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STUDIES OF THE URINARY EXCRETION OF RADIOACTIVE MATERIAL AFTER THE INJECTION OF N-ACETYL-D-GLUCOSAMINE-1-C-14 INTO THE HUMAN

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CHAPTER I

INTRODUCTION

Metabolism has been a major area of biochemical interest in this century. The study of metabolites is a key to the understanding of the natural processes of an organism as they occur in health and as they are altered in disease. Migration of metabolites is a complicating fact in such studies because, although metabolism occurs within cells, the metabolites often migrate to sites far removed from their origin.

Urine has received a large share of research attention since it is a readily available biological fluid that can be analyzed with hopes of providing information concerning metabolic changes in health and disease. The "obstacle of Donaggio" (1) was an early phenomenon that led to investigation of uromucoidal material. A violet precipitate is formed in normal human urine after addition of thionine and ammonium molybdate while the supernatant fluid is colorless. However, Donaggio observed that in some pathological urines the formation of this precipitate was inhibited. This inhibition was called "obstacle of Donaggio." Much research has been done on uromucoids in an attempt to establish a relationship between uromucoids and the "obstacle of Donaggio."

Tamm and Horsfall achieved the separation and characterization of a urinary protein which acted as an inhibitor for the hemagglutination reaction caused by influenza virus. This inhibitor was shown to be a mucoprotein. The discovery provided an impetus to investigators to expand the methods of isolation, characterization, detection, and determination of urinary mucoproteins.

Radioisotope methodology has been applied advantageously in many areas of metabolic studies. Since the most characteristic constituents of urinary mucoproteins are the hexosamines, labeled hexosamine would appear, a priori, to present the best opportunity for studies of these compounds. Recently radioactive D-glucosamine-1-C-14 and N-acetyl-D-glucosamine-1-C-14 have become available for laboratory studies. The objective of the investigation described below is to investigate the excretion of N-acetyl-D-glucosamine-1-C-14 and its metabolites in urine from normal and pathological human subjects.

CHAPTER II

REVIEW OF THE LITERATURE

General Reactions of Glucosamine

The chemical properties of glucosamine (2-amino-2-deoxy-D-glucose) are similar to those of neutral hexoses and primary amines (2). This dual character has often been utilized in the separation of this amino hexose from other compounds. Aqueous solutions of glucosamine as the free base are unstable, particularly in alkaline solutions and in the presence of oxygen. Glucosamine hydrochloride has a greater stability than glucosamine. Oxidation of the aldehyde group produces the corresponding 2-aminohexonic acid. If the oxidation process is continued, the amino group is lost and a mixture of dicarboxylic acids is produced. Since glycol-splitting agents such as sodium metaperiodate and lead tetraacetate cleave the carbon-carbon bond when the adjacent carbon atoms are substituted with either amino or hydroxyl groups, the reaction of glucosamine with these reagents is similar to that of glucose. Glucosamine forms the usual oxime, semicarbazone, cyanohydrin, and phenylosazone derivatives. Glycosides of glucosamine are observed frequently.

The amino group of glucosamine reacts readily with active carbonyl compounds to form Schiff's bases. N-acyl compounds are formed when glucosamine reacts with acyl chlorides and acid anhydrides. The amino group is alkylated to produce secondary and tertiary bases and

quaternary ammonium derivatives. Glucosamine condensed with 1-fluoro-2, 4-dinitrobenzene yields the colored derivative, N-(2,4-dinitrobenzyl)-D-glucosamine. This compound has been useful in the separation and characterization of the amino sugars from mixtures of other sugars. If ninhydrin or chloramine-T is reacted with D-glucosamine, oxidative degradation causes the loss of the C_2-amino group and the decarboxylation of the C_1-group to yield D-arabinose.

In aqueous alkaline solution, glucosamine has been found to undergo deamination eventually through a series of complex changes. Deamination also occurred in reaction with nitrous acid and condensation with phenylhydrazine or o-phenylenediamine.

Glucosamine does not give a positive Molisch reaction for carbohydrates. However, other amino sugars with the amino group further removed from the C_1-position give positive tests. Glucosamine usually does not react in other carbohydrate color reactions which occur with phenols, aromatic amines or similar compounds in the presence of strong acids.

The Elson-Morgan and Dische-Borenfreund tests have been of major importance in the quantitative determination of glucosamine and its detection on paper. In the Elson-Morgan reaction (3), the preparation containing hexosamine is heated in a weak alkaline solution of acetylacetone and a pyrrole is formed. A rose colored product is produced when this pyrrole is reacted with p-dimethylaminobenzaldehyde in alcoholic hydrochloric acid, while a purple colored product results after color development of the pyrrole from an N-acetyl hexosamine. Since several amino acids and reducing sugars interfere with the test, Boas (4) introduced a procedure using Dowex-50 column chromatography to separate hexosamine

from neutral sugars and other interfering chromogens. Glucosamine and galactosamine have been separated by Dowex-50 chromatography using preferential elution with 0.3 N hydrochloric acid (5).

Dische and Borenfreund (6) reported a sensitive analytical method for detecting as little as 5 μg of D-glucosamine. The method involves the deamination of 2-aminohexoses by nitrous acid to yield 2:5-anhydrosugars, which give a color reaction with indole in the presence of hydrochloric acid. Other sugars also reacted in this method. However, aminohexonic acids do not give the color reaction so one can distinguish between glucosamine and oxidized form of aminohexoses.

D-glucosamine has been distinguished from its N-acetyl derivative, N-acetyl-D-glucosamine. Morgan and Elson (7) modified the Elson-Morgan method for glucosamine by omitting acetylacetone from the alkaline solution. Furthermore, the absorption maxima for glucosamine at 512 and 500 mμ were different from those for N-acetyl glucosamine at 550 and 590 mμ. More recently, Reissig and co-workers (8) developed a buffered solution, pH 9.1, to detect N-acetyl-D-glucosamine by the Morgan-Elson procedure. Potassium tetraborate and potassium hydroxide react with N-acetyl hexosamines to produce chromogens in the presence of p-dimethylaminobenzaldehyde. Study of the absorption spectra in the Morgan-Elson reaction has been used to detect the presence of interfering substances such as glucose and lysine.

Occurrence of Glucosamine

Glucosamine or chitosamine occurs mainly in the animal kingdom (2). This nitrogenous sugar has been found frequently as a constituent of carbohydrate polymers, which have been termed mucosaccharides by

Stary (9), but which have been known commonly as mucopolysaccharides. The carbohydrate moiety of mucopolysaccharides consists of hexosamine residues in combination with either hexuronic acid or neutral hexose units. Mucopolysaccharide-protein complexes have been found in skeletal muscles of many species, bacterial protoplasm, antigens of many types, intercellular cementing substances, and a variety of biological lubricants. Biological function of mucopolysaccharides differ considerably from that of glycogen. Mucopolysaccharides have been known to be largely structural in function, whereas glycogen is a source of readily available energy. Turnover of mucopolysaccharides in the body has been estimated to be about 1000 times slower than that of glycogen and glucose (9).

Mucins are viscous, water-soluble mucosubstances secreted by the glands or membranes of many animal and bacterial species, including some types of worms. The high viscosity of mucins is due to the presence of glycoproteins, which contain varying amounts of firmly bound carbohydrates and water. Glucosamine has been found frequently as a component of the mucoprotein (10) comprising these mucosubstances. A major function of mucins appears to be the lubrication of moving parts, as in certain Annelida where mucins secreted by the organism facilitate its movement. Another important function is to provide protective or mechanical supports, for example in the nutrition and development of new cell walls. Examples of other mucins are submaxillary mucin, intestinal mucins, cervical mucins, respiratory mucins, ovomucoid, and frog spawn mucin (2,9).

Chitin has been the most readily available source of glucosamine. Chitin is synthesized predominantly during certain phases of growth of some lower animals. The exoskeletons of molluscs and the crustacean

group are formed by deposition of inorganic substances, mainly calcium carbonate, in a complex matrix of chitin-protein. This type of hard material may be analogous to urinary calculi found in mammals (2). Much effort has been given to improvement of methods for isolating glucosamine from chitin. When the amount of acid used for degradation is carefully controlled, the chief product is N-acetyl-D-glucosamine. This N-acetyl compound has also been obtained after using enzymes.

A variety of biological fluids contain bound glucosamine. Substances similar to the four main blood groups found in red blood cells, are also found in gastric mucins, saliva, cyst fluids, and urine. Purified A and O substance of hog gastric mucin have been hydrolyzed by acid to yield D-glucosamine (11). When red blood cells containing blood group substances were mixed with the specific antibodies of these blood groups, agglutination of the red cells occurred.

The globulin fractions of serum proteins contain bound glucosamine and other sugars. The carbohydrate bound to serum protein has been cleaved only by the hydrolytic action of acid, alkali, or enzymes that also split peptide bonds (12). The type of chemical bonds linking the hexosamine portion to the protein has been of great interest. Knowledge in this regard is still incomplete, but several suggestions have been made. Masamune (13) has postulated the existence of two types of carbohydrate-protein linkages, one being an O-glycosidic linkage between the hydroxyl group of serine and the hemiacetal hydroxyl group of a monosaccharide residue and the other being a N-glycosidic bond between the hemiacetal hydroxyl group of acetyl glucosamine and the free amino group of a terminal aspartic acid. Rosevear and Smith (14) have suggested an

amide linkage between the beta-carboxyl group of aspartic acid and the amino group of a hexosamine. They also have suggested an ester linkage of an acid group with an hydroxyl group of a hexosamine.

Shetlar and co-workers (15) reported the level of protein-bound hexosamine in normal sera to be 71 $\pm$ 4 mg per 100 ml, whereas Winzler (16) reported the slightly higher value of 83 $\pm$ 9 mg per 100 ml. The protein-bound hexosamine in the serum has been found to increase significantly in a number of pathological states. Cancer, rheumatoid arthritis, rheumatic fever, and tuberculosis are characterized by elevated serum glycoprotein and seromucoid levels (12). Boas and co-workers (17) demonstrated an increase in serum hexosamine levels following myocardial infarctions, surgical procedures, and an acute attack of gout. An increase in urinary excretion of hexosamine also followed these acute stresses.

Heparin is a polymer containing glucosamine and glucuronic acid. The nitrogen atom of glucosamine is partially sulfated (18), in contrast to the N-acetylation found in most glucosamine-containing compounds. This sulfated mucopolysaccharide is found in the tissues of many mammalian organs such as lung, heart, and liver. In 1916, McLean (19) reported the first isolation of heparin and demonstrated its potency in retarding the coagulation of whole blood. In addition to its anticoagulant properties, heparin is one of the most highly ionized polymeric substances synthesized by the mammalian body.

Occurrence of N-Acetyl Glucosamine

Hyaluronic acid is a polymeric mucosubstance or acid mucopolysaccharide comprised of a repeating disaccharide unit of N-acetyl-D-

glucosamine and glucuronic acid. The hexosaminidic linkages are beta (1→4) and the glucuronidic linkages are beta (1→3) (20). This substance has been found in synovial fluid, umbilical cord, and vitreous humor (21). Hyaluronic acid may be hydrolyzed enzymatically by the hyaluronidases from various sources and which are present in spermatozoa, urine, bacteria, and leeches. Hyaluronic acid appears to function as a lubricant and an intercellular cementing substance (21).

Mucoitin sulfates have been demonstrated to be sulfated mucopolysaccharides as found in gastric mucin and bovine cornea. The mucoitin sulfate from gastric mucin contains equimolar proportions of N-acetyl-D-glucosamine, glucuronic acid, and ester sulfate (22). This substance was found to exhibit a weak anticoagulant activity, which could be greatly enhanced by introducing additional sulfate esters (23). On the other hand, bovine cornea has been shown to have at least three distinct acid mucopolysaccharides (24), including chondroitin sulfate, a fraction resembling hyaluronic acid, and keratosulfate (which contained a sulfated N-acetyl glucosamine).

A variety of biological fluids have been found to contain bound N-acetyl glucosamine. Highly purified blood group A substance from human ovarian cyst fluid yielded an N-acetyl amino sugar on hydrolysis (25). N-Acetyl glucosamine has been reported in other mucosubstances (26) or protein-carbohydrate complexes such as seromucoid, orosomucoid, fetuin, haptoglobulin, ceruloplasmin, transferrin, bovine prothrombin, bovine fibrinogen, 7S gamma globulins, 19S gamma globulins, human follicle-stimulating hormone, human chorionic gonadotrophin, human thyroglobulin, ovalbumin, and ovomucoid. These glycoproteins contain varying amounts of

other sugar constituents such as N-acetyl galactosamine, sialic acid, fucose, galactose, mannose, and other hexoses.

Metabolism of Glucosamine and Acetyl Glucosamine

The question of direct utilization of hexosamines and acetyl-hexosamines has been approached experimentally by feeding experiments and injections. The free hexosamines and acetyl hexosamines were absorbed through the intestinal wall in varying degrees after forced feeding. In guinea pigs, glucosamine was absorbed from isolated, surviving intestine more slowly than was glucose (27). N-Acetyl-D-glucosamine-1-C-14 was shown to be absorbed by rats and utilized for incorporation into protein bound hexosamine (28). In rats, D-glucosamine and N-acetyl-D-glucosamine were absorbed from the intestine at the same rate if the animals had been treated with neomycin prior to feeding (28). Furthermore, an appreciable amount of radioactivity from absorbed N-acetyl-D-glucosamine-1-C-14 was found in the body tissues, although the rate of appearance was much slower than for glucose.

In man, Bergfield and Kapfhammer reported that intravenously injected glucosamine could be metabolized in amounts up to 100 mg per kg of body weight (29). A dose as high as 200 mg per kg failed to produce hyperglycemia in normal subjects. The addition of hexosamine in in vitro experiments with kidney, testes, brain and other tissues produced an increase in oxygen uptake. Glucosamine was deaminated to form ammonia, urea, and lactic acid. The relative rates of this degradation were: glucosamine > acetyl glucosamine > galactosamine (30). Serum glycoprotein synthesis in rats utilizing radioactive glucose has been studied by Spiro (31), who reported the presence of radioactive glucosamine bound

to plasma protein. Recently, Shetlar and co-workers have reported studies indicating efficient incorporation of radioactive glucosamine into mucopolysaccharides and glycoproteins, suggesting a relation of glucose and glucosamine. This provided a new approach to studies of metabolism of these compounds (12,32,33). When glucosamine-1-C-14 was injected intraperitoneally into rats and rabbits, it was efficiently incorporated into serum proteins (34), liver proteins (35), and a variety of rat tissues (35). Also, a substantial amount of radioactive material was excreted in the urine (35).

Tissue preparations have been utilized to gain additional information on the metabolism of glucosamine and N-acetyl glucosamine. These compounds have been found to be biosynthesized from glucose, the most abundant monosaccharide in nature (2). The formation of glucose-6-phosphate from glucose is an enzymatic step in the subsequent biosynthesis of amino sugars. Glucose-6-phosphate was converted to glucosamine-6-phosphate in the presence of glutamine in liver slices (36).

Purified preparations of pigeon liver (37) were found to acetylate a variety of amines including glucosamine and galactosamine. Glucosamine hydrochloride was readily phosphorylated by brain preparations (38), but N-acetyl glucosamine was unaffected. In this system, N-acetyl glucosamine was a competitive inhibitor of glucosamine phosphorylation.

Glucosamine-6-phosphate has been found to be converted into a bound acetyl glucosamine unit of a mucopolysaccharide (39,40,41,42). The reaction sequence is generally considered to be glucosamine-6-phosphate→ N-acetyl glucosamine-6-phosphate⇌N-acetyl glucosamine-1-phosphate→bound N-acetyl glucosamine unit. UDP-N-acetyl glucosamine is also converted by

an epimerase reaction to UDP-N-acetyl mannosamine, a precursor for sialic acids (43), and to UDP-N-acetyl galactosamine (42), a precursor of chondroitin sulfates when a suitable source of hexuronic acid is available.

Glucosamine-1-phosphate is converted to heparin by at least two enzymatic steps (44,45,12) involving a UDP-glucosamine pyrophosphorylase and one or more enzymes. The sequence producing bound glucosamine was glucosamine-1-phosphate→UDP-glucosamine→heparin.

Enzymes engaged in the liberation and degradation of glucosamine and N-acetyl glucosamine compounds of animal tissues and biological fluids have not been thoroughly studied. Homogenates of rat kidney contain an acetyl glucosaminidase capable of splitting the glycosidic linkages of acetyl glucosamine residues (46). A urinary hyaluronidase has also been reported (47).

Urinary Excretion of Dialyzable Glucosamine and Acetyl Glucosamine

Dialyzable glucosamine and acetyl glucosamine have been reported in the urine. However, the concentration of these compounds is not large (48) and, furthermore, they are accompanied by interfering salts which complicate analyses. The early reports of urinary hexosamine, which was determined after hydrolysis in hydrochloric acid, were mostly concerned with the non-dialyzable material (17,49,50). In 1956, Boas (51,52) obtained values for dialyzable hexosamine by subtracting the values of hydrolyzed non-dialyzable material from hydrolyzed centrifuged urine. His procedure employed Dowex-50 for tissue hexosamine determination (4). He reported that urinary excretion of hexosamine increased during pregnancy to twice the normal value for both dialyzable and non-dialyzable

material. The ratio of dialyzable to non-dialyzable hexosamine was 1:1 in American females and remained unchanged during pregnancy (Table 12).

The first determination of dialyzable hexosamine by a direct method was reported by Marcotte-Boy and co-workers (53). They fractionated the urine by dialysis and treated the dialyzable fraction with urease before separation on Dowex-50. Analysis of the water eluate from the column was not reported. The ratio of dialyzable to non-dialyzable hexosamine by this method was 1:3 for normal men and women (Table 12). These investigators obtained less dialyzable hexosamine than Hammerman and Hatch (50) reported by the hydrolytic method.

Pechan (54) first reported the isolation of N-acetyl hexosamine from urine dialyzates. The isolation procedure involved an ethanol precipitation of pooled urine from human males. The filtrate was retained and salts were removed with Zerolit 225-hydrogen form and Zerolit FF-acetate form. A charcoal-Celite column was used for fractionation and elution was carried out with water, aqueous ethanol and aqueous acetone. Ten fractions were obtained and chromatographed to yield several Elson-Morgan positive spots, whose purple color was characteristic of N-acetyl amino sugars.

Since unbound glucosamine and N-acetyl glucosamine have been found in urine, the question of renal clearance should be considered. Earlier work indicated that intravenously injected glucosamine could be metabolized by man in amounts up to 100 mg per kg of body weight (29). However, Carter and Peters (55) report that the renal clearance of exogenous glucosamine in dogs was equal to the glomerular filtration rate. In rats, exogenous glucosamine was also found to clear at the same rate

as inulin (56), a substance used to determine glomerular filtration rate. It was suggested, however, that glucosamine might undergo subsequent tubular reabsorption or secretion after glomerular filtration. Data on the renal clearance of N-acetyl glucosamine have not been published.

Boas studied the presence of hexosamine-containing compounds of low molecular weight from the lyophilized dialyzate of urine (51). In his preliminary report, at least three distinct fractions could be distinguished after chromatographic separation on cellulose columns. Pechan (54) was searching for a hexosamine-containing oligosaccharide or glycopeptide. He reported a total yield between 10 and 25 per cent of the dialyzable "hexosamine". A combination of three column chromatographic separations using urinary material soluble in ethanol was employed before paper chromatography in this determination. He also reported the presence of "N-acetyl amino sugars" on paper chromatograms. However, quantitative studies of these acetyl amino sugars have not been reported.

Urinary Excretion of Bound Glucosamine and Acetyl Glucosamine

Total amounts of urinary colloids have been reported to vary between 30 and 750 mg per 24 hours and the average concentration between 213 and 244 mg per liter (57). The amount varied somewhat with the isolation procedure used. Electrophoretic analysis of urinary protein from normal subjects (58) has indicated the presence of all of the components normally found in serum by free electrophoresis. However, only about one-third of the urinary proteins were derived from serum (59,60). The urinary mucoproteins have a remarkably high carbohydrate content of approximately 20 to 30 per cent of the total weight (61,62). The urinary

excretion of non-dialyzable hexosamine has also been observed to increase in calculous disease, pregnancy, and atherosclerosis (63).

