THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE MUCOPOLYSACCHARIDES AND GLYCOPROTEINS OF HUMAN COSTAL CARTILAGE

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

GEORGE H. STIDWORTHY

Oklahoma City, Oklahoma

1961

THE MUCOPOLYSACCHARIDES AND GLYCOPROTEINS

OF HUMAN COSTAL CARTILAGE

APPROVED BY

W R Shetlar

Arden T. Bever

DISSERTATION COMMITTEE

ACKNOWLEDGMENT

The author wishes to express his sincere appreciation to Doctor Marvin R. Shetlar for his counsel and assistance during the course of this research. Thanks are also due the Veterans Administration Hospitals of Oklahoma City, Oklahoma, and Martinsburg, West Virginia, where this research was conducted, and to my coworkers for their assistance and fellowship.

G.H.S.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

THE MUCOPOLYSACCHARIDES AND GLYCOPROTEINS OF HUMAN COSTAL CARTILAGE

CHAPTER I

INTRODUCTION

The study of the mucopolysaccharides of ground substance is one of long standing. In 1854 Boedecker reported that cartilage hydrolyzed with mineral acid contained a reducing substance which he named "Chondroitsaure" (1). Krukenberg in 1884 obtained fairly pure chondroitin sulfate by extracting tracheal cartilage with five to ten per cent sodium hydroxide (2). Morner (3) in 1889 prepared chondroitin sulfate by extraction with two and five per cent potassium hydroxide and precipitating with lead acetate and showed that all of the sulfur in chondroitin sulfate occurred as ester sulfuric acid. Schmiedeberg (4) in 1891 demonstrated that chondroitin sulfate contained hexosamine, hexuronic acid and acetic acid. However, it has been only in the past two decades that systematic study has revealed there are a variety of different mucopolysaccharides and that their presence and distribution varies in different tissues (5). Up to the present time six acid mucopolysaccharides have been identified in connective tissues. These compounds, which will be described in detail later, are the chondroitin sulfates, A, B and C; hyaluronic acid; chondroitin; and keratosulfate. Hyaluronic acid was

first described in 1934 (6); the chondroitin sulfates A, B and C, so designated, in 1951 (7); keratosulfate was isolated in 1953 (8) and chondroitin was identified in 1954 (9). This report is an extensive study of the mucopolysaccharides in one type of tissue, human costal cartilage, and their changes as elicited by the aging process.

Definitions

Connective Tissue. Connective tissues are those tissues which provide the continuity between other tissues. Essentially, connective tissue consists of a continuous fluid matrix in which lie an interlacing fabric of fibers of different compositions and structures. The matrix bathes or surrounds the connective tissue cells, from which the matrix components have originated.

Cartilage. Cartilage is a modified form of connective tissue. Like other connective tissues it is composed of cells and intercellular substance which, in turn, is composed of ground substance and of supporting fiber (10). The nature of the fiberous components of the intercellular material is the basis for the classification of cartilage into its three main types, hyaline, elastic and fibrous.

Costal cartilage is classified as hyaline cartilage and the twelve pairs of costal cartilage represents the largest amount of permanent hyaline cartilage in the body. Hyaline cartilage derives its name from the Greek hyalos, meaning glass; hyaline cartilage is translucent, bluish white and has a glossy appearance in the fresh state. It is a firm yet flexible tissue which can readily be cut with a knife.

Elastic cartilage, found in the external ear, the external audi-

tory and eustachian tubes and the epiglottis differs microscopically from hyaline cartilage. It is yellow, opaque, more flexible and elastic. Microscopically the cells are similar but the interstitial substance contains a network of branching fibers.

Fibrocartilage is found in small amounts in the intervertebral discs, articular cartilages, symphysis pubic and in places of attachment of tendons to bones. Fibrocartilage is a non-vascular, tendon-like tissue. The chondrocytes are surrounded by long parallel bundles in which is found little interstitial substance.

Type of cells. Microscopically the cellular components of cartilage vary in size and shape depending on their age and location. Chondrocytes located just beneath the perichondrium, the covering tissue, are small and flat, those further toward the center are spherical and larger and as older cells divide, they are found in clumps of two to eight. These clusters are called cell nests.

Ground Substance. Ground substance or interstitial substance is not really a substance but a multicomponent system. This matrix is a mixture of mucopolysaccharides, mucoproteins, glycoproteins, plasma proteins, collagen, lipid material and inorganic salts (11). The ground substance appears homogeneous except for fibrous capsules surrounding lacunae. The homogeneous nature of the intercellular substance is the result of masking of the fibrils by the matrix due to similarity of refractive index.

Classification of Nitrogen Containing Polysaccharides

No clear-cut classification system for the hexosamine containing

carbohydrate components of ground substance has been accepted by all investigators in this field. However, for purposes of understanding and communication, the classification proposed by Meyer (12) is largely used in this study. This classification is arbitrary, but represents the most commonly followed system in this field. Meyer divides the hexosamine containing polysaccharides into four groups: mucopolysaccharides, mucoproteins, mucoids and glycoproteins. The first three contain hexosamine as a major component (more than four per cent) while the fourth contains less than four per cent hexosamine.

Mucopolysaccharides are hexosamine containing polysaccharides in which all or nearly all of the nitrogen is contributed by hexosamine, therefore, these compounds contain little or no protein. These compounds have high molecular weight and are probably polydisperse. Mucopolysaccharides are divided into neutral and acid mucopolysaccharides since some have acid functions and others do not. The neutral group includes chitin, a polymer of N-acetylglucosamine and those polysaccharides which contain hexosamine linked to neutral sugars; the neutral polysaccharide of gastric mucosa, which is bound to a mucin; type XIV pneumococcus polysaccharide, which contain N-acetylglucosamine and galactose; and the Shiga-Kruse specific polysaccharide, which contains glucosamine, galactose and rhamnose.

The acid mucopolysaccharides contain, in addition to hexosamine, a carbohydrate with an acid function and are in turn divided into two groups, the simple acid mucopolysaccharides and the complex acid mucopolysaccharides. The first subgroup, the simple acid mucopolysaccharides, are those which are composed of only acetyl hexosamine and hexuronic acid. Two members of this group have been isolated: hyaluronic acid (6) which

is composed of N-acetylglucosamine and glucuronic acid (the disaccharide unit is hyalobiuronic acid 1-4 linkage) and chondroitin (8). Chondroitin is composed of N-acetylgalactosamine and uronic acid (the disaccharide unit is β glucuronido 1:3 N-acetylgalactosamine). These compounds are isomers and have similar properties.

The complex acid mucopolysaccharides include the sulphate esters or sulfomucopolysaccharides. Heparin, discovered by McLean (13) is the only member of this group in which the hexosamine is not acetylated. It is a polymer of glucosamine and glucuronic acid with an N-sulfate group (14), (15). Heparin also contains sulfate in addition to the N-sulfate; this sulfate is probably linked with the uronic acid portion of the molecule according to Wolfrom.

Mucoitin sulfate, first described by Levene (16) was reported to be an isomer of chondroitin sulfate found in almost all tissues. It is a polymer of N-acetylglucosamine and glucuronic acid. However, it is now felt by some investigators that this material was an artifact composed of a mixture of degraded hyaluronic acid and inorganic sulfate and does not exist, certainly not in abundance as has been previously stated (17). Meyer (18) and Wolfrom (19) have both studied the fraction and isolated glucosamine from it. It may be that they were studying heparin monosulfate or the heparitin sulfate of Jorpes and Gardell (20). However, the latter compound is dextro rotating while mucoitin sulfate was reported to exhibit a negative rotation.

Keratosulfate is the only member of this sulfomucopolysaccharide group which does not contain uronic acid. Keratosulfate is composed of N-acetylglucosamine, galactose and sulfate. The repeating unit of 4-O-D

galactopyranosyl-3-O-N acetylglucosamine-6-O-sulfate has recently been reported (21). It was isolated by Meyer's group in 1953 from bovine cornea (8). Meyer also reported keratosulfate had been isolated from calf bone (22) and more recently from rib cartilage as suggested by Stidworthy, Shetlar and Masters (23).

The chondroitin sulfates are the best characterized and probably the most important members of this group. There are three different chondroitin sulfate compounds which have been named "A", "B" and "C". Chondroitin sulfate "A" and "C" are closely related compounds and upon hydrolysis, yield N-acetylglucosamine, glucuronic acid and sulfate in equimolar ratios. Both are polydisperse polymers of the disaccharide chondrosine (3-O—(glycopyranosyluronic acid) -2-amino-2-deoxy galactose) (24). These compounds are distinguished by the solubility of their respective calcium salts and by their optical rotations. Chondroitin sulfate "A" precipitates in acetate buffer at an alcohol concentration of 30-40 per cent and "C" in an alcohol concentration of from 40-45 per cent. In neutral solution "A" has an optical rotation of $[\alpha]_D$ -28° to 32° while that of "C" exhibits a rotation of $[\alpha]_D$ -12° to -18°. Chemically the only difference between chondroitin sulfate "A" and "C" is the position of the sulfate group. In chondroitin sulfate "A" the sulfate is on the C4 carbon of the galactosamine while in "C" it is on the C6 position.

Chondroitin sulfate "B" is also characterized by optical rotation and solubility. Its calcium salt precipitates in 18-25 per cent ethanol and its rotation is $[\alpha]_D$ -60° to -70° (25).

In addition to these properties chondroitin sulfate "B" cannot be

enzymatically hydrolyzed by testicular hyaluronidase as can "A" and "C". While the uronic acid analysis by the CO_2 method of Tracey (26) showed the same uronic acid content in "B" as in "A" and "C", the carbazole method (27) indicated "B" to have less than half the amount of uronic acid. This indicated that the uronic acids were probably different. Hoffman in 1956 identified the uronic acid of chondroitin sulfate B as iduronic acid (28). Chemically chondroitin sulfate "B" is a (β -L-idio-pyranosyluronic acid) -3-O-N-acetylgalactosamine-4-sulfate polymer with 1-4 linkage (29).

Heparitin sulfate which yields N-acetylglucosamine glucuronic acid and SO_4 in equimolar ratios on hydrolysis is probably identical with mucoitin sulfate which has already been discussed. It was first described by Gardell and Jorpes in Sweden in 1948 (20). It differs from heparin in that it contains an N-acetyl group and is less highly sulfated.

The Mucoproteins. Mucoproteins are distinguished from mucopolysaccharide by the fact that the bulk of the nitrogen is contributed by amino acids. These compounds are high molecular weight and are complexes of mucopolysaccharides bound to proteins.

The Mucoids. The mucoids are carbohydrate containing high molecular weight proteins which contain more than four per cent hexosamine. The carbohydrate found of these compounds is not contributed by the acid mucopolysaccharide. Neutral mucoids which contain acetylglucosamine, galactose and in some cases mannose and fucose have been isolated from serum, urine, gastric mucosa and pseudomucinous cysts. Mucins which contain sialic acid in addition to the above listed carbohydrates are classified as acid mucoids.

Glycoproteins. The glycoproteins are compounds of the protein molecule with a substance or substances containing a carbohydrate other than nucleic acid (30). The term is usually restricted to proteins containing 1 per cent or more of carbohydrate. In compounds of this sort the carbohydrate is firmly linked to the protein.

Lipopolysaccharides. To the above classification of hexosamine containing compounds, a new group, the lipopolysaccharides, may now be added. Lipopolysaccharides are high molecular weight compounds which consist of a polysaccharide moiety linked to lipid. The carbohydrate moiety yields glucosamine, galactosamine, muramic acid, glucose, galactose, mannose, arabinose, and rhamnose upon hydrolysis. Lipopolysaccharides are typically constituents of gram-negative bacterial cell walls.

Distribution of Acid Mucopolysaccharides

The distribution of the acid mucopolysaccharides in connective tissues has been the subject of many investigations. Some tissues have been well studied and the polysaccharides constituents have been carefully isolated and characterized while in other cases the reported results have been based on chemical evidence gained by analysis of particular monomers and by analogy, the presence of a particular mucopolysaccharide or group of polysaccharides has been determined.

These studies make it apparent that ground substance is quite complex and that the constituents not only vary from tissue to tissue but also change within a given tissue with time and pathological conditions. The mucopolysaccharide constituents of tissues grouped according to Meyer (31) indicates the present status of these studied.

Group 1. Tissues which contain only hyaluronic acid. Those tissues from which only hyaluronic acid has been isolated are vitreous humor, synovial fluid, filterable fowl tumors and liposarcoma. Hyaluronic acid was identified by these criteria; hexosamine-uronic acid ratio of approximately one, and sulfate less than 0.5 per cent. Paper chromatography indicated only glucosamine and digestion by both testicular and pneumococcal hyaluronidase. Fractions which fulfilled these criteria had an optical rotation of $[\alpha]_D$ -70° to -80°.

Group II. Tissues which contain only chondroitin sulfate "A" and/or "C". Tissues from which only chondroitin sulfate "A" or "C", or a mixture of "A" and "C" have been isolated and identified are cartilage, adult bone, chondrosarcoma and chondroma. Chondroitin sulfates "A" and "C" were identified by the following criteria. On acid hydrolysis they yielded equimolar amounts of galactosamine, uronic acid, acetic acid and sulfate. The uronic acid values were the same either by carbazole or the CO_2 method. They were hydrolyzed by testicular hyaluronidase but resistant to bacterial hyaluronidase. Both gave the identical disaccharide, chondrosine. "A" and "C" were differentiated by solubility of the respective calcium salts and by their optical rotations. It is of interest that some chondrosarcomas contain only "A" while others contain only "C". Since this classification was made keratosulfate has been isolated from cartilage and rib bone and more recent studies indicate that at least some chondrosarcoma tissue also contain small amounts of this material.

Group III. Tissues which contain hyaluronic acid as a major component and chondroitin sulfate "C" as a minor constituent. These tissues are the umbilical cord, connective tissue from the main organ of

the electric eel and fibroblasts grown in tissue culture. All of these tissues are typical of primitive mesothelial structures. This mixture was identified and its distribution was based on rotation, digestion by testicular and bacterial hyaluronidase, solubility and paper chromatography of the hexosamines.

