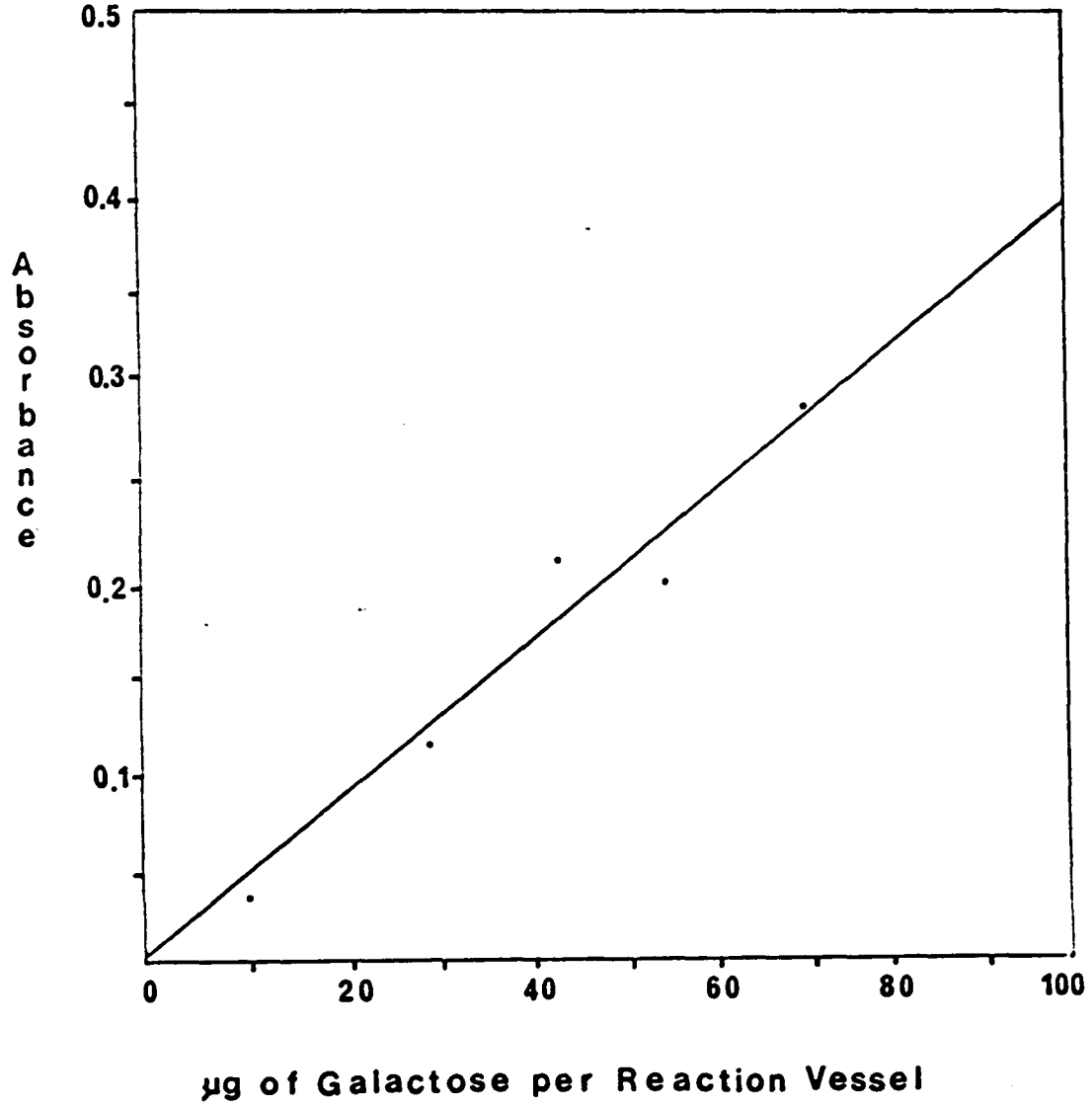


Figure V

Standard curve for the colorimetric analysis of hexose.



(137), employing a modification of the procedure of Meyer et al. The residue from the sulphate hydrolysis (A_3) was taken up in 1.0 ml of distilled water, and neutralized with sodium hydroxide to a pink coloration using phenolphthalein as an interval indicator. The volume was adjusted to 2.5 ml and 5.0 ml of absolute ethanol was added. The solution was then titrated with standard barium chloride (0.02 M) using four drops (about 0.2 ml) saturated aqueous potassium rhodizonate solution as indicator.

H. GAS-LIQUID CHROMATOGRAPHIC TECHNIQUES

1. Gas-Liquid Chromatography of Fatty Acids

Fatty material from the acetone (and chloroform-methanol), extracts were extracted with petroleum ether twice. The petroleum ether was evaporated from the extract. Approximately 30 mg of the fatty material from this extraction was methylated by the procedure of Metcalfe et al. (138) and passed through the chromatographic column.

The portion remaining after the extraction was saponified according to the AOAC using 50% KoH and 95% EtOH. This was again extracted with petroleum ether and the fatty residue methylated and chromatographed.

The conditions used for the chromatography were as follows:

Column: 5% Diethylene Glycol Succinate on Chromosorb W-AW-DMCS

Sensitivity = 3×10^{-9}

Carrier (He)= 20 cc/min

Hydrogen = 20 cc/min

Air = 175 cc/min

Program = Initial Temp. = 67°C
Initial Hold = 5 min
Temp. Rise = 2°C/min
Final Temp. = 187°C
Final Hold = Infinite

The program used was developed by Cook (139).

2. Gas-Liquid Chromatography of Amino Sugars

The acid eluate as per Section E.2., after drying, was dissolved in a 1.0 ml of distilled water. This solution was poured into a 13 x 100 mm screw-capped test tube (teflon) and dried over NaOH. The amino sugars in the dried material were silyated according to the method of Radhakrishnamurthy et al. (122). Hexamethyldisilazane reagent (HMDS) was prepared by adding 0.2 ml of hexamethyldisilazane to 1 ml of dimethylformamide. HMDS reagent (0.1 ml) was added to each tube. The mixture was heated in boiling water for two minutes. The

tube was cooled in cold water and 3 µl of the sample was injected into the chromatographic column. The conditions employed were as follows:

Column = 5% SE-30 on chromosorb W-AW-DMCS

Detector temp. = 210°C

Inlet temp. = 210°C

Sensitivity = 1×10^{-10}

Carrier (He) = 40 cc/min

Hydrogen = 40 cc/min

Air = 100 cc/min

Column temp. = 170°C (isothermal)

3. Gas-Liquid Chromatography of Uronic Acids

To the dried 2N HCl eluate from the Dowex-X8 column of E.2., 1.0 ml of absolutely anhydrous pyridine was added. The beakers were covered and allowed to stand for several hours. The contents of the beakers were then transferred to teflon capped 13 x 100 mm test tubes. The uronic acids were then silyated according to the method of Sweeley et al. (110). To the sample was added 0.2 ml of hexamethyldisilazane and then 0.1 ml of trimethylchlorosilane, with stirring each time. After the latter was added the tubes were shaken for approximately half an hour. The samples were taken to

dryness under nitrogen, and extracted with 2.5 ml of pentane. The pentane was transferred to 3 ml centrifuge tubes and taken to dryness under nitrogen. Pentane was again added (20-30 µl) and 2-4 µl taken for injection. The conditions employed were:

Column:	5% SE-30 on chromosorb W-AW-DMCS
Sensitivity:	1×10^{-10}
Carrier (He):	40 cc/min
Hydrogen:	40 cc/min
Air:	100 cc/min
Program:	Initial Temp. = 135°C Initial Hold = 4 min Temp. Rise = 0.8°C/min Final Temp. = 190°C Final Hold = 25 min

4. Gas-Liquid Chromatography of Neutral Sugars

The water eluate from the Dowex 1-X8 separation of Section E.2. was used for the neutral sugars. The procedure used was exactly the same as that used for uronic acid in Section G.3.

III. RESULTS AND DISCUSSION

A. THE EFFECT OF THE KIND OF VESSELS ON THE SENSITIVITY AND VARIABILITY OF COLORIMETRIC DETERMINATIONS

Because the colorimetric determinations could be performed easier when 13 x 100 mm teflon screw cap test tubes were used than when 10 ml volumetric flasks were used, tests were carried out to compare the sensitivity and variability of the results of the hexosamine determinations, when using these two vessels. The reason for the selection of the hexosamine determination for this test was that the variance of its results was larger than the variances of the other determinations.

Standard solutions of 10, 20, 50 and 60 μg of glucosamine-HCl per ml were prepared. Determinations were performed on 10 replicates for each sample concentration. The standard deviations of the absorbances were calculated. The mean values of the standard deviations are given in Table I. The variances of the absorbances of the solutions in the two types of vessels were compared by the F-test (Table II). As can be seen from Table II, the F-ratio appeared to be significant when the two kinds of vessels were compared at the

lower levels of 10 μg/ml and 20 μg/ml. There appeared to be no significant difference at the higher levels of 50 μg/ml and 60 μg/ml.

TABLE I

Average absorbance and standard deviation obtained when solutions of glucosamine hydrochloride at various concentrations were heated in 13 x 100 mm screw cap test tubes and in 10 ml volumetric flasks

Concentration	13 x 100 mm screw cap test tubes	10 ml volumetric flasks
10 μg/ml	0.0630 ± 0.0101	0.081 ± 0.0063
20 μg/ml	0.1680 ± 0.039	0.171 ± 0.0006
50 μg/ml	0.427 ± 0.064	0.377 ± 0.0496
60 μg/ml	0.494 ± 0.091	0.484 ± 0.091

TABLE II

Statistical comparison of data of Table I

Concentration	Comparison			
	Test tubes (degrees of freedom)	Volumetric flasks (degrees of freedom)	F value	P
10 μg/ml	7	9	3.29	0.1
20 μg/ml	7	9	15.92	0.1
50 μg/ml	7	9	0.60	0.1
60 μg/ml	7	9	1.00	0.1

B. COMPARISON OF METHODS FOR THE ISOLATION OF MUCOPOLYSACCHARIDES FROM SKIN TISSUE

Samples of skin tissue from mature Rhode Island Red chickens was digested by pepsin-trypsin or papain as in Section B of Analytical Methods, and the mucopolysaccharides were isolated by the two methods as in Section C(1) of Analytical Methods. All products of the two isolations were analyzed for hexosamine, hexose and uronic acid, the results of which are given in Tables III, IV and V, respectively. A statistical treatment, by means of the "t" test, of the data of these tables is given in Table VI.

A comparison of the contents of the pepsin-trypsin celite pad with the contents of the papain celite pad gives an indication of the effectiveness of the two enzymatic hydrolyses. Although some differences existed in the contents of hexosamine, hexose and uronic acid, these differences were not found to vary greatly. Taking into consideration the low values of these constituents in the pads, the indication exists that pepsin-trypsin and papain hydrolyze skin tissue equally well.

Another indication of the effectiveness of the enzymatic hydrolysis can be gathered from the total recoveries of hexosamine, hexose and uronic acid from 100 gm of tissue

TABLE III

Recovery of hexosamines (total mg/100 gm tissue)

	Pepsin-trypsin					Papain				
	I	II	III	Average	S.D.	I	II	III	Average	S.D.
"A"Celite Pad	16.9	19.1	25.9	20.6 ±	4.7	80.3	83.6	60.9	74.9 ±	12.3
1.2 volume Ethanol precipitate A_1	56.9	64.8	72.2	64.6 ±	7.7	80.5	78.5	83.6	80.9 ±	2.6
CPC precipitate A_2	42.9	31.8	40.9	38.5 ±	5.9	57.1	47.1	43.3	49.2 ±	7.1
CPC supernatant A_3	13.7	11.8	10.9	12.1 ±	1.4	3.3	2.7	3.2	3.1 ±	0.3
2.8 volume Ethanol precipitate A_4	61.2	50.6	64.2	58.7 ±	7.1	69.4	76.6	78.6	74.9 ±	4.8
CPC precipitate A_5	-	-	-	-	-	-	-	-	-	-
CPC supernatant A_6	-	-	-	-	-	-	-	-	-	-
2.8 volume Ethanol supernatant A_7	88.6	65.2	67.9	73.9 ±	12.8	109.3	81.0	89.1	93.1 ±	14.6
"B"										
CPC precipitate B_1	58.2	81.4	84.0	74.5 ±	14.2	74.0	77.9	87.7	79.9 ±	7.1
1.2 volume Ethanol precipitate B_2	60.3	75.5	81.7	72.5 ±	11.0	84.9	105.2	97.6	95.9 ±	10.3
2.8 volume Ethanol precipitate B_3	0.00	0.00	0.00	0.00±	-	0.00	0.00	0.00	0.00±	-
2.8 volume Ethanol supernatant B_4	0.00	0.00	0.00	0.00±	-	0.00	0.00	0.00	0.00±	-
1.2 volume Ethanol supernatant B_5	13.1	16.1	13.3	14.2 ±	1.7	7.7	6.2	8.0	7.3 ±	0.9
2.8 volume Ethanol precipitate B_6	53.7	49.6	55.7	53.0 ±	3.1	50.8	54.4	54.8	5.5 ±	4.5
2.8 volume Ethanol supernatant B_7	69.5	68.8	70.5	69.6 ±	0.8	84.8	81.7	70.8	79.1 ±	7.4

TABLE IV

Recovery of hexoses (total mg/100 gm tissue)