The two major mucoproteins which have been isolated from urine are the Tamm-Horsfall (T-H) mucoprotein and the uromucoid of Boyce and Swanson (64). Both of these urinary mucosubstances were isolated by precipitation methods. T-H mucoprotein was first isolated by precipitation in 0.58 M sodium chloride (65,66) and was found to inhibit the hemagglutination reaction of the myxoviruses. Normal urine contains approximately 25 mg per liter of T-H mucoprotein. This substance is responsible for over 99 per cent of the virus inhibitor present in urine. The crude T-H preparation was further treated with 0.025 M phosphate buffer at pH 6.8 and was fractionated to obtain a yield of 20 to 80 per cent of a soluble "inhibitory mucoprotein". Electrophoretic and ultracentrifugal studies of this apparently homogenous, phosphate-soluble component indicated that it was a threadlike molecule of 7×10^6 molecular weight (67). On the other hand, the phosphate-insoluble portion of "urinary mucoprotein" was demonstrated by Maxfield to have a molecular weight of 28×10^6 and thus was considered to be a tetramer of the smaller protein (68). This larger form has been examined in the electron microscope by Porter and Tamm (69). Both of these forms and polymers of even higher molecular weight were present in normal urine. A slow, spontaneous dissociation occurred to produce the smaller form (68).

The origin of T-H mucoprotein in the body is unknown. It is synthesized evidently in the mucous epithelia and in the mucous glands of the urinary tract (9). This mucoprotein has not been detected immunologically in serum (57). In rabbits, the renal tubular epithelium has

been shown by fluorescent antibody studies to be the site of production of T-H mucoprotein (70). This mucoprotein was present in urine obtained from the renal pelvis by cannulation in dogs and by catherization of the ureters in man. Hyaline casts in nephrotic urine consisted primarily of T-H mucoprotein (71). Such casts were formed within the distal tubules and collecting systems of the kidney; thus T-H mucoprotein must be formed either at that point or above. Maxfield (57) reported fluorescent antibody studies indicating that T-H mucoprotein was present at the brush border of the proximal tubules.

Gottschalk reported that carbohydrate constituents comprised 28 per cent of T-H mucoprotein and included the following: glucosamine 7.8 percent; galactosamine, 1.6; sialic acid, 9.1 (variable); galactose 5.4; mannose, 2.7; and fucose, 1.1 (61). This mucoprotein has been found to cause a cloudy formation (nubecula) in urine, adhere to surfaces, and coat the surface of salt crystals, thus preventing their growth and causing the formation of dendritic crystals. Variable results have been obtained when the T-H mucoprotein preparations were subjected to different types of electrophoresis (64,72,73). Maxfield (57) has suggested that this variation was probably due to the gelatinous properties of the materials, which hinder the flow of the threadlike macromolecules, and to the variable composition of the T-H mucoprotein in respect to the sialic acid component.

Albumin and the T-H mucoprotein, in an equimolar ratio form an insoluble complex (15), when isolated by salting out in 0.58 M sodium chloride. Since hexosamines have been demonstrated in the electrophoretically isolated globulin fractions of normal human plasma (74), their

presence in urinary alpha$_1$-, alpha$_2$-, beta-, and gamma-globulins would be likely. Boyce and co-workers (75) have shown that excretion of albumin and beta- and gamma-globulin was increased several-fold in patients with calculous disease.

Boyce and Swanson reported that uromucoid was similar to T-H mucoprotein (64). Uromucoid was obtained by ultrafiltration and extraction with barbital buffer, pH 8.6, and ionic strength 0.1. The yield of the precipitated uromucoid was two- to four-fold greater than that of T-H mucoprotein (76). Boyce suggested that fragments of T-H mucoprotein might be present in uromucoid. The composition of uromucoid and T-H mucoprotein differed in respect to amino acids (62), amino sugars and neutral hexose sugars. There is sufficient evidence to consider uromucoid and T-H mucoprotein as being distinctly different.

Injection of large doses of parathyroid hormone into rats or humans increases the serum level and the urinary excretion of seromucoid and glycoprotein (77).

An acid mucopolysaccharide having a molecular weight of 10,000 has been isolated from normal human urine by Di Ferrante and co-workers (78). They found this substance to be similar though not identical to chondroitin sulfate A or C, which has N-acetyl galactosamine as a constituent. Treatment of this substance with testicular hyaluronidase released a hexosamine-containing material of which 3 per cent was glucosamine and 28 per cent was galactosamine. Three bands with a mobility greater than albumin have been demonstrated by paper electrophoresis of ultrafiltered and dialyzed normal human urine at pH 8.6 (79).

A neutral non-dialyzable mucopolysaccharide was found by King

and Hyder (80) to be present in normal human urine. They found 26 mg of this substance was excreted per 24 hours, using an assay procedure which involved the heating of the non-dialyzable material with stannous chloride in 10 per cent hydrochloric acid. Glucosamine and galactose were found, but no protein, sulfate, or hexuronic acid could be demonstrated. This mucopolysaccharide was also reported to be increased in calculous disease (63).

CHAPTER III

MATERIALS AND METHODS

Radioactive Acetyl Glucosamine

N-Acetyl-D-glucosamine-1-C-14, prepared by the New England Nuclear Corporation, Boston, Massachusetts, was a gift from Pfizer & Co., Brooklyn, New York, who assayed the material for pyrogenic activity.

Aliquots of this preparation and an aqueous solution containing 10 µg each of D-glucosamine hydrochloride (Eastman) and N-acetyl-D-glucosamine (Pfizer) were applied to Whatman Number 1 chromatography paper and subjected to high voltage electrophoresis in a buffer system containing acetic acid, pyridine and water (10:1:289) (81). Double development descending chromatography was then applied in the second dimension, using a solvent mixture of butanol, acetic acid and water (12:3:5). Radioactivity was detected on these chromatograms by autoradiographic technique. The radioactive areas were removed from the paper strips and counted in a liquid scintillation system. No radioactive mannosamine was detected on the autoradiogram. The mixture of butanol, acetic acid and water will separate glucosamine from mannosamine (82). Comparable areas removed from control strips were counted and the counts per minute (cpm) for these areas were subtracted from the cpm for the areas without color development to give a net cpm. No further correction factor was applied to the net cpm values since color development with an Elson-Morgan reagent

(83) produced no quenching of radioactivity for either glucosamine or N-acetyl glucosamine.

Duplicate chromatograms were developed using similar aliquots and the same procedure as previously described. Each paper was then color developed by a dipping procedure using an Elson-Morgan reagent to check for hexosamine and N-acetyl hexosamine (83). Color developed strips were counted using the same procedure used for the strips without color development. The ratios of N-acetyl hexosamine to hexosamine for these areas detected by autoradiography and by color development, respectively, were calculated. The latter areas had two-fold greater counts, but the ratios of N-acetyl hexosamine to hexosamine differed by not more than 6 per cent. A major radioactive spot with the mobility of N-acetyl-D-glucosamine and a minor radioactive spot with the mobility of D-glucosamine were detected. Four additional areas were weakly radioactive but were not identified. The area coinciding with N-acetyl-hexosamine had 79.2 per cent of the total radioactive count and the area coinciding with D-glucosamine had 14.5 per cent. By difference, the unidentified radioactive compounds contained 6.3 per cent of the total applied radioactivity. The ratio of N-acetyl hexosamine to hexosamine for these color developed areas was calculated.

Separation of Hexosamines and Acetyl Hexosamines

The separation of glucosamine from galactosamine was accomplished using high voltage electrophoresis in one dimension and paper chromatography in the second dimension. Aliquots of the radioactive preparation were applied as before and 20 μg each of D-glucosamine hydrochloride and D-galactosamine hydrochloride (Eastman) were added as non-radioactive

"carriers". High voltage electrophoresis was performed as previously described, but the paper chromatography was carried out using a solvent system containing ethyl acetate, pyridine, butanol, butyric acid and water (10:10:5:1:5) (84). The resulting chromatograms were developed by an Elson-Morgan reagent (77) to reveal the "carriers". The rose colored areas were well separated. These areas were counted in the scintillation system and net cpm determined, as previously described, without correction for color quenching.

Several N-acetyl hexosamines were separated using high voltage electrophoresis followed by paper chromatography on borate treated paper. Aliquots of the radioactive preparation and 25 μg each of N-acetyl-D-glucosamine, N-acetyl-D-galactosamine (Nutritional Biochemicals Corp.) and N-acetyl mannosamine (Nutritional Biochemicals Corp.), added as non-radioactive "carriers", were applied. After high voltage electrophoresis was carried out, the paper chromatograms were dried and treated with a buffer (82) containing sodium tetraborate decahydrate (Fisher, reagent) and hydrochloric acid at pH 8.2. The borate-treated paper was developed by descending chromatography using a butanol, pyridine, and water solvent system (6:4:3). The resulting chromatograms were removed after a single development to locate N-acetyl mannosamine, using the Elson-Morgan reagent (83). On the other hand, a double development was necessary to separate completely N-acetyl glucosamine and N-acetyl galactosamine. The distances of migration from the origin were 14.5 cm and 16.3 cm, respectively, after the double development. The purple colored areas were counted in the liquid scintillation system and net cpm determined, as previously described, without correction for color quenching.

Determination of N-Acetyl Hexosamine and Hexosamine by Column Chromatography

Dowex-50 was used to remove most of the urinary pigments, salts, and some interfering chromogens (53) According to Marcotte-Boy and co-workers (53), the water eluate contains chromogens which interfere with the Elson-Morgan test and give falsely high values for hexosamines.

N-Acetyl-D-glucosamine (Pfizer, N.F.) and D-glucosamine hydrochloride (Eastman) were added separately to Dowex-50 columns and their elution patterns established. Five milliliters of Dowex AG 50W-X12, 100-200 mesh, hydrogen form (Bio-Rad Laboratories, Analytical Grade) were prepared according to the method of Boas (4). Aliquots of 0.050 and 0.100 mg of standards in 1.00 ml of aqueous solution (preserved by a crystal of thymol) were applied to the Dowex column and eluted with 20 ml of water, 40 ml of 1.0 N hydrochloric acid and 20 ml of 2.0 N hydrochloric acid. The water fractions were lyophilized. The acid fractions were frozen and evaporated under reduced pressure (mechanical vacuum pump) over silica gel and soda lime in a vacuum desiccator to remove the hydrochloric acid, which interferes with the Elson-Morgan reaction. The white, dried solids were dissolved in distilled water and aliquots taken for determinations.

The first 10 ml of the water eluate contained material that reacted with Morgan-Elson reagents as an N-acetyl amino sugar (8). Duplicate samples, with and without color development, were read at 585 mμ using a Beckman Model DB spectrophotometer. The second 10 ml of water gave readings similar to those obtained on water or acid solutions collected from untreated resin. The recoveries of known amounts of N-acetyl-D-glucosamine in the first water eluate from two samples were 80.6 per

cent and 85.5 per cent (average, 83.1 per cent). A correction factor of 1.20 was therefore applied when the N-acetyl hexosamine(s) in the dialyzable fraction of centrifuged urine was determined by this procedure.

Hexosamine analysis by the Elson-Morgan reaction (3) was performed on duplicate aliquots from the first 30 ml of the 1.0 N hydrochloric acid eluate. The samples were read at 530 mμ using a Beckman Model DB spectrophotometer. Recovery of standard D-glucosamine hydrochloride for two samples was 94.0 per cent and 98.4 per cent (average, 96.2 per cent). No correction factor was applied when the dialyzable hexosamine(s) of the supernatant fraction of urine was determined by this procedure.

Determination of Protein

The Lowry method (85) for determination of protein has been adapted by Marlow (86) for urinary protein. The procedure described by Marlow was modified in that the urine was dialyzed prior to determination. The protein samples were analyzed after dialysis to remove substances which interfere with this determination, for example, uric acid, guanine, xanthine, and most phenols (85). Some of the thymol preservative (5-methyl-2-isopropyl-1-phenol) had dissolved in the preparations, as evidenced by the odor, and apparently increased the background color development with the Folin reagent by 3- to 4-fold. The thymol was, therefore, removed by lyophilizing 1.0 ml aliquots of the non-dialyzable urine preparations. The lyophilized solids were then dissolved in distilled water and the Lowry reagent added. Similar aliquots of the protein standard (from human serum) were taken from an aqueous solution containing 0.050 ml of Labtrol (Dade, Inc., Miami) per 50 ml of solution. One milliliter

of the standard contained 0.067 mg of serum protein as reported by the vendor. Samples, standard, and samples blanks were read against a reagent blank in a Beckman Model DB spectrophotometer at 750 mμ and the results were expressed as mg of protein per ml of supernatant fraction.

High Voltage Electrophoresis-Paper Chromatography

Two-dimensional paper electrophoresis-chromatography was done using high voltage electrophoresis in one direction and paper chromatography in the other. Yang and Shetlar (87) found that the high voltage electrophoresis process can be used for desalting samples for subsequent paper chromatography. Their method was modified in that (a) the paper used was 46 by 57 cm, (b) a more dilute buffer was used (81), (c) the samples were spotted 16 cm from the right edge of the paper to permit a longer run and provide better resolution of individual compounds from their isomers, (d) an additional strip, 4.5 cm wide, for either a sample duplicate or standard was used to determine the migration in the high voltage electrophoresis dimension, and (e) the interval during which the potential of 3000 volts was applied was extended from 30 minutes to 50 minutes.

Aliquots of samples and standards separately dissolved in 10 per cent isopropanol were applied to Whatman Number 1 chromatography paper. Each aliquot of sample and standard was applied in two positions at 2.5 and 10.5 cm from the longer edge of the paper and 16 cm from the shorter edge. High voltage electrophoresis along the longer dimension of the paper was performed in a buffer containing acetic acid, pyridine, and water (10:1:289) (81), at pH 3.7 and using 64.4 v per cm length of the paper (Model ET 48, Servonuclear Corp.).

A rectangular area (4.5 by 57 cm) of each paper bearing the partially separated mixture of amino acids, glucosamine, and N-acetyl glucosamine was cut as a separate strip and developed by ninhydrin. The approximate migration of the samples with standards could then be estimated on the remainder of the paper without using a color developer. In appropriate experiments, samples with non-radioactive standards as "carriers" were applied at suitably separated positions along the line of migration but beyond the estimated migration. Descending paper chromatography, in various solvent systems, was used for development along the shorter dimension of the paper. The resulting chromatograms were dried in air before color development.

Low Voltage Electrophoresis

Aqueous aliquots of urinary non-dialyzable and ultrafiltered material were subjected to paper electrophoresis using a Beckman Model R Electrophoresis unit and two buffer systems. Aliquots of samples and of normal serum (as a standard) were applied to 3.0 by 30.6 cm filter paper strips (Schleicher and Schuell, catalog number 2043-A mg), which had previously been equilibrated with barbital buffer (ionic strength of 0.075, pH 8.6; Beckman, B-2) or an aqueous solution of tris-EDTA-boric acid buffer (conductivity of 3.0 millimho, pH 8.9; LKB - Produkter, Stockholm, GB-10, "High Resolution Buffer Salts"). A current of 2.5 ma was applied for 16 hours.

The strips were stained for protein using a solution containing 1 g of bromphenol blue dye (Beckman, B-4) in 1000 ml of methanol (Baker, reagent grade). The strips were exposed to ammonia fumes and measured densitometrically using a Beckman Model RB Analytrol with two 500 mμ

interference filters (blue range).

Measurements of Radioactivity

Autoradiography

Autoradiograms were prepared by attaching the paper chromatograms to 35.5 by 43.0 cm X-ray film (Kodak Blue Brand, par speed film) and permitting exposure of the film to the radioactivity for 21 days. The film was developed (General Electric, Supermix Med) for 3 minutes, rinsed with tap water, placed in a fixative bath (General Electric, Supermix Med) for 30 minutes, rinsed with tap water and dried in air. Those areas of the paper chromatograms which corresponded to the exposed areas on the film were outlined with pencil. The paper chromatograms were either dipped or sprayed with specific color developing reagents.

Liquid Scintillation Counting

Paper chromatograms. Either pencil marked areas or color developed spots were cut out as 3 by 4 cm rectangles, creased lengthwise, and placed in scintillation vials (Nuclear-Chicago Corp.). Comparable non-radioactive strips were cut from the same chromatograms and placed in separate vials to provide a background count. Twenty milliliters of scintillation solution was added. This solution contained 4 g of 2,5-diphenyloxazole (PPO, Nuclear-Chicago Corp.) and 50 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP, Nuclear-Chicago Corp.) per 1000 ml of toluene (Matheson, Coleman and Bell, spectroquality). The samples were counted five times for 10 minutes each in a Nuclear-Chicago Model 723 liquid scintillation unit at room temperature. The net cpm was determined by subtracting the cpm for the non-radioactive strips from the cpm

for the radioactive areas.

Solid material. One to three milligrams of dried solid material was dissolved in between 3.0 and 9.0 ml of Hyamine Hydroxide (p-(diisobutylcresoxyethoxyethyl) dimethylbenzylammonium hydroxide, Packard, 10-X) and 1.0 ml aliquots were pipetted into scintillation vials. Fifteen milliliters of the scintillation solution was added to the samples, which were counted five times for 20 minutes each. A sample containing 1.0 ml of Hyamine Hydroxide dissolved in 15 ml of scintillation solution was also counted and the cpm for this mixture was subtracted from the cpm for the samples to obtain the net cpm.

Gas Flow Counting

Urinary solids with a large sample thickness. An aliquot of the saline solution of N-acetyl-D-glucosamine-1-C-14 containing 0.216 μc was diluted to 50 ml with distilled water. Two-milliliter aliquots of this solution were transferred to aluminum planchets (Nuclear-Chicago Corp.), evaporated and desiccated over silica gel at room temperature for 24 hours. These samples were weighed to an accuracy of ±0.04 mg using a Mettler semi-micro analytical balance. Radioactivity of these desiccated solids was measured using a Nuclear-Chicago gas-flow counter system and the results expressed as cpm per sample. A 24-hour specimen of normal human urine (designated as FrO-24) was centrifuged after freezing and thawing. Various amounts of the supernatant fraction of urine were placed on the planchets containing the N-acetyl-D-glucosamine-1-C-14. After distilled water was added to bring the volume on each planchet to 2 ml, these were evaporated, dried, weighed and counted as before. The sample

weights were expressed as mg per ml of supernatant fraction. The radioactivity data were expressed as cpm per sample. The fractional self-absorption factor was calculated by dividing the cpm of the planchets containing the urine sample and radioactive acetyl-glucosamine preparation by the cpm of the planchets containing only the radioactive acetyl-glucosamine preparation. The fractional self-absorption factor was plotted versus the sample thickness in mg per sq cm. This graph (Fig. 2) was used for self-absorption correction of the counting results obtained for the supernatant fraction of urine.

Urinary solids with a small sample thickness. Aliquots of 1.0, 1.5, and 2.0 ml of non-dialyzable material were placed on weighed planchets and water was added to bring the total volume to 2.0 ml. The planchets and samples were dried as before and weighed before counting. The sample thickness was between 0.03 and 0.06 mg per sq cm. In this region of the self-absorption curve the correction factor was between 0.965 and 0.991. The maximum error of 3.5 per cent was well within the error of the counting procedure and, consequently, self-absorption corrections were not necessary for the non-dialyzable urinary material. The samples were counted from 10 to 30 minutes using the gas-flow counting system.

Injection of the Radioactive Material into Adult Human Males

The solution for injection was prepared by dissolving solid N-acetyl-D-glucosamine-1-C-14 (8.98 μc per mg) in physiological saline to obtain a concentration of 100 μc per 10 ml. The solution was autoclaved prior to injection.

Three adult males were used in this study. All had fasting

blood sugar values within normal limits. Subject Wi (clinical diagnosis: minimal degenerative arthritic change) was 52 years old and weighed 218 pounds. Subject Bo (clinical diagnosis: chronic brain syndrome) was 65 years old and weighed 120 pounds. Subject Da (clinical diagnosis: active, severe rheumatoid arthritis) was 36 years old and weighed 125 pounds.

Subject Wi received by intravenous injection 200 μc of N-acetyl-D-glucosamine-1-C-14. Subjects Bo and Da each received 100 μc. Blood samples were taken at various intervals for determination of radioactivity (88,89). No restriction of food or fluids was imposed before or after the injection.

The urine of the first subject (Wi) was collected at 24-hour intervals up to 96 hours. The urine of the second subject (Bo) was collected at the following intervals: 0-1, 1-2, 2-3, 3-4, 4-6, 6-8, 8-12, 12-24, 24-36, 36-48, and 48-72 hours. The urine of the third subject (Da) was collected at the following intervals: 0-2, 2-3, 3-4, 4-6, 6-8, 8-12, 12-24, 24-48, and 48-72 hours. The specimens were stored at $4^{\circ}C$. The volume of each specimen was measured and estimations of pH, glucose and protein were made with Combistix reagent strips (Ames Co., Inc.) (90). The values obtained for all specimens were within normal limits. A crystal of thymol (Mallinckrodt, N. F., crystals) was added as a preservative and the samples were then stored at $-20^{\circ}C$.