Group IV. Tissues which contain chondroitin sulfate "B" as the major component, hyaluronic acid as a minor component. Pig skin and legumentum nuchae both contain chondroitin sulfate "B" as a major component with hyaluronic acid as a minor component. Ligumentum nuchae, in addition contains chondroitin sulfate "A". Chondroitin sulfate "B" was identified by the following characteristics. It precipitated as the calcium salt in an alcohol concentration of less than twenty-five per cent. Acid hydrolysis produced equimolar amounts of galactosamine, uronic acid (by the CO_2 method), acetic acid and sulfate. The carbazole method gave only 50 per cent of the value for uronic acid obtained by the CO_2 method. The optical rotation was $[\alpha]_D$ -56° to -63°. Mild acid hydrolysis produced a disaccharide which differed from chondrosine.

Group V. Tissue which contain chondroitin sulfate "B" and "C" in approximately equal amounts. This group of tissues include adult connective tissue, tendon, and heart valves. The latter two tissues also have been shown to contain hyaluronic acid as a minor constituent.

Group VI. Tissues which contain keratosulfate as a major component, chondroitin as a minor component. Of this group Meyer states, "This group is represented only by bovine cornea. No other tissue has been found in which the major constituent is keratosulfate, nor has there been any evidence of the existance of an identifiable fraction with the

physical and chemical properties of chondroitin in any tissues other than cornea."

Group VII. Tissues which contain a conglomerate. Tissues in Group VII include: amyloid, bovine aorta and young bone. These tissues contain a mixture of the chondroitin sulfate, some only partially sulfated, hyaluronic acid, keratosulfate and compounds related to heparin.

From this distribution data, which is incomplete and subject to many modifications, it is apparent that different connective tissues vary greatly both with respect to the character and amount of mucopolysaccharides which they contain.

This dissertation is a study of one type of connective tissue, human costal cartilage. The purpose of this study has been to clarify and extend information on the acid mucopolysaccharides and glycoproteins of this particular tissue.

CHAPTER II

METHODS

The tissue used in this study was fresh human costal cartilage obtained at autopsy from patients varying in age from premature to eighty-eight years who had died from various pathological causes in the University and Veterans Administration Hospitals, Oklahoma City, Oklahoma. As soon as possible the cartilage was cleaned by freeing it from perichondrium and removing any calcified areas. This tissue was either stored in a deep freeze at -20°C., or was dried, depending on the subsequent analysis.

Tissue Preparation

Dried samples were prepared by cutting the tissue into fine chips with a surgical knife and drying these chips in a vacuum oven at 60°C. under line vacuum for periods ranging from 17-24 hours. The dried cartilage was ground in a micro model Wiley laboratory mill to pass through a 60 mesh sieve and stored at room temperature.

Samples used in extraction studies were kept frozen until they were used. Aliquot samples were dried *in vacuo* to determine the dry weight of the tissues. The results of analysis were calculated on the basis of this dry weight determination. This fresh or frozen tissue was diced and minced using, at different times, an electric meat grinder, a

Latapie mill, a Waring blender or a high speed Virtis homogenizer or in certain cases a combination of these methods. In all of these procedures the tissue was protected from overheating by using ice, a cold room and intermittent operations.

Chemical Methods

Uronic Acid

Uronic acid was determined by a modification of the carbazole method of Dische (27). Ten milligram samples of whole cartilage and from one to twenty milligram samples of extracted fractions were weighed on a micro balance and were placed in 16 x 150 millimeter ground glass stoppered test tubes. Ten milliliters of 85.7 per cent sulfuric acid (Dupont reagent grade H_2SO_4, specific gravity 1.84, diluted six to one with distilled water) were added and the result and mixture was hydrolyzed for 15 minutes in a boiling water bath, cooled on cold water and two milliliter aliquots pipetted into each of three glass stoppered centrifuge tubes. Twelve milliliters of the six to one sulfuric acid was added to each tube. The tubes were cooled in an ice bath and 0.4 milliliters of carbazole reagent (0.1 per cent recrystallized carbazole in absolute ethanol) was added to two of the tubes while the third which received 0.4 milliliters absolute ethanol served as a blank. The contents of the tubes were mixed by inversion and placed in an incubator at 37°C. for one hour and in a water bath at 30°C. for an additional hour. The samples were read in square cuvettes in a Coleman Model 14 spectrophotometer at 530 millimicrons against their respective blank. A standard of glucuronic acid lactone equivalent to 100 milligrams of glucuronic acid in two

milliliters of water was carried through the same procedure except for the initial hydrolysis.

Hexosamine

Hexosamine was determined by a modification (32) of the Elson-Morgan reaction. To a weighed amount, usually ten milligrams of cartilage or cartilage extract, was added two milliliters of distilled water and two milliliters of eight N hydrochloric acid. The samples were hydrolyzed for four to five hours in a boiling water bath using a funnel with a stirring rod inserted in its stem for reflux. The hydrolysates were cooled in an ice bath and filtered through Whatman No. 1 filter paper into ten milliliters volumetric flasks. The tube, filter paper and funnel were rinsed several times with distilled water. The volume was adjusted to ten milliliters and mixed. One or two milliliters of the hydrolysate was transferred into four 15 milliliter calibrated centrifuge tubes and was neutralized with 2.5 normal sodium hydroxide using phenolphthalein as an internal indicator. Standards of glucosamine hydrochloride of 50 and 100 micrograms were carried through the same procedure. All tubes were diluted to 3.5 milliliters with distilled water and to three of the tubes in each set was added two milliliters of acetyl acetone solution (0.2 milliliters of acetyl acetone dissolved in ten milliliters of 0.5 molar sodium carbonate, freshly prepared). To the fourth tube, which serves as the blank, was added two milliliters of 0.5 molar sodium carbonate. The samples were mixed by inversion and placed in an ice bath. Absolute alcohol was added to bring the volume to ten milliliters and two milliliters of Ehrlich's reagent (five grams of paradimethylaminobenzal-

dehyde in 190 milliliters of absolute alcohol and 190 milliliters of concentrated hydrochloric acid) was added to each tube. The contents were again mixed by inversion and incubated at 37.5°C. for 45 minutes. Each sample was read at 540 millimicrons against its respective blank in a Coleman Model 14 spectrophotometer. Values were calculated on the basis of glucosamine.

Hexose

Hexose was determined by a modification of the anthrone reaction reported by Shetlar, et al. (33). In this analysis hydrolysis was carried out as described above for hexosamines and in most cases on the same hydrolysate used in that determination. One milliliter of the hydrolysate, proportional to one-tenth of the original sample was pipetted into each of two glass stoppered centrifuge tubes and was diluted with two milliliters of distilled water. Standards were prepared with each set using 25 micrograms each of galactose and mannose in three milliliters water for each tube. The reagent blank contained only the three milliliters of distilled water. The tubes were cooled for 15 minutes in an ice bath and six milliliters of anthronic reagent (0.15 per cent anthrone in 95 per cent sulfuric acid, aged at least four hours) was added to each tube. The contents were mixed by inversion and allowed to react in a boiling water bath for 20 minutes. They were cooled in an ice bath for ten minutes and the optical density determined against the blank in a Coleman Model 14 spectrophotometer at 520 millimicrons.

Nitrogen

Nitrogen was determined in the micro Kjeldahl apparatus of Jenden

and Taylor (34). In the analysis of whole cartilage and extracts where sufficient material was available approximately 150 milligram amounts weighed on a micro balance, however, in certain cases amounts as low as five milligrams were used. These samples were digested with one milliliter of concentrated sulfuric acid to which one or two Henger granules were added. Digestion was continued at least one hour after the sample had cleared. The digest was transferred quantitatively into a ten milliliter volumetric flask and diluted to ten milliliters with distilled water. One to two milliliter aliquots of the digest were pipetted into the distillation flask. In cases where small amounts were used digestion was carried out in the same flask which was used for distillation and no transfer was necessary. The ground glass joint of the distillation flask was lubricated with 50 per cent sodium hydroxide and connected to the distillation apparatus. The contents were neutralized with 50 per cent sodium hydroxide. The amount varied with the procedure initially followed. A 50 milliliter Erlenmeyer flask to which exactly ten milliliters of 0.01 normal sulfuric acid and a drop of methyl red indicator had been added was placed under the condenser so that the condenser tip dipped into the acid. Steam was allowed to pass into the apparatus and distillation proceeded for two minutes after which the receiver was lowered and the system flushed for an additional minute. Approximately 0.01 normal sodium hydroxide was accurately standardized. It was dispensed by a microburette to titrate the partially neutralized sulfuric acid to a yellow-orange end point.

Chromatographic Procedures

Paper chromatography was used in this study for the isolation and

identification of the monomers of the polysaccharides both of the whole cartilage and of the fractions obtained by extraction procedures. Hydrolysis of these materials was catalyzed by the hydrogen form of Permutit Q, a polystyrene sulfonic acid cation exchange resin, prepared as described by Glegg, et al. (35). The resin was acid regenerated by stirring with an equal volume of 4.4 normal hydrochloric acid for two to three hours, washed repeatedly with distilled water in a separatory funnel until free of chloride ions and was then extracted in a large soxlet type extractor until the extract was clear. Amounts which ranged from ten to 100 milligram of sample, two milligrams of resin and five milliliters of water were placed in 16 x 125 millimeter culture tubes with screw caps and were incubated in an oven for 48 hours at 100°C. After cooling, the hydrolyzed mixture and resin were quantitatively transferred to a glass chromatography column (15 millimeter inside diameter, 300 millimeters in length). The liquid portion was allowed to pass through the column and was collected in 50 milliliter Erlenmeyer flasks. The resin was washed twice with ten milliliter portions of distilled water. This fraction was designated as the water eluate. The resin was then eluted with two ten milliliter portions of 0.5 normal hydrochloric acid and the fraction collected was designated as the hydrochloric acid eluate. Both fractions were evaporated to dryness in vacuo using either a Rinco evaporator or by lyophilization. The dried material was dissolved in an accurately measured amount of water proportional to the starting material, to give a solution equivalent to 100 milligrams of starting material per milliliter. One hundred microliter aliquots of these solutions were pipetted as spots onto 6 x 22½ inch sheets of chromatographic filter paper and developed at room

temperature in the butanol-pyridine-water system of Chargaff, et al. (36), using the descending technique. In the case of the water eluate chromatographic papers Whatman No. 3 millimeter of four or Schleicher and Schuel No. 589 red ribbon were used. The straw colored liquid was applied as spots one inch apart along a line of origin 2-3/4 inches from one of the narrow edges of the paper. On the center spot a known mixture of 50 micrograms each of glucuronic acid, galactose, mannose and fucose was applied. Development was allowed to continue until the solvent front had reached the lower edge of the suspended paper. The chromatograms were removed from the developing jar or chromatocab and air dried in a fume hood until the pyridine odor was negligible, then heat dried in an oven at 110°C. for a few minutes to remove the last traces of the solvent system. The carbohydrate components of this system were detected by spraying the chromatogram lightly, with 2-amino-biphenyl-phosphate (37). This reagent is prepared by dissolving three grams of 2-amino-biphenyl in 100 milliliters of glacial acetic acid to which 1.3 milliliters of 85 per cent reagent grade phosphoric acid has been added. The sprayed chromatogram was dried in a fume hood using a hot air blower and then heated in an oven at 110°C. for two to three minutes.

The hydrochloric acid eluate, which contained the hexosamines was subjected to the same chromatographic system except that Whatman No. 4 chromatographic paper and the technique of multiple development was used. After the initial 16 hour development the chromatogram was dried and redeveloped in the same direction for an additional 16 hour period. A 50 microgram glucosamine standard or after galactosamine became commercially available, one containing 50 micrograms each of glucosamine and galacto-

samine were run with each strip.

Quantitation of uronic acid and the hexosamines was achieved by a modification of this chromatographic separation and chemical analysis of the eluted material. The uronic acid content of the hydrolysate was determined by cutting out the stained uronic acid areas with a scissors, reducing these areas to small pieces and transferring these pieces to a glass stoppered 16 x 150 millimeter test tube. A paper blank was prepared in the same manner, using a sugar free area of comparable size from the sheet of paper. One milliliter of distilled water was added to each tube and the tubes were placed in a boiling water bath for one hour. The tubes were cooled in an ice bath and five milliliters of 0.4 per cent 2-amino-biphenylphosphate in glacial acetic acid was added. The tubes were returned to the boiling water bath for an additional hour, cooled, centrifuged and the uronic acid determined in a Beckman DU spectrophotometer at 369 millimicrons against the paper blank and compared to a 50 microgram uronic acid standard obtained from the same chromatogram.

Quantitation of glucosamine and galactosamine in the hydrochloric acid eluate was achieved by use of side markers of known glucosamine and galactosamine. In early work the galactosamine was obtained by the hydrolysis of chondroitin sulfate. These standards were applied as spots along the line of origin, one inch from the long edges of the paper. Duplicate samples of the eluate were applied 2-1/4 inches from the long edge of the paper. After double development the side markers were cut from the paper and stained as described above. The two unstained center sections of the paper corresponding in migration to the stained side markers which contained the separated amino sugars were placed in 15

milliliter graduated centrifuge tubes and analyzed by a modified Elson-Morgan reaction. To each tube was added 3.5 milliliters of distilled water and two milliliters freshly prepared acetyl acetone solution (two per cent acetyl acetone dissolved in 0.5 M sodium carbonate). The tubes were stoppered, mixed and placed in a boiling water bath for twenty minutes, cooled in an ice bath and diluted to ten milliliters with absolute ethanol. Two milliliters of Ehrlich's reagent (prepared by dissolving five grams paradimethylaminobenzaldehyde in 190 milliliters of absolute ethanol and adding 190 milliliters of concentrated hydrochloric acid) was added to each tube, carefully mixed by inversion and reacted in a 37.5°C. incubator for 45 minutes. Samples were centrifuged, decanted and read against a paper blank at 540 millimicrons in a Coleman Model 14 spectrophotometer. The concentrations of glucosamine and galactosamine were calculated on a percentage basis and equated to the total hexosamine value obtained by the chemical analysis of the whole fraction as described in the preceeding section.

Fractionation Studies

The fractionation of the carbohydrate material present in human costal cartilage was carried out on specimens taken from persons varying in age from term to eighty-five years. Three different fractionation procedures were followed to determine what mucopolysaccharide fractions were present in these tissues and to determine what changes occurred with the aging process.

Aqueous Extraction

The method by which Shattan and Schubert isolated a chrondroitin

sulfate-protein complex from bovine nasal cartilage (38) was followed with certain modifications to fit these experimental conditions as shown in Chart 1.