	Pepsin-trypsin					Papain				
	I	II	III	Average	S.D.	I	II	III	Average	S.D.
"A" Celite Pad	121.4	118.6	143.9	127.9	± 13.9	93.8	160.7	125.2	126.6	±33.5
1.2 volume Ethanol precipitate A_1	86.8	73.9	86.3	82.3	± 7.3	26.7	32.9	38.3	32.6	± 5.8
CPC precipitate A_2	9.4	15.4	11.4	12.1	± 3.1	3.9	4.1	6.6	4.7	± 1.5
CPC supernatant A_3	31.8	56.9	54.0	47.6	± 13.7	16.9	17.6	15.3	16.6	± 1.2
2.8 volume Ethanol precipitate A_4	215.8	164.1	165.3	181.7	± 29.5	126.2	129.6	115.6	123.8	± 7.3
CPC precipitate A_5	-	-	-	-	± -	-	-	-	-	± -
CPC supernatant A_6	-	-	-	-	± -	-	-	-	-	± -
2.8 volume Ethanol supernatant A_7	963.9	868.8	1006.1	946.3	± 70.3	1451.4	1806.3	2368.7	1875.5	±462.5
"B"										
CPC precipitate B_1	40.4	35.7	45.8	40.6	± 5.1	39.5	48.5	22.5	36.8	±13.2
1.2 volume Ethanol precipitate B_2	70.8	74.1	85.4	76.8	± 7.7	52.9	51.0	48.9	50.9	± 2.0
2.8 volume Ethanol precipitate B_3	0.00	0.00	0.00	0.00	± -	0.00	0.00	0.00	0.00	± -
2.8 volume Ethanol supernatant B_4	0.00	0.00	0.00	0.00	± -	0.00	0.00	0.00	0.00	± -
1.2 volume Ethanol supernatant B_5	57.3	52.7	40.9	50.3	± 8.5	32.8	57.8	75.7	55.4	±21.5
2.8 volume Ethanol precipitate B_6	191.1	148.6	125.0	154.9	± 33.5	152.7	172.8	124.6	150.0	±24.2
2.8 volume Ethanol supernatant B_7	811.1	1018.9	651.5	827.2	±184.2	837.5	800.9	982.2	873.5	±95.9

TABLE V

Recovery of uronic acid (total mg/100 gm tissue)

	Pepsin-trypsin					Papain				
	I	II	III	Average	S.D.	I	II	III	Average	S.D.
"A" Celite Pad	56.6	41.5	27.8	42.0	± 14.4	40.0	44.3	25.9	36.7	± 9.6
1.2 volume Ethanol precipitate A_1	81.2	81.4	84.6	82.4	± 1.9	59.2	67.6	81.8	69.5	±11.4
CPC precipitate A_2	39.9	39.3	44.8	41.3	± 3.0	46.4	39.7	43.4	43.2	± 3.4
CPC supernatant A_3	7.7	11.4	10.1	9.7	± 1.9	8.6	7.8	5.4	7.3	± 1.7
2.8 volume Ethanol precipitate A_4	23.1	15.9	28.4	22.5	± 6.3	28.4	26.1	22.6	25.7	± 2.9
CPC precipitate A_5	-	-	-	-	± -	-	-	-	-	± -
CPC supernatant A_6	-	-	-	-	± -	-	-	-	-	± -
2.8 volume Ethanol supernatant A_7	66.6	87.7	118.7	91.0	± 26.2	141.6	150.1	148.1	146.6	± 4.4
"B"										
CPC precipitate B_1	62.2	84.6	88.2	78.3	± 14.1	61.2	75.3	102.7	79.7	±21.1
1.2 volume Ethanol precipitate B_2	125.2	115.4	167.6	136.0	± 27.7	101.5	154.6	106.2	120.8	±29.4
2.8 volume Ethanol precipitate B_3	12.7	20.0	14.2	15.6	± 3.9	16.2	26.8	10.0	17.7	± 8.5
2.8 volume Ethanol supernatant B_4	11.0	15.3	18.9	15.1	± 4.0	13.5	21.2	2.6	12.4	± 9.3
1.2 volume Ethanol supernatant B_5	18.5	8.8	13.5	13.6	± 4.8	11.3	13.8	9.8	11.6	± 2.0
2.8 volume Ethanol precipitate B_6	17.5	20.2	15.7	17.8	± 2.3	27.9	27.6	34.0	29.8	± 3.6
2.8 volume Ethanol supernatant B_7	66.0	70.1	72.5	69.5	± 3.3	105.5	92.2	120.6	106.1	±14.2

TABLE VI

Statistical comparison of data of Tables III, IV and V

	Hexosamine		Hexose		Uronic acid	
	"t" value	S/N.S.	"t" value	S/N.S.	"t" value	S/N.S.
Celite Pad	1.3	N.S.	0.05	N.S.	0.4	N.S.
Pepsin-trypsin						
1.2 volume Ethanol precipitate A_1 vs CPC precipitate B_1	0.9	N.S.	6.7	S	0.41	N.S.
CPC precipitate A_2 vs 1.2 volume Ethanol precipitate B_2	4.0	S	11.2	S	4.9	S
2.8 volume Ethanol precipitate A_4 vs 2.8 volume Ethanol precipitate B_6	2.2	N.S.	0.9	N.S.	0.3	N.S.
Papain						
1.2 volume Ethanol precipitate A_1 vs CPC precipitate B_1	0.2	N.S.	0.4	N.S.	0.6	N.S.
CPC precipitate A_2 vs 1.2 volume Ethanol precipitate B_2	5.4	S	6.8	S	3.8	S
2.8 volume Ethanol precipitate A_4 vs 2.8 volume Ethanol precipitate B_6	4.3	S	1.5	N.S.	1.3	N.S.

(see Tables III, IV and V). Of particular importance was the total hexosamine. Pepsin-trypsin yielded 531.6 mg of hexosamine while papain yielded 618.8 mg. This was a 15% greater yield. Similarly, hexose was 21% higher after papain hydrolysis, as was uronic acid, which was 11% higher. This information indicated that the papain hydrolysis gave higher yields than the pepsin-trypsin hydrolysis. For convenience, papain hydrolysis is preferable. This is because the pepsin-trypsin digestion needs 72 hours for completion and two enzyme preparations, whereas, the papain digestion needs 16 hours and only one enzyme preparation.

It is thought that absolute ethanol unlike CPC, will indiscriminately precipitate long chain complex polysaccharides in solution. That is to say, ethanol will precipitate mucopolysaccharides and peptide-glycoprotein complexes together. A comparison of the precipitates "A_1" and "B_1" of both the pepsin-trypsin and papain hydrolysis would clarify this claim and specify if it is better to precipitate the mucopolysaccharides with 1.2 volumes of ethanol or CPC.

An examination of Table VI shows that for pepsin-trypsin, the levels of hexosamine and uronic acid in the 1.2 volume ethanol and CPC precipitates did not vary greatly, while that of hexose did. This then, does substantiate the

claim, and although the levels of hexosamine did differ slightly (10%) in favor of CPC, the levels of hexose varied to such a great extent (50% in favor of 1.2 volume ethanol precipitate) that it might be said that CPC did give a much purer mucopolysaccharide than did the 1.2 volume ethanol precipitate.

For the papain digestion, there seems to be no great difference of the values of hexosamine, hexose and uronic acid between the two methods of precipitation. This would indicate that equally pure mucopolysaccharide samples were obtained by the 1.2 volume ethanol precipitation and the CPC precipitation.

Although the ethanol and CPC seem to be equally good mucopolysaccharide precipitants, it must be said, that from the standpoint of practicality and convenience, CPC would be favored, as crude mucopolysaccharides are precipitated easier with CPC than with ethanol.

A comparison of the precipitates "A_2" and "B_2" would ascertain whether it was better to reprecipitate the ethanol precipitate with CPC or to reprecipitate the CPC precipitate with ethanol if both of these precipitations were used for maximum recovery and purity of the products (see Figure I of Analytical Methods). Table VI shows that the composition

of precipitates "A_2" and "B_2" are different in both cases, i.e., the pepsin-trypsin hydrolysis and the papain hydrolysis. From the standpoint of purity it seems better to reprecipitate with CPC. This is evident by the very low values of hexose as compared to those values for both hexosamine and uronic acid. Reprecipitation of the CPC precipitate (B_1) with ethanol, gave approximately 50% more precipitate than reprecipitation of the ethanol precipitate (A_1) with CPC; however, the purity of this ethanol precipitate (B_2) is not nearly as good as the CPC precipitate (A_2). This is evident by the very high values of hexose for "B_2". This is the case with both pepsin-trypsin and papain hydrolyses.

Considering the two criteria of quantity and purity, it is preferable in many studies to stress purity, since it is often more important to obtain a pure sample than to have a larger but less pure sample. The purer mucopolysaccharide products obtained by CPC were to be expected, since CPC is a much more specific precipitant for mucopolysaccharides than ethanol.

Analysis of the supernatant "A_3" from the reprecipitation by CPC to form "A_2" indicates the effectiveness of the precipitations to obtain "A_1" and "A_2". The values for both pepsin-trypsin and papain hydrolyses for hexosamine,

hexose and uronic acid all are very low, indicating that the precipitation first by ethanol and then CPC was quite effective. This is especially indicated by the hexosamine values.

Analysis of the precipitate "B_3" and the resulting supernatant "B_4" gives an indication of the amount of glycoprotein material, if any, that had been precipitated by the CPC and not removed by 1.2 volumes of ethanol. As can be seen from Tables III, IV and V, no glycoprotein material remained in either "B_3" or "B_4" and therefore, no appreciable glycoprotein was carried down with the CPC precipitate.

The 1.2 volume ethanol precipitate "B_5" is an indication of any mucopolysaccharides remaining in the solution after the "B_1" precipitation. The ratio of hexosamine to hexose to uronic acid is approximately 1:4:1 and this indicates that the material was derived mainly from glycoproteins and that most of the mucopolysaccharides had been removed.

The precipitates "A_4" and "B_6" were thought to contain the carbohydrate part of glycoprotein material originally found in the supernatants of the enzymatic digests. Thus, if 1.2 volumes of ethanol and CPC effectively removed all the mucopolysaccharide material from the supernatant, the carbohydrate contents of "A_4" and "B_6" should not be very different. Examination of Table VI shows that this was

the case for the pepsin-trypsin hydrolysis. For papain hydrolysis, however, a difference was found for the hexosamine values, but not for hexose or uronic acid. The ratio of hexosamine to hexose to uronic acid (low hexosamine:high hexose:low uronic acid), however, indicates that the precipitate contained very little mucopolysaccharide substances.

When the redissolved "A_4" precipitate was heated with CPC, no precipitate was formed. This indicated that the "A_4" precipitate did not contain a substantial amount of mucopolysaccharide materials.

An examination of Tables III, IV and V indicated that for both pepsin-trypsin and papain, the hexose contents of "A_7" and "B_7" were exceedingly high as compared to the hexosamine and uronic acid contents. Since the "A_7" and "B_7" were the materials left after completion of all the precipitations, it was thought that these high hexose values may be due to monosaccharides, dissacharides, or other very short chain carbohydrates that were either not removed by the acetone (and chloroform-methanol) extraction, or were liberated during the enzymatic hydrolysis.

C. THE EFFECT OF PROTEOLYTIC HYDROLYSIS UPON SULPHATED MUCOPOLYSACCHARIDES, WITH PARTICULAR EMPHASIS UPON LIBERATION OF SULFATE

In recent years, many workers have abandoned pepsin

and trypsin hydrolysis and favored papain hydrolysis of tissues before precipitating the mucopolysaccharides. The reason for this change has been the suspicion that the pepsin and trypsin hydrolysis, because of the very low pH and the long time period (72 hours) of hydrolysis, caused (a) extensive hydrolysis of the sulphate group of the mucopolysaccharide, and (b) depolymerization of the mucopolysaccharide.

It was, therefore, felt necessary to investigate the effects of the proteolytic hydrolysis of sulphated mucopolysaccharides by pepsin-trypsin, and by papain, from the standpoint of sulphate destruction and mucopolysaccharide depolymerization.

A solution of 2.5% chondroitin sulphate (A, B and C) and a solution of 1% heparin were each divided into two equal parts and subjected to either a pepsin-trypsin or papain hydrolysis. Each hydrolysate was divided into two equal parts. The mucopolysaccharide materials were precipitated from one part by the addition of 1.2 volumes of ethanol and from the second part by CPC. The schematic outline of this procedure may be seen in Figure II, Section C-2 of Analytical Methods.

The concentrations and total amounts of the materials before proteolytic digestion and after digestion and

precipitation are presented in Table VII.

It can be seen from these data that as a result of both pepsin-trypsin hydrolysis and papain hydrolysis, there was an exceptionally high loss of sulphate. As a result of the pepsin-trypsin treatment and precipitation, 25-40% sulphate of chondroitin sulphate, and 50-70% of the sulphate of heparin were lost. When papain was used as the proteolytic enzyme, 55-65% of the sulphate of the chondroitin sulphate and 50-70% of the sulphate of heparin were lost.

The results do not substantiate the hypothesis that the pepsin-trypsin treatment leads to an additional chemical hydrolysis of the sulphate group as a result of the low pH and the length of time of the enzymatic hydrolysis. The results indicated that papain hydrolyzes just as much if not more sulphate than pepsin-trypsin.

Table VIII shows the percentage recoveries of sulphate, hexosamine and uronic acid from the original material. The recovery of hexosamine was poor with losses as great as 53%. The recovery of uronic acids was also poor, with losses as great as 21%. The explanation of these poor recoveries may be found in the following reasons: (a) that both enzymatic systems used may have depolymerized the mucopolysaccharide material used, or (b) that precipitation

TABLE VII

Concentration (mg/gm) and total amounts (mg/100 ml of solution containing 2.5 gm of chondroitin sulphate or 1 gm of heparin) of sulphate, hexosamine and uronic acid found in the materials before proteolytic digestion and after digestion and precipitation.