A 24-hour specimen of urine was collected from a control subject (Fr), who did not receive an injection of the radioactive material. The specimen was stored at $4^{\circ}C$, the volume was measured, and estimations of pH, glucose, and protein were made as before. The values obtained for the specimen were within normal limits. A crystal of thymol was

added as a preservative and the sample was then stored at -20°C.

Fractionation of Urine

Centrifugation Procedure

The samples were thawed at room temperature and centrifuged at 4°C in an International refrigerated centrifuge at 1300 x g for 30 minutes. Usually 50 ml of the supernatant portion of urine was removed by a volumetric pipette from 100 ml of centrifuged sample. The remainder was reserved for further studies, a crystal of thymol was added and samples were stored at -20°C.

Treatment of sediments after centrifugation. Only the sediments of the urine specimens from subject Da were checked for radioactivity. The individual collections obtained between 8 and 24 hours contained no sediments, while those obtained between 24 and 72 hours contained sediments that dissolved after rinsing with saline three times. The sediments obtained in each of the five urine specimens collected in the initial 8 hour interval following isotope administration did not constitute greater than 0.03 per cent of the total urinary dry solids. It was decided to determine the radioactivity removed from these sediments by washing with (a) 5 ml of 0.9 per cent saline three times, (b) 50 per cent ethanol once (57), and (c) 95 per cent ethanol twice. Since the washings from the first and second saline rinses were highly contaminated, they were discarded. The washings from the third saline rinses were evaporated in air. The resulting pale yellow solids were suspended in 2 ml of distilled water, placed on planchets, and evaporated in air. The radioactivity was measured by a gas-flow counter system. The results were expressed as cpm

per ml of supernatant fraction. A similar procedure was performed on the 50 per cent ethanol washings. Finally, the washings from the first rinse with 95 per cent ethanol for each of the fractions obtained between 0 and 8 hours were combined, as were those for the second 95 per cent ethanol rinse. The samples were counted using the gas-flow counter system.

The sediments remaining after the rinsings were dried over silica gel in a desiccator for several days and then were weighed. A portion of each sediment was suspended in distilled water and 2.0-ml aliquots were placed on planchets, evaporated in air, and counted using the gas-flow counting system. Results were expressed as cpm per mg of sediment.

Radioactivity of the supernatant fraction of urine. Triplicate radioactivity measurements of the supernatant fraction of the urine specimens from Da were obtained. Two 1.0-ml samples and one 2.0-ml sample were placed on weighed planchets and distilled water was added if needed to bring the total volume to 2.0 ml. The samples on the planchets were evaporated and then desiccated over silica gel at room temperature for 24 hours. The samples were weighed and results were expressed as mg per ml of supernatant fraction. The samples were counted as previously described to obtain net cpm. A graph of the fractional self-absorption factor versus sample thickness (Fig. 2) was used to correct the counting results for self-absorption and the corrected results were expressed as cpm per ml of supernatant fraction.

Fractionation of the Supernatant Fraction of Urine

Dialysis. Usually 50 ml of the supernatant fraction of each

urine specimen were pipetted into Visking casings and dialyzed against 500 ml of distilled water at 4°C in a covered beaker containing a small crystal of thymol. The dialyzable material was removed after 24 hours and lyophilized and a second 500 ml of distilled water added for further dialysis. The dialyzable material obtained in the first four dialyses of the individual specimens was combined for lyophilization. The dialyzable material obtained in the first four dialyses of the individual specimens was combined for lyophilization. The dialyzable residue was transferred with distilled water to 50-ml volumetric flasks. The preparations were transferred to plastic bottles, a crystal of thymol added as a preservative, and stored frozen. The non-dialyzable material was subjected to two additional dialyses and these dialysates discarded.

Radioactivity and weight of the dialyzable material. Aliquots of dialyzable material were placed on planchets and counted, as previously described in this chapter. Correction for self-absorption was made as before, except that the correction curve was established using a comparably prepared fraction obtained from a non-radioactive human urine specimen (D-FrO-24). The weight was expressed as mg of dialyzable material per ml of supernatant fraction. The activity was expressed as cpm per ml of supernatant fraction. A general excretion pattern for this dialyzable material from the subject with active rheumatoid arthritis is presented in Figure 3 for comparison with the corresponding non-dialyzable material.

Hexosamine in the dialyzable material. In this section the material will be presented from two categories, first, hexosamine content

without hydrolysis and second, hexosamine content with hydrolysis.

Hexosamine content without prior hydrolysis. The unhydrolyzed, dialyzable material gave a positive Elson-Morgan test. The concentrations of this free hexosamine in urine samples from Da and a non-radioactive, 24-hour urine (Fr) were determined by a modified Elson-Morgan method after column chromatography on Dowex-50 resin, as previously described.

Aliquots containing between 80 and 100 mg of dialyzable solids dissolved in between 2 and 5 ml of water were applied to the Dowex-50 column. The column was eluted as previously described for the standard samples of D-glucosamine hydrochloride and N-acetyl-D-glucosamine. The residue remaining after lyophilization of the water eluate had a brown, char-like color. The acid fractions were frozen and evaporated over silica gel and soda lime in a vacuum desiccator under reduced pressure (mechanical vacuum pump). The residue from the acid eluate was pale brown. The dried solids were dissolved in distilled water and aliquots taken for hexosamine determination.

The first 10 ml of the water eluate, which contains neutral hexoses, also contained material that reacted with Morgan-Elson reagents as an N-acetyl amino sugar (8). Marcotte-Boy and co-workers (53), in their determination of urinary hexosamine, evidently discarded the water eluate from Dowex-50. Values for urinary N-acetyl hexosamine were calculated as mg of N-acetyl hexosamine per ml of supernatant fraction.

An Elson-Morgan reaction for hexosamines was obtained for the first 30 ml of 1.0 N hydrochloric acid eluate. Values were expressed as mg of hexosamine per ml of supernatant fraction.

Hexosamine content after hydrolysis. The dialyzable fractions from two specimens of Da urine were separated by Dowex-50 chromatography. The water eluates were hydrolyzed in 3 N hydrochloric acid at 100°C for 7 hours. The samples were filtered through glass wool, frozen, and evaporated to dryness under reduced pressure over silica gel and soda lime. The solids were dissolved in distilled water. Aliquots of the water eluates before and after hydrolysis were taken for hexosamine assay.

Aliquots of these hydrolyzed water fractions were rechromatographed on Dowex-50. The amount of Elson-Morgan interfering chromogenic material in the hydrolyzed water fraction was 0.0128 ± 0.0010 mg of hexosamine per ml of supernatant fraction (D-DaO-2) compared with 0.0122 ± 0.0004 mg of hexosamine per ml of supernatant fraction (D-Da2-3). The net values of rechromatographed hydrolysate and hydrolyzed water eluate were also similar (0.0480 and 0.0472, respectively). Since hydrolysis appeared to have no effect on the amount of interfering chromogens, then analysis for these chromogens was performed on the unhydrolyzed material only and was used in calculations for both unhydrolyzed and hydrolyzed samples.

An experiment was designed to check the stability of this conjugated hexosamine by the Dowex-50 treatment, since there was a brown char-like residue from the water eluate after Dowex-50 treatment of the dialyzable material. Hydrolysis of the water eluate from a sample (D-DaO-2) followed by Dowex-50 treatment of an aliquot led to a value of 0.0288 mg of hexosamine per ml of supernatant fraction. This was not considered to be appreciably different from the value of 0.0297 obtained by Dowex-50 treatment followed by hydrolysis. If destruction of the

residual hexosamine occurred, the amount was apparently negligible and some other substance was possibly decomposed.

The remaining seven specimens of the Da urine and a non-radioactive, 24-hour centrifuged specimen of urine (FrO-24) were similarly separated into dialyzable and non-dialyzable material. The dialyzable material was fractionated by Dowex-50 and the water eluates hydrolyzed and assayed for hexosamine. The values for the Elson-Morgan chromogenic material in the water eluate and the N-acetyl hexosamine values were subtracted from the values obtained after hydrolysis. The net value was termed "conjugated hexosamine" and expressed as mg of conjugated hexosamine per ml of supernatant fraction.

Urinary excretion of radioactive dialyzable materials. Shetlar and co-workers (91) found that during the first 24 hours, 15.0, 13.1, and 26.2 per cent of the injected radioisotope was excreted in the urine of Wi, Bo, and Da, respectively. In the present study, the assay of the dialyzable fraction from several urine specimens verified the presence of several mg of hexosamine and N-acetyl hexosamine per liter of supernatant fraction. Since the radioactivity could be detected and measured, the ratio of N-acetyl-D-glucosamine to D-glucosamine and the ratio of dialyzable free hexosamine (yields a direct Elson-Morgan reaction) to dialyzable conjugated hexosamine were calculated.

A convenient method was chosen involving preparations which had not been desalted before high voltage electrophoresis-paper chromatography. Aliquots of the dialyzable material obtained from the Da urine specimens contained between 81 and 97 mg of solids in a volume between 2 and 5 ml. These aliquots were frozen and evaporated in a vacuum over

silica gel and 0.60 ml of 10 per cent isopropanol was added to dissolve most of the residue. The concentration was 0.154 to 0.170 mg of solid per μl of isopropanol. Since the samples had not been desalted, only 40 μl of the isopropanol mixture, containing about 6.5 mg of dialyzable material, could be readily applied to the chromatogram. On the other hand, if the sample were desalted by Dowex-50, several-fold more sample could be applied easily. It was calculated that from 6 to 10 μg of hexosamine would be present in the samples which were not treated with Dowex-50. About 2 μg provided an easily detected spot; hence, 20 μg each of non-radioactive D-glucosamine hydrochloride and N-acetyl-D-glucosamine were also applied to act as "carriers". Aliquots of a mixture containing at least the minimum detectable amounts of amino acids were also applied.

When high voltage electrophoresis was applied along the long axis of the paper, as previously described, N-acetyl-D-glucosamine migrated 1 cm from the origin toward the cathode and was easily separated from D-glucosamine hydrochloride, which migrated 27 cm toward the cathode. After electrophoresis, urinary samples, D-glucosamine hydrochloride, N-acetyl-D-glucosamine, D-galactosamine hydrochloride, and 20 amino acids were applied and developed by paper chromatography along the short axis of the paper using the butanol-acetic acid-water solvent system previously described. Autoradiograms were prepared to determine whether radioactive amino acids were present.

The chromatograms were analyzed by the Elson-Morgan reaction, using the procedure described by Smith (83), and were dried in air for 4 hours. The Elson-Morgan positive areas were counted in a liquid scintil-

lation system, as described previously, and net cpm calculated. Values for the 0-8 hour specimens were not corrected for quenching. The values for the 8-72 hour specimens were not utilized in this phase of study because of the low levels of radioactivity.

The solvent system containing ethyl acetate, pyridine, n-butanol, butyric acid, and water (10:10:5:1:5) afforded a better separation of glucosamine and galactosamine (84). Aliquots of the Da urine from the 0-3 hour specimens and standard D-glucosamine hydrochloride were applied and subjected to high voltage electrophoresis and then paper chromatography using this solvent system. These chromatograms were analyzed by the Elson-Morgan reaction (83) and the rose colored areas counted in a liquid scintillation system. The net cpm were calculated without correction for color quenching.

A third solvent system was applied to separate N-acetyl hexosamines on borate-treated chromatography paper. Aliquots of the Da urine from the 0-3 hour specimens and 25 μg each of N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, and N-acetyl mannosamine were applied to borate-treated chromatography paper (82) and subjected to high voltage electrophoresis and then paper chromatography, using a solvent system of butanol, pyridine and water (6:4:3). The purple-colored areas which were localized when these chromatograms were analyzed by the Elson-Morgan reaction (83) were discretely separated by intervals of 2 to 3 cm. These areas were counted in a liquid scintillation system and the net cpm were calculated without correction for color quenching.

Non-dialyzable material fractionated from supernatant urine. The non-dialyzable material of the supernatant fraction of the urine specimens

from Wi and Bo were transferred to 50- or 100-ml volumetric flasks and diluted to volume with distilled water. The solutions were stored at -20°C in plastic bottles to which a crystal of thymol had been added. They were thawed as few times as possible.

Aqueous solutions of the non-dialyzable material of these urines were lyophilized and the resulting pale-tan solids were desiccated over calcium sulfate for several days at 4°C. Weights of this material were expressed as mg of non-dialyzable material per ml of supernatant fraction. The range was between 0.06 and 0.39 mg of solid material per ml of supernatant fraction for the nine Da specimens. Samples of the solid material were dissolved in distilled water, usually in concentrations of 0.2 mg of non-dialyzable material per ml of preparation. These solutions were stored frozen.

Radioactivity of the non-dialyzable material. Aliquots in triplicate of the non-dialyzable material were placed on planchets, desiccated, and weighed before counting, using a gas-flow counting system. The net cpm values were corrected for self-absorption, using the curve obtained for the supernatant fraction as a close approximation (Fig. 2), and the results were expressed as cpm per ml of supernatant fraction.

Protein determination of the non-dialyzable material. Aliquots of the non-dialyzable material from the Da urine were lyophilized to remove the interfering thymol preservative. The dried samples were redissolved in distilled water and analyzed by the Lowry method (85), using Marlow's adaptation (86). The results were expressed as mg of protein per ml of supernatant fraction.

Hexosamine content of the non-dialyzable material. Total hexosamine content of the non-dialyzable material in all Da specimens was determined by hydrolysis and the modified method of Boas (4), but without the use of Dowex-50. Three- to six-milliliter aliquots, containing between 0.4 and 1.0 mg of non-dialyzable material, were acidified with concentrated hydrochloric acid until the concentration was 4 N in respect to the mineral acid. The acidified samples were heated in closed tubes for 5 hours at 100°C and filtered through glass wool to remove the coarse black particles. The filtrates were frozen and evaporated under reduced pressure over silica gel and soda lime in a vacuum desiccator. The residue was dissolved in distilled water and aliquots taken for hexosamine determination by the Elson-Morgan procedure, using D-glucosamine hydrochloride as a standard. Results were expressed as mg of hexosamine per ml of supernatant fraction.

Treatment of a sample by modifying the Elson-Morgan procedure in omitting the acetyl acetone reagent gave very low values for the non-dialyzable material in comparison with the values obtained for the corresponding dialyzable material.

Analysis of a sample containing water saturated with thymol gave a negligible value. A variety of hydrolytic conditions were utilized on two specimens of non-dialyzable material. No significant difference was observed among hydrolyses performed with 2 N hydrochloric acid for 15 hours, 3 N hydrochloric acid for 7 hours, 4 N hydrochloric acid for 5 hours, and 4 N hydrochloric acid for 7 hours. Marcotte-Boy and co-workers (53) used 3 N hydrochloric acid for 7 hours. Although Boas (4) found it necessary to use Dowex-50 for a variety of tissue hydrolysates,

Marcotte-Boy and co-workers found it unnecessary to use Dowex-50 for hydrolysates of the non-dialyzable material from urine. This question was resolved by subjecting duplicate aliquots of the non-dialyzable fraction of a urine sample to hydrolysis. The hexosamine value for the Elson-Morgan reactive material of a non-resin-treated hydrolysate from this sample (ND-Da24-48) was 0.0361 mg per ml of supernatant fraction. An aliquot of this hydrolysate was fractionated by Dowex-50. The hexosamine value of the acid eluate from the Dowex-50 column was 0.0376 mg per ml. The value of 0.0023 mg per ml for the Elson-Morgan reactive material obtained in the water eluate was considered as indicative of interferring chromogenic material and was deducted from 0.0376 mg per ml. Hence, the net hexosamine value for the Elson-Morgan reactive material subjected to Dowex-50 chromatography was 0.0353 mg per ml, compared with 0.0361 mg per ml obtained by assay without Dowex-50 chromatography.

Identification of radioactive hexosamines and amino acid in the non-dialyzable material. Aliquots containing 250 to 650 cpm from the same hydrolysates used for hexosamine analysis were develooed by the high voltage electrophoresis-paper chromatography technique using 20 amino acids, glucosamine hydrochloride, galactosamine hydrochloride, and mannosamine hydrochloride as non-radioactive "carriers". Autoradiograms were made before the paper chromatograms were developed using the diethylamine-ninhydrin method (92). By this technique, twelve of the amino acids had distinctive colors after diethylamine-ninhydrin treatment for a few hours. There was only one radioactive spot which had the staining characteristics and mobility of glucosamine. Standard mannosamine hydrochloride migrated further than either glucosamine hydrochloride or galactosamine hydro-

chloride and thus apparently could be excluded as a radioactive compound in the urine. Lysine, histidine and arginine were not radioactive. Since the areas occupied by galactosamine hydrochloride and glucosamine hydrochloride were overlapping on the chromatogram separated in the butanol-acetic acid-water solvent system, a second solvent system, containing ethyl acetate, pyridine, n-butanol, butyric acid and water was used to separate these two hexosamines (84).

Fractionation of Urine by 0.58 M Sodium Chloride

The Tamm-Horsfall preparation. An effort was made to determine whether radioactivity could be detected in the material isolated according to the method of Tamm and Horsfall (65). Since sufficient urine containing the radioactivity was not available in the first 24-hour specimen, a sample from the second 24-hour specimen of the Da urine and two 24-hour specimens of Wi urine were used for these studies. The Tamm-Horsfall (T-H) mucoprotein has been reported to be excreted in concentrations of about 25 mg per liter, as determined by precipitation in 0.58 M sodium chloride (66). To insure sufficient material for the isolation, 200-ml aliquots of several 24-hour specimens of urine (U-Da24-48, U-Wi0-24, U-Wi24-48) were used. The samples were diluted with equal volumes of distilled water. When 13.6 g of sodium chloride (analytical grade) were added to make the concentration 0.58 M in respect to sodium chloride, the urine became cloudy after a few hours. After 16 hours at 4°C, the supernatant fractions were removed by pipette until a volume of about 40 ml remained. Each precipitate and mother liquor was centrifuged at 4°C in an International refrigerated centrifuge at 1270 x g for 30 minutes. The individual supernatant fractions were combined with the

corresponding decanted supernatant fraction and the residue was rinsed with 20 ml of cold, 0.58 M saline. The saline wash was removed by centrifuging and the procedure was repeated two additional times. The saline washes were added to the corresponding supernatant fractions and the combined fractions designated as Sol ND-Da24-28, Sol ND-WiO-24, and Sol ND-Wi24-48.

The insoluble material was suspended in 200 ml of distilled water and dialyzed against 1 liter of distilled water at 4°C. Dialysates were changed and discarded twice daily for 5 days. Finally, the preparation was lyophilized and desiccated over calcium sulfate at atmospheric pressure for 24 hours. The salt-insoluble preparations were designated as T-H mucoprotein (TH-Da24-48, TH-WiO-24, TH-Wi24-48). The white material was weighed and was slightly hygroscopic.

Aliquots of this white material were dissolved in Hyamine Hydroxide to provide uniform concentrations of 0.33 mg per ml. These samples were counted by the liquid scintillation procedure and no correction was made for quenching. Results were expressed in cpm per mg of dry solids.

Paper electrophoresis was performed on aliquots of the Tamm-Horsfall mucoproteins, using a Beckman Model R Paper Electrophoresis System. Distilled water, 0.10 ml, was added to 2 mg of solids. The solids were dispersed and formed a white, viscous liquid. Aliquots of 0.020 ml of the dispersion (20 mg per ml) were placed on filter paper strips. Aliquots of 0.006 ml of normal serum were also applied for identification of albumin and globulins. Other aliquots of urinary samples were added to non-radioactive aliquots of normal serum to serve as "carriers". Low

voltage electrophoresis was done either in barbital buffer or in tris-EDTA-boric acid buffer.

The strips were stained to demonstrate protein. Densitometric curves for the samples and standard serum were recorded on paper, using a Beckman Model RB Analytrol and the results are summarized in Table 11.

The stained strips were cut into rectangles to include single bands with a minimum of the areas which failed to stain with bromphenol blue. These rectangles were counted in a liquid scintillation system. The values for radioactivity were not corrected for quenching and were expressed as cpm per mg of dry solids.