Approximately 150 grams of a fresh-frozen cartilage sample was shaved into small chips and was ground in an electric meat grinder then "homogenized" in a Waring blender.

The sample was kept cold by using ice made from distilled water in chilled distilled water. The homogenizer and meat grinder were operated intermittently to avoid warming. All centrifugations were carried out in the cold. The resultant homogenate was quantitatively transferred from the homogenizer with distilled water and brought up to a volume of 4000 milliliters, 30 milliliters of toluene were added to prevent bacterial contamination. The homogenate allowed to stand in the refrigerator for six days. The supernatant liquid was separated by decantation after centrifugation, and was precipitated with two volumes of alcohol. A white, flocculent precipitate formed immediately and was aged in the refrigerator for three days, centrifuged and the supernatant fraction from this alcohol precipitation was added 240 milliliters of an aqueous solution of potassium acetate (one gram of potassium acetate per milliliter of water). This salt caused a second precipitation of white flocculent material. The solution was stored in the refrigerator for two days and the precipitate was collected by centrifugation. The alcohol and potassium acetate precipitates were dried *in vacuo*. The residual cartilage was extracted two additional times in the same manner for periods of four and five days respectively and the resultant fractions precipitated as described above. Each of the fractions were dried, ground to pass through a 60 mesh screen

Chart 1

Cartilage Extraction by Mild Aqueous Solutions and Fractionation of Extracts

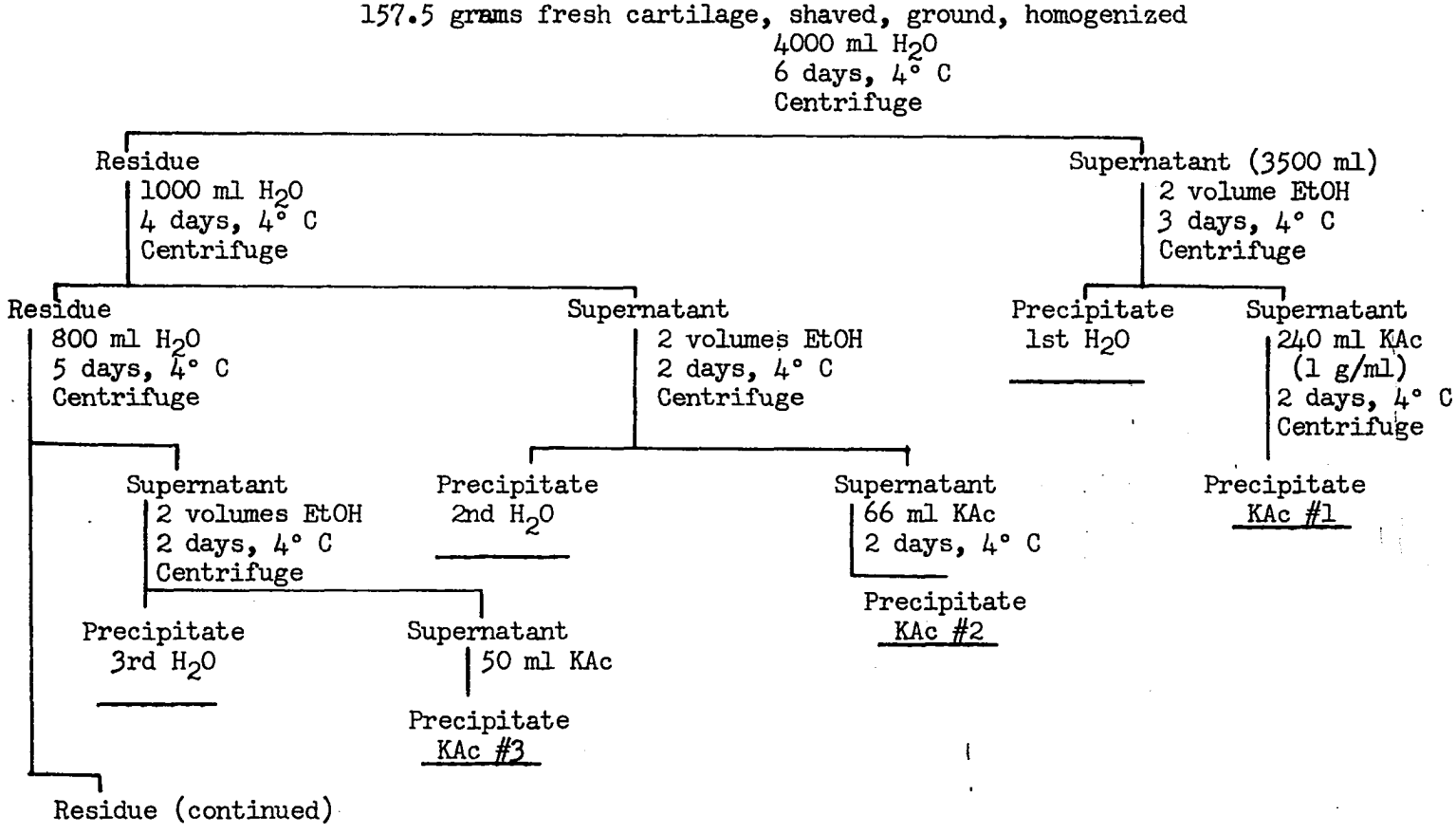
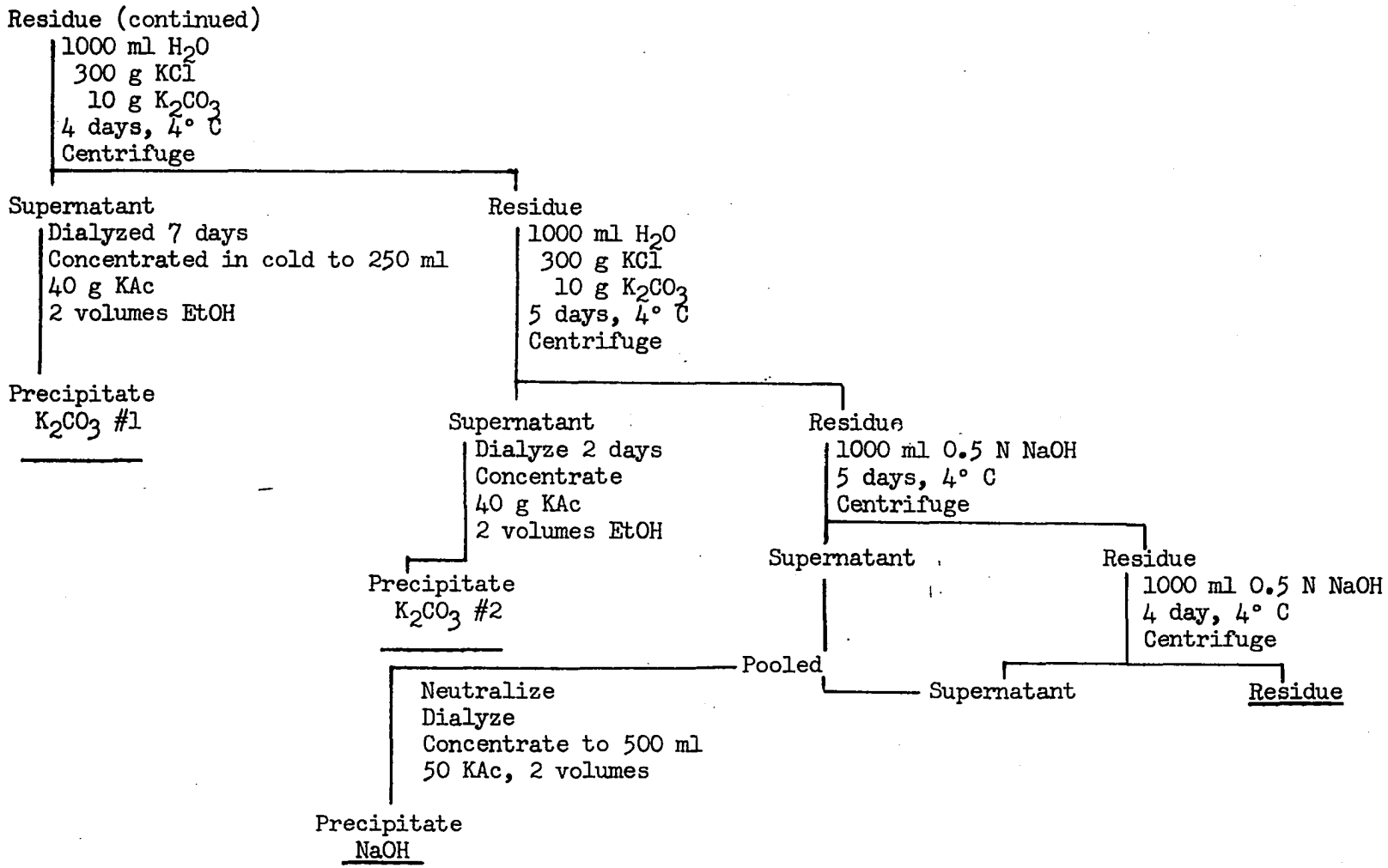


Chart 1 (continued)



and were analyzed separately. The residual cartilage was then subjected to a further extraction with potassium chloride and potassium carbonate as described by Einbender and Schubert (39). It was extracted in the cold four days in a liter of water which contained 300 grams potassium chloride and ten grams potassium carbonate. The supernatant was removed by centrifugation and decantation. It was dialyzed for seven days against cold running tap water and concentrated to approximately 250 milliliters using a rotating evaporator with a vacuum pump utilizing a double dry ice trap. The mucopolysaccharides were precipitated with a mixture of 40 grams of potassium acetate in two volumes of alcohol and the residue was re-extracted and the supernatant reprecipitated by the same method. To the unextracted material one liter of 0.5 normal sodium hydroxide was added. After three days the supernatant was removed, neutralized with hydrochloric acid and dialyzed four days. Following an additional extraction the supernatant fractions were pooled and the precipitation procedures carried out as in the preceding step. The residual material was dried under vacuum.

Sodium Hydroxide Extraction

Fresh frozen cartilage samples weighing between 2.2 to 14 grams were cut into small shavings and were homogenized in a high speed Virtis homogenizer in 0.5 normal sodium hydroxide. They were transferred quantitatively to 250 milliliter centrifuge bottles using the sodium hydroxide solution for transfer. The volume was brought to 100 milliliter with the same solution. The samples were extracted five days in the refrigerator and the supernatant removed by centrifugation and decantation. The resi-

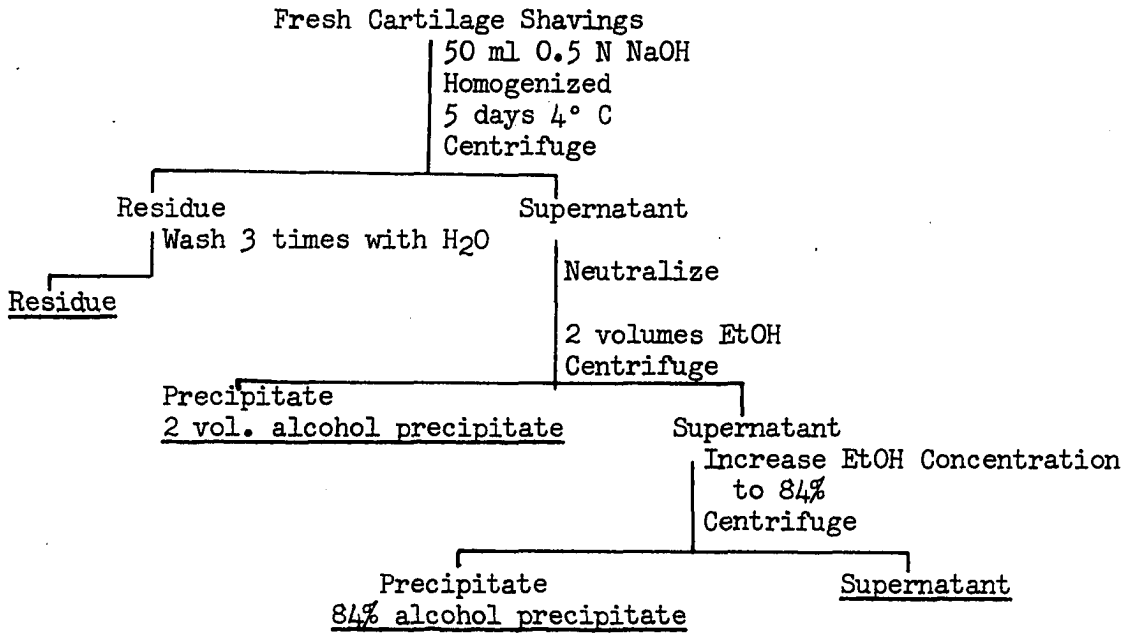
dues were washed with cold water, and were divided using lyophilization in the original 250 milliliter centrifuge bottles. The washings were added to the supernatant solutions which were then neutralized with hydrochloric acid diluted to a volume of 200 milliliters of ethyl alcohol. The precipitate was collected by centrifugation and after standing in the cold overnight, the addition of 638 milliliters of alcohol to the decanted supernatant brought the alcohol concentration to 84 per cent and brought about a second precipitation. After standing in the refrigerator overnight these solutions were again centrifuged. The precipitate was dried by lyophilization and the supernatant reduced in volume in a rotating evaporator and finally dried by lyophilization. This procedure is outlined in Chart 2.