	Pepsin-Trypsin						Papain					
	Sulphate		Hexosamine		Uronic Acid		Sulphate		Hexosamine		Uronic Acid	
	Conc	Total	Conc	Total	Conc	Total	Conc	Total	Conc	Total	Conc	Total
Chondroitin Sulphate Solution	176.0	440.0	92.50	231.0	79.3	198.2	232.8	583.0	91.5	229.5	77.6	198.0
1.2 vol Alc ppt	109.8	268.0	77.25	188.2	67.7	165.0	115.5	199.0	102.7	177.7	90.8	157.1
CPC ppt	98.4	326.0	32.98	109.3	61.5	242.0	81.10	272.5	64.2	216.0	52.6	177.0
Heparin Solution	630.0	630.0	179.5	179.5	39.8	39.8	564.0	564.0	183.8	183.8	50.7	50.7
1.2 Alc ppt	64.10	190.0	94.4	175.2	25.2	46.9	161.7	158.1	138.1	135.2	41.9	41.0
CPC ppt	151.3	321.0	72.9	154.7	26.3	56.8	127.8	294.0	77.2	177.5	22.6	52.0

TABLE VIII

Percentage recoveries of sulphate, hexosamine and uronic acid found in the materials after proteolytic digestion and precipitation

	Pepsin-Trypsin			Papain		
	Sulphate	Hexosamine	Uronic Acid	Sulphate	Hexosamine	Uronic Acid
Chondroitin Sulphate Solution	100	100	100	100	100	100
1.2 vol Alc ppt	60	81	83	34	77	79
CPC ppt	74	47	122	47	94	89
Heparin solution	100	100	100	100	100	100
1.2 vol Alc ppt	30	98	117	28	74	80
CPC ppt	50	86	142	52	96	102

by CPC, or ethanol did not in fact completely precipitate the mucopolysaccharide material.

D. FATTY ACID ANALYSIS OF EXTRACTS (ACETONE AND CHLOROFORM-METHANOL) BY GAS-LIQUID CHROMATOGRAPHY

The pooled skin extracts from several birds were placed on a rotatory evaporator and the solvents were removed. The oily extract was then shaken with petroleum ether and the ether removed from the solution on the rotatory evaporator. The remaining material was methylated as in Section H-1 of the Analytical Methods and chromatographed.

The material remaining after the petroleum ether extraction was saponified according to the AOAC method, extracted with petroleum ether and the fatty residue methylated and chromatographed.

It was thought that extraction of the acetone and chloroform-methanol extract of the tissues by petroleum ether would contain the triglycerides and free fatty acids, but not the fatty acids which are a part of the glycolipids or lipoproteins.

The AOAC saponification step was carried out to ascertain whether or not all of the fatty acids and tryglycerides had been removed from the extracts.

Table IX gives the retention times in minutes of the fatty acids C_8-C_{20} inclusive, in addition to C_6, C_{18}^-, $C_{18}^=$ and $C_{18}^{\equiv}$. Inspection of Table IX shows that all the fatty acids come off the gas chromatographic column in uniform or regular intervals.

TABLE IX

Retention time (min) of individual fatty acids chromatographed as in Section H-1 of Analytical Methods

Fatty Acid	Retention time (min)
C_6	12.1
C_8	20.6
C_9	26.6
C_{10}	32.5
C_{11}	38.0
C_{12}	43.4
C_{13}	48.4
C_{14}	53.2
C_{15}	57.9
C_{16}	62.5
C_{17}	66.8
C_{18}	73.8
C_{18}^-	75.1
$C_{18}^=$	79.3
$C_{18}^{\equiv}$	80.5
C_{19}	86.8
C_{20}	91.9

Table X gives the areas and the percentages of individual fatty acids extracted before saponification, by the petroleum ether from the acetone (and chloroform-methanol)

extract of skin. Inspection of Table X shows that the C_{16}, C_{16}^{-}, C_{18}, C_{18}^{-} and $C_{18}^{=}$ fatty acids predominated. Of major importance was the C_{18}^{-} fatty acid which seemed to account for slightly over 50% of the total fatty acids present. $C_{18}^{=}$ came second, accounting for 19.8%, and C_{16} a close third with 19.0%. C_{16}^{-} accounted for 5.4%, C_{18} for 4.8% and finally C_{14} accounted for 0.8% of the total fatty acids present.

TABLE X

Individual fatty acids (and their percentage of the total area) found in the petroleum ether extract of skin tissue

Fatty Acid	Area (cm^2)	% of Total Area
C_{14}	0.14	0.8
C_{16}	3.48	19.0
C_{16}^{-}	0.99	5.4
C_{18}	0.88	4.8
C_{18}^{-}	9.18	50.1
$C_{18}^{=}$	3.64	19.8

Table XI gives the amounts of fatty acids found in the acetone (and chloroform-methanol) extract from comb tissue. As can be seen, a much wider range of fatty acids were present in the comb extract than in the skin extract. C_{10}, C_{12}, C_{13}, C_{14}^{-}, C_{15}, C_{17}, C_{17}^{-}, C_{19}, C_{20}, C_{21} and C_{22} are the fatty acids which were found in the comb and absent in the skin. The percentages of these fatty acids, however,

were very small; none of them being greater than 1% of the total area. As with skin, the predominant fatty acid appeared to be C_{18}^{-} which was 45.6% of the total fatty acid content. In skin C_{10} and $C_{18}^{=}$ were found in equal quantities, but with comb, C_{16} accounted for 25.7% of the total while $C_{18}^{=}$ accounted for only 9.3% of the total. C_{16}^{-} fatty acid amounted to 12.0% of the total while C_{18} fatty acid amounted to 4.2% of the total.

TABLE XI

Individual fatty acids (and their percentage of the total area) found in the petroleum ether extract of comb tissue

Fatty Acid	Area (cm^2)	% of Total Area
C_{10}	0.14	0.3
C_{12}	0.15	0.3
C_{13}	0.06	0.1
C_{14}	0.33	0.7
C_{14}^{-}	0.10	0.2
C_{15}	0.18	0.4
C_{16}	12.21	25.7
C_{16}^{-}	5.70	11.9
C_{17}	0.15	0.3
C_{17}^{-}	0.09	0.2
C_{18}	1.98	4.2
C_{18}^{-}	21.70	45.6
$C_{18}^{=}$	4.40	9.3
C_{19}	0.06	0.1
C_{20}	0.03	0.2
C_{21}	0.08	0.2
C_{22}	0.20	0.5

Table XII shows the amounts of fatty acids found in the extract from leg bones. The fatty acid distribution of the leg bones was quite similar to the distribution of the fatty acids of comb.

TABLE XII

Individual fatty acids (and their percentage of the total area) found in the petroleum ether extract of leg bone

Fatty Acid	Area (cm^2)	% of Total Area
C_{13}	0.11	0.2
C_{14}	0.30	0.7
C_{16}	11.40	25.5
C_{16}^{-}	3.55	7.9
C_{17}	0.07	0.2
C_{17}^{-}	0.05	0.1
C_{18}	1.33	2.9
C_{18}^{-}	21.18	47.4
$C_{18}^{=}$	6.44	14.4
C_{19}	0.14	0.3
C_{21}	0.08	0.2

No traces of C_{10}, C_{12}, C_{20} or C_{22} fatty acids were found in leg bone, although they were found in comb. Traces, however, of C_{13}, C_{14}, C_{17}, C_{17}^{-}, C_{19} and C_{21} were found in leg bone as in the comb. The C_{18}^{-} fatty acid again accounted for the largest portion of the total (47.4%) and C_{16} again was accounted the second largest (25.5%). The relative amounts of C_{16}^{-} and $C_{18}^{=}$ were in reverse order than the order found in the comb, $C_{18}^{=}$ accounting for 14.4% and C_{16}^{-}

for 7.9% in the leg bone. In the leg bone C_{18} accounted for 3.0%.

Fat extracts of comb tissue and leg bone appeared to contain far more trace fatty acids than the extracts of skin. Comb extract contained larger amounts of trace fatty acids than leg bone extract.

In all three tissues, C_{18}^{-} and C_{16} accounted for nearly 70% of the total fatty acids present, with C_{18}^{-} present in nearly twice the quantity as C_{16}. Again in all three cases, C_{16}^{-}, C_{18} and $C_{18}^{=}$ accounted for 28% of the remaining fatty acids present. The only great difference among the three tissues was the amount of C_{16}^{-}, C_{18} and $C_{18}^{=}$. In skin C_{16}^{-} accounted for 5.4%, in comb for 12.0% and in leg bone for 7.9%. C_{18} of skin accounted for 4.8%, in comb for 4.2% and in leg bone for 3.0%. $C_{18}^{=}$ in skin accounted for 19.8%, in comb for 9.3% and leg bone for 14.4%.

The material not extractable by petroleum ether was treated again with alcoholic KOH and petroleum ether. The ether extract was treated as the original extract and gas-chromatographed. (This step as mentioned before was carried out in order to determine whether or not complete removal of all fatty acids, tryglycerides, etc. was obtained by the previous steps.) When this unsaponifiable material was chromatographed, no peaks of any fatty acids were found,

thus indicating that the previous extraction had removed all the free fatty acids.

E. GAS-LIQUID CHROMATOGRAPHIC STUDIES OF THE COMPONENTS OF MUCOPOLYSACCHARIDES

1. Selection of Liquid Phase to be Used for Resolution of Carbohydrates

The separation of any compound by gas-chromatography depends to a great extent upon the liquid phase that the components are separated upon. Many liquid coatings are now available, and possess a variety of properties. For example, a liquid coating may either be polar or non-polar.

Because of this dependence of separation upon the liquid phase, it was felt necessary to first investigate the separation of a mixture of several silyated carbohydrates by several liquid phases in order to choose the most appropriate liquid phase.

A mixture of α-xylose, α-fucose, α-glucose, α-galactose, α-mannose, glucosamine-HCl, N-acetyl glucosamine, α-glucuronic and α-galacturonic acid was prepared and trimethylsilyated. This mixture was chosen as it was felt that it was representative of the components of mucopolysaccharides.

The liquid phases used were as follows:

1. 10% neopentyl glycol sebacate (a polar liquid phase).
2. 3% silicone fluid GEXF 1150 (a non-polar liquid phase).
3. 1.5% silicone fluid GEXF 1150 + 1.5% neopentyl glycol sebacate.
4. 5% silicone gum rubber SE-52 (a non-polar liquid phase).
5. 5% silicone gum rubber SE-30 (a non-polar liquid phase).

In all cases the solid support used was chromosorb W-AW-DMCS. The chromatograph was run isothermally, as a proper program could only be selected after a proper liquid phase had been chosen.

Figures VI and VII are reproductions of the gas chromatograms of the mixture of silyated carbohydrates obtained with the various liquid phases. The various numbers above each diagram correspond to the liquid phases listed previously. Response was plotted against retention time in minutes.

Diagram 1 of Figure VI shows that the 10% neopentyl glycol sebacate liquid phase was only able to separate five of the original nine carbohydrates injected. The carbohydrates not separated were: α-glucuronic acid, α-galacturonic acid, glucosamine-HCl and N-acetyl glucosamine.