Material obtained using the Tamm-Horsfall preparation from one 24-hour specimen of Fr urine was analyzed for the presence of amino acids. An aqueous aliquot of 1.0 mg of this material was acidified with concentrated hydrochloric acid until the concentration was 6 N and the solution was placed in a sealed glass tube. The tube and contents were heated at 110°C for 22 hours. The tube was opened and heated to dryness at 70°C under reduced pressure (mechanical vacuum pump) to remove the acid. The residue in the tube was dissolved with 0.500 ml of a sodium citrate buffer (93) having a pH of 2.875. An aliquot equivalent to 0.80 mg of the original material was applied to a Technicon Amino Acid Analyzer (Technicon Laboratories, Chauncey, New York) (94). All of the amino acids known to be present in the Tamm-Horsfall preparation (95) were detected except cysteine, methionine, and valine, while the presence of arginine and histidine were doubtful. Cysteine and methionine are known to be present in low amounts in the Tamm-Horsfall preparation. The detection of tryptophane was not attempted.

The 0.58 M sodium chloride soluble fraction of urine. The salt soluble fractions (designated as Sol ND-Da24-28, Sol ND-Wi0-24, Sol Ni-Wi24-48) were dialyzed, the non-dialyzable material was lyophilized, desiccated over calcium sulfate for 24 hours and then weighed. The urine fraction contained hygroscopic, tan solids.

Aliquots of these fractions of urine soluble in 0.58 M sodium chloride were measured for radioactivity and were subjected to low-voltage electrophoresis. These aliquots were dissolved in Hyamine Hydroxide to yield uniform concentrations of 0.33 mg per ml. These samples were counted by the liquid scintillation procedure and no correction was made for quenching. Results were expressed as cpm per mg of dry solids.

For low-voltage paper electrophoresis, 8 mg of the solids was dissolved in 0.10 ml of distilled water to form a tan colored solution. Aliquots of 0.020 ml of this solution (80 mg per ml) were applied to paper strips. Some of these strips also contained normal serum, which served as a "carrier". Aliquots of normal serum were also applied separately to serve as controls. Electrophoresis either in barbital buffer or in tris-EDTA-borate buffer and staining were performed simultaneously with the samples from the salt insoluble fraction. These stained strips were measured densitometrically using the Beckman Model RB Analytrol. The strips were cut into bands and counted by the liquid scintillation procedure. The values obtained for radioactivity were not corrected for quenching and were expressed as cpm per mg of dry solids.

Fractionation of Urine by Ultrafiltration

The radioactivity on the residue obtained when a urine specimen was subjected to ultrafiltration was compared with that in the non-

dialyzable fraction of this urine specimen. Fifty-milliliter aliquots of a 0-24 hour specimen of urine (U-Wi0-24) were placed in an ultrafiltration unit under a pressure of 40 lbs per sq in of nitrogen at 4°C. Ultrafiltration through a disc of dialysis membrane (Visking Corp.) was completed within 48 hours. The residue was rinsed three times with distilled water by applying ultrafiltration pressure and the residue was transferred with distilled water to a lyophilizing bottle. The lyophilized material weighed 2.8 mg (11 mg per 200 ml).

The ultrafiltered residue readily dissolved in 0.10 ml of distilled water to form a tan colored solution. Aliquots of 0.010 ml (2.8 mg per 0.100 ml) were applied to paper strips with and without applied aliquots of normal human serum, which served non-radioactive "carriers". Aliquots of normal human serum were used as a standard. The strips were then subjected to low voltage electrophoresis in two different buffer systems and the strips were stained for protein and measured densitometrically using the Beckman Model RB Analytrol. The strips were cut into bands and counted in a liquid scintillation system. The values obtained for radioactivity were not corrected for quenching and were expressed as cpm per mg of dry solids.

Studies on Hexosamine Conjugated Material Found in the Dialyzable Fraction

Recovery of Radioactive Material Containing Conjugated Hexosamine

The material contained in the areas with measurable radioactivity (Fig. 6, areas 1 and 2) were used for further studies. As previously described, 5.0 ml of Da urine from the 4-6 hour specimen, which had been

dialyzed to remove the non-dialyzable material, were subjected to column chromatography using Dowex-50. The water eluate was lyophilized and applied to five sheets of Whatman 3 MM chromatography papers for high voltage electrophoresis and paper chromatography using the butanol, acetic acid, and water solvent system. A standard solution containing a mixture of 20 amino acids and N-acetyl neuraminic acid in amounts detectable by ninhydrin and thiobarbituric acid developers was applied to two of the papers. The two radioactive areas were located before staining by autoradiography on each paper; an exposure for a period of four days was used. Rectangles (2.5 x 3 cm) containing the areas with radioactive material were cut from each of the five papers; all of the no. 1 areas were sewn together with cotton thread (Clark, O.N.T. 40, best 6 cord) which had been soaked in distilled water, and treated with distilled water by descending capillary flow. The no. 2 areas were similarly combined and treated. Two hundred milliliters of eluate were collected, evaporated to dryness *in vacuo* at 40°C, transferred to a weighing bottle by using six separate rinses of 0.5 ml of distilled water, lyophilized, and weighed. The material eluted from radioactive areas 1 and 2 weighed 3.0 and 17.9 mg, respectively. The paper rectangles which originally contained this material were counted in a liquid scintillation system. The net cpm was less than 10 cpm for each paper.

Amino Acids in the Eluted Material from the Radioactive Areas

An experiment was done to verify that free amino acids were not present in the eluted material from radioactive areas 1 and 2. One sample of the water eluate from Dowex-50 and a mixture of 20 amino acids was

developed by high voltage electrophoresis and paper chromatography. The radioactive areas 1 and 2 were cut from the paper and then ninhydrin was applied to the remainder of the paper. The material in radioactive area 1 migrated 0.5 cm farther than cystine and 4.0 cm less than serine, the second nearest of the remaining amino acids behaving as meutral compounds. The material in radioactive area 2 migrated 1.0 cm less than aspartic acid while glutamic acid was farther removed.

Aliquots of 0.95 and 5.4 mg of material from radioactive areas 1 and 2 were separately dissolved in 0.80 and 1.00 ml of 6 N hydrochloric acid and sealed in glass tubes. The tubes and contents were heated at 110°C for 22 hours. The tubes were opened and heated to dryness at 70°C under reduced pressure (mechanical vacuum pump). The contents of each tube were dissolved separately with 0.500 ml of a sodium citrate buffer (93) having a pH of 2.875 and centrifuged to remove a dark brown residue. Aliquots of the supernatant fraction equivalent to 0.38 and 2.1 ml of the original material from radioactive areas 1 and 2 were applied to a Technicon Amino Acid Analyzer (Technicon Laboratories, Chauncey, New York) (94). Several amino acids and considerable ammonia were detected. The quantitative data for the amino acids were not calculated since the hydrolyzed samples contained interfering substances which produced an irregular base line printed by the recorder.

Aliquots of material from radioactive areas 1 and 2, containing 0.98 and 7.90 mg in 0.55 and 2.20 ml of 3.0 N hydrochloric acid, respectively, were heated in screw capped glass tubes at 100°C for 7 hours, and used for hexosamine assay and the detection of radioactivity in hexosamine and amino acids. The tubes were opened and the contents were

evaporated at room temperature under reduced pressure (mechanical vacuum pump). The contents of each tube were dissolved separately in 2.00 ml of distilled water and aliquots taken for hexosamine assay and radioactivity studies. For the latter studies, 1.00 ml aliquots were evaporated as before. These aliquots containing 0.49 and 3.95 mg of hydrolyzed material from radioactive areas 1 and 2, respectively, were dissolved in two successively applied portions of 0.10 ml of distilled water and applied to Whatman 3 MM chromatography paper. A standard misture of 20 amino acids, glucosamine, and galactosamine were also applied at the origin. High voltage was applied in one dimension and then the same standard mixture was applied at the side and in front of the areas of the electrophoretic run. Paper chromatography was done in the second dimension using a solvent mixture of butanol, acetic acid, and water. The side strip containing the standard mixture was removed and developed with ninhydrin to aid in location of the amino acids and hexosamines. The corresponding areas without color development, containing amino acids and hexosamines, were cut into rectangles and counted using a liquid scintillation system.

Hexosamine in the Eluted Material from the Radioactive Areas

Studies for the hexosamine contained in the eluted material from radioactive areas 1 and 2 were done using a hydrolysis in 3 N hydrochloric acid for 7 hours followed by high voltage electrophoresis and paper chromatography as described above. Areas corresponding to various amino acids and hexosamines were cut into rectangles and counted in the liquid scintillation system. Net values of 126 and 638 cpm for the hexosamine area from the hydrolysate of areas 1 and 2, respectively, were obtained. No

correction was made for self absorption. The paper strips were rinsed five times with toluene (Matheson, Coleman and Bell, spectroquality) to remove the fluorescing material and treated with 75 ml of distilled water by a descending capillary flow to remove the hexosamine. The water eluates were evaporated under reduced pressure at 40°C. The residues were dissolved in 0.100 ml of distilled water. Each solution and 20 μg each of D-glucosamine hydrochloride and D-galactosamine hydrochloride standards were applied to Whatman 3 MM chromatography paper and developed using a solvent mixture containing ethyl acetate, pyridine, butanol, butyric acid and water (10:10:5:1:5) (84) to separate the glucosamine and galactosamine. Areas containing glucosamine and galactosamine were cut into rectangles and counted before color development in a liquid scintillation system giving net values of 47 and 121 cpm for the glucosamine contained in radioactive areas 1 and 2. Only background count was obtained for the corresponding galactosamine areas. No correction was made for self-absorption. These paper rectangles were removed from the glass vials and treated with a ninhydrin developer to verify the presence of the hexosamine.

Hexosamine was assayed as previously described using the Elson-Morgan reaction on aliquots of hydrolysate from 0.24 and 2.0 mg of material contained in radioactive areas 1 and 2, respectively. Results (p. 68) were expressed as mg of conjugated hexosamine per ml of supernatant fraction and as per cent of conjugated hexosamine in eluted material.

Hexose in the Eluted Material from the Radioactive Areas

The amount of bound hexose contained in the eluted material from

the radioactive areas was determined by the tryptophane-sulfuric acid method (96). Aliquots of 0.24 and 1.05 mg of material eluted from radioactive areas 1 and 2 were compared with a standard containing a mixture of 0.025 mg of mannose and 0.025 mg of galactose dissolved in 0.50 ml of distilled water. Samples and standard were read against a reagent blank in a Beckman Model B spectrophotometer at 500 mμ and the results were expressed as per cent and μmoles of bound hexose.

Sialic Acid in the Eluted Material from the Radioactive Areas

An experiment was done to verify that free N-acetyl neuraminic acid was not present in the eluted material from the radioactive areas 1 and 2. Sixty thousandths mg of N-acetyl neuraminic acid (Sigma Chemical Co., sialic acid from egg Type III) was added as a locating agent to one of the five chromatographic papers serving to recover the radioactive material containing conjugated hexosamine. After the high voltage electrophoresis, an additional 0.060 mg of N-acetyl neuraminic acid was applied to the paper but beyond electrophoretic migration in the first dimension before applying the solvent mixture of butanol, acetic acid, and water for separation in the second dimension. The spot of the N-acetyl neuraminic acid applied along the side of the paper was developed using thiobarbituric acid in a spray reagent (97) and had migrated 19.5 cm from the origin. The corresponding area within the two-dimensional realm of the sample and the N-acetyl neuraminic acid was cut into the form of a rectangle from the paper and counted using a liquid scintillation system giving a net value of 154 cpm. No correction was made for self-absorption. However, there was no indication of radioactivity in this

area as detected by the appropriate autoradiogram. The paper rectangle was removed from the glass vial and developed using the thiobarbituric acid reagent. The resulting red spot was separated from the material in radioactive area 2 by an interval of 0.8 cm. The material in radioactive area 1 migrated 1.0 cm towards the cathode whereas N-acetyl neuraminic acid migrated 2.0 cm towards the anode during high voltage electrophoresis.

The amount of bound sialic acid contained in the eluted material from the two radioactive areas was determined by an assay using thiobarbituric acid (98). Aliquots of 0.24 and 1.05 mg of material from radioactive areas 1 and 2, respectively, were dissolved in 0.20 ml of 0.1 N sulfuric acid and heated at 80°C for 1 hour to liberate the bound sialic acid (99). A similar treatment was given to 0.100 mg of a sialic acid preparation (Nutritional Biochemicals Corporation, N-acetyl neuraminic acid, assay sialic acid 18.3%) serving as a standard. Samples and standard were read against a reagent blank in a Beckman Model B spectrophotometer at 549 mμ and the results were expressed as per cent and μmoles of bound sialic acid.

An attempt was made to determine if any of the bound sialic acid was radioactive. An aliquot containing 0.24 mg of material from radioactive area 1 was made to a volume of 1.00 ml having concentration of 0.1 N hydrochloric acid and heated at 80°C for 1 hour. This solution was evaporated at room temperature under reduced pressure (mechanical vacuum pump). The residue was dissolved in 0.10 ml of distilled water and applied to Whatman 3 MM chromatography paper. Sixty thousandths mg of standard N-acetyl neuraminic acid (Sigma Chemical Co.) in aqueous solution

was applied separately. The sample and standard were subjected to high voltage electrophoresis as previously described but for a period of 90 minutes to afford a better separation of neutral and acidic components. The area containing the N-acetyl neuraminic acid was cut from the paper as a rectangle and counted before color development in a liquid scintillation system. The cpm for this area was similar to that of a blank. The area containing N-acetyl neuraminic acid was developed as a red spot using a method for the detection of sialic acid on paper (97). N-acetyl neuraminic acid migrated 2.0 cm towards the anode during the high voltage electrophoresis.

CHAPTER IV

RESULTS

Distribution of Radioactivity in Fractions of Centrifuged Urine

Radioactivity in the Supernatant Fraction

It was usually necessary to centrifuge the radioactive urines to remove any sediment that formed upon standing. The supernatant fraction was further fractionated by dialysis into dialyzable and non-dialyzable material. Corrections for self-absorption were significant for the supernatant fraction and its dialyzable component. The urine of a subject (Da) with active rheumatoid arthritis was checked for radioactivity in the supernatant fraction in this regard for correction of self-absorption. The values for cpm per ml of the supernatant fraction of centrifuged urine agreed reasonably well with the sum of these values for the dialyzable and non-dialyzable components (Table 4). Furthermore, the amount recovered in these two components approximated closely the original weights of the supernatant fraction (Table 4). The major portion of radioactivity was found in the supernatant fraction (Tables 1,2). The supernatant fraction of Wi, Bo, and Da contained 4.5 per cent, 17.4 per cent, and 25.7 per cent, respectively, of the injected dose, as measured by gas-flow counting (calculated using Table 6).

Distribution of radioactivity in urinary dialyzable and non-dialyzable material. There was considerable variation in individual excretion of the injected dose in regard to radioactivity of the components in the supernatant fraction. The dialyzable material excreted in all cases was 93 per cent or more of the total urinary radioactivity and the average was 96.0 per cent. Most of this excretion of radioactivity in dialyzable molecules occurred within the first 24 hours following the intravenous injection. The rate of excretion of dialyzable material decreased rapidly with time (Tables 5,6). The rate of excretion of non-dialyzable material also decreased, but not so rapidly (Tables 5,6). The percentage of radioactivity in the dialyzable material excreted during the second, third, and fourth 24-hour periods after injection decreased to a plateau value of about 80 per cent (Table 6).

Urinary excretion of the radioactive non-dialyzable material was much lower than the dialyzable material. The values for the non-dialyzable material were calculated from Table 6 to be 0.0030 per cent, 0.0035 per cent, and 0.0030 per cent of the injected dose for subjects Bo, Wi and Da, respectively. The variation in values for the non-dialyzable material was much less than for the dialyzable material. The percentage excretion of radioactive non-dialyzable material was 4.4 per cent, 6.5 per cent, and 1.2 per cent (average, 4.0 per cent) of the total radioactivity excreted in the urine of subjects Wi, Bo and Da, respectively. The percentage excretion based on total radioactivity excreted of this non-dialyzable material increased from about 3 per cent during the first 24 hours after injection to about 20 per cent during the second, third, and fourth 24-hour periods for Wi and Da. Thus, the relative

proportion of radioactivity in the non-dialyzable material tended to increase as the time interval after radioisotope injection increased while that in the dialyzable material tended to decrease.

The radioactivity of the dialyzable material in the urine of subjects Bo and Da was at a maximum within 2 hours and dropped very rapidly within the initial 6-hour interval after injection (Tables 4,5). A graph of radioactivity versus time for dialyzable material from these men gave a hyperbolic curve as shown by the graph given for one of the men (Fig. 3).

The excreted radioactivity of the non-dialyzable material was more irregular than that of the dialyzable material for the three individuals (Tables 4,5). The maximal total radioactivity in the non-dialyzable material occurred in the interval between 2 and 3 hours after isotope injection in subjects Bo and Da. A less pronounced maximum was also seen in subject Da between 4 and 6 hours (Table 4). The curve for the non-dialyzable material did not cross the curve for dialyzable material when radioactivity was plotted versus time for the three subjects, as shown for Da in Figure 3. The maximal specific radioactivity (cpm per mg) for the non-dialyzable material occurred between 3 and 4 hours after isotope injection (Fig. 4). Protein and hexosamine of the non-dialyzable material obtained from the urine of subject Da were determined.

Radioactivity in the Sediment of Centrifuged Urine

Samples of the sediment fraction from urine specimens obtained in the interval between 0 and 8 hours after isotope injection of subject Da were selected for study because more than 90 per cent of the total

radioactivity found in the urine was excreted during this time. Ethanol was used to remove certain urinary biocolloids, which are soluble in 50 per cent ethanol (57). The radioactivity of the 50 per cent ethanol soluble material in the urine sediments (Supernatant R-2) was negligible compared with the radioactivity of the non-dialyzable material (Table 1). The radioactivity of the labeled biocolloids in the sediment was low. On the other hand, the radioactivity of the sediments after washing (Sediment, R-4) was greater than for the ethanol soluble material in the urine sediments (Supernatants R-2 and R-3) (Table 2). The ethanol insoluble material did not appear hygroscopic.

Distribution of Radioactive Hexosamine in Urine

Radioactive Glucosamine in the Dialyzable Fraction of Urine

The distribution of radioactive free hexosamine in the dialyzable fraction was determined using urine samples from subjects Wi and Da. After paper chromatographic separation in a solvent system containing butanol-acetic acid-water (4:1:2), only one radioactive area was obtained in the hexosamine region, as determined by autoradiograms (Fig. 5). This technique distinctly separated mannosamine from glucosamine and galactosamine, although glucosamine migrated only slightly farther than galactosamine. Nevertheless, no radioactivity was present in the appropriate area for mannosamine. A second solvent system containing ethyl acetate-pyridine-butanol-butyric acid-water (10:10:5:1:5) (84) separated glucosamine and galactosamine. Localization of the individual amino sugars was facilitated by the addition of non-radioactive "carriers". The following values for cpm in the glucosamine and galactosamine areas

were obtained using aliquots of the radioisotope solution injected and of two urine specimens collected from subject Da.

	Injected radioisotope solution	Urine specimens	
		Da0-2	Da2-3
glucosamine, cpm	760	292	599
galactosamine, cpm	22	9	21

The ratios of glucosamine to galactosamine are 34.1, 32.8, and 29.2 for the injected radioisotope solution and two urine specimens, respectively. It therefore appears that the proportions of radioactive glucosamine and galactosamine in the urine specimens approximated those of the injected hexosamine solution.

The quantity of D-glucosamine-1-C-14 excreted in the urine can be estimated. The calculated amount of D-glucosamine-1-C-14 injected was approximately 1.6 mg, based on the specific activity value provided by the vendor after correction for radioactive impurities. About 8 per cent of this glucosamine or 0.13 mg was excreted within the first 8 hours in the urine of subject Da (Table 8). Hence, the major portion of the injected radioactive glucosamine was not accounted for in these studies on the urine. The excretion of radioactive glucosamine was 2.7-fold greater than that of radioactive N-acetyl hexosamine.

Radioactive Glucosamine in the Non-Dialyzable Fraction

Hydrolysates of radioactive non-dialyzable material from the urine of subjects Wi and Da were subjected to high voltage electrophoresis and paper chromatography. Subsequent localization of the individual sugars was facilitated by the addition of non-radioactive "carriers:. Two different solvent systems were used, as previously described, to

separate glucosamine, galactosamine, and mannosamine. There was only one radioactive area, as demonstrated by autoradiography, and this area had the staining characteristics and mobility of glucosamine. Lysine, histidine, and arginine, which migrated into the vicinity of the amino sugars on the chromatograms, were not radioactive.