Enzymatic Digestion

Cartilage samples were treated with the proteolytic enzymes pepsin and trypsin for removal of protein to facilitate the extraction of mucopolysaccharides. The procedure followed was a modification of that reported by Dyrbye and Kirk (40). Their method is derived from methods described by Meyer, et al. (25), (41) and by Pearce and Watson (42). Fresh or frozen cartilage samples of up to 50 grams cartilage were weighed and ground in a Waring blender containing approximately 250 milliliters of cold distilled water for two to three hours of intermittent grinding. The homogenates were transferred to a liter Erlenmeyer flask with repeated water washings and brought to a volume of approximately 500 milliliters with distilled water. The pH of the homogenates were adjusted to pH 1.5 and of 50 milligrams pepsin (pepsin powder N.F.) were

Chart 2

Cartilage Extraction by Sodium Hydroxide and Fractionation of Extracts



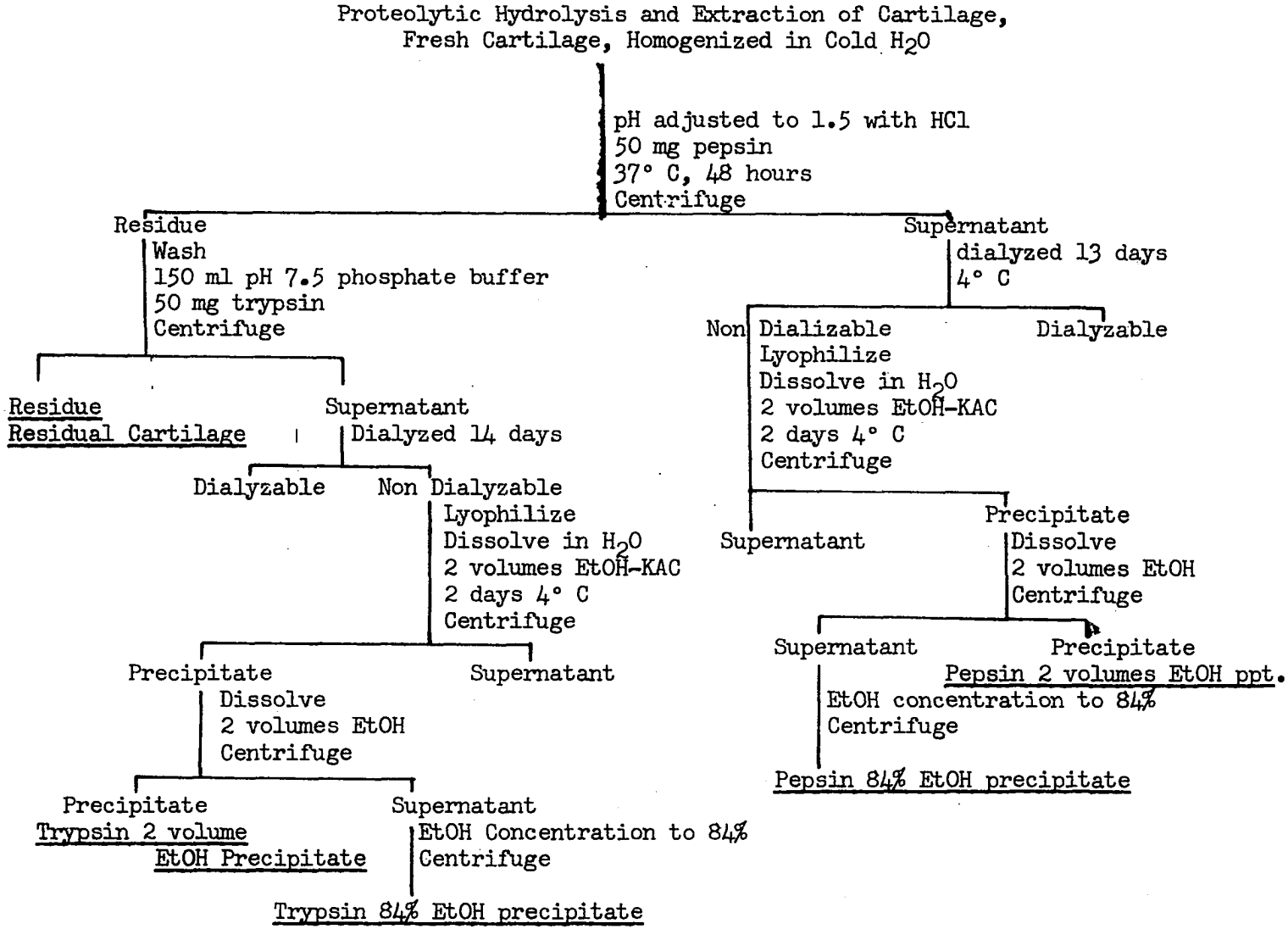
added. One milliliter of toluene was layered onto the mixtures to prevent bacterial action and the flask was placed in a 37°C. incubator oven for 48 hours. The solution was neutralized with sodium hydroxide and the supernatant solution removed by centrifugation and decantation. This clear, viscous liquid was dialyzed against distilled water and the dialysate collected and concentrated after periods of two, six and thirteen days. The non-dialyzable fraction was dried by lyophilization. To the residue from the pepsin digest was added 150 milliliters of pH 7.5 phosphate buffer and 50 milligrams of trypsin. The same procedure of enzymatic digestion was followed as previously described. The dialysates were analyzed chemically for hexosamine and were subjected to paper electrophoresis. Since no carbohydrate was found in these fractions they were discarded. The non-dialyzable fractions were dissolved in water and precipitated with a precipitation mixture composed of one gram potassium acetate in 100 milliliters of ethyl alcohol which had been acidified with one milliliter of glacial acetic acid. Two volumes of this mixture were added to the non-dialyzable fractions, which were then mixed by inversion and refrigerated for two days. The samples were centrifuged and the supernatant discarded. The precipitates were dissolved in one normal acetic acid and precipitation was repeated. The precipitates were redissolved and precipitated with two volumes ethyl alcohol and allowed to stand in the cold for at least two days. The precipitate was collected by centrifugation and the supernatant fraction brought to an alcohol concentration of 84 per cent and reprecipitated in the cold for at least two days. The precipitates were dried *in vacuo*, and the supernatant alcohol fractions reduced in volume using a Rinco

evaporator and dried by lyophilization. The residual cartilage was removed from the dialysis tubing and lyophilized. This procedure is outlined in Chart 3.

Purification of the fraction which precipitated in 84 per cent alcohol was carried out by enzymatic digestion and precipitation with the aliphatic ammonium salt cetyltrimethylammonium bromide. One hundred milligrams of papain and two hundred milligrams of the material to be purified were added to five milliliters of pH 6.5, 0.1 M phosphate buffer which contained 0.005 M disodium ethylenediaminetetraacetate and 0.005 M cysteine hydrochloride. The digestion was allowed to proceed overnight at 37°C. and the digested material was dialyzed against several changes of distilled water for seventy-two hours. The resultant nondialyzable fraction was lyophilized and adjusted to pH 9.5 by the addition of 10 milliliters sodium borate 0.05 M. Five hundred milligrams of cetyltrimethylammoniumbromide were added and the mixture was shaken mechanically for one hour and allowed to stand at 4°C. overnight. The precipitate was collected by centrifugation and treated three times with five milliliters of sodium iodide, five per cent in absolute ethyl alcohol with mixing, centrifuging and decanting between each treatment. The precipitate was dissolved in 500 microliters of distilled water and reprecipitated with ten milliliters of absolute alcohol. This procedure is outlined in Chart 4.

All of the fractions obtained from these procedures were analyzed chemically for uronic acid, hexose, hexosamine, and nitrogen as previously described. Each fraction was also hydrolyzed using Permutit Q and subjected to paper chromatography and the individual carbohydrate monomers

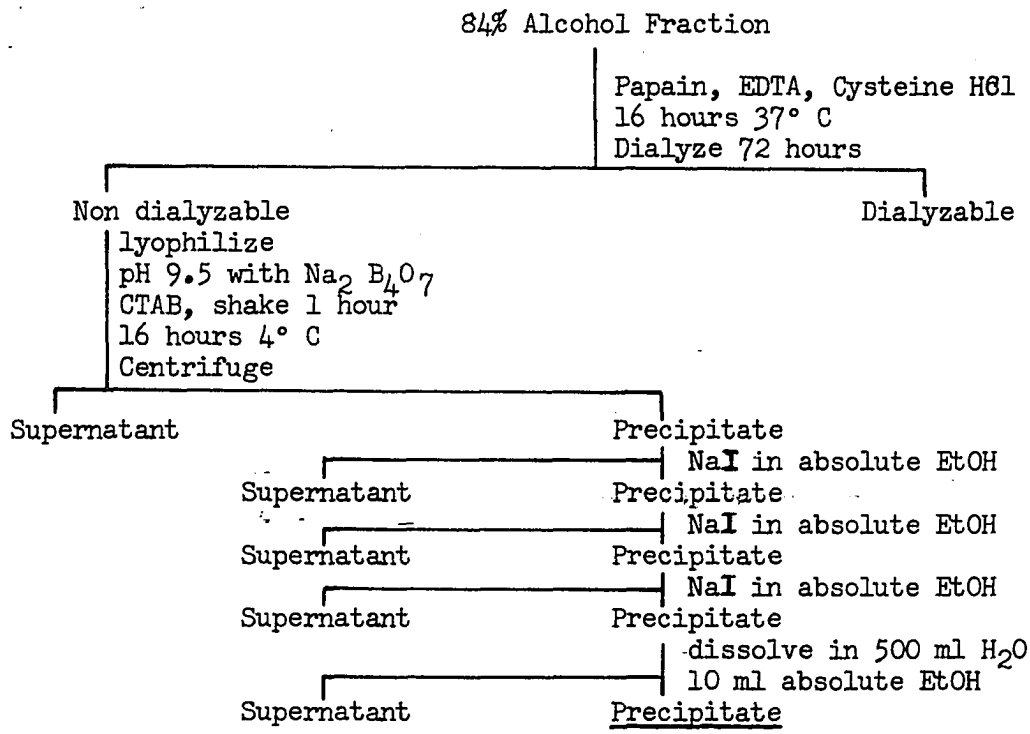
Chart 3


Proteolytic Hydrolysis and Extraction of Cartilage,
Fresh Cartilage, Homogenized in Cold H_2O
pH adjusted to 1.5 with HCl
50 mg pepsin
37° C, 48 hours
Centrifuge
Residue
Wash
150 ml pH 7.5 phosphate buffer
50 mg trypsin
Centrifuge
Residue
Residual Cartilage
Supernatant
Dialyzed 14 days
Dialyzable
Non Dialyzable
Lyophilize
Dissolve in H_2O
2 volumes EtOH-KAC
2 days 4° C
Centrifuge
Precipitate
Dissolve
2 volumes EtOH
Centrifuge
Supernatant
Precipitate
Trypsin 2 volume
EtOH Precipitate
Supernatant
EtOH Concentration to 84%
Centrifuge
Trypsin 84% EtOH precipitate
Supernatant
dialyzed 13 days
4° C
Non Dializable
Dialyzable
Lyophilize
Dissolve in H_2O
2 volumes EtOH-KAC
2 days 4° C
Centrifuge
Supernatant
Precipitate
Dissolve
2 volumes EtOH
Centrifuge
Supernatant
Precipitate
Pepsin 2 volumes EtOH ppt.
EtOH concentration to 84%
Centrifuge
Pepsin 84% EtOH precipitate

quantitatively estimated as previously described. In addition, all of the fractions which could be made soluble in buffer solutions were subjected to paper electrophoresis using the Spinco electrophoretic system with a series of different buffers. Barbitol buffer, pH 8.5, $\Gamma/2$ 0.075 and borate buffer, pH 8.9, $\Gamma/2$ 0.1 were used for all studies. In addition, phosphate buffer, pH 7.6, $\Gamma/2$ 0.1, citrate buffer, pH 5.9, $\Gamma/2$ 0.03 and acetate buffer, pH 4.8, 0.1 were also used. The mucopolysaccharides were visualized by staining the strip with toluidine blue or alcian blue.

Chart 4

Isolation of Keratosulfate from 84% Alcohol Fractions by Proteolytic Hydrolysis and Cetyltrimethylammonium Bromide Precipitation



CHAPTER III

RESULTS

The results of these experiments are summarized in a series of figures, graphs and tables. Table 1 is a compilation of the uronic acid, hexose and hexosamine analysis of whole dried costal cartilage from a series of patients of different ages. The uronic acid levels of this group are plotted graphically in Chart 5. From these data it can be seen that the carbohydrate composition of human costal cartilage changes with the aging process. The uronic acid values decrease progressively during the aging process while the hexosamine or the hexose content does not reflect this change. If one assumes that the uronic acid is a reflection of the chondroitin sulfate then excess hexosamine over that which can be accounted for by chondroitin sulfate must be present. These conclusions made by Shetlar and Masters (43) who suggested that the hexosamine in excess of that bound to uronic acid was a constituent of a neutral polysaccharide which increased in concentration until maturity was reached.

Cartilage samples selected from this series were hydrolyzed with Permutit Q cation exchange resin and separation of the carbohydrate monomers was achieved by paper chromatography. Figures 1 and 2 are representative paper chromatograms obtained from the aqueous and acid eluates of these cartilage hydrolysates respectively.

TABLE 1

The Carbohydrate Content of Human Costal Cartilage
Samples Taken from Persons of Different Age Levels

AGE (Years)	Uronic Acid	Hexos-amine	Hexose	AGE (Years)	Uronic Acid	Hexos-amine	Hexose
Prem.	76.6	78.5	97.2	32	29.2	69.2	101.8
	80.0	85.2	132.8		35.0	77.0	100.1
	78.8	79.5	85.6	35	31.4	72.0	113.0
	77.8	82.3	86.6	36	29.2	74.3	113.3
	73.5	79.6	90.6	45	27.7	75.6	91.0
	64.9	77.0	77.1		22.2	67.5	110.4
Term	73.0	89.0	94.8	47	18.5	55.8	73.2
	76.6	85.0	112.0	50	31.7	76.2	94.3
	73.2	72.0	72.5	53	19.9	69.0	95.8
	62.1	69.6	63.8	55	16.2	54.1	84.1
	75.9	84.5	74.8	56	16.8	65.4	100.0
1/3	70.2	71.0	114.5	58	17.8	62.8	89.6
1	53.4	63.9	77.6	59	17.0	66.2	99.6
1-5/6	52.4	60.5	81.0	60	19.4	58.4	91.6
3-1/2	48.2	62.3	74.0		18.8	61.4	92.8
6	36.9	60.0	93.4	61	16.4	60.0	93.2
11	36.1	61.8	95.3	62	14.8	49.4	86.1
14	31.4	45.9	63.0	63	17.7	62.6	96.5
24	40.7	78.7	90.1	67	16.8	61.8	91.8
	39.2	71.4	99.4		15.0	52.4	70.7
31	29.2	68.4	80.3	70	13.4	53.6	74.7
				76	10.8	46.6	80.0
				79	15.8	51.4	96.7
				80	12.8	57.4	78.0
				85	7.6	36.9	66.1
				88	12.3	52.0	77.8

* All values are expressed as milligrams per gram of dried cartilage.