Diagram 2 of Figure VI shows that the 3% silicone

Figure VI

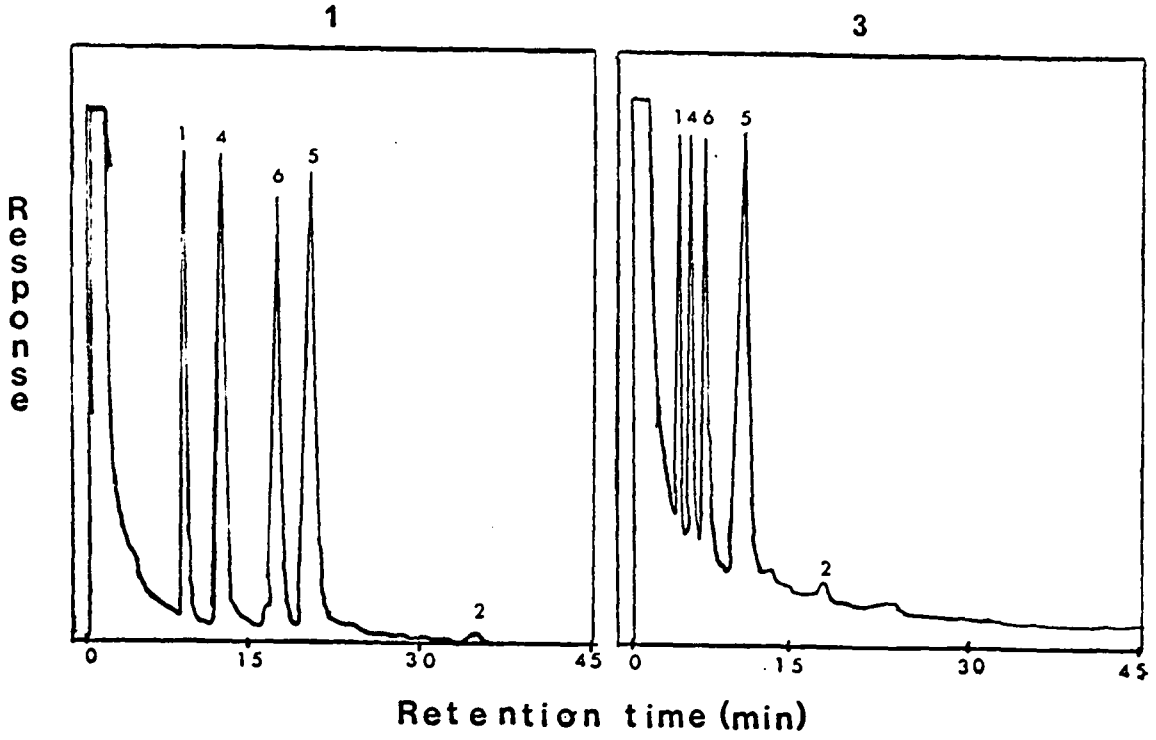
Figure VI

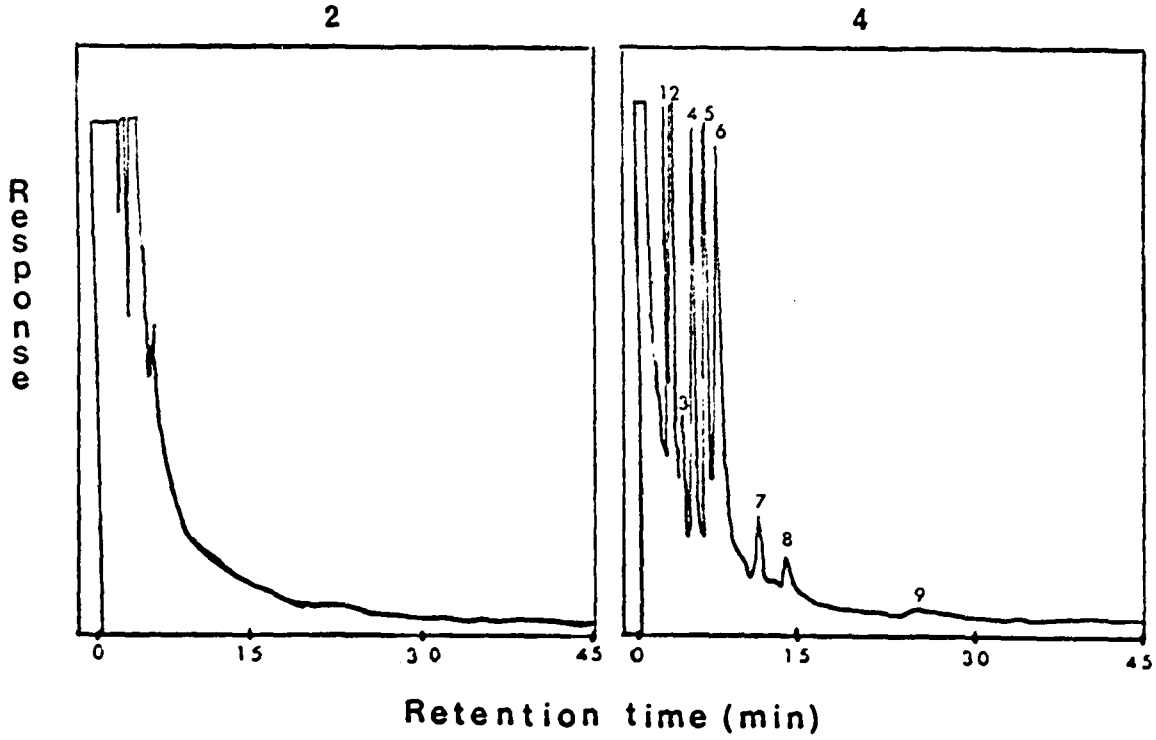
Gas chromatograms of a mixture of carbohydrates on liquid phases of:

1. 10% neopentyl glycol sebacate.
2. 3% silicone fluid GEXF 1150.
3. 1.5% silicone fluid GEXF 1150 + 1.5% neopentyl glycol sebacate.
4. 5% silicone gum rubber SE-52.

Peak numbers and corresponding carbohydrate:

1. α-fucose
2. α-xylose
3. α-glucuronic acid
4. α-mannose
5. α-galactose
6. α-glucose
7. α-galacturonic acid
8. glucosamine-HCl
9. N-acetyl glucosamine


1
3
Response
1
4
6
5
2
1 4 6
5
2
0
15
30
45
Retention time (min)


2
4
Response
12
4 5
6
3
7
8
9
0
15
30
45
Retention time (min)

fluid GEXF liquid phase was unable to separate any of the carbohydrates. They all came off the column in one large peak at the beginning. Because of this it was impossible to ascertain the identities of the carbohydrates.

Diagram 3 gave a chromatogram very similar to Diagram 1, except that the carbohydrates were much more closely packed. The same carbohydrates were separated in Diagram 3 as in Diagram 1.

Diagram 4 shows that a liquid phase of 5% silicone gum rubber SE-52 was able to separate completely the mixture of carbohydrates. The peaks, however, were very closely packed together.

Figure VII which was a liquid phase of 5% silicone gum rubber SE-30 shows a completely separated mixture. The peaks were well spaced and each peak distinct.

The 10% neopentyl glycol sebacate liquid phase was judged unacceptable, as it was not able to separate α-glucuronic acid, α-galacturonic acid, glucosamine-HCl, or N-acetyl glucosamine. This liquid phase was too polar, and retained these substances too strongly.

The 3% silicone fluid GEXF 1150 liquid phase was rejected, as it had too little retention power, and consequently all the carbohydrates emerged from the column in one

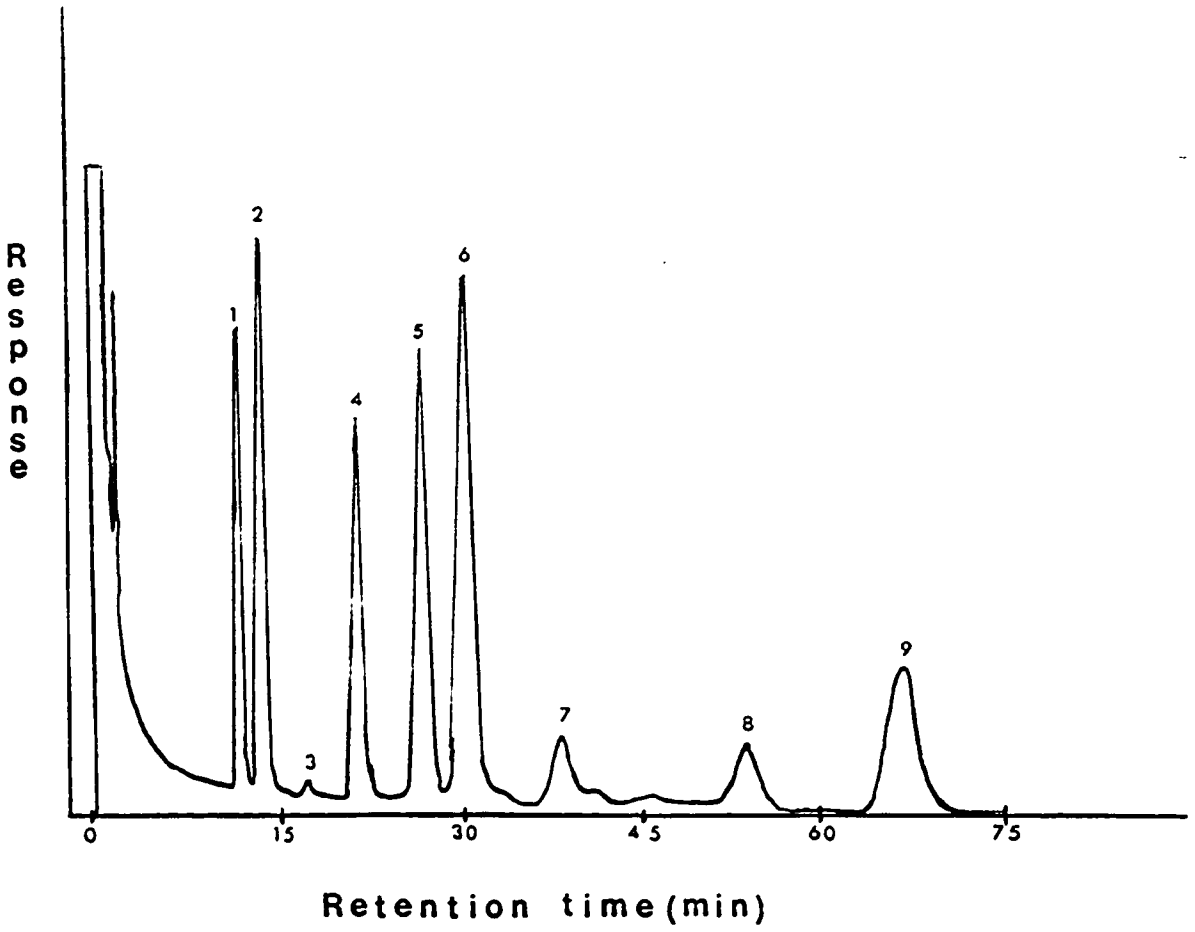
Figure VII

Figure VII

Gas chromatogram of a mixture of carbohydrates on a liquid phase of silicone gum rubber SE-30 (5%)

Peak numbers and corresponding carbohydrate:

1. α-fucose
2. α-xylose
3. α-glucuronic acid
4. α-mannose
5. α-galactose
6. α-glucose
7. α-galacturonic acid
8. glucosamine-HCl
9. N-acetyl glucosamine


Response
1
2
3
4
5
6
7
8
9
0
15
30
45
60
75
Retention time (min)

large peak.

The mixture of 1.5% silicone fluid GEXF 1150 + 1.5% neopentyl glycol sebacate was rejected for the same reasons as the 10% neopentyl glycol sebacate, i.e., it could not separate four of the carbohydrates of the mixture.

Although the 5% silicone gum rubber SE-52 liquid phase was able to separate all the carbohydrates it was not used because the peaks were all too closely packed together.

The 5% silicone gum rubber SE-30 liquid phase was the coating which was used for the separations. All the carbohydrates of the mixture were resolved, and the peaks were well separated.

2. Selection of a Program for the Separation of Carbohydrates

After an appropriate liquid phase had been found, it was next decided to develop a program which would lead to a more distinct resolution of the carbohydrate components.

With this objective in mind, a racemic mixture of fucose, xylose, mannose, galactose, glucose, glucuronic acid, galacturonic acid, glucosamine-HCl and galactosamine-HCl was prepared for the development of the program.

The program tried first was a program with a temperature range of 135°C-190°C, an initial hold of 4 min and a

temperature gradient of 1°C/min to 0.5°C/min. A second program with a temperature range of 145°C-190°C, an initial hold of 4 min and a temperature gradient of 1°C/min was also tried.

At this time several points must be made in order to clarify the discussion. When the program selection was begun, it was found that a much better chromatogram could be produced if the hexosamines were chromatographed separately, as the hexosamines had the same retention times as several neutral and acid monosaccharides. For this reason, hexosamines were chromatographed by a separate isothermal run.

It was found that several extraneous peaks were obtained directly after the solvent peak. Considerable attention was paid to these peaks until it was found that they had no significance or relation to the rest of the chromatogram, but were simply contaminants.

During the testing, it was found that β-mannose and β-galactose could not be separated. Later testing with liquid-phase mixtures indicated that it was impossible to separate them, so the area of the individual β-peaks were estimated by measuring their α-peaks, and presuming that the α and β peaks were in a constant ratio in the solution (which was in fact the case).

It was found that it was better to begin programmed

operation at a temperature of 135°C and hold for 4 min instead of at 145°C and hold for 4 min. The reasons for this were that if programmed operation was begun at 145°C, too little space remained between the extraneous peaks and the carbohydrate peaks, and often the two could not be distinguished. Using 135°C as the initial temperature, there existed sufficient space between the two series of peaks. Also using 145°C as the start, it was found that β-fucose, which came off very early in the program could not be separated, however, if 135°C was the starting point, it could be separated.

In view of these two points it was decided that 135°C with an initial hold of 4 min was to be used. It now remained to find the best programming rate. Programming rates ranging from 1°C/min to 0.5°C/min were tried, and it was found that the best rate was 0.8°C/min. Rates above 0.8°C/min gave peaks too closely packed together, while rates below 0.8°C/min gave peaks which were too broad and diffuse.

A final hold of 25 min at 190°C was found necessary to keep the columns clean, even though most of the pertinent carbohydrates emerged from the column as 190°C was reached. The program that was then decided upon was as follows:

Initial temperature: 135°C
Final temperature: 190°C

Program rate: 0.8°C/min
Initial hold: 4 min
Final hold: 25 min

3. The Effect of Heating the Reactants Before and During the Trimethylsilyation Reaction

It was found during the preliminary investigations that many of the carbohydrates were insoluble in pyridine, which was one of the reactants. Many papers suggested heating the carbohydrates in pyridine for dissolution. With this in mind an experiment was devised to determine the effects of heating the reactants.

Several standard carbohydrates were added to pyridine and heated in boiling water for 2 minutes. The trimethylsilyl reagents were then added and the solutions again heated for 2 minutes in boiling water. The reactants were dried and the trimethylsilyl derivatives extracted with pentane and injected into the chromatograph.

Examination of the chromatograms showed that a great number of additional peaks were produced when the reactants were heated.

The results of this experiment indicated that it was not advisable to heat the reactants before or during silyation, but it was preferable to dissolve the carbohydrate in

pyridine by constant agitation at room temperature for a minimum period of one hour.

4. Studies on the Release of Component Carbohydrates from Mucopolysaccharides, and Elimination of Substances Interfering with their Gas-Chromatographic Analysis

In order to analyze the mucopolysaccharides for their components it is first necessary to hydrolyze the mucopolysaccharide samples into their simpler components: neutral sugars, uronic sugars and the amino sugars. Hydrolysis of the mucopolysaccharide samples was attempted using four different hydrolysis procedures. The acids and their concentrations used, the temperatures and the times of heating are given in Table XIII.

TABLE XIII

Acid concentrations, times, temperatures, and amounts of acids used to hydrolyze a 15-30 mg mucopolysaccharide sample

	Dowex-50X8 in 0.075N HCl	Formic Acid 90%	1N HCl	1N H_2SO_4
Time	20 hours	20 hours	3 hours	3 hours
Temperature	100°C	100°C	100°C	100°C
Amount of acid	5 mls	3 mls	3 mls	3 mls

The second step after the hydrolysis of the mucopolysaccharide samples to their simpler components was the separation of these components into their neutral, basic and acidic fractions. This was attempted by passing the hydrolysates through a column of Dowex-50X8 in the H^+ form and elution with distilled water and 2N HCl. It was presumed that (1) the amino sugars (basic sugars) would be eluted with 2N HCl and (2) the neutral sugars and acidic sugars (uronic sugars) would be eluted together by distilled water. After the eluates were dried, they were silyated and chromatographed.