The maximum urinary excretion of radioactive non-dialyzable material occurred in the interval between 3 and 4 hours after injection of the radioisotope into subjects Bo and Da. Protein and hexosamine determinations were done on the urine of subject Da. The specific radioactivity based on protein (cpm per mg of protein) was at a maximum during the interval between 3 and 4 hours after isotope injection (Table 9). The specific radioactivity based on total non-dialyzable material (cpm per mg of non-dialyzable material) (Table 4) and that based on hexosamine (cpm per mg of hexosamine) (Table 10) were also at a maximum during the interval between 3 and 4 hours. The average ratio of hexosamine to protein was calculated as 0.2 (Table 10). The values of cpm per mg of non-dialyzable material, cpm per mg of protein, and cpm per mg of hexosamine were plotted versus time (Fig. 4).

Radioactive Hexosamine in the Fractions Obtained Using the Tamm-Horsfall Procedure

The radioactivity of the glucosamine-containing Tamm-Horsfall mucoprotein was studied using urine specimens. The Tamm-Horsfall preparation represented less than 3 per cent by weight of the total non-dialyzable solids (Table 11) and the percentage of radioactivity varied between 3.3 per cent and 8.5 per cent of that present in the total non-dialyzable solids. However, the specific radioactivity (cpm per mg) of the Tamm-

Horsfall preparation was at least twice that of the remaining non-dialyzable solids for subject Wi and was 1-1/2-fold greater for subject Da. The Tamm-Horsfall preparation was subjected to low voltage electrophoresis and a radioactive band was obtained which did not migrate, but stained with bromphenol blue (Table 11).

The amount of dry solids in the urine specimens soluble in 0.58 M sodium chloride (salt soluble) was much greater than that in the insoluble fraction containing the Tamm-Horsfall mucoprotein (Table 11). Protein comprised 44.5 per cent of the non-dialyzable material from the second 24-hour urine specimen of subject Da (Table 9). Approximately 3 per cent of the non-dialyzable material (Tables 4 and 6, Da24-48) was contained in the Tamm-Horsfall mucoprotein, of which 73 per cent was calculated to be protein (61). Thus the urine contained 42.3 per cent of protein other than this mucoprotein. Two 24-hour specimens from subject Wi and one 24-hour specimen from subject Da yielded salt soluble fractions of non-dialyzable material containing more than 90 per cent of the total radioactivity (Table 11). However, as mentioned previously, the specific radioactivity (cpm per mg) of the salt soluble non-dialyzable material was lower than that of the corresponding Tamm-Horsfall mucoprotein. Electrophoresis using barbital buffer demonstrated a band at the origin and a very broad band at the $alpha_1$- and the $alpha_2$-globulin region, which stained with bromphenol blue. A band at the origin and a much sharper band at the $alpha_2$-globulin region were obtained by electrophoresis using tris-EDTA-boric acid buffer. Radioactive areas with the mobility of albumin were noted in the salt insoluble and the salt soluble fractions of the urine from subjects Wi and Da. Radioactive

areas with the mobility and staining characteristics of alpha$_2$- and beta-globulins were detected (Table 11) in the salt soluble fractions of urine from subject Wi. In addition, a third component with the mobility and staining characteristics of alpha$_1$-globulin was observed in the salt soluble fraction of urine from subject Da.

Radioactive Hexosamine in the Residue from Ultrafiltered Human Urine

When serum was added to specimens of ultrafiltered human urine and aliquots of this mixture subjected to low-voltage electrophoresis using buffer systems of barbital and tris-EDTA-boric acid, several bands of material reacting with bromphenol blue were observed. These bands had measurable radioactivity in the gamma-, beta-, alpha$_2$-, and alpha$_1$-globulin, and the albumin regions (Table 11). In addition, a radioactive band was observed at the origin. The results were very similar to those previously presented for the non-dialyzable fraction of these urine specimens.

Distribution of Radioactive N-Acetyl Hexosamine in the Dialyzable Fraction of Urine

The distribution of radioactive free N-acetyl hexosamine in the dialyzable fraction of urine was determined using a combination of high voltage electrophoresis and two different solvent systems for subsequent paper chromatography. The solvent system containing butanol, acetic acid and water separated the N-acetyl hexosamines in the non-desalted urinary samples of subjects Wi (Fig. 5) and Da (Table 8) into N-acetyl mannosamine and probably a mixture of N-acetyl glucosamine and N-acetyl galactosamine. The results obtained on chromatography of these samples

with non-radioactive "carriers" were compared with those obtained on chromatography combined with electrophoresis. The N-acetyl glucosamine, N-acetyl galactosamine and N-acetyl mannosamine contained in urine samples from subject Da were separated employing a butanol-pyridine-water solvent system on borate treated paper (82) and appropriate non-radioactive "carriers".

The following values for cpm were obtained for aliquots of the injected radioisotope solution and for urine specimens obtained from subject Da:

	Injected radioisotope solution	Urine specimens	
		Da0-2	Da2-3
N-acetyl glucosamine, cpm	3,650	3,020	858
N-acetyl galactosamine, cpm	144	1,275	508
N-acetyl mannosamine, cpm	46	53	35

The ratio of the radioactivity of the N-acetyl glucosamine to that of N-acetyl galactosamine in the injected solutions was 25.3, whereas that of the urine specimens was 2.36 and 1.69, respectively. The ratio of the radioactivity of the N-acetyl glucosamine to that of N-acetyl mannosamine in the injected solution was 79.3, whereas that of the urine specimens was 57.0 and 24.7, respectively.

The urinary excretion of N-acetyl glucosamine-1-C-14 can be determined assuming that approximately 9 mg was contained in the injected solution, based on the calculations previously described. About 18 per cent or 1.6 mg of the injected radioactivity was excreted, mainly as N-acetyl glucosamine and N-acetyl galactosamine (Table 8), within the first 8 hours in the urine of subject Da. The average rate was 0.2 mg per

hour during this interval. The excretion ratio of radioactive N-acetyl hexosamine (N-acetyl glucosamine and N-acetyl galactosamine) to glucosamine was 1.7 for subject Wi and 2.3 for subject Da. The urinary excretion of radioactive glucosamine was 2.7-fold greater than that of radioactive N-acetyl glucosamine and N-acetyl galactosamine, since 5.5-fold more of the acetyl glucosamine was injected.

Distribution of Radioactive Hexosamine Conjugate in the Dialyzable Fraction

The dialyzable fraction of centrifuged urine obtained from subject Da in the interval between 4 and 6 hours after radioisotope injection was fractionated and subsequently desalted using Dowex-50 column chromatography. This specimen was selected since an assay (Table 7) indicated a high concentration of hexosamine conjugated material was present. The water eluate was subjected to high voltage electrophoesis and paper chromatography using a solvent mixture of butanol, acetic acid and water. Autoradiographic technique demonstrated five radioactive areas. One discrete area (Fig. 6, area 1) exhibited a low mobility by high voltage electrophoresis and paper chromatography. Its mobility, as determined by high voltage electrophoresis, was about the same as N-acetyl glucosamine (Fig. 6, AcGN). By paper chromatography in the butanol-acetic acid-water system, the mobility was less than that of glucosamine. The radioactive areas were counted and the ratio of the radioactivity in the N-acetyl hexosamine area to that of the radioactive material in area 1 was 0.6. The radioactive material from area 1 was harvested from five paper chromatograms and eluted with water. Hydrolysis of an aliquot in 3 N hydrochloric acid yielded only one radioactive area having the same

mobility as glucosamine in the butanol-acetic acid-water system. The specific radioactivity of the glucosamine area without correction for self-absorption was 250 cpm per mg of original area 1 material and 9100 cpm per mg of hexosamine. The latter value was derived from the Elson-Morgan assay for hydrolyzed material contained in radioactive area 1. This hydrolyzed material did not contain detectable amounts of radioactive material with the mobilities of amino acids.

Another discrete area (Fig. 6, area 2) exhibited a slightly greater mobility than radioactive area 1 by high voltage electrophoresis and paper chromatography. Its mobility as determined by high voltage electrophoresis was similar to the mobility of aspartic acid and sialic acid but by paper chromatography (butanol, acetic acid, and water) moved less than aspartic acid or sialic acid and greater than the radioactive material in radioactive area 1. The ratio of the radioactivity of the N-acetyl hexosamine area to that of the radioactive material from area 2 was 2.4. Hydrolysis of an aliquot of the radioactive material eluted from five paper chromatograms yielded only one radioactive area having the same mobility as glucosamine. The specific radioactivity of this glucosamine area without correction for self-absorption was 161 cpm per mg of original area 2 material and 35,000 cpm per mg of hexosamine. This latter value was derived from the Elson-Morgan assay for hydrolyzed material contained in radioactive area 2. This hydrolyzed material did not contain detectable amounts of radioactive material with the mobility of amino acids.

Occurrence of Hexosamine and Its Metabolites in Urine

Hexosamine and its Metabolites in the Dialyzable Fraction of Supernatant Urine

Free urinary hexosamine, that is, the material giving a positive Elson-Morgan reaction without hydrolysis, was assayed in the acid eluate following Dowex-50 fractionation and radioactive glucosamine was identified by autoradiography and two dimensional technique of high voltage electrophoresis-paper chromatography. Excretion of free hexosamine from subject Fr over a 24-hour period was 30.6 mg, while from subject Da the average for three 24-hour periods was 31.8 mg (Tables 7,12). These values were compared with those for non-dialyzable hexosamine (Table 10). Values of the ratio of dialyzable free hexosamine to non-dialyzable hexosamine from subject Da averaged 2.4:3 for three 24-hour periods. The dialyzable free hexosamine was also compared with the total dialyzable hexosamine, including free, acetylated, and conjugated hexosamine (Table 7). The percentage excretion of free hexosamine in the total dialyzable hexosamine for a control subject (Fr) was 51.0 per cent, in contrast to that of an average of 33.4 per cent for three 24-hour periods of the subject Da.

Hexosamine in the Non-Dialyzable Fraction

The hexosamine in the non-dialyzable fraction of urine was determined after hydrolysis and thus included total hexosamine. The urinary excretion of non-dialyzable hexosamine for three successive 24-hour periods in subject Da averaged 41.4 mg (Tables 10,12), but varied as much as 20 mg for individual determinations. The percentage of hexosamine in

the total non-dialyzable solids averaged 8.3 per cent for three successive 24-hour periods, which had maximum differences of about 1 per cent from the average. The former result indicated a variation in the amount of bound hexosamine excreted in the urine but the latter result indicated a rather consistent content of bound hexosamine in the non-dialyzable material.

Occurrence of N-Acetyl Hexosamine in the Dialyzable Fraction of Supernatant Urine

An assay of free urinary N-acetyl hexosamine, that is, the material giving a positive Morgan-Elson reaction, was obtained following Dowex-50 chromatography of the dialyzable fraction of supernatant urine (Table 7). The 24-hour urinary excretion of N-acetyl hexosamine was 10.4 mg for one specimen from subject Fr and an average of 19.7 mg from three successive 24-hour specimens from subject Da. As mentioned previously, radioactive N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, and N-acetyl mannosamine were identified in the urine of subject Da. The amount of free acetyl hexosamine excreted per hour for 24-hour periods was always less than the amount of free hexosamine and also less than the hexosamine conjugate in his urine and that of subject Fr (Table 13). The urinary excretion rate was calculated to be 0.4 mg per hr. for a 24-hour specimen of urine from subject Fr and 0.6 to 1.1 mg per hr. for that from subject Da. Excretion of free N-acetyl hexosamine followed an irregular pattern, as did also free hexosamine, dialyzable hexosamine conjugate, and bound hexosamine. As expected, less variation in the rate of excretion (mg per hr) was realized when the urinary excretion was calculated on the basis of 24-hour intervals (Table 13).

The values for dialyzable N-acetyl hexosamine in 24-hour specimens of urine from subjects Fr and Da were 0.0138 mg per ml (one 24-hour specimen) and 0.0102 mg per ml (average of three 24-hour specimens), respectively, and were thus present in the lowest concentration of the three dialyzable, hexosamine-containing materials (Table 7). The percentage of N-acetyl hexosamine excreted in the total dialyzable solid material varied between 0.030 per cent and 0.051 per cent of three 24-hour specimens of urine from subject Da. However, the percentage of N-acetyl hexosamine excreted relative to the dialyzable hexosamine for 24-hour periods was 17.4 per cent for subject Fr and 19.2 per cent (17.9 to 19.9 per cent) for subject Da.

Occurrence of Hexosamine Conjugate in the Dialyzable Fraction of Supernatant Urine

A hexosamine analysis was performed following hydrolysis of water eluates obtained by Dowex-50 fractionation of dialyzable material from supernatant urine. There was a large increase in the amount of Elson-Morgan reacting material contained in the water eluate after hydrolysis. The amount was several-fold in excess of the Elson-Morgan interfering chromogens and calculated hexosamine resulting from hydrolysis of N-acetyl hexosamine (100). No significant increase was observed in the hexosamine content of the acid eluates after hydrolysis.

One assay using the urine of subject Fr and nine assays using the urine of subject Da yielded evidence for the presence of conjugated hexosamine (Table 7). Only once during the excretion interval of subject Da did the amount of conjugated hexosamine fail to exceed the amount of free hexosamine. This occurred in the interval between 4 and 6 hours

following injection of the radioisotope solution. The maximum excretion rate of the conjugate occurred during the interval between 8 and 12 hours following injection. This maximum excretion rate was from 3- to 8-fold greater than that at any other interval between 0 and 24 hours.

The 24-hour urinary excretion of dialyzable conjugated hexosamine was 19.0 mg for subject Fr and an average of 50.8 mg for three successive 24-hour specimens from subject Da. The concentrations of dialyzable conjugated hexosamine in 24-hour specimens of urine from subjects Fr and Da were 0.0207 mg per ml and 0.0258 mg per ml, respectively. The percentage of dialyzable conjugated hexosamine in the total dialyzable hexosamine was 31.7 per cent and 50.8 per cent, respectively. The maximum variation in the latter average of subject Da was about 8 per cent. On the other hand, the percentage of dialyzable conjugate material in the total dialyzable solid material varied between 0.053 per cent and 0.139 per cent for three successive 24-hour specimens of urine from this subject.

Studies were made on the composition of the material eluted from radioactive areas 1 and 2 (Fig. 6, areas 1 and 2). The eluted material from radioactive area 1 contained 14.7 per cent hexose, 2.9 per cent hexosamine, and 0.44 per cent sialic acid or 2.40, 0.47, and 0.041 μmoles of hexose, hexosamine, and sialic acid, respectively. The presence of aspartic acid, glutamic acid, threonine, serine, glycine, alanine, valine, isoleucine, leucine, lysine, and histidine was detected by the amino acid analyzer. Proline, cystine, methionine, tyrosine, phenylalanine, and arginine were not found. A very large amount of ammonia was also detected. The eluted material from radioactive area 2 contained 3.8 per

cent hexose, 0.45 per cent hexosamine, and 0.06 per cent sialic acid or 3.76, 0.44, and 0.063 μmoles of hexose, hexosamine, and sialic acid, respectively. The presence of aspartic acid, glutamic acid, threonine, serine, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, and arginine were detected. Proline, cystine, and methionine were not found. The assay of hexosamine by the Elson-Morgan method for the material in radioactive areas 1 and 2 was 0.0208 and 0.0204 mg per ml of the supernatant fraction of urine. The total value in mg per ml of supernatant fraction of urine for the two areas was 0.0412 compared to 0.0430 (Table 7) as determined for the water eluate from Dowex-50.

CHAPTER V

DISCUSSION

Non-Dialyzable Hexosamine Containing Components Found in Human Urine

Following intravenous administration of the solution containing N-acetyl-D-glucosamine-1-C-14, most of the radioactivity excreted in the first 24 hours was in dialyzable molecules, identified as glucosamine, N-acetyl glucosamine and N-acetyl galactosamine. This suggests that glucosamine, N-acetyl glucosamine, and N-acetyl galactosamine are rapidly cleared from the blood. Also, Shetlar and co-workers (89) found the highest level of dialyzable radioactivity in the serum of these men to be lower than the highest level of bound radioactivity, even as early as 5 minutes after injection. As high as 30% of the injected dose of N-acetyl glucosamine-1-C-14 and glucosamine-1-C-14 was efficiently incorporated into serum proteins of the control subjects and the subject with active rheumatoid arthritis (89). These radioactive serum proteins were probably either degraded or excreted over a long period of time; some eventually may have been excreted in the urine. The radioactive non-dialyzable material excreted in the urine consisted of about 0.03% of the injected dose.

The excretion of non-dialyzable radioactive material was not as variable as that of the dialyzable material. No appreciable difference

was observed between two control subjects and a subject with active rheumatoid arthritis in this respect. The change in rate of excretion of the radioactivity in the non-dialyzable material was much less rapid than in the dialyzable material (Table 6). It is suggested that the non-dialyzable material contained radioactive serum proteins being excreted in the urine at a continuing rate. Furthermore, the level of bound radioactivity in serum was found to drop with time (91).

The radioactive dialyzable material was excreted in the urine of a control subject and the subject with active rheumatoid arthritis very rapidly. The highest excretion occurred during the first two hours in both cases, suggesting a very rapid clearance from the kidneys. On the other hand, the maximum excretion of radioactive non-dialyzable material occurred during the interval between 2 and 3 hours after injection. This slower rate of excretion suggests an incorporation of radioactive material into tissue, which is eventually sloughed and excreted, and/or not excreted as rapidly as dialyzable molecules.

Only radioactive glucosamine was recovered from the hydrolysates of non-dialyzable material. This compound may have been in the non-dialyzable material as either bound glucosamine or bound N-acetyl glucosamine, which yields glucosamine following vigorous hydrolytic procedures. However, the hydrolytic procedure used would probably destroy all sialic acid if it were present (61), and consequently the presence of radioactive sialic acid can not be excluded. No radioactive amino acids were recovered 6 hours after injection of the radioactive preparation. This indicates no appreciable metabolism of either glucosamine or N-acetyl glucosamine to yield bound amino acids was discernible by study of urine

excretion products.

Two types of gamma-globulin (101) are known to have bound N-acetyl glucosamine. The maximum radioactivity for serum proteins occurred 8 hours after injection (88) in two control subjects and the subject with active rheumatoid arthritis. Also, the radioactivity of the dialyzable material in the serum dropped sharply as the serum protein radioactivity increased, suggesting possible incorporation of N-acetyl glucosamine. The radioactivity of the serum proteins diminished after 8 hours (88) and this would not exclude the possibility of urinary excretion of serum proteins or metabolites derived from them.

The average percentage of bound hexosamine in the total non-dialyzable solids from the urine of a subject with active rheumatoid arthritis was 8.3% (Table 10). The average percentage of protein was 42.5%, or the protein to hexosamine ratio is 5.1 (Table 9). Hammerman and co-workers (49) obtained average values of 10% hexosamine and 50% protein in urinary colloids of normal subjects. Their ratio of protein to hexosamine was calculated to be 5.0. Their ratio was not considered to be greatly different from that of the subject with active rheumatoid arthritis. However, the amount of bound hexosamine found in the non-dialyzable fraction of urine from this subject was higher (Table 10). Hence, the urine of the subject with active rheumatoid arthritis contained a greater amount of non-dialyzable solids in comparison due to increased amounts of non-dialyzable hexosamine.

The data from radioactivity measurements and determinations involving the non-dialyzable material (Table 4) and protein (Table 9) suggested non-dialyzable material and/or protein were excreted with a high

specific radioactivity. Approximately four-fold greater specific radioactivity was obtained for hexosamine (Table 10, Fig. 4). Moreover, glucosamine was the only radioactive compound detected after hydrolysis of the non-dialyzable solids. This amino sugar may have been incorporated as an amino sugar, acetyl hexosamine, or N-sulfated hexosamine into glycoproteins, glycopeptide polymers, acid mucopolysaccharides, or neutral mucopolysaccharides.

In specimens collected within 8 hours after injection from the subject with active rheumatoid arthritis, the ratios of total hexosamine to total protein were 0.20 or higher (Table 10). However, in the Tamm-Horsfall mucoprotein there is 9.4% hexosamine and 72% protein (61), so this ratio is 0.13. Moreover, this subject with active rheumatoid arthritis might be expected to exhibit a marked increase of hexosamine in the non-dialyzable material since an increased excretion of non-dialyzable hexosamine compounds has been observed in inflammatory disease in which the level of protein-bound carbohydrates is increased (9). Also, additional hexosamine may have been hydrolyzed from bound N-acetyl glucosamine.