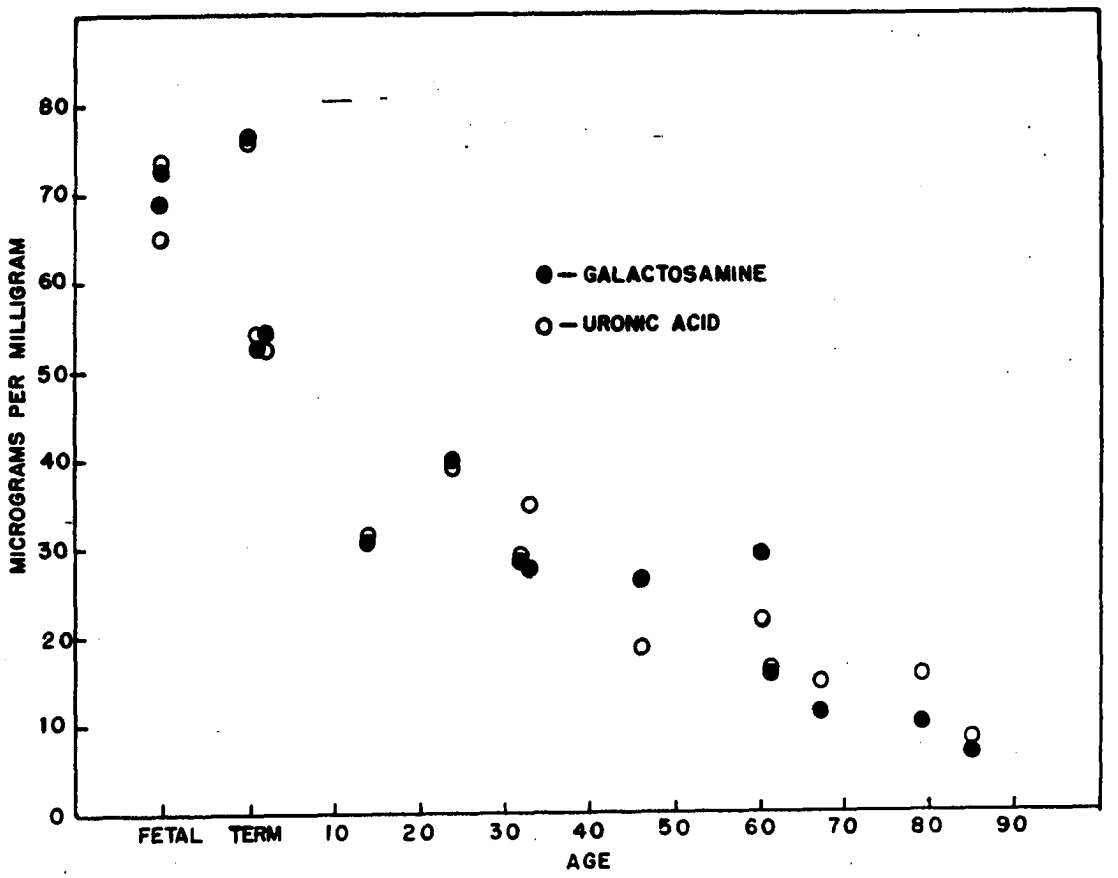


Chart 5

Changes in Galactosamine and Uronic Acid Levels in Human Costal Cartilage as Affected by Aging

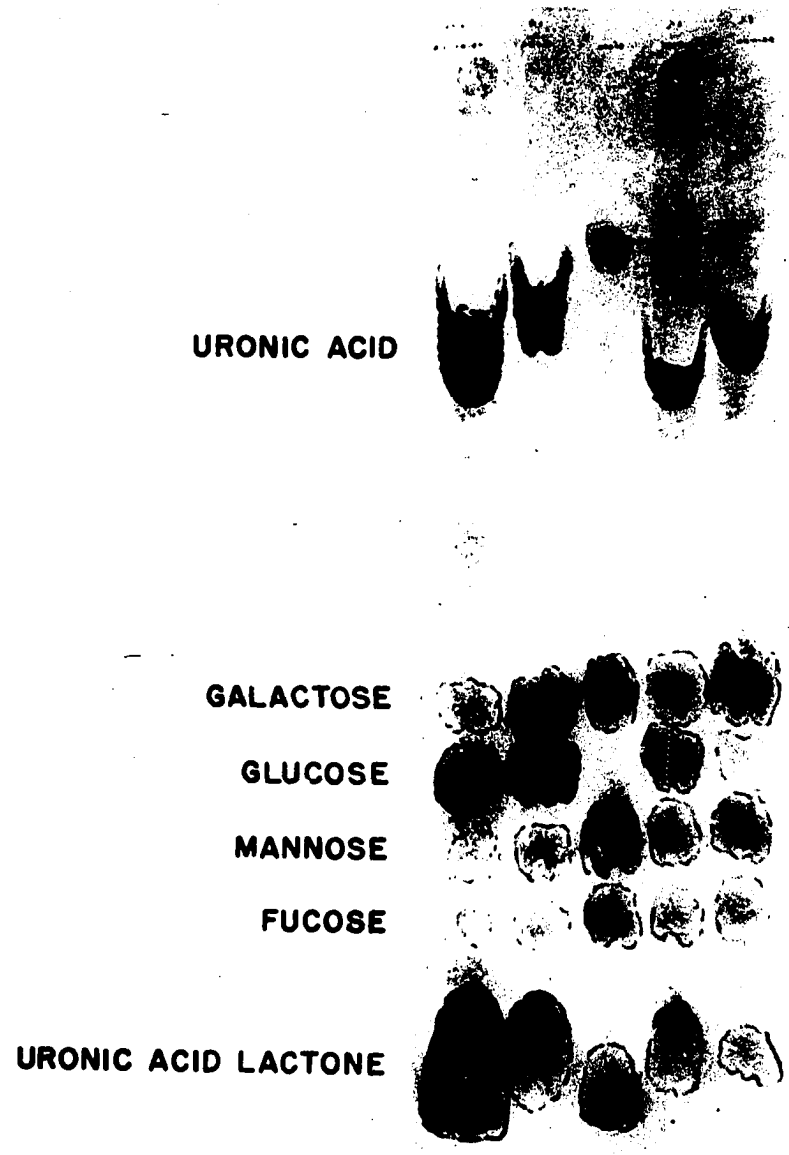


Figure 1

Paper Chromatogram of Uronic Acid, Hexoses and the Methylpentose of Hydrolyzed Human Costal Cartilage

Left to right - term, 14 years, standard mix, 32 years, and 88 years

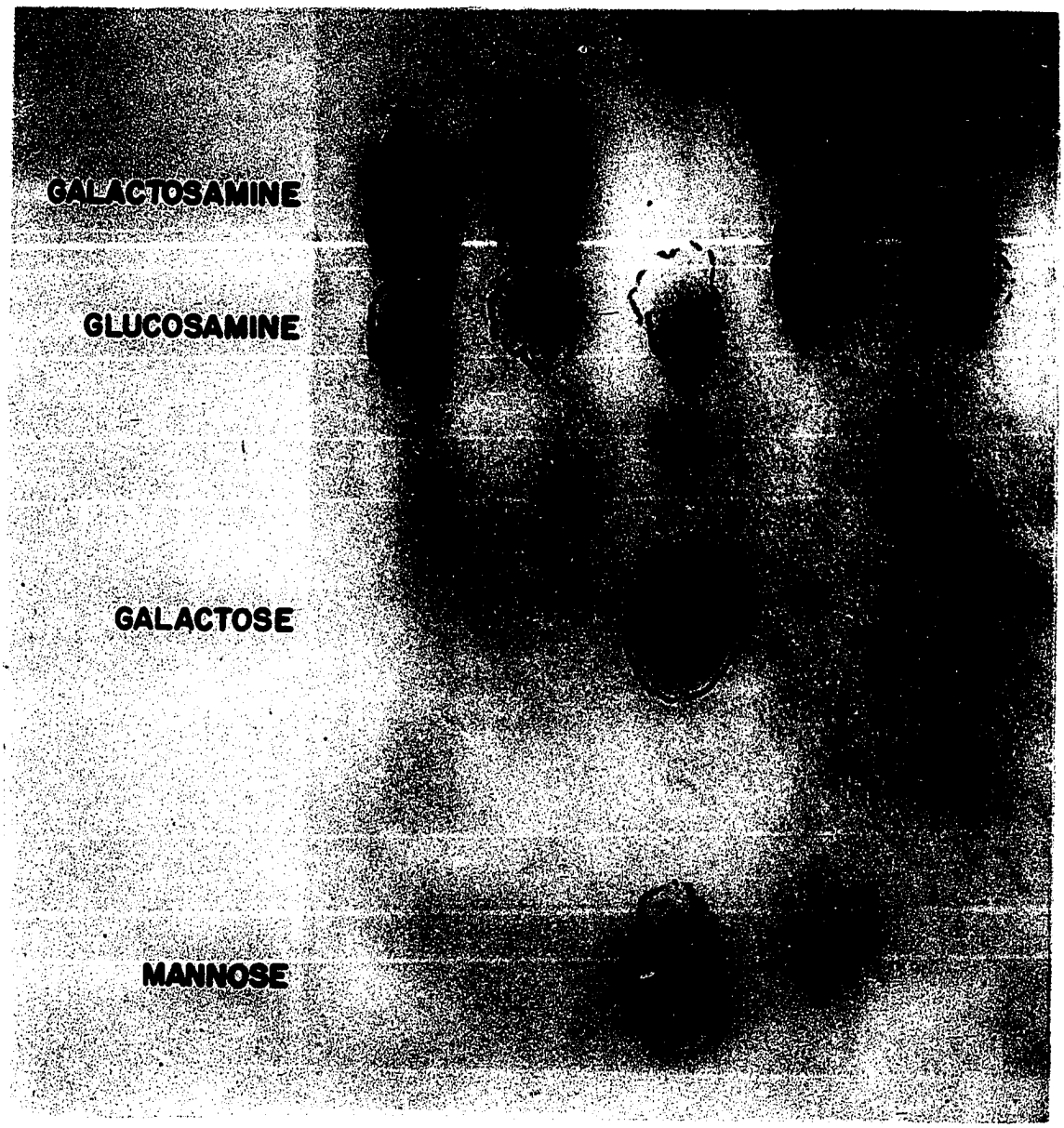


Figure 2

Paper Chromatogram of the Hexosamines of Hydrolyzed Human Costal Cartilage

Left to right - term, 14 years, standard mix, 32 years, and 88 years

Figure 1 shows by visual comparison the decrease in uronic acid which occurs during aging. Figure 2 indicates that while galactosamine decreases at a rate corresponding to uronic acid, the glucosamine shows an increased concentration during aging. Chemical analysis from paper chromatograms, carried out as previously described, is indicated by Table 2 and Charts 5 and 6. The values obtained by analysis for uronic acid by paper chromatography and 2-aminobiphenyl analysis compare favorably with those obtained by the carbazole method. The values for uronic acid also compare closely with those obtained for galactosamine. Chart 5 indicates that these substances apparently occur in equimolar ratios. Chart 6 indicates the changes of galactosamine and glucosamine with age and also demonstrates that the ratio of glucosamine to galactosamine is a reflection of the physiological age of the individual from which the sample was taken. It is suggested that the glucosamine at least in part is a component of keratosulfate.

The aqueous cartilage extraction procedure resulted in the separation of six different fractions from the tissue sample since each of the successive aqueous fractions were treated separately. The results of this extraction and the subsequent re-extraction of the residue, first with potassium chloride-potassium carbonate, then with sodium hydroxide is shown in Table 3. It is apparent from this table that over half of the original material is extracted by water in the first four days and that very little additional material is leached out in subsequent extractions. Recovery on a weight basis is very close to theoretical.

The composition of the individual fractions from this set of extractions is indicated in Table 4. From these data it is noted that from

TABLE 2

Summary of Uronic Acid and Hexosamine Analysis of Cartilage from Individuals of Different Ages

Age	Uronic Acid		Hexosamine		
	Carbazole	Paper[1]	Total	Galactosamine[2]	Glucosamine[2]
Prem.	73.2	72.4	77.0	72.6	4.4
Prem.	64.9	63.3	72.0	68.9	3.1
Term	79.5	80.3	84.5	76.7	7.8
1 Yr.	53.4	51.2	63.9	52.6	11.3
2	52.4	62.1	60.5	54.2	6.3
14	31.7	34.5	47.7	30.9	16.8
24	39.1	41.6	71.4	39.9	31.5
31	29.2	32.5	66.6	28.5	38.1
32	35.0	35.5	77.0	27.6	49.4
47	18.5	22.8	57.4	26.8	30.6
60	21.8	20.8	58.4	29.4	29.0
61	16.5	17.1	59.5	15.9	43.6
67	15.0	17.5	52.4	11.4	41.0
79	15.8	16.5	59.6	10.1	49.5
85	8.4	12.5	36.9	6.5	30.4

* All values are expressed as milligrams per gram of dry cartilage.

1 Determined by reaction with 2 aminobiphenyl reagent after elution from paper chromatogram.

2 Determined after elution from paper chromatogram.

TABLE 3

Description of Fractions Obtained from Serial Extraction of Human Costal Cartilage from a 35 Year Old Man

	Description	Weight	% of Original/
Original Cartilage	Fresh, slightly tan	157.5 wet (58.27 dry)	--
1st H_2O extraction	Granular, "off-white"	20.3	34.8
2 vol. alcohol ppt	Fine white powder	11.8	20.2
potas. aceta. ppt			
2nd H_2O extraction			
2 vol. alcohol ppt	Powdery, light tan	0.36	0.6
potas. aceta. ppt	More granular, light tan	0.90	1.5
3rd H_2O extraction			
2 vol. alcohol ppt	Less dense, flaky, tan	0.04	0.1
potas. aceta. ppt	Granular, tan	0.19	0.3
1st $KCl-K_2CO_3$ extract	Heavy white powder	0.78	1.3
2nd $KCl-K_2CO_3$ extract	Heavy white powder	0.89	1.5
NaOH extract	White, fine powder	4.27	7.3
Residual cartilage	Tan, granular	20.10	34.4
	ACCOUNTED FOR:	59.73	102.0

/ Calculated on dry weight basis.