Both the 1N H_2SO_4 (which was first neutralized with Ba CO_3 to eliminate the H_2SO_4, before passing through Dowex-50X8) and 90% formic acid hydrolysis were judged unacceptable, as no peaks were obtained from their 2N HCl eluates, and the peaks obtained from their distilled water eluates could not be resolved.

Chromatograms were obtained from both the water eluates and the 2N HCl eluates of the 1N HCl hydrolysis and the Dowex-50X8 in 0.075N HCl hydrolysis, which had many peaks which could be resolved. The 2N HCl eluate chromatograms of both hydrolysates had no interfering peaks, but the chromatograms of the water eluates of these two hydrolyses had interfering peaks, a far greater number being found from the 1N HCl

hydrolysis than the Dowex-50X8 hydrolysis.

Since there was more interference in the water eluate of the 1N HCl hydrolysis than with the Dwoex-50X8 in 0.075N HCl hydrolysis, it was felt that future efforts for mucopolysaccharide analysis should be performed using Dowex-50X8 in 0.075N HCl as the hydrolyzing agent.

It was postulated that the unidentified peaks were due either to interfering substances that were not retained by the Dowex-50X8 resin, or To degradation produced by the component sugars caused by the acid, which concentrated when the water eluates were dried. A set of experiments were then devised to determine the cause of the interference.

Standard carbohydrates of ribose, fucose, xylose, mannose, galactose, glucose, glucuronic acid and galacturonic acid were suspended in 0.02N HCl solution and each split into three parts. One portion was freeze dried, one dried over NaOH, and the third neutralized with $BaCO_3$ and then dried over NaOH. Freeze drying was used because it was thought that the HCl would sublimeand therefore during the operation no concentrated HCl liquid solution would be formed to cause the decomposition of the sugars. Neutralization by $BaCO_3$ would create a neutral solution before drying. The portion dried directly over NaOH would serve as the "standard", as

the HCl would be concentrated as to the concentration of constant boiling HCl as the solution dried. When the chromatograms of each portion were compared, it was found that they were all exactly the same, i.e., they had no interference and each gave the same number of peaks. It was concluded from this experiment that concentration of the HCl during drying did not in fact degrade the carbohydrate components, and that the unidentified peaks previously found were due to interfering substances originating from the hydrolysis and not retained by the Dowex-50X8 resin.

It was then necessary to find a method to remove interfering substances from the hydrolysates after hydrolysis. The initial step of passing the raw hydrolysate through the Dowex-50X8 resin was retained, as this was a simple procedure to separate neutral sugars together with the acidic sugars from the basic sugars. Also the chromatogram of the basic sugar eluate (2N HCl eluate) showed no interference, even though amino acids were present. This was due to the fact that hexamethyldisilazane was not a powerful enough silyating reagent to silyate the amino acids, but was powerful enough to silyate the basic sugars.

The problem still remained to remove the interference from the water eluates. It was suggested that perhaps the

method suggested by Simkin et al. (132) to remove from the water eluates, substances which interfered with the paper chromatography of sugars, may be applied to this case. This method consisted basically of passing the water eluate from the Dowex-50X8 resin through Dowex-2X8 in the (HCO_3^-) form. Since Dowex-2X8 was not available, Dowex-1X8 in the HCO_3^- form was used instead. The neutral sugars were then eluated with 20 ml of distilled water and the acidic sugars with 15 ml of 2N HCl. It was thought that the Dowex-1X8 resin would retain the interfering substances.

To test the effectiveness of the separation of neutral sugars and acidic sugars by Dowex-1X8, samples of galactose, mannose, glucuronic acid and galacturonic acid were prepared and eluated through Dowex-1X8 as previously described. The separation was found to be very effective, as the water eluate contained only galactose and mannose, while the 2N HCl eluate contained only glucuronic acid and galacturonic acid.

It was now deemed necessary to make preliminary investigations using crude mucopolysaccharide samples (i.e., 1.2 volume ethanol precipitates, CPC precipitates, etc.) to test the effectiveness of the proposed separation procedure.

Samples were heated with 5 ml of Dowex-50X8 in 0.075N HCl for 20 hours at 100°C. The basic sugars were eluated

from the Dowex-50X8 resin by elution with 2N HCl and the neutral sugars together with the acidic sugars with water. The water eluate was passed through Dowex-1X8 and the neutral sugars eluated with water, while the acidic sugars were eluted with 2N HCl. After drying, each eluate was chromatographed.

It was found that the basic sugar eluate gave distinct amino sugar peaks with no interference.

The neutral sugar eluate from Dowex-1X8 gave distinct peaks for the neutral sugars; however, there were two peaks that could not be identified.

The acidic sugar eluate from Dowex-1X8 gave distinct peaks for glucuronic acid and galacturonic acid, but also gave many unidentified peaks.

One of these peaks was thought to be due to iduronic acid, but because of the lack of a standard, a positive identification could not be made. Similarly it could not be positively said that the other peaks were due to actual carbohydrate components or to interference. This method was adapted for the gas-chromatographic analysis of mucopolysaccharide samples. Details are given in sections H (2), (3), and (4) of Analytical Methods.

5. Gas-liquid Chromatographic Separations of Standard Carbohydrates

Gas-liquid chromatographic separations were carried out using standard carbohydrates, in order to determine their retention times. The carbohydrates used and their corresponding retention times are given in Table XIV.

Figure VIII shows the chromatogram obtained from neutral sugars. It can be seen that β-mannose and β-galactose emerged from the chromatographic column as a single peak. Individual samples of mannose and galactose were chromatographed under the same conditions, and the percentage of the total areas formed by the β peaks and the α peaks (also ℘ peak for galactose) were measured. Thus by measuring the α peaks for mannose and galactose (also ℘ peak for galactose) the amounts of β-mannose and β-galactose could be determined.

Figure IX shows the chromatogram of standard uronic sugars. As can be seen from the figure, the α and β anomers of glucuronic acid were determined. However, attempts to distinguish the individual peaks due to galacturonic acid were unsuccessful. The only thing that could be determined was that the sum of the four peaks gave the total amount of galacturonic acid.

Figure X gives the chromatogram of the amino sugars.

As can be seen from the figure, both the α and β uronic pairs of mannosamine, galactosamine and glucosamine were resolved.

6. Gas-liquid Chromatographic Analysis of the Components of the Mucopolysaccharide samples Isolated from Skin Tissue

The mucopolysaccharide samples which were isolated by the procedures outlined in section C (1) of the Analytical Methods, were hydrolyzed and the hydrolysates analyzed by Gas-Liquid Chromatographic techniques using the method developed in section E (4) of the Results and Discussion.

The mucopolysaccharide samples that were analyzed were "A_1", "A_2", "A_4", "B_1", "B_2", "B_3", "B_5", and "B_6" of both pepsin-trypsin and papain hydrolyses (see Figure 1 of the Analytical Methods for descriptions of the mucopolysaccharide samples).

Table XV is a list of the ratios of the neutral sugars found in each sample. Mannose was taken as equal to unity.

From Table XV it appears that reprecipitation of the 1.2 volume ethanol precipitate with CPC, or of the CPC precipitate with 1.2 volumes of ethanol, led to products that contained a higher proportion of xylose, fucose and ribose, and slightly richer in galactose than the original precipitates.

TABLE XIV

Retention time in minutes of standard carbohydrates

Neutral Sugars		Uronic Sugars		Amino Sugars	
Carbohydrate	Retention Time	Carbohydrate	Retention Time	Carbohydrate	Retention Time
δ^{p}-fucose	30.5	α-glucuronic acid	47.6	α-mannosamine	19.0
δ^{p}-xylose	31.7			α-galactosamine	20.9
ribose	32.3	β-glucosamine acid	49.7	β-galactosamine	22.7
α-fucose	35.3			α-glucosamine	25.6
α-xylose	39.0	galacturonic acid	61.0	β-mannosamine	27.2
β-fucose	40.4			β-glucosamine	32.9
		galacturonic acid	62.0		
β-xylose	45.2				
α-mannose	51.8	galacturonic acid	67.6		
δ^{p}-galactose	53.9				
α-galactose	57.6	galacturonic acid	76.6		
α-glucose	60.4				
β-mannose	62.6				
β-galactose	62.6				
β-glucose	72.9				

Figure VIII

Figure VIII

Gas chromatogram of standard neutral sugars.
Peak number and corresponding neutral sugar.

(1) ℘-fucose
(2) ℘-xylose
(3) ribose
(4) α-fucose
(5) α-xylose
(6) β-fucose
(7) β-xylose
(8) α-mannose
(9) ℘-galactose
(10) α-galactose
(11) α-glucose
(12) β-mannose and β-galactose
(13) β-glucose

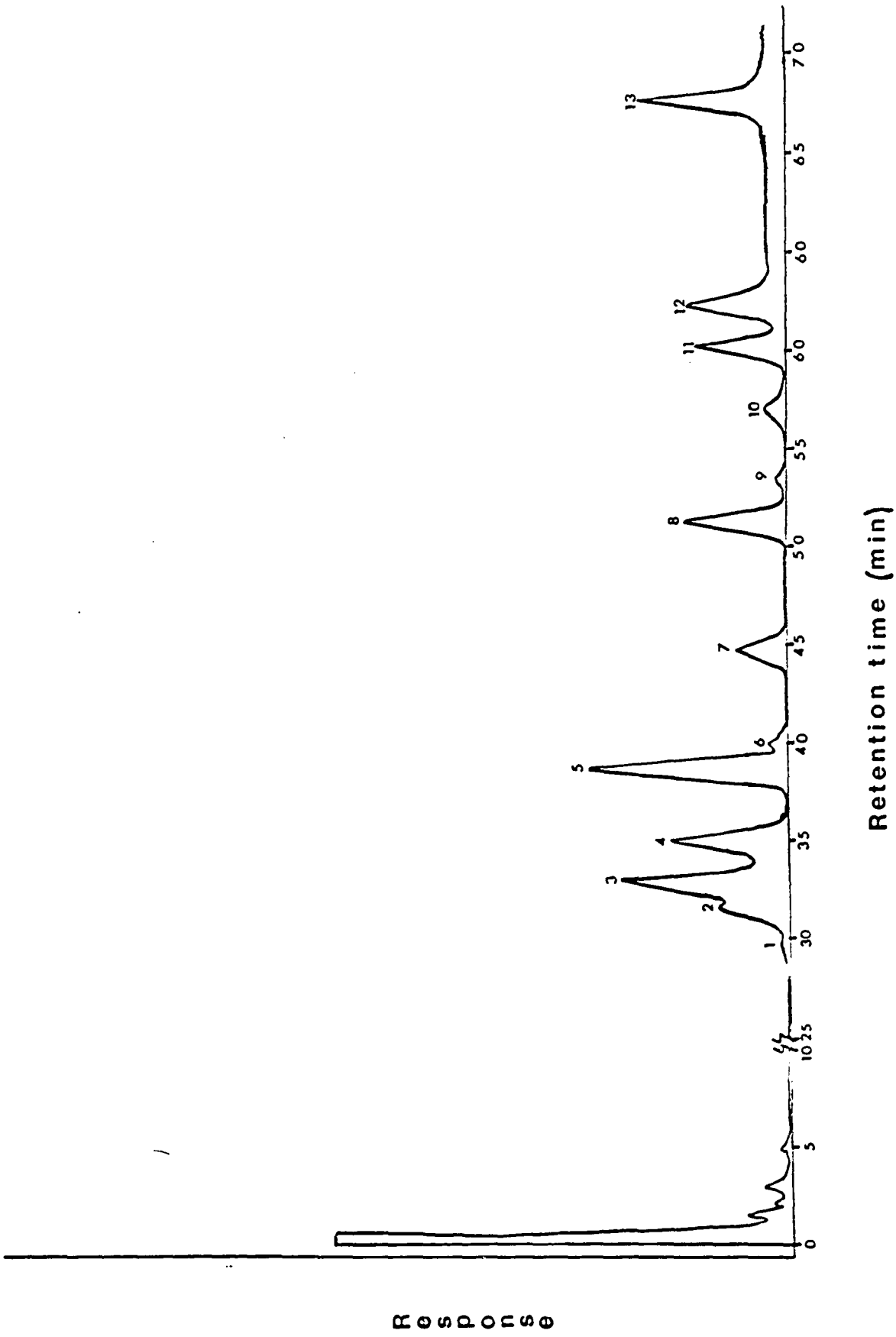

Response
Retention time (min)
0
5
10
25
30
35
40
45
50
55
60
65
70
1
2
3
4
5
6
7
8
9
10
11
12
13

Figure IX

Figure IX

Gas chromatogram of standard uronic acids.
Peak number and corresponding uronic sugar.