The Tamm-Horsfall carbohydrate-protein complex has been reported by Gottschalk (61) to contain 7.8% glucosamine and 1.6% galactosamine. The protein content of the non-dialyzable solids totaled 44.5% (Table 9). Thus, the non-dialyzable solids contained approximately 42.3% of protein other than that in the Tamm-Horsfall fraction. Nevertheless, the specific radioactivity of the Tamm-Horsfall preparation was very high (Table 11) for the control subject and the subject with active rheumatoid arthritis, suggesting a large contribution of radioactivity from this

mucoprotein. The band which did not migrate during electrophoresis was assumed to be the Tamm-Horsfall homogenous mucoprotein.

Tamm and Horsfall (65) and Boyce and Swanson (64) suggested the possible biosynthesis of two mucoproteins in the urinary tract of man. Either of these two glucosamine-containing mucosubstances might become radioactive after the injections of glucosamine-1-C-14. Another possible source of radioactivity in the non-dialyzable fraction is the serum proteins which may filter through the glomerulus and fail to be reabsorbed sufficiently by the proximal tubules (102). Some of the serum proteins are glycoproteins, and have been shown to be radioactive in rats and rabbits given radioactive glucosamine (34,103). Albumin was also radioactive, but this radioactivity was less than 3% of the total radioactivity in the serum proteins.

The data from serum proteins from humans (89) and sheep and urinary mucoprotein (a protein similar to Tamm-Horsfall mucoprotein) from sheep (104,105) were selected for comparison with the data in Table 11 and the graph in Figure 4, regarding urinary proteins and specific radioactivity. Mia and Cornelius (104) injected sheep intravenously with C-14 labeled glucosamine and glycine. Serum protein fractions and ovine urinary mucoprotein were isolated and assayed for radioactivity. According to Cornelius (105) both the polysaccharide groups and peptide chain of ovine urinary mucoprotein became radioactive after injection. The specific activity of this mucoprotein reached a maximum at about 1 hour with glucosamine-1-C-14 and at 5 hours with glycine-2-C-14. However, the serum proteins of these sheep attained maximum radioactivity at approximately 8 hours after injection with both radioactive materials. It

appeared from their data that the polysaccharide groups were formed more rapidly and evidently were attached to preformed peptide chains, which are synthesized at a slower rate (105). Cornelius (105) concluded that once the mucoprotein is formed, it is excreted without any exchange or degradation of its polysaccharide groups. A maximum specific radioactivity of hexosamine and protein (Fig. 4) was obtained between 3 and 4 hours after injection of the isotope solution containing a small amount of D-glucosamine-1-C-14. As previously mentioned, the findings of Shetlar and co-workers (89) indicate the incorporation in vivo of radioactive N-acetyl glucosamine and glucosamine into serum proteins of the three men. The radioactivity in these proteins attained a maximum 8 hours after injection and then gradually disappeared from the blood stream. Only about 5% of the total radioactivity excreted in the urine 24 hours after injection of mainly N-acetyl-D-glucosamine-1-C-14 was in the Tamm-Horsfall fraction (Table 11), while the remaining major portion of the radioactivity was in the salt soluble fraction.

The Tamm-Horsfall preparation contained other material, failing to stain with bromphenol blue and migrating towards the anode, which may have been an acid mucopolysaccharide, containing hydrolyzable acetyl hexosamine. The urinary acid mucopolysaccharide as isolated by Di Ferrante and co-workers (78) contained 31.7% hexosamine. A neutral mucopolysaccharide, as King and Hyder's glucosamine and galactose conjugate (80), merits some attention. This fraction would probably migrate not far from the origin in paper electrophoresis under the particular conditions of pH employed and possibly would not stain with bromphenol blue. However, the solubility of these two mucopolysaccharides in 0.58 M

sodium chloride is not available.

The fraction of urine soluble in 0.58 M sodium chloride had a higher percentage of protein than the Tamm-Horsfall fraction. Paper electrophoresis of this salt soluble fraction demonstrated four radioactive protein-containing materials, one remaining at the origin and the others migrating less than albumin. A neutral mucopolysaccharide would probably migrate either not far from the origin or in the opposite direction from albumin. A radioactive fraction migrating beyond albumin was detected in paper electrophoresis. The acid mucopolysaccharides of urine migrate beyond albumin according to Di Ferrante and co-workers (78). It is suggested that the urinary proteins are derived from plasma proteins and constitute a large portion of the radioactive non-dialyzable material. This suggestion would be in agreement with the findings of Boyce and co-workers (58) indicating urinary protein contains all of the components normally found in serum by electrophoresis.

Hexosamine and Hexosamine-Containing Components in the Dialyzable Fraction of Human Urine

Radioactive mannosamine was not found to be present in the dialyzable fraction of urine from a control subject or a subject with active rheumatoid arthritis. Radioactive galactosamine was found to be excreted in very small amounts in the latter subject. In comparing ratios of radioactive glucosamine to radioactive galactosamine, no appreciable difference was observed between these hexosamines present in the injection solution and the urine specimens (page 57). Since glucosamine was excreted nearly 3 times faster than N-acetyl glucosamine in the subject with active rheumatoid arthritis, this suggests variations in

transport mechanisms of tubular secretion or tubular reabsorption.

The amount of dialyzable free hexosamine in urine from a control subject did not differ appreciably from that of a subject with active rheumatoid arthritis. However, the percentage excretion of free hexosamine in the total dialyzable hexosamine was only 0.6 as great (28.5% ÷ 51.0%) from this subject than it was in the control subject. The 2.4:3 ratio of the dialyzable free hexosamine to non-dialyzable hexosamine from the subject with active rheumatoid arthritis is in contrast to the value of 1:3 reported by Marcotte-Boy and co-workers (53). They employed urease and Dowex-50 separation on unhydrolyzed dialyzable material, but the analysis of the water eluate was not reported. Only hexosamines were assumed to be present in their acid eluate and neither acetyl hexosamines nor conjugated hexosamines would have been included as dialyzable hexosamines. On the other hand, Hammerman and Hatch (50) obtained a 3:1 ratio of dialyzable to non-dialyzable hexosamine by an hydrolytic method (Table 12) on the entire dialyzable fraction. They apparently reported a value including free, acetylated and conjugated dialyzable hexosamine material. If the data on the subject with active rheumatoid arthritis were calculated according to the experimental procedure of Hammerman and Hatch, the ratio would be 2.4:1 (Table 12), and agrees favorably with that obtained by these investigators. The urine had a somewhat greater "hexosamine" content than average male adults.

Radioactive N-acetyl hexosamine was isolated from the dialyzable fraction of urine from a control subject, while N-acetyl-D-glucosamine-1-C-14 and radioactive N-acetyl galactosamine were isolated from a subject with active rheumatoid arthritis. The galactosamine isomer appeared as

early as 2 hours after intravenous injection and appeared in increasing amounts as compared with its glucosamine isomer. There are at least two interconversions between N-acetyl glucosamine and N-acetyl galactosamine (42). The radioactivity values of dialyzable N-acetyl galactosamine were very high compared with those of the non-dialyzable material. Moreover, enzyme systems which liberate and decompose N-acetyl galactosamine are present in animal tissues (9). Two dialyzable radioactive materials were isolated from the urine specimen collected between 4 and 6 hours after injection from the subject with active rheumatoid arthritis (Fig. 6). It is conceivable that these materials may contain units of bound glucosamine or N-acetyl glucosamine derived from free glucosamine or N-acetyl glucosamine which were present in decreasing amounts at least 2 hours after injection.

It is suggested that the term "N-acetyl hexosamine" (Table 7) includes N-acetyl glucosamine, N-acetyl galactosamine and only a trace amount of N-acetyl mannosamine since these isomers were identified as radioactive urinary excretion products from the urine of a subject with active rheumatoid arthritis. This subject was injected with N-acetyl-D-glucosamine-1-C-14 and D-glucosamine-1-C-14 and the three N-acetyl isomers were isolated. The maximum excretion of N-acetyl hexosamine per hour occurred during the interval between 2 and 3 hours after injection. This does correspond to the maximum excretion of radioactivity in the non-dialyzable fraction (Table 4), although no correlation is suggested.

Consistent values were obtained for urinary N-acetyl hexosamine when expressed as a percentage of the dialyzable hexosamine for a control subject and a subject with active rheumatoid arthritis (Table 7). The

remaining free hexosamine and conjugate hexosamine in the total dialyzable hexosamine was compared by an appropriate ratio (Table 14). Ratios of acetyl hexosamine were 54% to 133% higher for a subject with active rheumatoid arthritis than for a control subject; there was much less free hexosamine in the urine of the former subject. The converse of this relation would apply for the ratio of acetyl hexosamine to conjugated hexosamine.

The water eluate from Dowex-50 as fractionated from the dialyzable fraction contained material from two radioactive areas with low mobility on paper chromatograms (Fig. 6, areas 1 and 2). Each material was either neutral or acidic since each was eluted from the hydrogen form of Dowex-50 in the water fraction. The mobility of the radioactive material from area 1 in high voltage electrophoresis, was similar to that of other neutral compounds such as glucose, N-acetyl glucosamine, and the monobasic amino acids. However, the mobility of the radioactive material from area 2 was similar to that of acidic compounds as aspartic acid and sialic acid. The hexosamine analysis for material in these areas compared favorable with that of the water eluate which would contain this material as fractionated from Dowex-50.

The hexosamine conjugate analysis for 24-hour specimens indicated less variation between the total dialyzable hexosamine and the total dialyzable material. A more meaningful comparison is suggested for hexosamine conjugate and total dialyzable hexosamine. The percentage of N-acetyl hexosamine in total dialyzable hexosamine was fairly constant (Table 7), and suggested a fairly constant relation for the sums of conjugated and free hexosamine. Ratios of conjugated and free hexosamine

to total hexosamine dialyzable material were about 0.81 for a control subject and a subject with active rheumatoid arthritis (Table 14). The ratios of conjugated to free hexosamine were 190% to 360% higher in the subject with active rheumatoid arthritis than in a control subject. This apparent correlation might have a diagnostic significance in health and disease. Assuming a relation does exist between free hexosamine and the conjugates, it could be postulated that (a) the conjugates could be degraded to increase the free hexosamine in the mammalian body operating in conditions of health, and (b) the degradation of the conjugates could be inhibited in certain pathological conditions. Still another consideration involves the postulation that one or more hexosamine-containing polymers could be degraded by cleavage of a bond to release the conjugates in increasing amounts in certain pathological conditions. On the other hand, this degradation system could function in various organisms but not be related markedly to disease states.

Some chemical and physical characteristics of the two dialyzable hexosamine conjugates have been ascertained by their behavior during chromatographic treatments and by their assay on chemical composition. The conjugates contained in radioactive areas 1 and 2 are non basic, water soluble materials since they were eluted from the hydrogen form of Dowex-50 in the water fraction. The conjugate contained in radioactive area 1 is essentially neutral at pH 3.7 while the conjugate contained in radioactive area 2 is acidic as established by migration resulting from high voltage electrophoresis. Only radioactive glucosamine and/or N-acetyl glucosamine is contained in both conjugates since radioactive glucosamine was the only radioactive hexosamine obtained after their hydrolysis in acid. Other naturally occurring material identical

in chemical composition but which is non-radioactive is likely present. The glucosamine is apparently substituted or conjugated on the nitrogen atom and conjugated with other molecular units since hydrolysis was a prerequisite step before reaction with the Elson-Morgan reagents. The substitution might involve acetyl or sulfate groups. The conjugation of the glucosamine with other molecular units might include an amide linkage between the carbonyl group of aspartic acid and the amino group of the glucosamine or an ester linkage between either aspartic acid or glutamic acid with an hydroxyl group of glucosamine (14). The bound hexosamine contained in the material eluted from both radioactive areas was high in comparison to the bound sialic acid. The molar ratio of sialic acid to hexosamine for material eluted from radioactive areas 1 and 2 was 0.09 and 0.14, respectively.

The value for the bound hexose contained in the material eluted from radioactive areas 1 and 2 were high in terms of the content of hexosamine and sialic acid. The molar ratio of hexose to hexosamine was 5.1 and 8.6 while that of hexose to sialic acid was 58 and 60 for material eluted from radioactive areas 1 and 2, respectively. This may have been due to some inadvertent elution of cellulose material from the chromatography paper. The hexose to hexose linkage is well known. It is suggested also that hexose to protein linkage contained in either the neutral or the acidic hexosamine conjugate could be an O-glycosidic bond between the hemiacetal hydroxyl group of a hexose residue and the hydroxyl group of serine or threonine (13).

The data indicate about one-fourth of the conjugated hexosamine excreted in the urine of a subject with active rheumatoid arthritis is

contained in a more neutral conjugated material of low molecular weight. This conjugated hexosamine is glucosamine and/or N-acetyl glucosamine. The neutral conjugated material also contains bound components of hexose, sialic acid, aspartic acid, glutamic acid, threonine, serine, glycine, alanine, valine, isoleucine, leucine, lysine, and histidine.

The data also indicate about one-fourth of the conjugated hexosamine excreted in the urine of a subject with active rheumatoid arthritis is contained in acidic conjugated material of low molecular weight. This conjugated hexosamine is glucosamine and/or N-acetyl glucosamine. This acidic conjugated material also contains bound components of hexose, sialic acid, and the same amino acids as contained in the more neutral conjugated material plus the additional amino acids of tyrosine, phenylalanine, and arginine.

CHAPTER VI

SUMMARY

An exploratory study was done on the radioactive urinary products of three human males who were intravenously injected with N-acetyl-D-glucosamine-1-C-14.

The urinary excretion of radioactive dialyzable and non-dialyzable material was measured for three men, one of whom had active rheumatoid arthritis. Of the total radioactivity excreted during the first 24 hours, 96.0% was in the dialyzable fraction. The excretion of radioactive dialyzable and non-dialyzable material occurred mostly in the first 24 hours, but the change in the excretion rate of non-dialyzable material was much less rapid as compared with the dialyzable material.

N-acetyl-D-glucosamine-1-C-14 and D-glucosamine-1-C-14 were incorporated into macromolecules *in vivo*, excreted via the urinary tract, and found in the non-dialyzable fraction from centrifuged urine. On hydrolysis, radioactive glucosamine was identified in non-dialyzable material.

Radioactive glucosamine and lesser amounts of radioactive galactosamine were isolated from the dialyzable fraction of urine from a control subject and a subject with active rheumatoid arthritis. Labeled mannosamine was not present. The excretion of glucosamine was 2.7 times greater than N-acetyl glucosamine.

No radioactive amino acids were isolated from either the dialyzable or non-dialyzable urine fraction.

The specific radioactivity of the non-dialyzable material and its protein and bound hexosamine components was maximum before that of the serum proteins. Biosynthesis of non-dialyzable material in the human urinary system is suggested. Radioactive material isolated from 24-hour specimens according to the procedure of Tamm-Horsfall for urinary mucoprotein was obtained in low yields, with the radioactivity varying between 3% and 8% of the total non-dialyzable solids.

Radioactive, non-dialyzable material was isolated from the 0.58 M sodium chloride soluble fraction of centrifuged urine.

The method of Marcotte-Boy, utilizing Dowex-50 for the urinary dialyzable fraction, was applied to the study of the N-acetyl hexosamine and N-substituted hexosamine conjugated material found in human urine. Radioactive N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, and a trace amount of N-acetyl mannosamine were isolated in the water eluate from Dowex-50 and identified by the two dimensional technique of high voltage electrophoresis-paper chromatography. N-acetyl-D-galactosamine was found in the urine of a subject with active rheumatoid arthritis. Two radioactive materials exhibiting approximately as much radioactivity as N-acetyl-D-glucosamine-1-C-14, were isolated and harvested from the same Dowex-50 fraction of the dialyzable fraction of human urine by using an elution technique on chromatography paper.

The average urinary excretion of dialyzable N-acetyl hexosamine over a 24-hour period was 10.4 and 19.7 mg in two of the subjects. The average concentration of N-acetyl hexosamine in these urines was 11.5

and 10.3 mg per liter of centrifuged urine. N-acetyl hexosamine in the dialyzable fraction represented 17.3 to 19.9% of the total dialyzable hexosamine.

The average hexosamine excreted as the dialyzable conjugated material was 19.0 and 48.7 mg per 24-hour period for a control subject and a subject with active rheumatoid arthritis. The average concentration of hexosamine in the conjugated material was 21 and 26 mg per liter of centrifuged urine. The conjugated hexosamine represented 31.7 and 50.8% of the total dialyzable hexosamine.

The sum of the percentages of free and conjugated hexosamine in urine from four 24-hour samples of a control subject and a subject with active rheumatoid arthritis was fairly constant at 80.1 to 82.9% of the total dialyzable hexosamine. The ratio of free hexosamine in total dialyzable hexosamine in the urine of the subject with active rheumatoid arthritis to that of a control subject was 0.6, whereas that for conjugated hexosamine was 1.6.

Two materials containing radioactive glucosamine were isolated using the dialyzable urine fraction from a subject with active rheumatoid arthritis. Bound components of amino acids, hexose, and sialic acid were also found in each material. One material was water soluble and moved in an electric field towards the cathode at pH 3.7. The other material was also water soluble but moved in an electric field towards the anode at pH 3.7.

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APPENDIX A

TABLE 1

RADIOACTIVITY OF URINE FRACTIONS FROM A SUBJECT WITH ACTIVE RHEUMATOID ARTHRITIS (Da)

Specimen	cpm x 10^{-3} per hour[a]									
	0-2[b]	2-3	3-4	4-6	6-8	8-12	12-24	0-24[c]	24-48	48-72
Total Supernatant I	6,330	3,220	911	850	167	127	23.2	817	33.5	20.2
Dialyzable Material	6,400	3,460	1,030	590	102	78.3	13.0	798	12.8	12.4
Non-Dialyzable Material	17.4	50.3	16.9	24.8	2.3	9.8	2.5	9.4	4.3	1.6
Sediment, R-4[d] (after washings)	1.34	1.41	0.60	1.76	0.38	----	----	---	----	----
Washings										
Supernatant R-1, saline	2.67	1.41	0.60	3.52	0.76	----	----	---	----	----
Supernatant R-2, 50% ethanol	0.99	0.03	0.00	0.10	0.00	----	----	---	----	----
Supernatant R-3, 95% ethanol	----------		0.54	----------		----	----	---	----	----

[a]Cpm per ml of supernatant fraction was multiplied by ml per specimen times 10^{-3} and divided by the number of hours and represents total counts per hour during the collection.

[b]Urine specimen collected in the interval between 0 and 2 hr after isotope injection.

[c]Values for 0-24 hr period only are calculated.

[d]See flow sheet (Fig. 1) for complete explanation of fractions.

TABLE 2

RADIOACTIVITY OF SEDIMENT FROM CENTRIFUGATION OF URINE FROM A SUBJECT WITH ACTIVE RHEUMATOID ARTHRITIS (Da)

Specimen	0-2[a]	2-3	3-4	4-6	6-8
ml of Supernatant Fraction	188	71.5	32.0	82.5	90.0
Sediment, R-4[b]					
mg dry weight	80.0	32.5	7.5	38.0	9.0
cpm per mg dry weight	33.4	43.4	80.1	92.7	84.4
cpm per ml supernatant fraction	14.2	19.7	18.8	42.7	8.4
Washings (cpm per ml supernatant fraction)					
Supernatant R-1, saline	11.0	11.0	5.6	2.74	5.9
Supernatant R-2, 50% ethanol	5.35	0.34	0.04	1.00	0.03
Supernatant R-3, 95% ethanol	----------		1.17	----------	

[a]Urine specimen collected in the interval between 0 and 2 hr after isotope injection.

[b]See flow sheet (Fig. 1) for complete explanation of fractions.