TABLE 4

Composition* of Mucopolysaccharide Fractions Obtained From Costal Cartilage Obtained From a 35 Year Old Man by Mild Aqueous and Alkaline Extraction Procedures

	Uronic Acid	Hexosamine			Hexose	Nitrogen
		Total	Galactosamine/	Glucosamine/		
Original Cartilage	34.8	73	33.4	39.6	98.4	113.6
1st H_2O extraction						
2 vol. alcohol	16.6	55	19.1	35.9	66.5	101.2
potas. aceta.	64.3	113	65.8	47.2	116.5	52.1
2nd H_2O extraction						
2 vol. alcohol	18.2	36	18.0	18.0	64.7	98.0
potas. aceta.	52.0	89	57.1	31.9	107.4	51.7
3rd H_2O extraction						
2 vol. alcohol	16.7	34	-//	-//	63.5	-//
potas. aceta.	37.6	72	42.0	30.0	87.4	51.1
1st KCl-K_2CO_3 extract	53.3	67	58.8	6.2	60.5	82.3
2nd KCl-K_2CO_3 extract	41.3	58	49.9	7.1	46.6	59.6
NaOH extract	94.4	134	101.2	32.8	104.2	62.6
Residual Cartilage	2.7	18	-**	18.0	52.6	117.0

* All values expressed as milligrams per gram dry weight.
/ Determined by paper chromatography.
// Not sufficient material.
** Not detectable chromatographically.

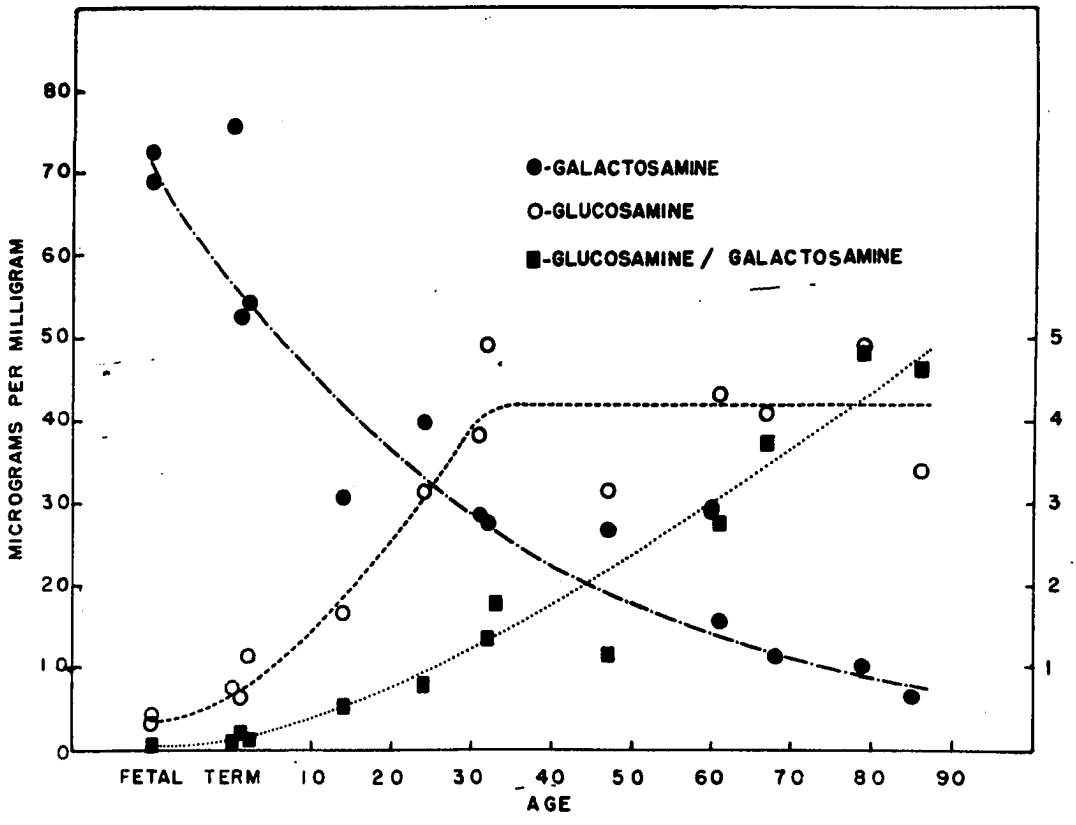


Chart 6

Changes of Galactosamine and Glucosamine Levels in Human Costal Cartilage As Affected by Aging

the aqueous extraction a mixture of mucopolysaccharide are obtained. This mixture contains chondroitin sulfate and the glucosamine containing mucopolysaccharide both protein bound and free. Paper chromatography on the hydrolysates of these fractions indicates that each of these fractions contain uronic acid, galactosamine, glucosamine, and galactose which are constituents of these two mucopolysaccharides. Paper chromatography shows, in addition, the presence of fucose, mannose and glucose. The presence of these latter monosaccharides indicates that other polysaccharide material may be present in these fractions. The mannose, fucose and parts of the galactose may also be contributed by tissue glycoproteins similar to those found in serum which may be present in cartilage. That some of the chondroitin sulfate is firmly bound and requires strong alkaline extraction is indicated by the sharp rise in uronic acid and correspondingly in chondroitin sulfate in the sodium hydroxide extraction. It is also of interest that the residual material which is very low in uronic acid and showed no galactosamine and only a slight amount of fucose chromatographically still contained an appreciable amount of glucosamine, galactose, glucose and mannose.

Electrophoresis carried out on the soluble fractions indicated that no fraction was electrophoretically pure, that each fraction contained material which migrated at the same rate as chondroitin sulfate, material which migrated a rate somewhat less than that material and that those fractions which were precipitated with two volumes of ethanol contained some residual material always remained at the line of origin.

Since this procedure was extremely long and tedious, the less complicated method of weak sodium hydroxide extraction and alcohol pre-

cipitation was followed on a series of four cartilage samples obtained from persons 2, 14, 31 and 67 years old. The data obtained by this procedure is presented in Table 5. The data on original cartilage constituents are included here with the additional hexose and nitrogen figures added. The sodium hydroxide extracted fraction which precipitates with two volumes (63 per cent alcohol) contains the major portion of the uronic acid of the original sample in all cases and, as indicated by the galactosamine, all of the chondroitin sulfate (See Figure 3). However, this fraction does not represent pure chondroitin sulfate since appreciable hexose is present as well as some glucosamine. The protein value of from three to four per cent indicates that the protein is very low in this fraction.

The material which precipitates in 84 per cent ethanol is apparently very high in keratosulfate as shown in Figure 3. This fraction contains only a slight contamination of chondroitin sulfate since uronic acid, determined chemically is very low and both uronic acid and galactosamine were either very faint or absent as measured by paper chromatography. This fraction also has an appreciable amount of hexose. Galactose was always present in greatest concentration as indicated by paper chromatography, however, mannose and glucose were also present. The methyl pentose, fucose, was also present in each chromatogram. Nitrogen values of from 6 to 8 per cent indicate that protein or peptide are also found in this fraction. Analysis of the supernatant fraction indicates that some carbohydrate material is present which does not precipitate in 84 per cent alcohol. This fraction was not dialyzed and may contain some mono, di, or oligosaccharides, however, paper chromatography of unhydrolyzed supernatant

Table 5

Comparison of Mucopolysaccharide Composition of Human Costal Cartilage from Persons of Different Ages After Extraction with Dilute Sodium Hydroxide and Selective Precipitation with Ethyl Alcohol

	AGE			
	22 mo.	14 years	31 years	67 years
dry/wet %	31.75	31.25	45.45	56.01
wet weight ext. (gram)	2.17	7.84	11.20	13.99
Chemical analysis*				
Original Cartilage				
Uronic Acid	52.4	31.7	29.2	15.0
Hexosamine	60.5	47.7	66.6	52.4
galactosamine	54.2	30.9	28.5	11.4
glucosamine	6.3	16.8	38.1	41.0
Hexose	80.3	70.5	82.5	68.8
Nitrogen	98.9	118.2	112.7	110.5
2 vols alcohol ppt.				
Uronic Acid	98.6	140.4	106.5	0.7
Hexosamine	172.0	152.9	126.2	21.4
galactosamine	103.1	138.3	126.2	6.2
glucosamine	68.9	13.6	0	15.2
Hexose	150.0	106.7	124.5	23.5
Nitrogen	36.4	33.0	44.9	28.0
84% alcohol ppt				
Uronic Acid	0.8	1.3	4.2	0.0
Hexosamine	114.0	135.0	118.9	25.0
galactosamine	0.0	detectable	0.0	0.0
glucosamine	114.0	130.0	118.9	25.0
Hexose	135.3	184.4	183.5	39.4
Nitrogen	81.2	65.0	63.3	63.0
Supernatant				
Uronic Acid	0.1	0.1	0.7	0.0
Hexosamine	7.9	5.1	8.4	1.9
galactosamine	7.9	5.1	8.4	---
glucosamine	---	---	---	---
Hexose	8.0	8.1	18.5	12.0
Nitrogen	7.8	7.0	11.2	10.3
Residual Cartilage				
Uronic Acid	0.6	2.1	1.6	2.9
Hexosamine	7.6	10.5	28.7	30.6
galactosamine	2.4	0.0	6.4	0.0
glucosamine	5.2	10.5	22.3	30.6
Hexose	58.3	50.6	53.3	48.5
Nitrogen	129.9	121.5	117.3	113.1

* Expressed as milligrams per gram of dry weight

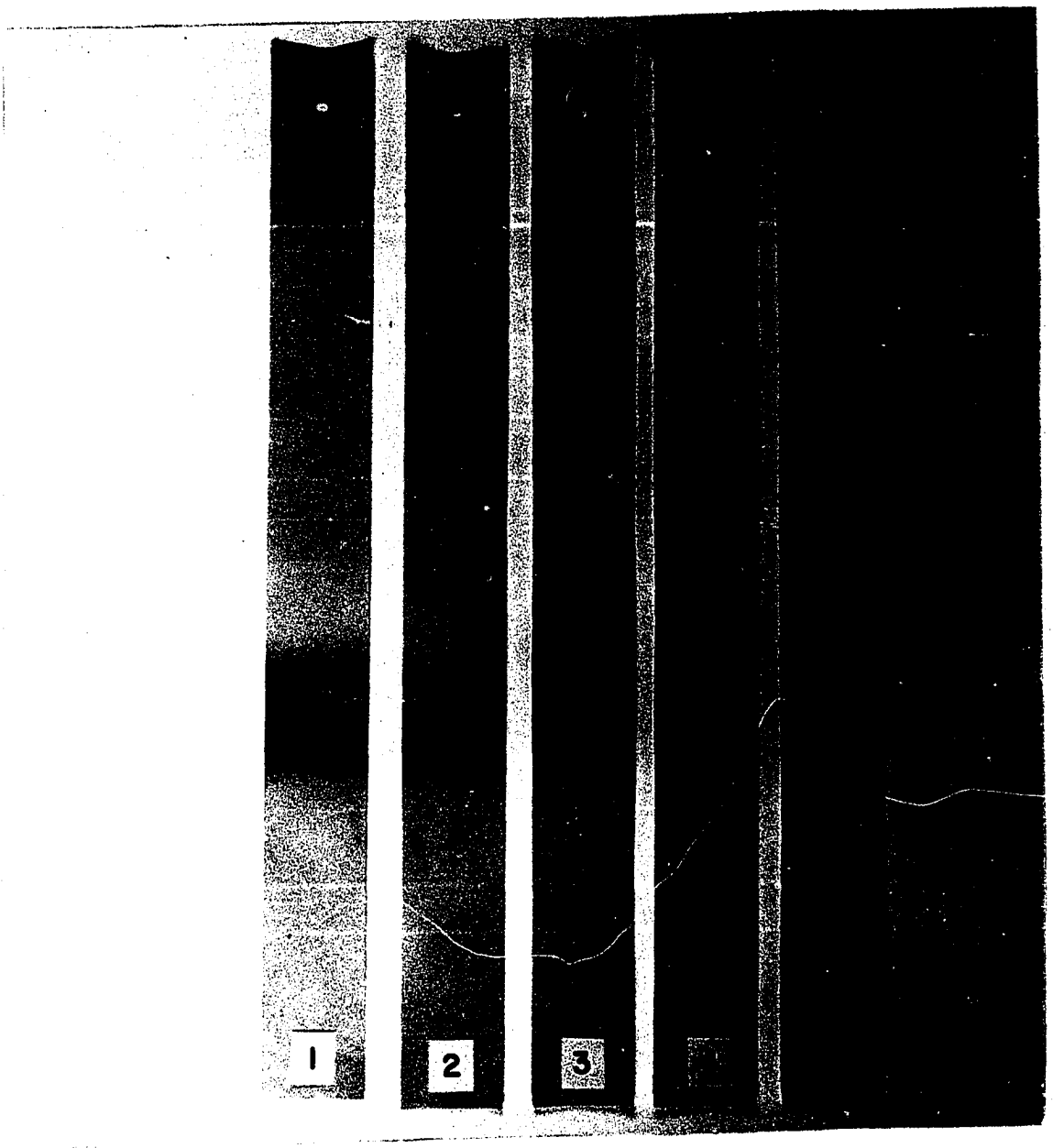


Figure 3

Paper Electrophoretic Strips of 84 Per Cent Ethanolic Fractions Before Purification, Barbitol Buffer pH 8.6, Ionic Strength .075. No.1 - 22 mo., No.2 - 14 years, No.3 - Keratosulfate Standard, No.4 - 31 years, and No.5 - 67 years.

fraction did not indicate any of these were present. Appreciable nitrogen is also present in this fraction. The residual cartilage from this extraction still contains considerable carbohydrate material only a small portion of which can be contributed by chondroitin sulfate. From these data it is evident that an increasing portion of glucosamine per milligram of remaining cartilage is found in progressively older cartilage. This apparently is bound very tightly to the residual protein. The rather large amount of hexose present in this fraction is composed of glucose, mannose, and galactose. Fucose was also present in these fractions.

Purification of the keratosulfate-rich fraction by papain digestion, dialysis and cetyltrimethylammonium bromide precipitation was carried out on each of the four 84 per cent fractions obtained from persons 2, 14, 31, and 67 years old by sodium hydroxide extraction and alcohol precipitation. No essential difference was found in these fractions after purification as determined by chemical analysis (Table 6). Hexosamine values varied from 205.8 to 239.2 micrograms per milligram while hexose values ranged from 224.0 to 263.0 micrograms per milligram. No uronic acid was found either chemically or by paper chromatography. All of the hexosamine was glucosamine as shown by paper chromatography. Fucose was noted to be present in trace amount. Electrophoretically the isolated fractions migrated at a rate comparable to a sample of pure keratosulfate obtained from Dr. Karl Meyer (Figure 4). By the procedure mannose, glucose and protein were removed and lost. Calculation of the amount of keratosulfate isolated by this procedure indicates that this constituent increases with age (Table 7).