(1) α-glucuronic acid
(2) β-glucuronic acid
(3) galacturonic acid
(4) galacturonic acid
(5) galacturonic acid
(6) galacturonic acid

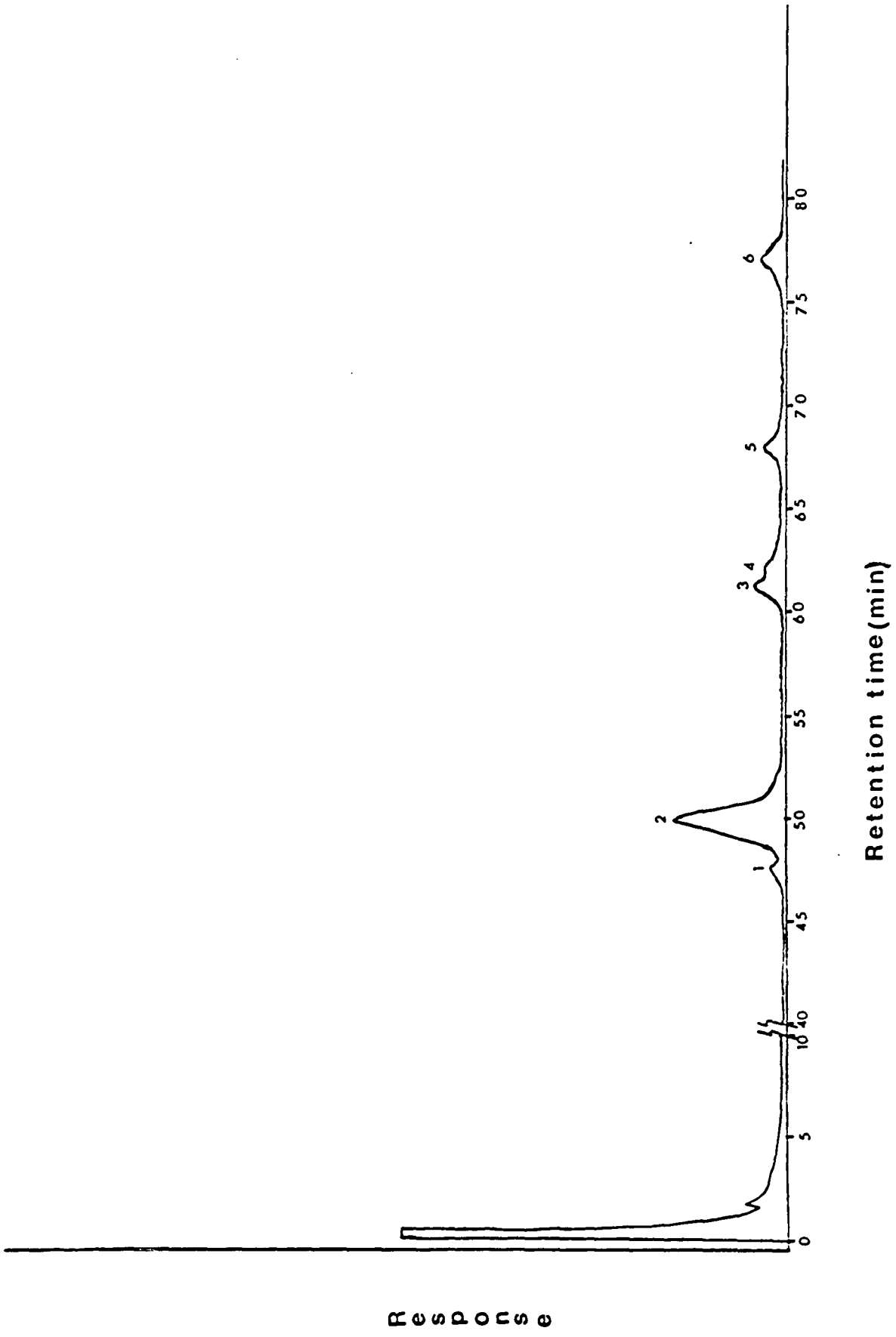

Response
Retention time(min)
0
5
10
40
45
50
55
60
65
70
75
80
1
2
3
4
5
6

Figure X

Figure X

Gas chromatrogram of standard amino sugars.
Peak number and corresponding amino sugar.

(1) α-mannosamine
(2) α-galactosamine
(3) β-galactosamine
(4) α-glucosamine
(5) β-mannosamine
(6) β-glucosamine

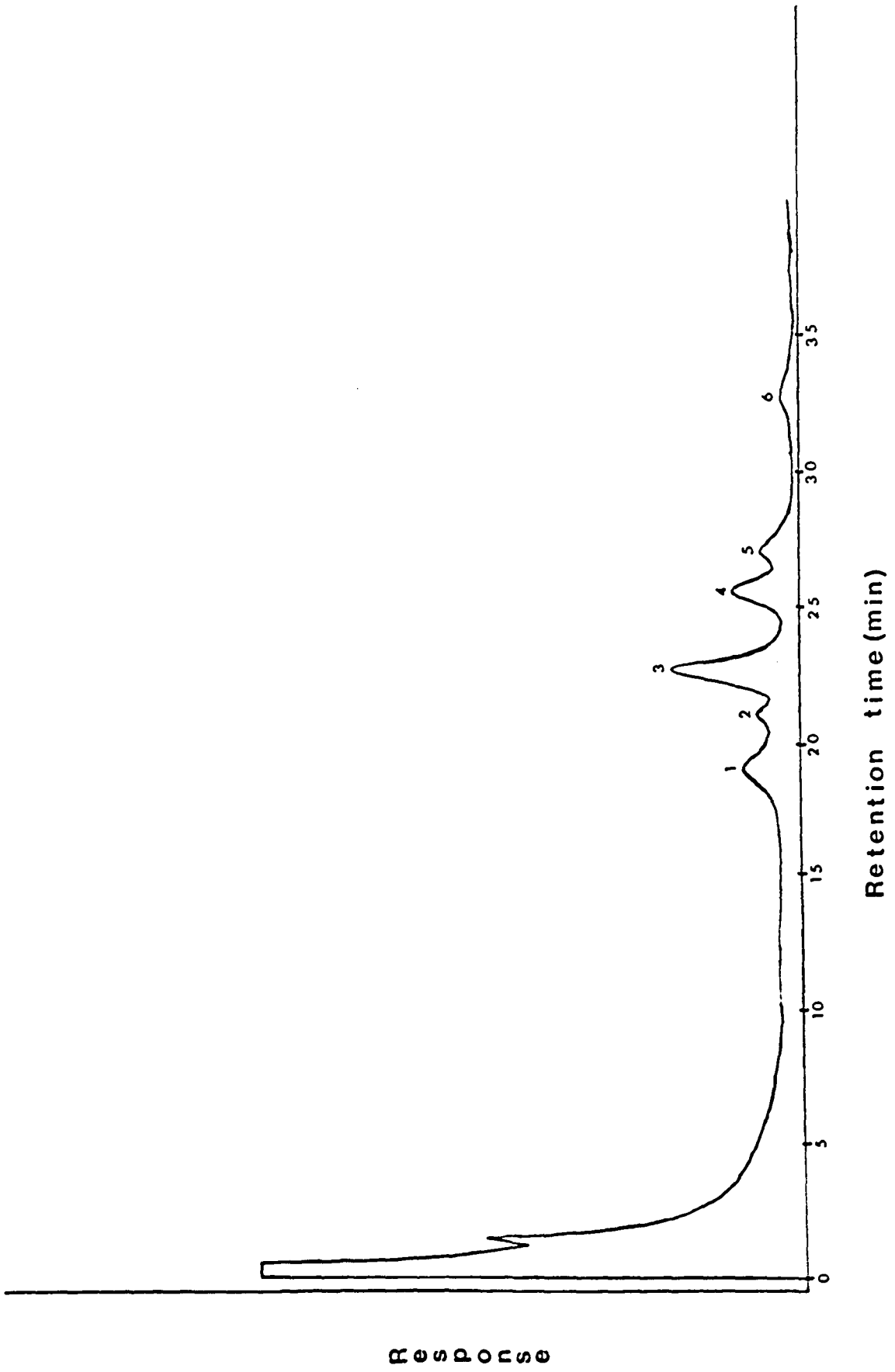

Response
Retention time (min)
0
5
10
15
20
25
30
35
1
2
3
4
5
6

TABLE XV

Ratios of neutral sugars. Mannose = 1.

Sample	Mannose	Galactose	Glucose	Xylose	Fucose	Ribose
Pepsin-Trypsin						
A_1	1	1.98	1.16	1.04	0.82	0.48
A_2	1	2.47	0.17	4.42	4.05	5.73
A_4	1	0.88	0.53	0.17	0.10	0.01
B_1	1	1.95	1.54	1.12	0.77	1.40
B_2	1	2.58	1.50	4.68	0.85	1.23
B_3	-	-	-	-	-	-
B_5	1	2.09	1.17	0.62	0.70	0.85
B_6	1	1.19	0.36	0.17	0.09	0.03
Papain						
A_1	1	2.55	0.56	0.30	0.78	2.08
A_2	1	2.86	0.74	3.73	4.34	8.68
A_4	1	0.98	0.52	0.11	0.07	0.06
B_1	1	2.33	3.03	0.72	0.56	1.36
B_2	1	2.42	1.98	5.39	3.44	5.32
B_3	-	-	-	-	-	-
B_5	1	1.97	4.32	0.21	0.20	0.48
B_6	1	0.88	0.63	0.12	0.07	0.07

It also appears that the other samples, precipitated with ethanol or CPC, produced mucopolysaccharide substances which contained a higher proportion of galactose and glucose and a smaller proportion of xylose, fucose and ribose than the original precipitates.

The proportion of glucose of the mucopolysaccharide

samples were found to be much higher than expected. It was though that these high levels of glucose were due to glycogen precipitated along with the mucopolysaccharide samples.

Precipitate B_3 contained no neutral sugars in either the papain or pepsin-trypsin hydrolyses.

Table XVI is a list of the ratios of the uronic acids found in each sample. Galacturonic acid was taken as equal to unity. There were no uronic acids found in samples A_1, A_4, B_3, B_5 or B_6. Uronic acids were found in samples A_2, B_1 and B_2. Glucuronic acid values were found to be very high in these three samples.

Values were given for iduronic acid. These peaks were not positively identified, because of lack of a suitable standard, however, on the basis of elution pattern given in previously published papers it was considered that these peaks were due to iduronic acid.

Table XVII is a list of the ratios of the amino sugars found in each sample. Galactosamine was taken as equal to unity. Samples A_4, B_3, B_6 and B gave no peaks for amino sugars in the products obtained after both pepsin-trypsin and papain hydrolyses. After pepsin-trypsin the precipitates A_1, A_2 and B_1 obtained after pepsin-trypsin hydrolysis gave peaks for amino sugars, while the precipitates A_1, A_2 and B_2

TABLE XVI

Ratios of uronic acids

Sample	Galacturonic	Glucuronic	Iduronic
Pepsin-trypsin			
A_1	-	-	-
A_2	1	6.8	0.9
A_4	-	-	-
B_1	1	1.7	0.6
B_2	1	3.6	0.4
B_3	-	-	-
B_5	-	-	-
B_6	-	-	-
Papain			
A_1	-	-	-
A_2	1	9.3	3.5
A_4	-	-	-
B_1	1	7.0	0.5
B_2	1	1.9	0.4
B_3	-	-	-
B_5	-	-	-
B_6	-	-	-

obtained after papain hydrolysis gave peaks for amino sugars. Glucosamine seemed to be the most prevalent amino sugar.

It is unfortunate that the method gives only the ratio of each group of constituents and does not permit a reliable relation of the ratios of the neutral sugars to those of uronic acids and of amino sugars. In order for the method to give concentration values so that the ratios of

TABLE XVII

Ratios of amino sugars

Sample	Galactosamine	Glucosamine
Pepsin-trypsin		
A_1	1	1.13
A_2	1	2.10
A_4	-	-
B_1	1	2.21
B_2	-	-
B_3	-	-
B_5	-	-
B_6	-	-
Papain		
A1	1	1.90
A2	1	2.10
A4	-	-
B1	-	-
B2	1	1.90
B3	-	-
B5	-	-
B6	-	-

the three could be compared, each sample must first be taken to dryness, and the samples must then be silyated and the neutral sugar and uronic acid samples taken to dryness and dissolved in the same amount of pentane (e.g. 10 µl; for injection equal amounts should be used). For silyation of the amino sugars, 200 µl of solution must be used and this cannot be taken to dryness, and thus the only way that this can be

put on the same quantitative basis is to inject such a quantity to be a fraction of the amount injected for the other two fractions (e.g. 3 times the total amount) and decrease or increase amplification of a corresponding amount to achieve the same ratio. Under the conditions of the present method the exact amount of the dry matter of each injection is not known.

IV. SUMMARY

1. Hexosamine determinations were carried out in 13 x 100 mm screw cap test tubes and 10 ml volumetric flasks, in order to determine in which vessel the results have the least variability and the method most sensitive. From the various concentrations of hexosamine tried, a significant difference was obtained at the lower concentrations (10 μg/ml and 20 μg/ml. The results obtained in the two kinds of vessels when the more concentrated solutions (50 μg/ml and 60 μg/ml) were used were not appreciably different. Because the manipulation of test tubes was easier, the screw cap tubes were used for the subsequent colorimetric analysis.

2. Avian skin was hydrolyzed by pepsin and trypsin, and by papain in order to compare the effectiveness of these two enzymatic systems for the isolation of mucopolysaccharides from tissues. It was found (from the yields of hexosamine, hexose and uronic acid) that the two procedures were equally good. For convenience, papain was preferred.

3. It was found that CPC and absolute ethanol gave crude mucopolysaccharide precipitates of the same approximate composition in characteristic components. Again, from the

standpoint of convenience, the CPC was preferred, as it was easier to use. When the mucopolysaccharide samples were re-precipitated by CPC or absolute ethanol, it was found that the CPC gave a purer precipitate. Both absolute ethanol and CPC should be considered quite effective mucopolysaccharide precipitants, as all precipitations obtained from the mother liquor after the initial ethanol or CPC precipitation, contained very little mucopolysaccharide material.

4. Pepsin-trypsin and papain hydrolyses were compared in samples of chondroitin sulfate and heparin from the standpoints of (1) possible splitting or losses of the sulphate group of mucopolysaccharides and (2) depolymerization of the mucopolysaccharide. Sulphate losses were very great in both cases, which showed that during pepsin-trypsin and papain hydrolysis, sulphate is split to great and equal extents.