TABLE 3

SELF ABSORPTION OF RADIOACTIVITY OF N-ACETYL-D-GLUCOSAMINE-1-C-14 BY SUPERNATANT AND DIALYZABLE FRACTIONS OF HUMAN URINE IN THE GAS-FLOW COUNTING SYSTEM

N-Acetyl-D-Glucosamine-1-C-14 cpm per sample	7950	8060	8920	8620	8850	8840	8710	8780	8640	8180	8360
ml of Supernatant Fraction	0.1	0.2	0.3	0.5	0.7	0.9	1.0	1.3	1.5	1.7	2.0
NAcGN-1-C-14[a] + Fr0-24[b]											
mg dry weight	6.04	11.88	17.68	29.01	41.25	53.75	60.23	78.45	91.0	102.0	120.8
cpm per sample	4460	3780	3370	2860	2380	2190	2130	1790	1780	1560	1340
Fractional self-absorption factor	0.552	0.443	0.377	0.311	0.269	0.247	0.245	0.204	0.205	0.193	0.163
N-Acetyl-D-Glucosamine-1-C-14 cpm per sample	54,520	55,950	56,400	56,700	57,300	57,800	58,100	58,050	56,900		
ml of Dialyzable Material[c]	0.2	0.4	0.5	0.7	0.9	1.0	1.3	1.7	2.5		
NAcGN-1-C-14 + Fr0-24[c]											
mg dry weight	9.98	19.31	24.12	33.65	40.82	47.84	59.81	82.0	118.1		
cpm per sample	31,950	25,850	12,600	10,400	20,700	17,200	16,300	13,300	11,800		
Fractional self-absorption factor	0.580	0.465	0.452	0.359	0.361	0.298	0.280	0.229	0.207		

[a] N-Acetyl-D-Glucosamine-1-C-14.

[b] Fraction of 24-hr urine specimen from control subject (Fr) who did not receive the injected isotope.

[c] Urinary aliquots from a preparation containing 1:1 ratio of dialyzable material from urine (Fr).

TABLE 4

RADIOACTIVITY OF SUPERNATANT, DIALYZABLE, AND NON-DIALYZABLE FRACTIONS OF URINE FROM A SUBJECT WITH ACTIVE RHEUMATOID ARTHRITIS (Da)

Specimen	0-2[a]	2-3	3-4	4-6	6-8	8-12	12-24	0-24[b]	24-48	48-72
ml Supernatant Fraction	188	71.5	32.0	82.5	90.0	693	1025	2182	1740	2090
Supernatant Material										
mg/ml supernatant fraction	24.3	26.6	31.4	41.6	33.8	15.8	8.9	----	26.2	25.9
cpm per ml supernatant fraction	67,320	44,970	28,470	20,560	3,720	734	272	----	462	232
Dialyzable Material										
mg/ml supernatant fraction	23.9	26.5	31.3	40.9	32.4	15.2	8.5	----	23.9	23.8
cpm per ml supernatant fraction	68,060	48,380	32,180	14,290	2,270	452	152	----	176	142
excreted, cpm x 10^{-3}	12,800	3,460	1,030	1,180	204	313	156	19,140	306	297
Non-Dialyzable Material										
mg dry weight	26.3	20.5	5.70	30.0	5.67	129	129	347	676	441
mg/ml supernatant fraction	0.14	0.29	0.18	0.36	0.06	0.20	0.13	----	0.50	0.20
cpm per ml supernatant fraction	185	704	527	601	50.9	56.2	29.5	----	58.9	18.2
excreted, cpm x 10^{-3}	34.8	50.3	16.9	49.6	4.58	39.0	30.2	225	102	38.0
cpm x 10^{-3} per mg dry weight	1.32	2.46	2.96	1.65	0.80	0.30	0.23	0.65	0.15	0.086

[a]Urine specimen collected in the interval between 0 and 2 hr after isotope injection.

[b]Values for 0-24 hr period only are calculated.

TABLE 5

RADIOACTIVITY OF DIALYZABLE AND NON-DIALYZABLE FRACTIONS OF URINE FROM CONTROL SUBJECTS

Specimen	0-1[a]	1-2	2-3	3-4	4-6	6-8	8-12	12-24	0-24[b]	24-48	48-72	72-96
Bo, ml Supernatant Fraction	44	30.8	96	68	77	68	89	470	973			
Dialyzable Material												
cpm per ml supernatant fraction	46,540	2,670	2,200	1,390	750	2,250	2,120	1,032	---			
excreted, cpm x 10^{-3}	2,050	82.2	212	94.4	57.8	153	188	485	3,320			
Non-Dialyzable Material												
cpm per ml supernatant fraction	838	726	735	408	54.6	125	140	106	---			
excreted, cpm x 10^{-3}	36.8	22.4	70.6	27.7	4.2	8.5	12.4	49.6	232			
Wi, ml Supernatant Fraction									885[c]	1285	820	1000
Dialyzable Material												
cpm per ml supernatant fraction									26,700	756	571	344
excreted, cpm x 10^{-3}									23,630	971	468	344
Non-Dialyzable Material												
cpm per ml supernatant fraction									1,220	204	175	117
excreted, cpm x 10^{-3}									1,080	262	144	117

[a] Urine specimen collected in the interval between 0 and 1 hr after isotope injection.
[b] Values for 0-24 hr period only are calculated.
[c] Values for 0-24 hr period and subsequent intervals are measured.

TABLE 6

RADIOACTIVITY EXCRETED IN DIALYZABLE AND NON-DIALYZABLE FRACTIONS OF URINE

Specimen	Dialyzable				Non-Dialyzable			
	0-24	24-48	48-72	72-96	0-24	24-48	48-72	72-96
Excretion, cpm x 10^{-3}								
Wi[a], 200 microcuries	23,630	971	468	344	1,080	262	144	117
Bo[a], 100 microcuries	3,320	---	---	---	232	---	---	---
Da[b], 100 microcuries	19,140	306	297	---	225	102	38.0	---
Percentage of total radioactivity excreted								
Wi	95.6	78.8	76.6	74.5	4.4	21.2	23.4	25.5
Bo	93.5	---	---	---	6.5	---	---	---
Da	98.8	74.9	88.7	---	1.2	25.1	11.3	---
Excretion Rate cpm x 10^{-3} per hr								
Wi	985	40.5	19.5	14.3	45.0	10.9	6.0	4.9
Bo	138	---	---	---	10.0	---	---	---
Da	798	12.8	12.4	---	9.4	4.3	1.6	---

[a] Control subjects

[b] Subject with rheumatoid arthritis

TABLE 7

URINARY HEXOSAMINE CONTENT OF DIALYZABLE MATERIAL FROM A SUBJECT WITH ACTIVE RHEUMATOID ARTHRITIS (Da) AND A CONTROL SUBJECT (Fr)

Specimen	Da 0-2[a]	2-3	3-4	4-6	6-8	8-12	12-24	0-24[b]	24-48	48-72	Fr 0-24
ml Supernatant Fraction	188	71.5	32.1	82.5	90.0	693	1025	2184	1740	2090	905
Hexosamine, mg/ml supernatant fraction[c]	0.0061	.0182	.0172	.0528	.0169	.0085	.0071	.0101	.0286	.0114	0.0338
mg excreted	1.15	1.30	0.55	4.35	1.52	5.88	7.28	22.0	49.7	23.8	30.6
mg excreted/hr	0.58	1.30	0.55	2.18	0.76	1.47	0.61	0.92	2.07	0.99	1.27
% of total dialyzable material	0.025	0.069	0.055	0.129	0.352	0.056	0.083	0.067	0.119	0.048	----
% of total dialyzable hexosamine	17.0	27.9	21.5	42.1	31.1	15.3	33.8	24.7	36.9	28.5	51.0
N-Acetyl hexosamine, mg/ml supernatant fraction	0.0097	.0189	.0213	.0296	.0180	.0075	.0043	.0080	.0153	.0072	0.0138
mg excreted	1.83	1.35	0.68	2.44	1.62	5.19	4.42	17.5	26.7	15.0	10.4
mg excreted/hr	0.92	1.35	0.68	1.22	0.81	1.30	0.37	0.73	1.11	0.62	0.43
% of total dialyzable material	0.041	0.071	0.068	0.073	0.056	0.049	0.051	0.053	0.064	0.030	----
% of total dialyzable hexosamine	27.3	28.9	26.7	23.6	33.2	13.5	20.5	19.7	19.9	17.9	17.4

TABLE 7 CONTINUED ON PAGE 98

TABLE 7--Continued

Specimen	0-2[a]	2-3	3-4	4-6	6-8	8-12	12-24	0-24[b]	24-48	48-72	Fr 0-24
					Da						
	ml										
Supernatant Fraction	188	71.5	32.1	82.5	90.0	693	1025	2184	1740	2090	905
Conjugated hexosamine, mg/ml supernatant fraction	0.0199	.0283	.0413	0.430	.0194	.0396	.0096	.0227	.0333	.0215	0.0207
mg excreted	3.74	2.02	1.32	3.55	1.75	27.5	9.82	49.7	57.9	44.9	19.0
mg excreted/hr	1.87	2.02	1.32	1.77	0.87	6.86	0.82	2.02	2.41	1.87	0.79
% of total dialyzable material	0.083	0.107	0.132	0.105	0.060	0.261	0.112	0.053	0.139	0.090	----
% of total dialyzable hexosamine	55.7	43.2	51.8	34.3	35.7	71.3	45.6	55.7	43.1	53.6	31.7
Total mg dialyzable hexosamine excreted	6.72	4.68	2.55	10.3	4.89	38.5	21.5	89.2	134	83.6	59.9
ratio of dialyzable to non-dialyzable	2.5	2.2	4.3	3.0	8.4	4.7	2.8	3.5	2.1	2.3	----

[a]Urine specimen collected in the interval between 0 and 2 hr after isotope injection.

[b]Values for 0-24 hr period only are calculated.

TABLE 8

RADIOACTIVITY OF HEXOSAMINES ISOLATED BY HIGH VOLTAGE ELECTROPHORESIS-PAPER CHROMATOGRAPHY FROM HUMAN URINE AND RADIOACTIVE N-ACETYL-D-GLUCOSAMINE-1-C-14

Specimen	0-2[a]	2-3	3-4	Da[b] 4-6	6-8	8-12	12-24	24-48	48-72	Wi[c] 0-24	De-vel-oped[d] NAcGN[e]	Un-de-vel-oped NAcGN[e]
Electrophoresis-Chromatography												
Hexosamine, cpm	1270	799	253	144	24.0	4.6	0.8	1.9	1.1	374	624	1320
Acetyl hexosamine, cpm	3380	1683	809	282	44.9	1.7	4.8	5.8	2.0	641	3230	7210
Acetyl hexosamine/ hexosamine	2.65	2.11	3.19	1.96	1.86	---	---	---	---	1.71	5.21	5.47
Chromatography												
"Hexosamine"[f], cpm	332	302	146	168	24.7	6.0	2.2	5.1	2.6	105	---	---
Hexosamine, cpm	1049	736	221	212	20.6	7.1	6.3	-0.4	3.9	209	---	---
Acetyl hexosamine, cpm	2480	1255	554	262	50.1	9.5	7.1	7.4	0.9	354	---	---
Acetyl hexosamine/ "hexosamine"	7.47	4.15	3.79	1.55	2.03	---	---	---	---	3.36	---	---
Acetyl hexosamine/ hexosamine	2.36	1.71	2.50	1.24	2.43	---	---	---	---	1.69	---	---

[a] Urine specimen collected in the interval between 0 and 2 hr after isotope injection.
[b] Subject with active rheumatoid arthritis.
[c] Control subject.
[d] Areas located by the Elson-Morgan reaction (83).
[e] Radioactive N-acetyl-D-glucosamine.
[f] "Hexosamine" unidentified Elson-Morgan positive spot.

TABLE 9

PROTEIN ANALYSIS OF URINE FROM A SUBJECT WITH ACTIVE RHEUMATOID ARTHRITIS (Da)
BY THE LOWRY METHOD AND THE CALCULATED NON-PROTEIN VALUES

Specimen	0-2[a]	2-3	3-4	4-6	6-8	8-12	12-24	0-24[b]	24-48	48-72
ml Supernatant Fraction	188	71.5	32.0	82.5	90.0	693	1025	2182	1740	2090
Protein mg/ml supernatant fraction	0.0696	.1420	.0980	.0180	.0297	.0790	.0494	.0068	.0173	0.0843
mg excreted	13.1	10.2	3.13	14.9	2.67	54.7	50.7	149	300	176
mg excreted/hr	6.5	10.2	3.13	7.43	1.34	10.6	3.27	6.22	12.5	7.3
% of non-dialyzable material	49.7	49.8	55.0	49.6	47.1	42.3	39.2	43.1	44.5	39.9
cpm x 10^{-3} (non-dialyzable per mg protein	2.66	4.93	5.38	3.34	1.72	0.92	0.77	1.51	0.34	0.22
mg protein/mg non-dialyzable hexosamine	4.76	4.83	5.10	4.37	4.55	6.75	6.79	5.95	4.79	4.88

[a] Urine specimen collected in the interval between 0 and 2 hr after isotope injection.

[b] Values for 0-24 hr period only are calculated.

TABLE 10

URINARY HEXOSAMINE CONTENT OF NON-DIALYZABLE MATERIAL IN URINE FROM A SUBJECT WITH ACTIVE RHEUMATOID ARTHRITIS (Da)

Specimen	0-2[a]	2-3	3-4	4-6	6-8	8-12	12-24	0-24[b]	24-48	48-72
ml Supernatant Fraction	188	71.5	32.0	82.5	90.0	693	1025	2182	1740	2090
Hexosamine, non-dialyzable										
mg/ml supernatant fraction	0.0146	.0295	.0192	.0412	.0065	.0117	.0076	.0116	.0361	0.0173
mg excreted	2.74	2.15	0.60	3.40	0.58	8.11	7.81	25.3	62.9	36.1
mg excreted/hr	1.37	2.15	0.60	1.70	0.29	2.03	0.65	1.05	2.62	1.50
% total of non-dialyzable solids	10.4	10.3	10.8	11.3	10.3	6.27	5.90	7.30	9.29	8.17
cpm x 10^{-3} (non-dialyzable) per mg hexosamine	12.8	23.9	28	15	7.8	4.62	3.87	8.91	1.63	1.06
mg non-dialyzable hexosamine/ mg protein	0.210	.207	.196	.229	.219	.148	.154	.169	.209	0.205

[a]Urine specimen collected in the interval between 0 and 2 hr after isotope injection.

[b]Values for 0-24 hr period only are calculated.

TABLE 11

FRACTIONATION OF SAMPLES FROM 24-HOUR SPECIMENS OF URINE
BY THE METHOD OF TAMM AND HORSFALL

	Material Insoluble in 0.58 M NaCl			Material Soluble in 0.58 M NaCl		
	Da24-48[a]	Wi0-24[b]	Wi24-48[b]	Da24-48[a]	Wi0-24[b]	Wi24-48[b]
mg dry solids	3	≪1[c]	<1[c]	103	62	72.6
mg dry solids/liter	15	≪5[c]	<5	515	310	363
cpm per 200 ml sample	272	1,150	1,550	5,845	36,260	15,470
cpm per mg dry solids	90.7	≫1,150[c]	>1,550[c]	56.8	585	213
cpm per ml supernatant fraction	1.36	5.8	7.3	29.2	181	77.4
Total activity, cpm	2,370	5,090	9,316	50,854	160,400	99,400
% total activity	4.5	3.3	8.5	95.5	96.7	91.5

Band	Electrophoretic Mobility[d]	Radioactivity by Liquid Scintillation Da24-48[f]	Electrophoretic Mobility[d]	Radioactivity by Liquid Scintillation			
				Da24-48[f]	UFR[e] 0-24[f]	0-24[f]	24-48[g]
1	-	0-	gamma-globulin	6-	6	3	3
2	Origin	22+	origin	9+	8	21+	7+
3	-	23-	beta-	41+	6	10	12+
4	-	14-	$alpha_2$-	37+	5	23+	9
5	-		$alpha_1$-	31++	10	16	11
6	albumin	-	albumin	0+	19	71	30
7	-	6-	-	8-	-	-	-

[a]Urine specimen from a subject with active rheumatoid arthritis.
[b]Urine specimen from a control subject.
[c]Estimated values.
[d]Based on mobility of components of added serum.
[e]UFR-residue after ultrafiltration.
[f]B-2 buffer
[g]LKB buffer
[+]Stained with bromphenol blue, in separate experiments without serum carrier.
[-]No reaction with bromphenol blue, in separate experiments without serum carrier.

TABLE 12

COMPARISON OF URINARY HEXOSAMINE VALUES REPORTED BY DIFFERENT INVESTIGATORS IN DIALYZABLE AND NON-DIALYZABLE FRACTIONS FROM HUMAN URINE

	Male				Female			
	Dialyzable mg/24 hr	Non-dialyzable mg/24 hr	Total mg/24 hr	D:ND[a]	Dialyzable mg/24 hr	Non-dialyzable mg/24 hr	Total mg/24 hr	D:ND[a]
Boas (52)								
Normal, 8 subjects					40	40	80	1:1
Pregnant, 8 patients					80	80	160	1:1
Hammerman & Hatch (50)								
Normal, 8 subjects	87	28.9	---	3:1	----	----	----	---
Marcotte-Boy et al. (53)								
Normal (9 male, 8 female)	17.6	62	81	1:3	12.8	52.6	66.8	1:4
This Investigation								
Da (average of 3)[b]								
N-acetyl hexosamine	19.7							
Hexosamine	31.8							
Conjugated hexosamine	48.7							
Total	100.7	41.4	151	2.4:1				
Fr[c]								
N-acetyl hexosamine	10.4							
Hexosamine	30.6							
Conjugated hexosamine	19.0							
Total	60.0	----	---	---				

[a]Ratio of dialyzable to non-dialyzable material.
[b]Subject with active rheumatoid arthritis.
[c]Control subject

TABLE 13

RATES OF EXCRETION OF DIALYZABLE AND NON-DIALYZABLE HEXOSAMINE IN THE URINE FROM A SUBJECT WITH ACTIVE RHEUMATOID ARTHRITIS (Da) AND A CONTROL SUBJECT (Fr)

Specimen	Da 0-2[a]	2-3	3-4	4-6	6-8	8-12	12-24	0-24[b]	24-48	48-72	Fr 0-24
mg hexosamine excreted/hr	0.57	1.30	0.55	2.17	0.76	1.47	0.61	0.92	2.07	0.99	1.27
mg acetyl hexosamine excreted/hr	0.92	1.35	0.68	1.22	0.81	1.30	0.37	0.73	1.11	0.62	0.43
mg conjugated hexosamine excreted/hr	1.87	2.02	1.32	1.78	0.87	6.86	0.82	2.02	2.41	1.87	0.79
mg dialyzable hexosamine excreted/hr	3.36	4.68	2.55	5.17	2.44	9.63	1.79	3.72	5.59	3.49	2.50
mg non-dialyzable hexosamine excreted/hr	1.37	2.15	0.60	1.70	0.29	2.03	0.65	1.05	2.62	1.50	----

[a]Urine specimen collected in the interval between 0 and 2 hr after isotope injection.

[b]Values for 0-24 hr period only are calculated.

TABLE 14

RATIOS OF DIALYZABLE HEXOSAMINE COMPONENTS OF URINE FROM A SUBJECT WITH ACTIVE RHEUMATOID ARTHRITIS (Da) AND A CONTROL SUBJECT (Fr)

Specimen	Da 0-2[a]	2-3	3-4	3-6	6-8	8-12	12-24	0-24[b]	24-48	48-72	Fr 0-24
N-acetyl hexosamine/hexosamine	1.60	1.04	1.24	0.56	1.07	0.88	0.59	0.80	0.54	0.52	0.34
N-acetyl hexosamine/conjugate	0.49	0.67	0.51	0.69	0.93	0.19	0.45	0.36	0.46	0.33	0.55
N-acetyl hexosamine/total dialyzable hexosamine	0.27	0.29	0.27	0.24	0.33	0.13	0.21	0.20	0.20	0.18	0.17
Hexosamine/total dialyzable hexosamine	0.17	0.28	0.21	0.42	0.31	0.15	0.34	0.25	0.37	0.28	0.51
Conjugate/hexosamine	3.27	1.55	2.41	0.82	1.15	4.67	1.35	2.25	1.17	1.88	0.62
(Hexosamine & conjugate)/total dialyzable hexosamine	0.73	0.71	0.73	0.76	0.67	0.86	0.79	0.80	0.80	0.82	0.83
Conjugate/total dialyzable hexosamine	0.56	0.43	0.52	0.34	0.36	0.71	0.46	0.56	0.43	0.54	0.32

[a]Urine specimen collected in the interval between 0 and 2 hr after isotope injection.

[b]Values for the 0-24 hr period are calculated.