Enzymatic digestion of fresh costal cartilage was carried out on

TABLE 6

Composition* of Eighty-four Per Cent Alcohol Fractions Purified by Proteolytic Hydrolysis and Cetyltrimethylammonium Bromide Precipitations

	AGE			
	22 mo.	14 years	31 years	67 years
Hexosamine	205.8	221.5	239.2	209.1
Hexose	230.6	242.7	263.0	224.0
Uronic Acid	-	-	-	-
Nitrogen	34.	30.	32.	35.

* Expressed as milligram per gram dry weight.

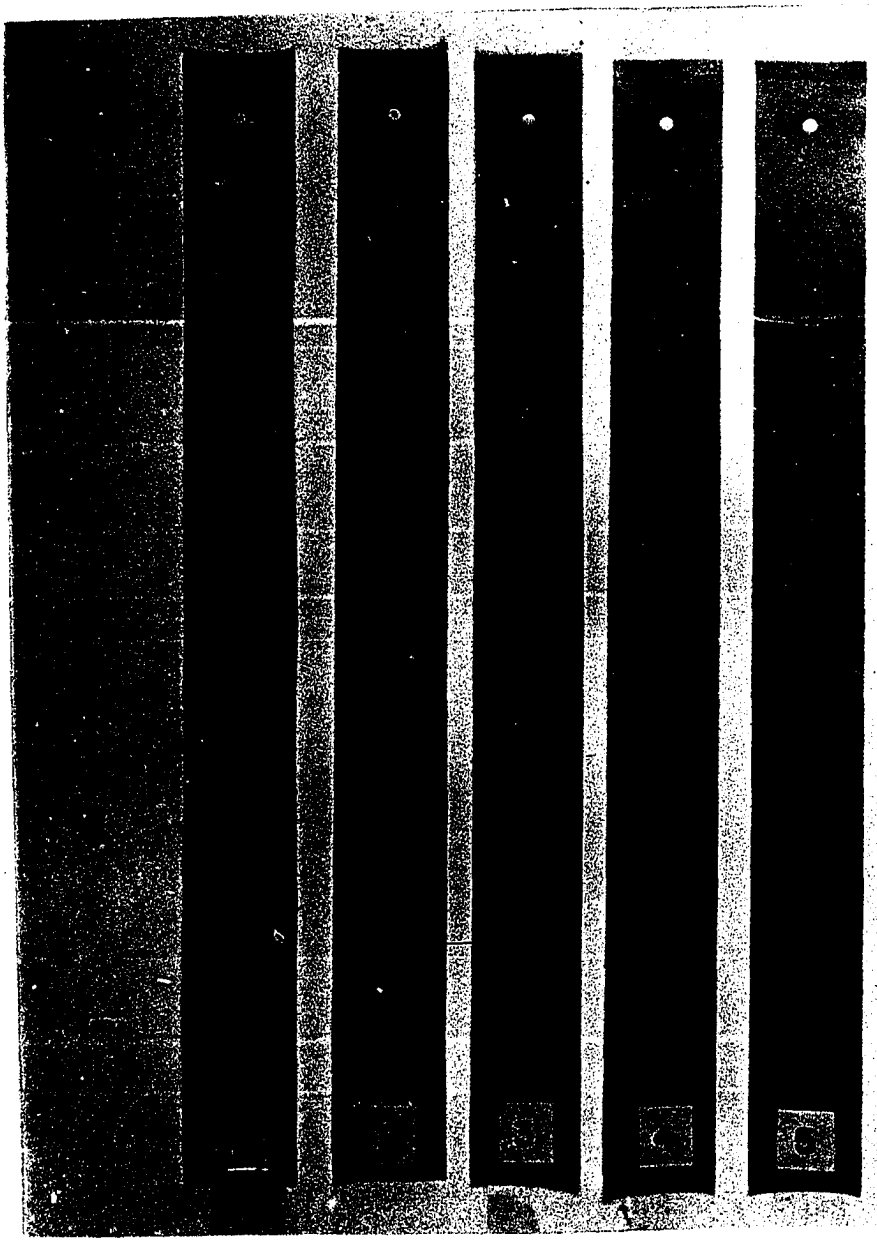


Figure 4

Paper Electrophoretic Strips of Purified "Keratosulfate Fraction" from the 84 Per Cent Ethanolic Fraction. No.1 - 22 months, No.2 - 14 years, No.3 - Keratosulfate Standard, No.4 - 31 years, and No.5 - 67 years

TABLE 7

Keratosulfate Concentration[1] in Human Costal Cartilage Obtained from Persons of Different Ages

	AGE (years)			
	1-5/8	14	31	67
Theoretical[2]	22.5	40.3	109.0	125.2
Amount Recovered[3]	17.5	35.3	89.6	105.4

1 Expressed as milligrams per gram dry weight.

2 Calculated for hexosamine in excess of uronic acid.

3 Calculated from yield of eighty four per cent alcohol fraction corrected to chemical analysis of purified keratosulfate.

specimens obtained from persons who died at term, 21 years, 34 years, and 85 years, respectively. A summary of the data obtained from these analyses is given in Table 8. The water content of cartilage can again be seen to decrease with aging and from the whole cartilage analysis the decrease in uronic acid is also evident. The greatest portion of carbohydrate material is taken into solution by the pepsin digestion and the material solubilized is non-dialyzable through ordinary dialyzing tubing. The fraction which precipitates in two volumes of alcohol contains nearly all of the uronic acid from the original material. This fraction does not contain protein to any appreciable extent. It can be seen, however, that this fraction also contains appreciable hexose. The pepsin digest fraction which is insoluble in 84 per cent alcohol is composed of keratosulfate and hexose in excess of that expected from an equimolar combination with hexosamine. Carbohydrate is also present in the supernatant fraction.

Additional proteolytic hydrolysis apparently releases more mucopolysaccharide material into solution. The residual fractions obtained are apparently composed of the same mucopolysaccharides but the quantity is greatly reduced even when this is expressed on the basis of micrograms per milligram of material extracted. There is apparently a greater release of glycoprotein and tightly bound mucopolysaccharides from cartilage by this method when compared with the earlier extractions. The residual material remaining after enzymatic hydrolysis was much smaller than that found after sodium hydroxide extraction.

Table 8

Composition of Fractions Obtained by Enzymatic Hydrolysis and Alcohol Precipitation of the Hydrolysate

	AGE			
	Term.	21 years	34 years	85 years
Ratio dry/wet weight	30.26	39.78	44.62	58.23
Tissue extracted	7.30	41.72	50.00	50.00
Chemical Analysis*				
Original Cartilage				
Uronic Acid	73.2	43.6	29.7	8.4
Hexosamine	80.3	67.2	61.5	36.9
galactosamine	74.6	40.7	32.0	6.5
glucosamine	5.7	16.5	29.5	30.4
Hexose	103.6	116.7	110.4	108.4
Nitrogen	110.7	122.1	110.3	119.6
Pepsin Treated, Non Dialyzable				
2 vols Alcohol precipitate				
Uronic Acid	113.6	136.7	72.9	12.0
Hexosamine	118.7	165.3	84.3	20.7
galactosamine	118.7	143.1	79.8	14.3
glucosamine	0	22.2	3.5	6.4
Hexose	142.1	144.6	142.1	84.0
Nitrogen	30.7	37.8	31.6	25.4
84% Alcohol Precipitate				
Uronic Acid	5.3	2.4	3.7	0.0
Hexosamine	89.4	115.6	110.2	98.4
galactosamine	---	---	---	---
glucosamine	89.4	115.6	110.2	98.4
Hexose	127.2	185.9	174.3	116.9
Nitrogen	9.1	7.6	6.7	6.3
Supernatant				
Uronic Acid	0.2	1.8	2.3	0.0
Hexosamine	4.3	5.1	7.6	2.1
galactosamine	4.3	5.1	7.6	2.1
glucosamine	---	---	---	---
Hexose	5.4	7.9	8.9	13.2
Nitrogen	8.2	6.9	8.4	7.6
Trypsin Treated, Non Dialyzable				
2 vols Alcohol precipitate				
Uronic Acid	21.3	23.5	18.9	1.4
Hexosamine	18.7	17.4	20.1	3.8
galactosamine	14.9/	17.4	20.1	3.8
glucosamine	3.8/	0	0	0
Hexose	21.2	20.6	18.4	24.8
Nitrogen	3.1	2.8	3.0	3.7

(continued)

Table 8 (continued)

	AGE			
	Term.	21 years	34 years	85 years
84% Alcohol precipitate				
Uronic Acid	0.0	0.0	0.0	0.0
Hexosamine	12.3	19.2	17.6	24.1
galactosamine	0.0/	0.0	0.0	0.0
glucosamine	12.3/	19.2	17.6	24.1
Hexose	44.3	39.6	40.2	17.1
Nitrogen	5.7	5.4	6.7	8.3
Supernatant				
Uronic Acid	0.0	0.0	0.0	0.0
Hexosamine	3.4	7.1	2.0	3.6
galactosamine	3.4	7.1	2.0	3.6
glucosamine	---	---	---	---
Hexose	4.2	4.1	7.3	5.6
Nitrogen	10.2	11.5	10.9	6.1
Residual Cartilage	//			
Uronic Acid	---	0.8	1.2	2.6
Hexosamine	---	7.8	6.3	12.7
galactosamine	---	---	---	---
glucosamine	---	7.8	6.3	12.7
Hexose	---	8.5	10.7	6.1
Nitrogen	---	7.6	9.1	5.4

* Expressed as milligrams per gram of dry weight

/ Estimated visually

// Not sufficient material to transfer and analyze

CHAPTER IV

DISCUSSION

This study of the mucopolysaccharides of human costal cartilage confirms that this tissue contains in its ground substance appreciable quantities of carbohydrate containing material and that the composition of this tissue changes during aging both with respect to water content and to mucopolysaccharide concentrations. Young human cartilage contains a large amount of chondroitin sulfate which declines slowly with age. Karl Meyer in his studies of the chondroitin sulfates of different tissues states that the chondroitin sulfate found in rib cartilage is a mixture of the A and C variety and that C is predominant over A (30). In the present study no attempt was made to characterize the chondroitin sulfate fractions obtained. Electrophoretically they each have the same mobility.

The presence of keratosulfate in rib cartilage was suggested in the early phases of this study (23) and subsequent extractions clearly showed that this mucopolysaccharide is found in increasing amounts during the aging process up to maturity after which no appreciable change occurs.

These studies also indicate the presence of carbohydrate containing material other than that which can be accounted for in these two acid mucopolysaccharides. The presence of fucose, mannose and of galactose, which can be accounted for in keratosulfate, indicate that other mucopolysaccharides probably similar to keratosulfate but with fucose or mannose

replacing galactose may also be present in this tissue. It is important to note that in no case was keratosulfate isolated free from these other mucopolysaccharides and that electrophoretically the band which migrated more slowly than chondroitin sulfate was usually diffuse. This would indicate that while these compounds have similar solubility properties that they have slightly different electrophoretic mobilities. This is an analogous situation to that found in the gamma globulin fraction of whole serum. If we assume that the glucosamine, the hexoses and the methyl pentose which we observe chromatographically are contributed by serum mucoids or glycoprotein material only, then these materials must be considered as separate polysaccharide moieties bound loosely to protein since they are found in fractions which are low in total protein.

When the keratosulfate-rich fraction was purified by enzymatic hydrolysis and cetyltrimethylammonium bromide precipitation, both mannose and protein were lost (Figure 6). This does not preclude the possibility that mannose might be a constituent of a keratosulfate-like compound or that it might have been present as a glycoprotein contaminant.

The comparison which can be made of data obtained from aqueous extract of cartilage as compared to that obtained from sodium hydroxide extraction and enzymatic hydrolysis indicates that the protein complex with acid mucopolysaccharides is broken by the 0.5 normal sodium hydroxide extraction and also by proteolytic hydrolysis. However, this is apparently not true of tissue mucoids and glycoproteins where evidence indicated that binding is very firm and rigorous hydrolysis is necessary to release the carbohydrate from the protein. It appears rather that the carbohydrate found in the residual cartilage, that which is left after alkaline extrac-

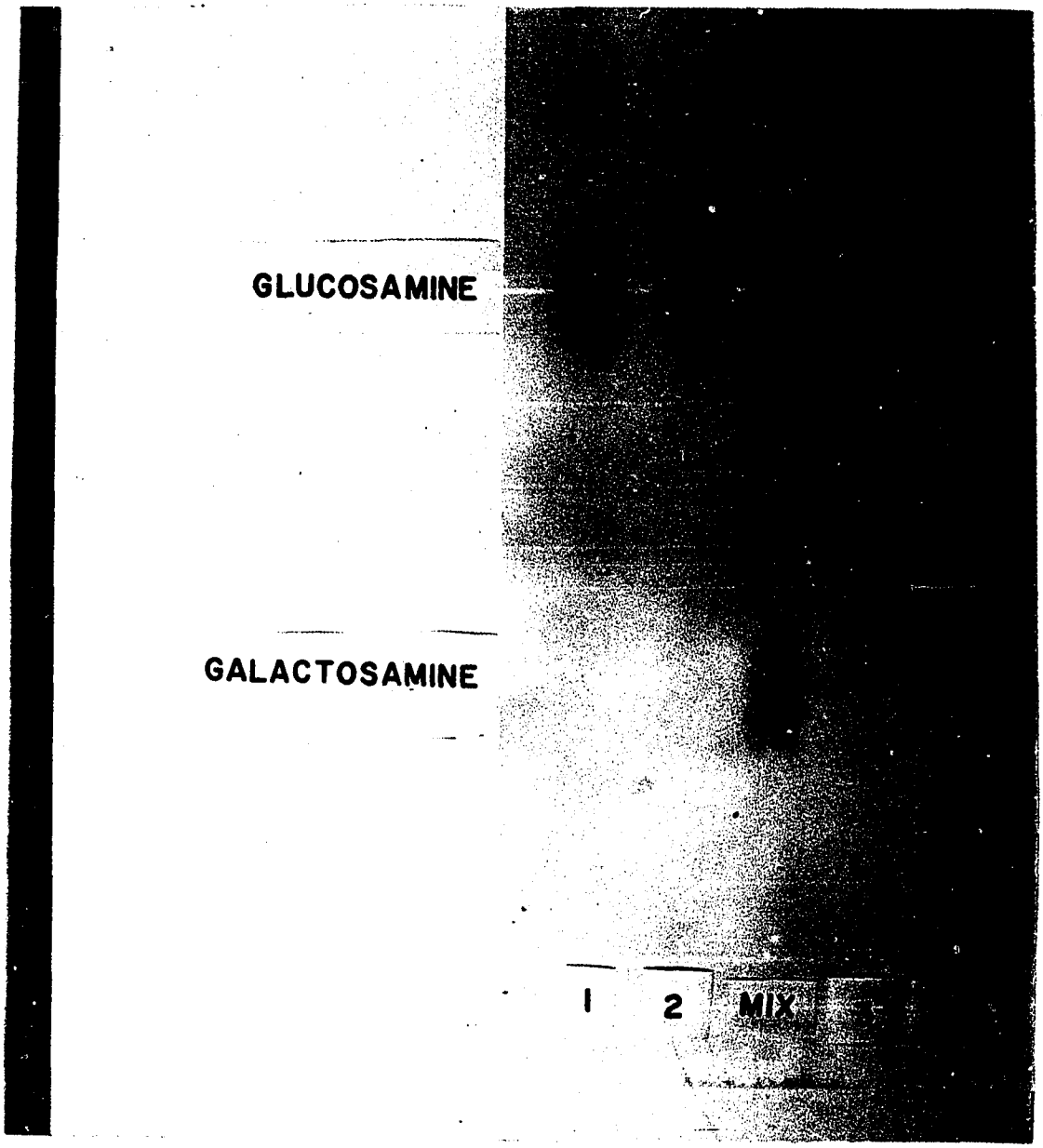


Figure 5

Paper Chromatograms of Hexosamine in Hydrolysates of "Keratosulfate" Fraction. Conditions: Whatman No. 4 paper butanol-pyridine-water system, stained with 2 amino biphenylphosphate. Left to right - 22 months, 14 years, standard mix, 31 years, and 67 years.