5. For both hydrolyses, mucopolysaccharide recoveries were poor. It was thought that these poor recoveries may be due to (1) depolymerization of the mucopolysaccharide material or (2) precipitation of the mucopolysaccharide material by CPC or ethanol did not in fact completely precipitate the mucopolysaccharide material.

6. The fatty acids from skin tissue, bone and comb tissue

were studeid by gas-liquid chromatography. In all three tissues, C_{18}- and C_{16} acids accounted for 70% of the total fatty acids, with C_{18}^{-} present in twice the amount as C_{16}. C_{16}^{-}, C_{18} and $C_{18}^{=}$ acids accounted for 28% of the remaining fatty acids present, the only difference being in their proportions in the various tissues.

7. The components of the mucopolysaccharides were studied. The resolution of neutral sugars, amino sugars and uronic acids were performed separately. It was found that the liquid coating which gave the best separation of the components was silicone gum rubber SE30 coated to a concentration of 5% on a solid packing of chromosorb W-AW-DMCS.

A program was developed for the separation of the neutral sugars and uronic acids which consisted of:

Initial temperature:	135°C
Initial hold:	4 min
Temperature gradient:	0.8°C/min
Final temperature:	190°C
Final hold:	25 min

The amino sugars were separated using an isothermal run at 170°C. During silyations, it was found that no heat should be applied, as this led to many unresolved peaks.

8. A study of the release of the component carbohydrates from mucopolysaccharides, and elimination of substances

interfering with their gas-chromatographic analysis was carried out. It was found that the hydrolysate from the Dowex-50X8 in 0.075N HCl hydrolysis produced chromatograms with the least interferences. In subsequent tests, this method was used for hydrolysis. For removal of interfering substances, it was found best to separate the hydrolysate first into its amino sugar fraction and a second fraction of neutral sugars and uronic acids by the use of Dowex-50X8, and then separate the neutral sugars from the uronic acids by passing this fraction through Dowex-1X8. It was found best to dry all fractions before silyation.

9. The elution sequences of the standard carbohydrates were established using standards for the neutral sugars, amino sugars and uronic acids. It was found that the retention times for β-galactose and β-mannose were identical. All the amino sugars were well separated and easily identifiable. Of the uronic acids detected, the retention time for iduronic acid could not be established, because of lack of a suitable standard. It was tentatively identified by use of elution data from previously published papers. Galacturonic acid instead of giving two peaks gave a total of four peaks.

10. Mucopolysaccharide samples previously extracted from avian skin (see Chapter II, C) were analyzed using the

gas-chromatographic technique developed (see Chapter II, H). It was found that all the samples contained mannose, galactose, glucose, xylose, fucose and ribose in varying amounts. Sample B_3 (2.8 volume ethanol precipitate) contained no neutral sugars, amino sugars or uronic acids. Glucose was found to be quite high, probably due to the presence of glycogen in the tissue precipitates. Only samples A_2 (CPC precipitate), B_1 (CPC precipitate), and B_2 (1.2 volume ethanol precipitate) contained uronic acids, the greatest amount being glucuronic acid. Glucosamine was found to be present in samples A_1 (1.2 volume ethanol precipitate), A_2 (CPC precipitate), and B_1 (CPC precipitate) of the pepsin-trypsin hydrolysis and B_2 (1.2 volume ethanol precipitate) of the papain hydrolysis in approximately twice the quantity of galactosamine. A method was suggested so that the ratios of neutral sugar to amino sugar to uronic acids could be determined.

REFERENCES

1. Joint recommendations of the physiological and biochemical committees on protein nomenclature.
   J. Biol. Chem. 4: XLVIII (1908).

2. Levene, P.A.
   "Hexosamines and Mucoproteins."
   Longmans, Green and Co., London (1925).

3. Meyer, K.
   The chemistry and biology of mucopolysaccharides and glycoproteins.
   Symposia on qualitative biology, 6: 249 (1938).

4. Stacey, M.
   The chemistry of mucopolysaccharides and mucoproteins.
   Advances in carbohydrate chemistry, 2: 161 (1946).

5. Kent, P.W. and Whitehouse, M.W.
   "Biochemistry of the Amino Sugars."
   Butterworths, London (1955).

6. Betleheim-Jevons, F.R.
   Protein-carbohydrate complexes.
   Advances in protein chemistry, 13: 35 (1958).

7. Winzler, R.J.
   "Ciba Foundation Symposium on the chemistry and biology of mucopolysaccharides."
   Churchill, London (1958).

8. Jeanloz, R.W.
   The nomenclature of mucopolysaccharides.
   Arth. Rheu. 3: 233 (1960).

9. Jeanloz, R.W. and Balays, E.A.
   "The Amino Sugars."
   Chemistry of Amino Sugars.
   Academic Press, New York, Vol. Ia (1969).

10. Neufeld, E.F. and Ginsberg, V.
    Methods in Enzymology - Complex carbohydrates.
    Academic Press, New York. Vol. VIII (1966).

11. Wood, D.F.
M.Sc. Thesis, McGill University (1965).

12. Brimacomb, .S. and Webber, J.M.
"Mucopolysaccharides."
Biochim. Biophys. Acta Library. Vol. 6 (1964).

13. Stacey, M. and Barker, S.A.
"Carbohydrates of Living Tissues."
Van Nostrand Co., Ltd. (1962).

14. Hilborn, J.C.
M.Sc. Thesis, McGill University (1968).

15. Pamer, T., Glass, G.B.T. and Horowitz, M.I.
Purification and characterization of sulphated glycoproteins and hyaluronidase-resistant mucopolysaccharides from dog gastric mucosa.
Biochem. 7: 3821 (1968).

16. Jeanloz, R.W. and Balays, E.A.
"The Amino Sugars" - Distribution and Biological Role.
Academic Press, New York. Vol. IIA (1965).

17. Jeanloz, R.W. and Balays, E.A.
"The Amino Sugars" - Metabolism and Interactions.
Academic Press, New York. Vol. IIB (1966).

18. Fischer, G. and Boedeker, D.
Ann. Chem. 117: 111 (1861).
Cited from "Mucopolysaccharides" by Brimacomb, J.S. and Webber, J.M.
Biochim. Biophys. Acta Library. Vol. 6 (1964).

19. Levene, P.A. and LaForge, F.B.
On chondroitin sulphuric acid I.
J. Biol. Chem. 15: 69 (1913).

20. Furth, O. and Bruno, T.
Chondroitinsulfuric acid I.
Biochem. 2. 294: 153 (1937).

21. Bray, H.G., Gregory, J.E. and Stacey, M.
Chemistry of tissues.
Biochem. J. 38: 142 (1944).

22. James, S.P., Smith, F., Stacey, M. and Wiggins, L.
A constitutional synthesis of chondrosamine.
Nature, 156: 308 (1945).

23. Levene, P.A.
Chondrosin.
J. Biol. Chem. 140: 267 (1941).

24. Davidson, E.A. and Meyer, K.
Structural studies on chondroitin H_2SO_4 (II) glucuronic linkage.
J. Am. Chem. Soc. 77: 4796 (1955).

25. Weissmann, B., Rapport, M.M., Linker, A. and Meyer, K.
Isolation of the aldobionic acid of umbilical cord hyaluronic acid.
J. Biol. Chem. 205: 205 (1953).

26. Jeanloz, R.W. and Stoffyn, P.J.
Chemical structure of β-heparin.
Federation Proc. 17: 249 (1958).

27. Stoffyn, P.J. and Jeanloz, R.W.
The identification of the uronic acid component of dermatan sulfate (β-heparin, chondroitin sulfate B).
J. Biol. Chem. 235: 2507 (1960).

28. Jeanloz, R.W., Stoffyn, P.J. and Tremege, M.
Chemical structure of β-heparin.
Federation Proc. 16: 201 (1957).

29. Hoffman, P., Linker, A., Lippman, V. and Meyer, K.
The structure of chondroitin sulfate B from studies with flavobacterium enzymes.
J. Biol. Chem. 235: 3066 (1960).

30. Ori, S.F. .
Infrared spectroscopic studies of some polysaccharides.
Biochim. Biophys. Acta, 14: 173 (1954).

31. Meyer, K.
Chemical structure of hyaluronic acid.
Federation Proc. 17: 1075 (1958).

32. Jeanloz, R.W.
Proc. 3rd intern. Congr. Biochem., Brussels, 1955.
Academic Press, New York (1956), pg. 65.

33. Hirano, S. and Hoffman, P.
Hexosaminidic linkage of hyaluronic acid.
J. Org. Chem. 27: 395 (1962).

34. Jeanloz, R.W. and Jeanloz, D.A.
The degradation of hyaluronic acid by methanolysis.
Biochem. 3: 121 (1964).

35. Flowers, H.M. and Jeanloz, R.W.
The synthesis of the repeating unit of hyaluronic acid.
Biochem. 3: 123 (1964).

36. Preston, B.W., Davis, M. and Ogston, A.G.
The composition and physiochemical properties of hyaluronic acids prepared from ox synovial fluid and from a case of mesothelioma.
Biochem. J. 96: 449 (1965).

37. McLean, J.
Am. J. Physiol. 41: 250 (1916).
Cited by Foster and Huggard (1955).

38. Jorpes, J.E. and Bergström, S.
Heparin.
Physiol. Chem. 244: 253 (1936).

39. Danishefsky, I., Eiber, H.B. and Carr, J.J.
Investigations on the chemistry of heparin. I. Desulfation and acetylatin.
Arch. Biochem. Biophys. 90: 114 (1960).

40. Foster, A.B., Olanesen, A.H., Stacey, M. and Webber, J.M.
Identity of the uronic acid in heparin.
Chem. Ind. (London) 143 (1961).

41. Cifonelli, J.A. and Dorfman, A.
Structural studies on heparin and heparitin.
Biochem. Biophys. Res. Commun. 4: 328 (1961).

42. Danishefsky, I., Eiber, H.B. and Langhoty, E.
Investigations on the chemistry of heparin.
J. Biol. Chem. 237: 1413 (1962).

43. Foster, A.B., Harrison, R., Inch, T.D., Stacey, M. and Webber, J.M.
Periodate oxidation of heparin and related compounds.
Biochem. J. 80: 12P (1961).

44. McKusick, V.A. et al.
Medical genetics.
Medicine, 44: 145 (1965).

45. Linker, A., Hoffman, P., Sampson, P. and Meyer, K.
Heparin sulfate.
Biochim. Biophys. Acta, 29: 443 (1958).

46. Linker, A. and Sampson, P.
The enzymatic degradation of heparitin sulfate.
Biochim. Biophys. Acta, 43: 366 (1960).

47. Knecht, J., Cifonelli, J.A. and Dorfman, A.
Structural studies on heparitin sulfate of normal and hurler tissues.
J. Biol. Chem. 242: 4652 (1967).

48. Cifonelli, J.A. and Dorfman, A.
Uronic acid of heparin.
Biochem. Biophys. Res. Comm. 7: 41 (1962).

49. Meyer, K., Linker, A., Davidson, E.A. and Weissman, B.
The mucopolysaccharides of bovine cornea.
J. Biol. Chem. 205: 611 (1953).

50. Hirano, S., Hoffman, P. and Meyer, K.
Structure of keratosulfate of bovine cornea.
J. Org. Chem. 26: 5064 (1961).

51. Rollett, A.
In moleschott unt ersuchurgen yur maturlitre des menschen und der thiere.
7: 190 (1860).
Cited by P.A. Levene, "Hexosamines and mucoproteins."
Longmans, Green and Co., Inc. London, 1925.

52. Ogston, A.G. and Stainer, J.E.
On the state of hyaluronic acid in Synovial Fluid.
Biochem. J. 46: 364 (1950).

53. Meyer, K.
Mucoids and glycoproteins.
Advances in Protein Chemistry, 2: 249 (1945).

54. Meyer, K. and Smyth, E.M.
On Glycoproteins. VI. The preparation of chondroitin sulphuric acid.
J. Biol. Chem. 119: 307 (1937).

55. Woodin, A.M.
The corneal mucopolysaccharides.
Biochem. J. 51: 319 (1952).

56. Dische, Z. and Robert, L.
Protein linked carbohydrate of corneal stroma.
Federation Proc. 21: 172 (1962).

57. Blix, A. and Snellman, O.
Arkir. Keni. 19(a), No. 32 (1945).
Cited by Stacey and Barber (1962).

58. Robert, L. and Dische, Z.
Analysis of a sulphate sialofucoglucosamino galacto manosidoglycan from corneal stroma.
Biochem. Biophys. Res. Comm. 10: 209 (1963).

59. Odelli, T.T. Jr. and Andeson, B.
Isolation of a sulphated mucopolysaccharide from blood platelets.
Proc. Soc. Exptl. Biol. Med. 94: 151 (1957).

60. Seno, N. and Anno, K.
The presence of hyaluronic acid in whale cartilage.
Biochim. Biophys. Acta, 49: 407 (1962).

61. Anderson, A.J.
Relation between sialic acid and the mucopolysaccharides protein complexes in human cartilage.
Biochem. J. 82: 372 (1962).

62. Pearce, R.H. and Watson, E.M.
The mucopolysaccharides of human skin.
Can. J. Research, E.27: 43 (1949).

63. Meyer, K. and Chaffer, E.
The mucopolysaccharides of skin.
J. Biol. Chem. 138: 491 (1941).

64. Meyer, K., Davidson, E., Linker, A. and Hoffman, P.
The Acid Mucopolysaccharide of Connective Tissue.
Biochim. Biophys. Acta, 21: 506 (1956).

65. Kaplan, D., and Meyer, K.
Mucopolysaccharides of aorta at various ages.
Proc. Soc. Exptl. Biol. Med. 105: 78 (1960).

66. Loeuvi, G. and Meyer, K.
The acid mucopolysaccharides of embryonic skin.
Biochem. and Biophys. Acta, 27: 453 (1958).

67. Linker, A., Coulson, W.F. and Carves, W.H.
Cardiovascular studies on copper-deficient swine.
VI. The mucopolysaccharide composition of aorta and cartilage.
J. Biol. Chem. 239: 1690 (1964).