APPENDIX B

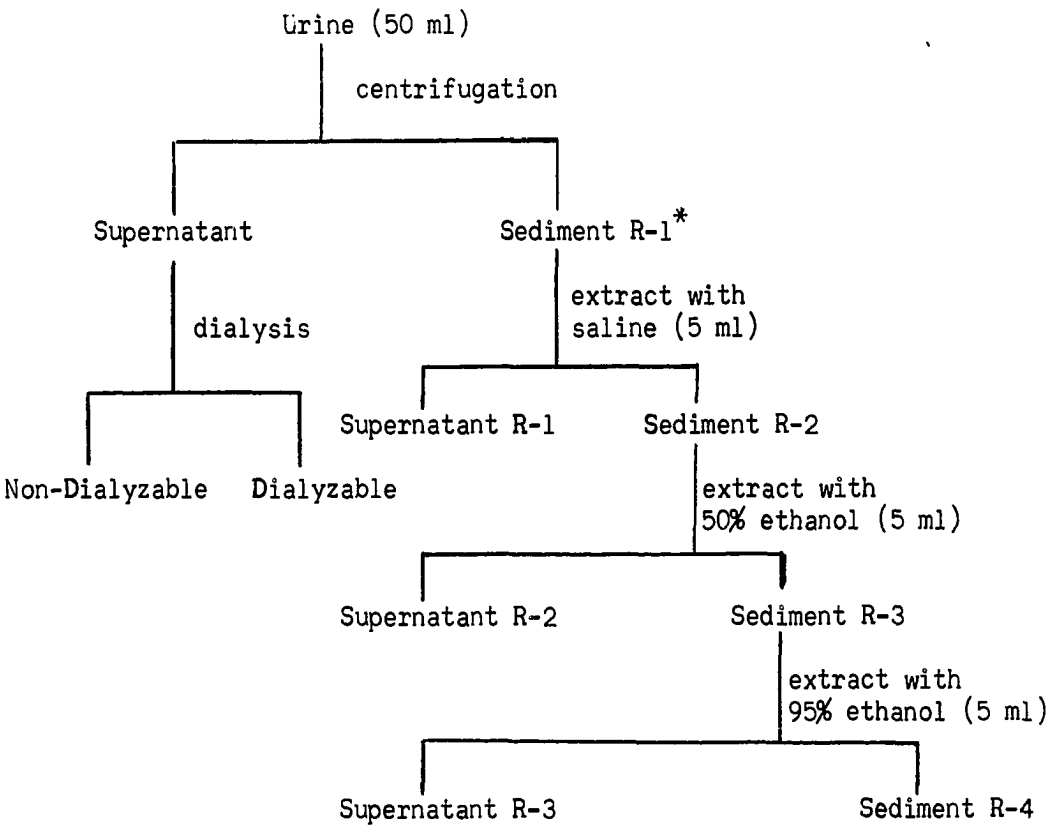

Figure 1. Flow diagram for fractionation of urine specimens.

*Subfractionation of sediment R-1 was performed only for subject with active rheumatoid arthritis (Da).

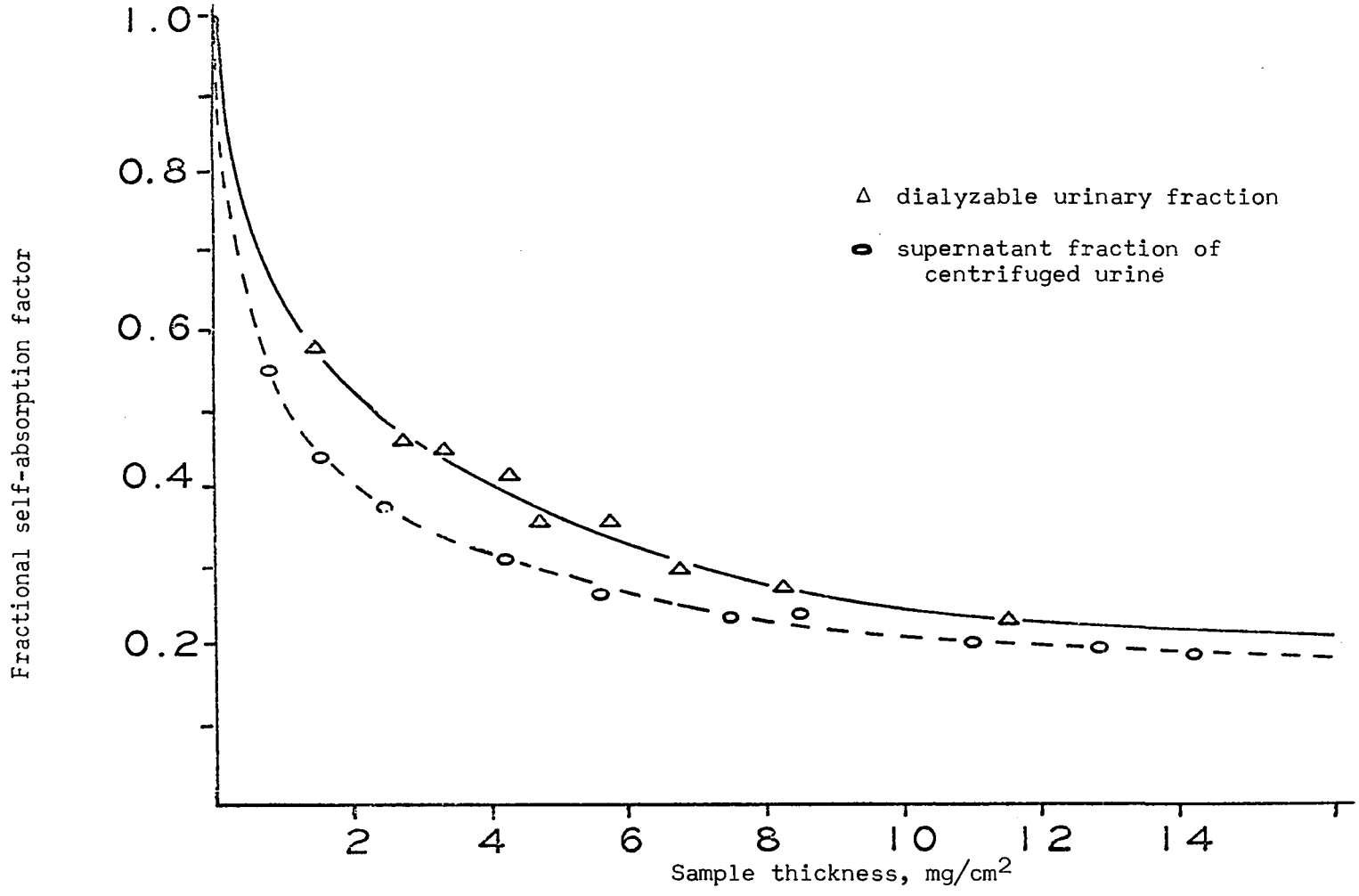

Figure 2. Self absorption curve prepared by adding N-acetyl-D-glucosamine-1-C-14 to various amounts of human urine fractions

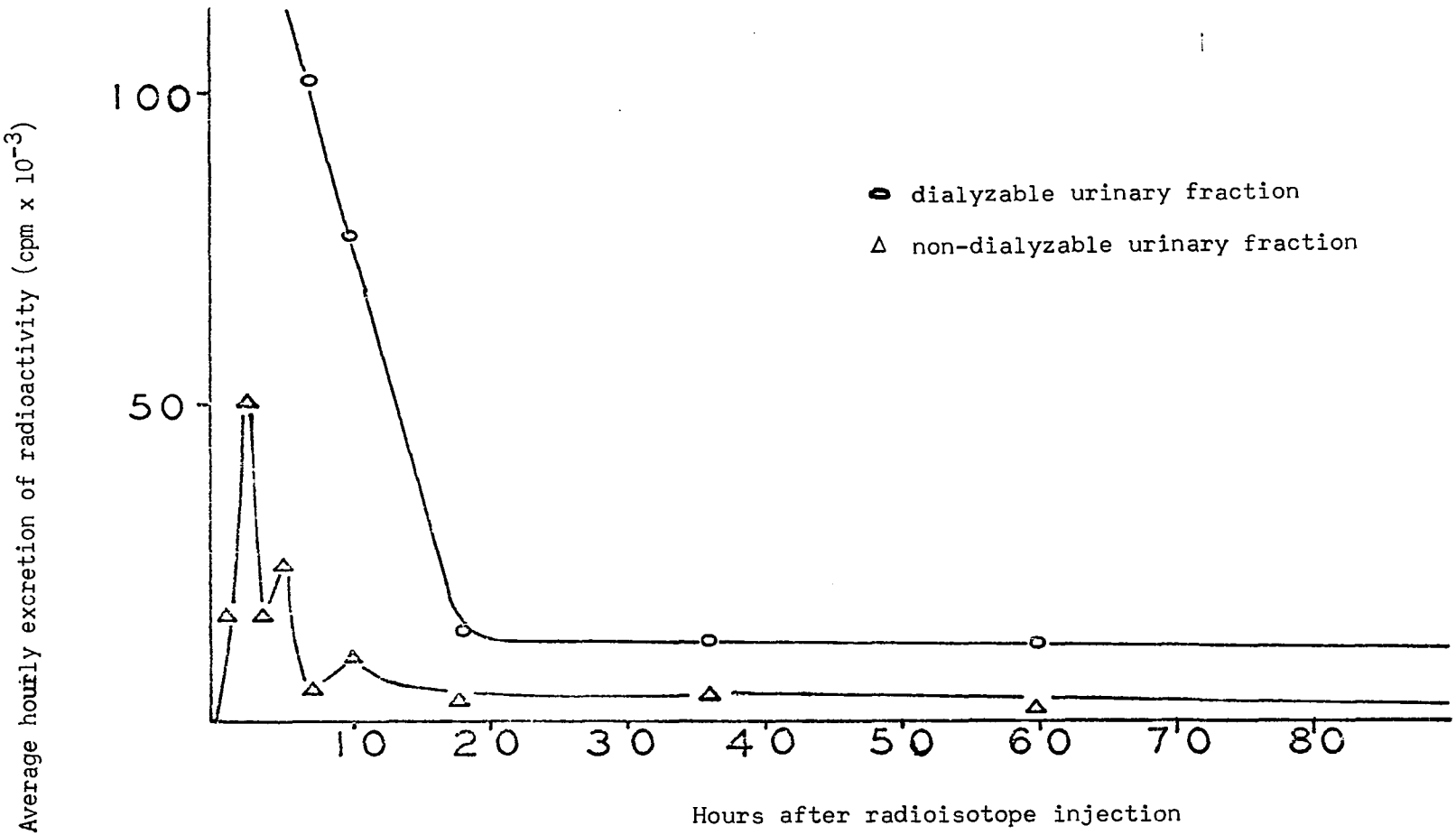

Figure 3. Excretion pattern for radioactive dialyzable and non-dialyzable material of urine specimens from a subject with active rheumatoid arthritis (Da)

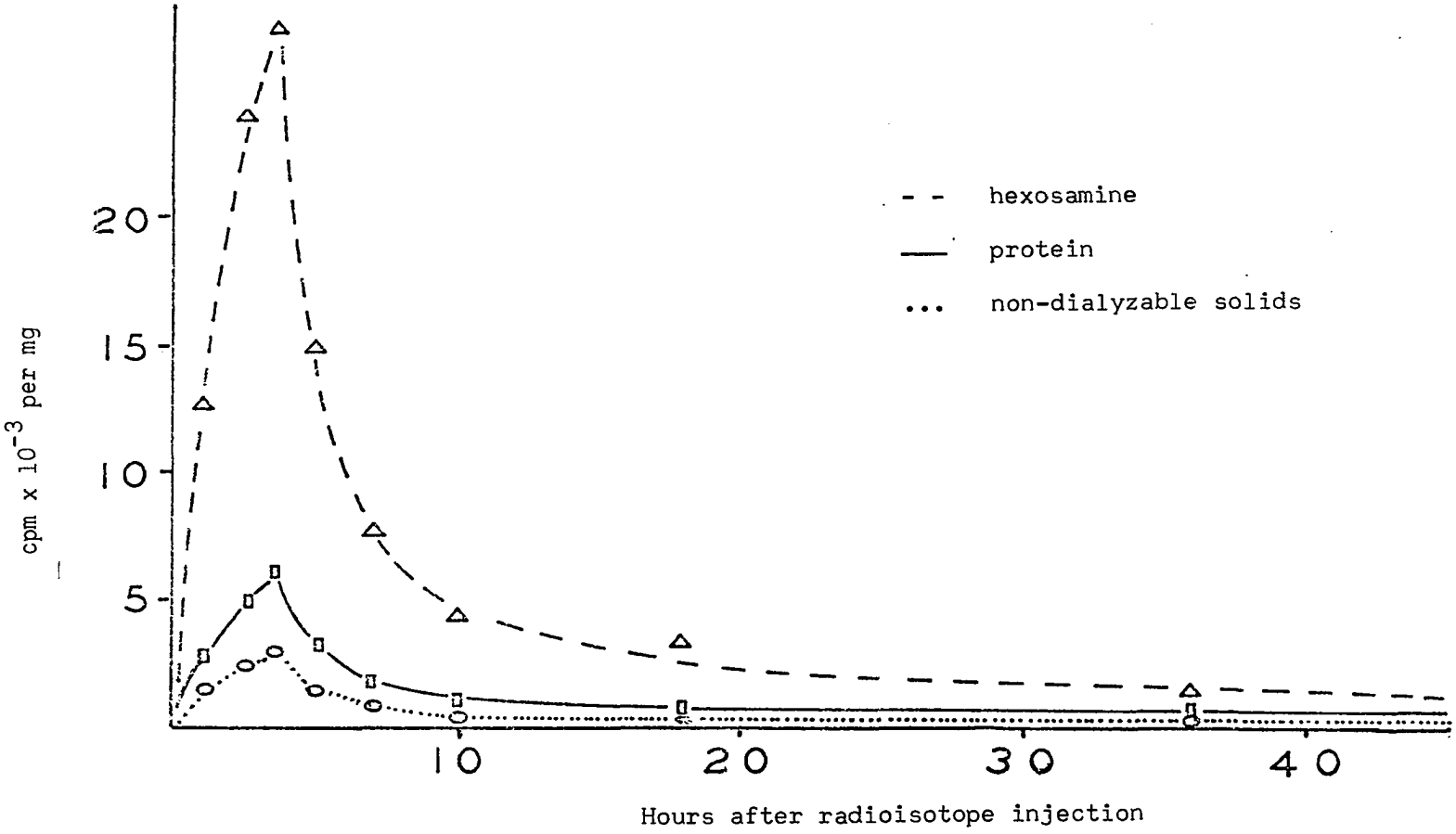

Figure 4. Comparison of the amount of specific radioactivity of hexosamine, protein, and non-dialyzable material in the urine from a subject with active rheumatoid arthritis (Da)

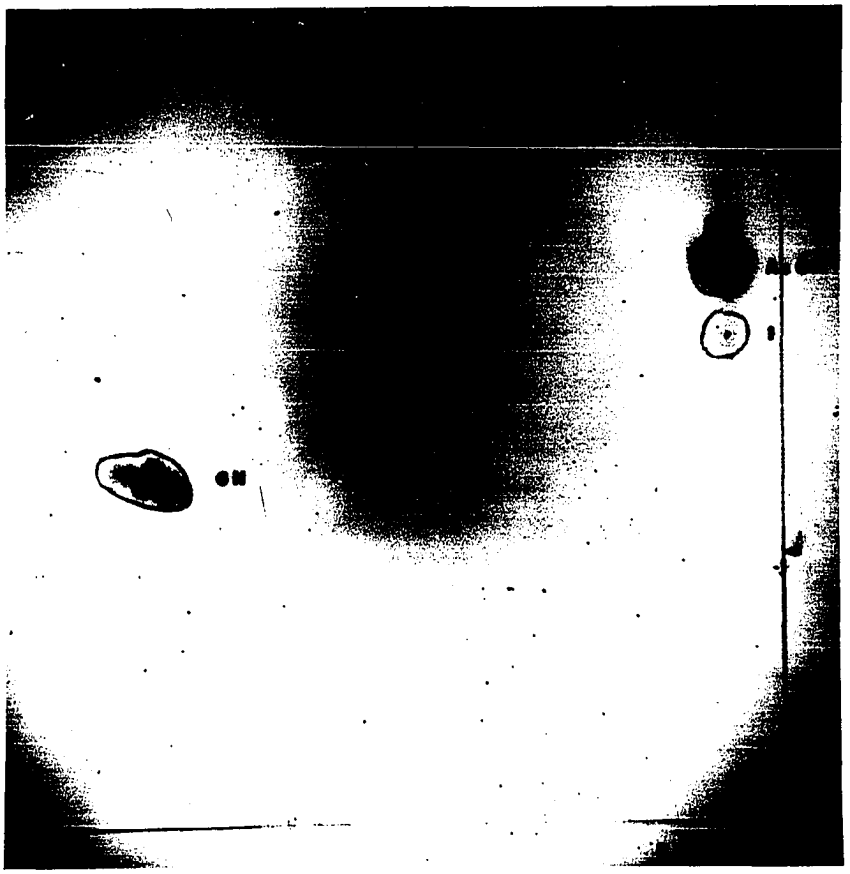

Figure 5. *Autoradiogram of radioactive material isolated from dialyzable material*.

GN - glucosamine
1 - unidentified
AcGN - N-acetyl glucosamine
2 - unidentified

*Dialyzable material obtained from the human urine of a control subject (Wi) and isolated by high voltage electrophoresis-paper chromatography.

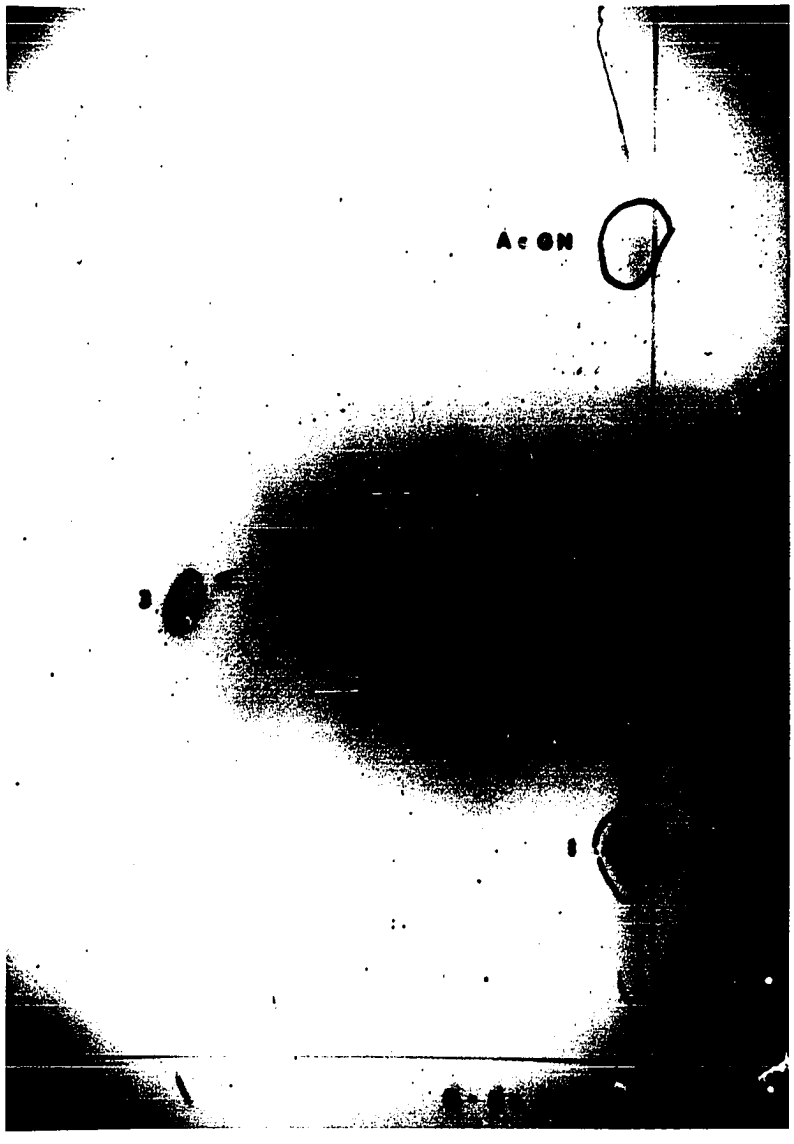

Figure 6. Autoradiogram of radicactive material isolated from dialyzable material* fractionated by Dowex-50 column chromatography.

origin	151 cpm
area 1	647
area 2	168
area 3	77
AcGN (N-acetyl glucosamine)	395

*Dialyzable material in water eluate obtained from the urine of a subject with active rheumatoid arthritis and isolated by high voltage electrophoresis-paper chromatography.