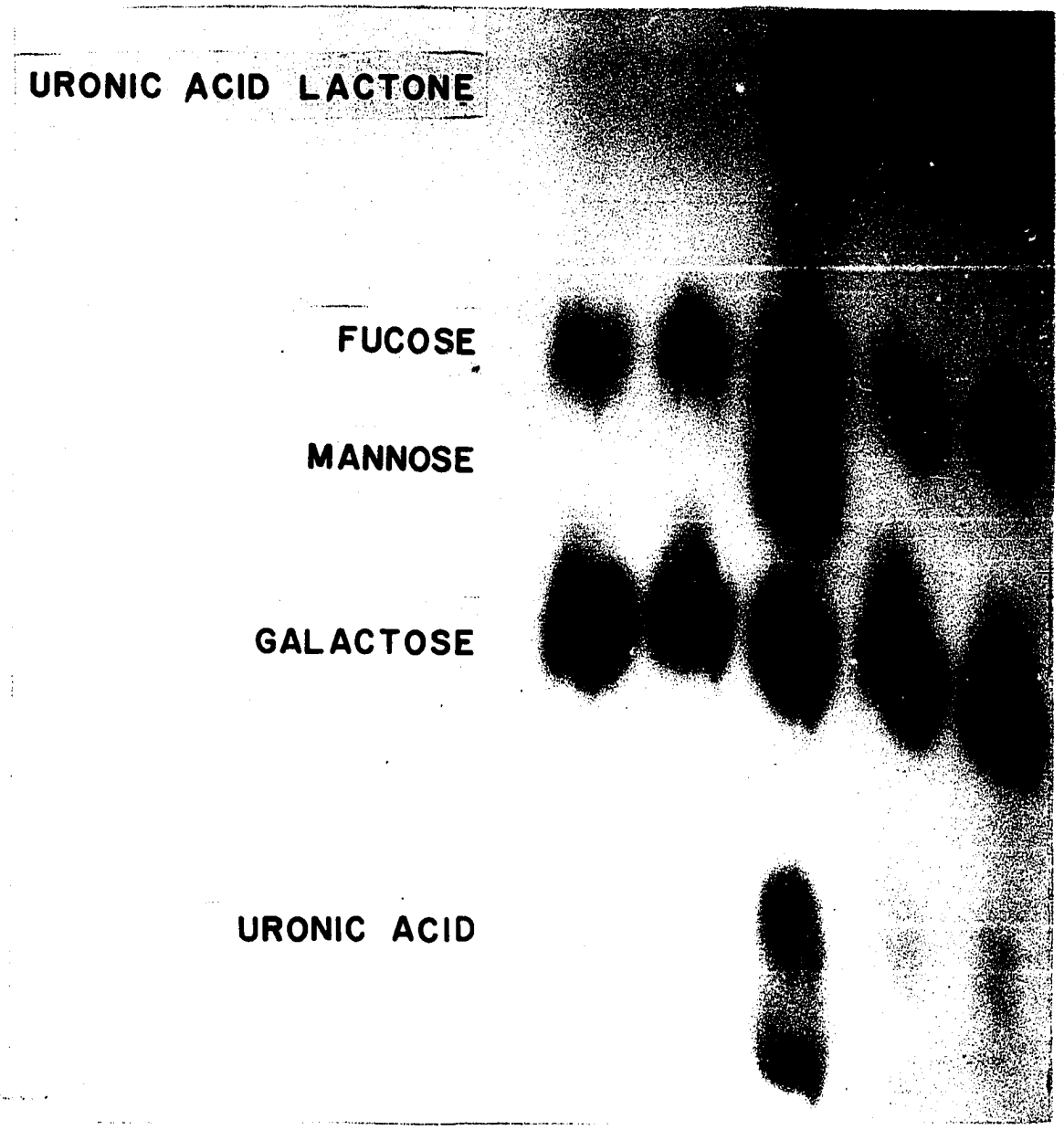


Figure 6

Paper Chromatograms of Galactose and Fucose in Hydrolysates of "Keratosulfate" Fraction. Conditions: Whatman No. 4 paper, butanol-pyridine-water system, stained with 2-amino biphenyl phosphate. Left to right - 22 months, 14 years, standard mix, 31 years, and 67 years.

tion represents tissue glycoprotein and tissue mucoids since this fraction appears to be tightly bound and insoluble in any of the extraction procedures used. Enzymatic hydrolysis of the protein does apparently release some of this material but when solubilized it cannot be distinguished from that which has previously been extracted. Another possibility is that the keratosulfate and keratosulfate like mucopolysaccharides containing mannose and fucose are bound in different type of binding and to different protein moieties which could account for some of these differences. It has been shown by Schubert's group that the protein moiety which is found in the water extractable chondromucoprotein is distinct from collagen as measured by hydroxyproline content. However, since recovery is never complete, this does not preclude the possibility that some of the chondroitin sulfate is not bound with collagen. The methods used in this study did not in any case yield a fraction of chondroitin sulfate completely free from protein or some other contaminating material since none of these fractions which contained chondroitin sulfate yielded a hexosamine or uronic acid value equal to that expected calculated for this mucopolysaccharide. The highest per cent yields were found in the pepsin digest, which had been exhaustively dialyzed. Schubert also reports (37) that chondroitin sulfate and chondromucoprotein exhibit nearly identical electrophoretic mobilities over a wide pH range of from five to ten pH units and that it is not possible to distinguish one from the other. However, in citrate buffer it is possible to separate these materials at a higher pH value, the chrondroprotein splits into two peaks. Having observed the same effect in this study, it seems likely that pH extreme causes the chondroitin sulfate-protein bond to be broken.

The fact that electrophoretic mobility is constant over a wide pH range indicates that this mobility is not a property of the protein per se but rather by the presence of an anionic material which does not reflect the pH change. This is probably the chondroitin sulfate itself.

CHAPTER V

SUMMARY AND CONCLUSIONS

The mucopolysaccharides and glycoproteins of human costal cartilage as influenced by age have been investigated. Analysis of total uronic acid, hexosamine and hexose content in the costal cartilage obtained from forty-seven individuals, ranging in age from premature through eighty-eight years of age, showed that while uronic acid concentration decreased with increasing age that total hexosamine and hexose values did not reflect this change. The hexosamine fraction was separated chromatographically into glucosamine and galactosamine. Quantitative analysis of these fractions showed the galactosamine levels compared closely to the values obtained for total uronic acid. It is assumed that these two carbohydrate monomers are a reflection of chondroitin sulfate and that the amount of this mucopolysaccharide decreases during aging. Glucosamine was considered to be a component of one or more other mucopolysaccharides or of glycoprotein.

Isolation of the mucopolysaccharide components of cartilage was carried out using extraction procedures ranging from the mildest, water, through treatment with mild alkaline salt solutions, and finally, treatment with dilute sodium hydroxide. Enzymatic digestion with proteolytic enzymes was also used to remove protein material. The resultant extracted

material was subjected to selective precipitation which indicated the presence of chondroitin sulfate and several unidentified fractions which contained galactose, mannose, fucose and glucosamine as identified by paper chromatography and paper electrophoresis.

From the latter fractions keratosulfate was isolated by papain digestion followed by precipitation with cetyltrimethylammonium bromide. This material as well as chondroitin sulfate was confirmed by comparison with known pure samples of these mucopolysaccharides.

Comparison of these isolated, purified fractions from the costal cartilage obtained from persons of different ages indicated that while chondroitin sulfate levels decrease during the aging process that the level of keratosulfate tends to increase. The other hexosamine containing materials, which were not identified, are probably glycoprotein.

BIBLIOGRAPHY

1. Boedecker, E. J., Ann. Chem. Pharm., 117, 11 (1854).
2. Krukenberg, C. F. W., Z. Biol., 20, 168 (1884).
3. Morner, C. T., Z. Physiol. Chem., 20, 357 (1895).
4. Schmiedeberg, O., Arch. F. Exper. Path. u. Pharmakol, 28, 355 (1891).
5. Klemper, F., Harvey Lectures, 49, 100 (1953-54).
6. Meyer, K., and Palmer, J. W., J. Biol. Chem., 107, 629 (1934).
7. Meyer, K., and Rappart, M. M., Science, 124, 174 (1956).
8. Meyer, K., Linker, A., Davidson, E. A., and Weissman, B., J. Biol. Chem., 205, 611 (1953).
9. Davidson, E. A., and Meyer, K., J. Biol. Chem., 211, 605 (1954).
10. Maximow, A. W., and Bloom W. A Textbook of Histology. Philadelphia and London: W. B. Saunders Co., 1948.
11. Follis, R. H., Jr., and Benthrong, M., Bull. Johns Hopkins Hosp., 85, 281 (1949).
12. Meyer, Karl. Mucoprotein and Mucoid. Some Conjugated Proteins. A Symposium. Edited by W. H. Cole. New Brunswick, N. J.: Rutgers University Press, 1953.
13. McLean, J., Am. J. Physiol., 41, 250 (1916).
14. Jorpes, J. E., Bostrom, H., and Mutt, V., J. Biol. Chem., 183, 607 (1950).

15. Wolfrom, M. L., Montgomery, R., Korabinos, J. V., and Rathgeb, P., J. Am. Chem. Soc., 72, 5796 (1950).

16. Levene, P. A. Hexosamine and Mucoproteins. London: Longmans, Green and Co., Inc., 1925.

17. Smith, H., and Gallop, R. C., Biochem. J., 53, 666 (1953).

18. Meyer, K., Smyth, E. M., and Palmer, J. W., J. Biol. Chem., 119, 73 (1937).

19. Wolfrom, M. L., and Rice, F. A. H., J. Am. Chem. Soc., 69, 1833 (1947).

20. Jorpes, J. E., and Gardell, S., J. Biol. Chem., 176, 267 (1948).

21. Hirano, S., Hoffman, P., and Meyer, K., Fed. Proc., 19, 146 (1960).

22. Meyer, K. The Mucopolysaccharides of Bone. Ciba Foundation Symposium on Bone Structure and Metabolism. Boston: Little, Brown and Co., 1956.

23. Stidworthy, G., Masters, Y. F., and Shetlar, M. R., J. Gerontol. 13, 10 (1958).

24. Davidson, E. A., and Meyer, K., J. Am. Chem. Soc., 76, 5686 (1954).

25. Meyer, K., and Chaffee, E., J. Biol. Chem., 138, 491 (1941).

26. Tracey, M. V., J. Biol. Chem., 43, 185 (1948).

27. Dische, Z., J. Biol. Chem., 167, 189 (1947).

28. Hoffman, P., Linker, A., and Meyer, K., Science, 124, 1252 (1956).

29. Jeanloz, R. W., and Stoffyn, P. J., Fed. Proc., 17, 249 (1958).

30. Committee on Protein Nomenclature, American Physiological Society and American Society of Biological Chemists, J. Biol. Chem., 4, 18 (1908).

31. Meyer, K., Davidson, E., Linker, A., and Hoffman, P., Biochemica et Biophysica Acta, 21, 506 (1958).

32. Shetlar, M. R., Foster, J. V., Kelly, K. H., and Everett, M. R., Proc. Soc. Exp. Biol. Med., 69, 507 (1948).

33. Shetlar, M. R., Anal. Chem., 24, 1844 (1952).

34. Jender, D. J., and Taylor, D. B., Anal. Chem., 25, 685 (1953).

35. Glegg, R. E., Eidinger, D., and Leblonde, C. P., Science, 118, 614 (1953).

36. Chargaff, E., Levine, C., and Green, C., J. Biol. Chem., 175, 67 (1948).

37. Timell, T. E., Glaudemans, C. P. J., and Currie, A. L., Anal. Chem., 28, 1916 (1956).

38. Shattan, J., and Schubert, M., J. Biol. Chem., 211, 565 (1954).

39. Einbender, J., and Schubert, M., J. Biol. Chem., 185, 725 (1950).

40. Dyrbye, M., and Kirk, J. E., J. Gerontol., 12, 20 (1957).

41. Meyer, K., Linker, A., Davidson, E. A., and Weissmann, B., J. Biol. Chem., 205, 611 (1953).

42. Pearce, R. H., and Watson, E. M., Canad. J. Research, 27, 43 (1949).

43. Shetlar, M. R., and Masters, Y. F., Proc. Soc. Exp. Biol. Med., 90, 31 (1955).