68. Oppeinheimer, T., Heritier, N., Watkins, O. and Willhite, M.
Mucopolysaccharides in animal tissues.
Career Res. 26: 229 (1966).

69. Bertelsen, S. and Marcker, K.
Isolation of hyaluronic acid and chondroitin sulphuric acid from human aortas.
Acta Pharmacol. Toxicol. 18: 1 (1961).

70. Shiller, S., Slover, G.A. and Dorfman, A.
A method for the separation of acid mucopolysaccharides. Its application to the isolation of heparin from the skin of rats.
J. Biol. Chem. 236: 983 (1961).

71. Fabianek, J., Kerp, A. and Pigman, W.
Comparative study of acid mucopolysaccharides in dermal connective tissues of some mammals.
Comp. Biochem. Biophys. 14: 21 (1965).

72. Fabianek, J., Herp, A. and Martinez, L.
Acid mucopolysaccharides and hexosamine metabolism in dermal connective tissue of adrenalectomized rats.
Nature, 208: 1212 (1965).

73. Slack, H.G.B.
The metabolism of sulphated polysaccharides in limb atrophy in the rat.
Biochem. J. 60: 112 (1955).

74. Searls, R.L.
Isolation of mucopolysaccharides from the pre-cartilaginus embryonic chick limb-bird.
Proc. Soc. Exptl. Biol. Med. 118: 1172 (1965).

75. Antonopoulos, C.A., Engfeldt, B., Gardell, S., Hyert-quist, S.O. and Solheim, K.
Isolation and identification of glycosaminoglycas from fracture callus.
Biochim. Biophys. Acta, 101: 150 (1965).

76. Prane, G., Gardell, S. and Antonopoulos, C.A.
On the glycosaminoglycas of human retuobulbar tissue.
Exptl. Eye. Res. 5: 79 (1966).

77. Pearce, R.H. and Mathieson, J.M.
Quantitative isolation of purified acidic glyco-saminoglycas from rat skin.
Can. J. Biochem. 45: 1565 (1967).

78. Bollet, A.J., Anderson, D.V. and Simpson, W.F.
Measurement of tissue acid mucopolysaccharides.
J. Clin. Invest. 37: 858 (1958).

79. Scott, J.E.
In "Methods of Biochemical Analysis." Vol. 8, Ed. D. Glick.
Interscience, New York. 1960, pg. 145.

80. Snellman, O.
"Evaluation of Extraction Methods for Acid Tissue Polysaccharides," in R.E. Tunbridge, ed., Connec-tive Tissue, Blackwells Scientific Publication, Oxford, 1957.

81. Barker, S.A., Cruickshank, C.N.D. and Webb, T.
Mucopolysaccharides in rat skin. I. Isolation and identification.
Carbohydrate Res. 1: 52 (1965).

82. Jarpes, J.E. and Gardell, S.
On heparin monosulphuric acid.
J. Biol. Chem. 176: 267 (1948).

83. Smith, H. and Gallop, R.C.
The acid mucopolysaccharides of hog gastric mucosa.
Biochem. J. 53: 666 (1953).

84. Kuhn, R., Bielig, H.J. and Dann, O.
Invert soaps. I. Action of invert soaps on proteins.
Ber. 73B: 1080 (1940).

85. Jaques, L.B., Mankhouse, F.C. and Stewart, M.
Determination of heparin in the blood.
J. Physiol. (London), 109: 41 (1949).

86. Scott, J.E.
The reaction of long-chain quaternary ammonium salts with aridic polysaccharides.
Chem. and Ind. (London), 168 (1955).

87. Stacey, M. and Barker, S.A.
Structure of a polysaccharide synthesized by a streptococcus isolated from a ropy fermentation - value of infrared spectroscopy in polysaccharide studies.
J. Clin. Pathol. 19: 314 (1956).

88. Pearce, R.H., Mathieson, J.M. and Grimmer, B.J.
Fractionation of Anionic Glycosaminoglycans by Ion-Exchange Chromatography.
Anal. Biochem. 24: 141 (1968).

89. Kavamoto, S., Date, N. and Hasegava, E.
The interfering substance in the determination of acid mucopolysaccharides by means of cetyltrimethylammonium bromide. I. Removal of tissue nucleic acid.
Kyoto Furistu Ika. Daigaku Zasski, 77: 794 (1968).

90. Harris, G.S. and Fraser, J.R.E.
Extraction and measurement of glycosaminoglycas in serum and cell culture medium: a system with the use of an alipatic quaternary ammonia salt.
J. Lub. Clin. Med. 74: 527 (1969).

91. Bishop, C.T. and Glick, D.
"Methods of Biochemical Analysis."
Vol. 10, Interscience Publishers, Inc.
New York, N.Y. 1962, pg. 1.

92. Van der Heuvel, W.J.A.
Gas chromatographic separations of sugars and related compounds as acetyl derivatives.
Biochem. Biophys. Res. Commum. 4: 399 (1961).

93. Makita, M. and Wells, W.W.
Quantitative analysis of fecal bile acids by gas-liquid chromatography.
Anal. Biochem. 5: 523 (1963).

94. McInnes, A.G., Bull, D.H., Cooper, F.P. and Bishop, C.T.
Separation of carbohydrate derivatives by gas-liquid partition chromatography.
J. Chromatography, 1: 556 (1958).

95. Kircher, H.W.
Gas-liquid partition chromatography of methylated sugars.
Anal. Chem. 32: 1103 (1960).

96. Jones, J.K. and Perry, M.B.
The gas-liquid partition chromatography of carbohydrate derivatives.
Can. J. Chem. 40: 1339 (1962).

97. Gurne, S.W., Jones, J.K.W. and Perry, M.B.
Analysis of sugar mixtures by gas-liquid partition chromatography.
Chem. Ind. (London), 255 (1961).

98. Wells, W.W., Sweely, C.C. and Bently, R.
In "Biomedical Applications of Gas Chromatography,"
Szymanski, H.A. (ed.).
Plenum Press, New York, 1963, pg. 169.

99. Berry, J.W.
In "Advances in Chromatography," vol. 2. Giddings, J.C. and Keller, R.A. (eds.).
Marcel Dekker, New York, 1962, pg. 271.

100. Bishop, C.T.
In "Advances in Carbohydrate Chemistry."
Vol. 19, Wolfrom, M.L. (ed.).
Academic Press, New York, 1964, pg. 95.

101. Gunner, S.W., Jones, J.K.W. and Perry, M.B.
The gas-liquid partition chromatography of carbohydrate derivatives.
Can. J. Chem. 39: 1892 (1961).

102. Sawardeker, J.S., Sloneker, J.H. and Jeanes, A.
Quantitative determination of monosaccharides as their alditol acetates by gas-liquid chromatography.
Anal. Chem. 37: 1602 (1965).

103. Crowell, E.P. and Burnett, B.B.
Determination of the carbohydrate composition of wood pulps by gas chromatography of the alditol acetates.
Anal. Chem. 39: 121 (1967).

104. Kim, J.H., Shone, B., Liao, T. and Pierce, J.A.
Analysis of neutral sugars by gas-liquid chromatography of alditol acetates.
Anal. Biochem. 20: 258 (1967).

105. Jones, H.G., Jones, J.K.W. and Perry, M.B.
The gas-liquid partition chromatography of carbohydrate derivatives.
Can. J. Chem. 40: 1559 (1962).

106. Bishop, C.T., Cooper, F.P. and Murray, R.K.
Reactions of carbohydrate derivatives during gas-liquid chromatography.
Can. J. Chem. 41: 2245 (1963).

107. Perry, M.B. and Webb, A.C.
Analysis of 2-amino-2-deoxyhexoses by gas-liquid partition chromatography.
Can. J. Biochem. 46: 1163 (1968).

108. Hedgley, E.J. and Overend, W. .
Trimethylsilyl derivatives of carbohydrates.
Chem. Ind. (London), 378 (1960).

109. Bentley, R., Sweeley, C.C., Makita, M. and Wells, W.W.
Gas chromatography of sugars and other polyhydroxy compounds.
Biochem. Biophys. Res. Commun. 11: 14 (1963).

110. Sweeley, C.C., Bentley, R., Makita, M. and Wells, W.W.
Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances.
Am. Chem. Soc. 85: 2497 (1963).

111. Perry, M.B. and Hulyalkar, R.K.
The analysis of hexuronic acids in biological materials by gas-liquid partition chromatography.
Can. J. Biochem. 43: 573 (1965).

112. Birkhofer, L., Ritter, A. and Bentz, F.
Silyation of glucose and several glucose derivatives - Gentiobiose synthesis via silylglucose.
Chem. Ber. 97: 2196 (1964).

113. Klebe, J.E.
New silyl donors.
J. Am. Chem. Soc. 86: 3399 (1964).

114. Birkhofer, L., Ritter, A. and Giessler, W.
Disilyated carboramides.
Angen. Chem. Intern. Ed. Eng. 2: 96 (1963).

115. Wells, W.W., Katagi, T., Bently, R. and Sweeley, C.C.
Gas chromatography of sugar phosphates.
Biochim. Biophys. Acta, 82: 408 (1964).

116. Hashizune, T. and Sasaki, Y.
Gas chromatography of sugar phosphate.
Anal. Biochem. 15: 346 (1966).

117. Karkkainen, J., Yehtonin, A. and Nikkari, T.
Determination of glucosamine and galactosamine by gas chromatography.
J. Chromat. 20: 457 (1965).

118. Horowitz, M.I. and Delman, M.R.
Gas chromatography of hexosaminitols.
Ibid. 21: 300 (1966).

119. Sawardeker, J.S., Sloneker, J.H. and Dimler, R.J.
Detection and determination of anhydroglycose by gas chromatography.
Ibid. 21: 260 (1966).

120. Perry, M.B.
The separation, determination and characterization of 2 amino-2 deoxy-D-glucose and 2 amino-2 deoxy-D-galactose in biological materials by gas-liquid partition chromatography.
Can. J. Biochem. 42: 451 (1963).

121. Karkkainen, J.E., Haahti, E.O. and Lehtonen, A.A.
Thin layer and column chromatography of carbohydrates as T.M.S. with applications to mucopolysaccharide analysis.
Anal. Chem. 38: 1316 (1966).

122. Radhakrishnamurthy, B., Dalfers, E.R. and Berenson, G.S.
Determination of hexosamines by gas-liquid chromatography.
Anal. Biochem. 17: 545 (1966).

123. Marshall, W.E. and Kunemerson, F.A.
The carbohydrate constituents of human serum β-lipoprotein: galactose, mannose, glucosamine and sialic acid.
Archives of Biochem. Biophys. 98: 271 (1962).

124. Sweeley, C.C. and Walber, B.
Determination of carbohydrates in glycolipids and gangliosides by gas-chromatography.
Anal. Chem. 36: 1461 (1964).

125. Karkkainen, J., Lehtonen, A. and Nikkari, T.
Determination of glucosamine and galactosamine by gas-chromatography.
J. Chromatog. 20: 457 (1965).

126. Lehtonen, A.A., Karbbainen, J.K. and Haakti, E.O.
Detection of iduronic acid in mucopolysaccharide mixtures.
Anal. Biochem. 16: 526 (1966).

127. Oates, M.D.G. and Schrager, J.
The use of gas-liquid chromatography in the analysis of neutral monosaccharides in hydrolysates of gastric mucopolysaccharides.
Biochem. J. 97: 697 (1965).

128. Lehtonen, A., Karkkainen, J. and Haahti, E.
Gas-chromatographic characterization of the electrophoretically separated fractions of acid mucopolysaccharides.
J. Chromatog. 24: 179 (1966).

129. Oates, M.D.G. and Schrager, J.
The determination of sugars and amino sugars in the hydrolysates of mucopolysaccharides by gas-liquid chromatography.
J. Chromatog. 28: 232 (1967).

130. Huttunen, J.K. and Miettinen, T.A.
Quantitative determination of urinary neuraminyl oligosaccharides by gas-liquid chromatography.
Anal. Biochem. 29: 441 (1969).

131. Lehnhardt, W.F. and Winzler, R.J.
Determination of neutral sugars in glycoproteins by gas-liquid chromatography.
J. Chromatog. 34: 471 (1968).

132. Simkin, J.L., Skinner, E.R. and Seshadri, H.S.
Studies on an acidic glycoprotein-containing fraction isolated from guinea-pig serum.
Biochem. J. 90: 316 (1964).

133. Anastassiadis, P.A. and Common, R.H.
Liberation of hexosamine, hexuronic acid, and hydroxyproline from tissues by resin hydrolysis.
Can. J. Biochem. Physiol. 36: 413 (1953).

134. Anastassiadis, P.A., Maw, W.A. and Common, R.H.
Studies on the glycoproteins of domestic fowl. The hexosamine content of certain tissues of the sexually immature pullet and some effects thereon of the gonadal hormones.
Can. J. Biochem. Physiol. 36: 413 (1958).

135. Dische, Z.
A new color reaction for the determination of sugars in polysaccharides.
Methods of Biochemical Analysis, 2: 313 (1955).
Ed. Glick, D. Interscience Pub., Inc., New York, N.Y.

136. Fairbairn, W.J.
A modified anthrone reagent.
Chem. Ind. 72: 86 (1953).

137. Alicino, J.F.
Microvolumetric method for determination of sulphur in organic compounds.
Anal. Chem. 20: 85 (1948).

138. Metcalfe, L.D., Schmitz, A.A. and Pelka, J.R.
Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis.
Anal. Chem. 38: 514 (1966).

139. Cook, H.
M.Sc. Thesis, McGill University, 1970.