mals seemed to be prolonged indicating that a direct antineoplastic effect might play an additional role. The adrenals were not affected by the chemical carcinogens.

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Urinary Protein and Carbohydrate III. Age Differences in Acid Mucopolysaccharides in Human Beings. (32271)

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Urinary acid mucopolysaccharides (AMP) have been of interest to investigators since Brante(1) reported that Hurler's syndrome was an acid mucopolysaccharidosis. At that time, the presence of AMP in urine of normal persons was in doubt. Later, Kerby(2) used Astrup's method(3) to precipitate AMP from normal urine and to determine the normal level of excretion. She also used paper chromatography to prove that chondroitin sulfate was the principal AMP present in normal urine. Heremans *et al*(4), using electrophoretic techniques, demonstrated 3 AMP components in normal urine. Shortly thereafter, both the Dorfman(5) and Meyer(6) groups identified chondroitin sulfate B (CSB) and heparitin sulfate (HS) in the urine in gargoylism (Hurler's syndrome). They(6) also reported that chondroitin sulfate A and C (CSA and CSC) were the principal AMP in the urine of normal children. Recently King (7), using chromatographic techniques, obtained evidence for the presence of HS in normal urine. This finding was confirmed by Di Ferrante(8), Linker(9), Berenson *et al* (10) and Teller(11). The latter authors also believed that in addition to CSA and CSC there were CSB(8,9,10,11) and keratosulfate (KS)(10) present in normal urine. Furthermore, both King(7) and Kelley *et al*(12) revealed the presence of neutral sugar in AMP of urine. The complexity of the composition of urinary AMP and its relation to pathological conditions deserves further investigation.

Most work with urinary AMP has been done with cetyl trimethyl-ammonium bromide (CTAB) precipitates(13) with further enzymic digestion and alcoholic precipitation. Under these conditions, the natural forms of the urinary AMP are altered or destroyed. Previously, we reported a simple method(14) for fractionation of urinary carbohydrate-protein complexes (CPC). By this method, all components were obtained in their natural forms and suitable for further fractionation. The AMP were quantitatively recovered in the AMP-glycopeptide (AMP-GP) fraction.

The present study deals with 1) fractionation of urinary AMP-GP and their enzymic digests; 2) chemical analysis of the composition of individual subfractions; and 3) age differences in the excretion of urinary AMP.

Methods. The starting material for our analysis of urinary AMP-GP was obtained by the previously reported method(14). The AMP-GP fraction was dialyzed against running tap water overnight at room temperature and then against distilled water at 5° until free of $(NH_4)_2SO_4$. The resulting solution was evaporated to dryness under reduced pressure at 10°. For the fractionation of AMP-GP fraction, pooled samples from several subjects were used. In the fractionations after enzymic digestion of AMP-GP fractions, urine samples from individual subjects were used. Some of the samples from children may not have been complete 24 hour collections.

Separation of urinary AMP-GP fraction on Dowex 1-X2 (200-400 mesh) column, chloride form. Samples were dissolved in 2 ml of water and applied to the column. All chromatographic methods used were those reported by Schiller *et al*(15), except that one sample was eluted with a linear gradient of NaCl. The latter was obtained with a 2-chamber constant level device. Water was the starting eluting solvent and 4 M NaCl was the limiting solvent. The recovery of AMP standards* was 95% for hyaluronic acid (HA), 109% for chondroitin sulfate (CSH), 82% for heparin and 86% for heparitin sulfate. Fifteen ml fractions were collected. Aliquotes were taken from each fraction for determination of peptides and uronic acid.

Enzymic digestion of urinary AMP. To approximately 240 mg of dried AMP-GP fraction, a mixture of 6 ml 0.2 M Tris buffer, pH 8, 0.48 ml 0.04 M $CaCl_2$, 0.48 ml of 95% ETOH and 24 mg of pronase (B grade, California Corp. for Biochemical Research) were added. The mixture was incubated at 50° for 18 hours and then dialyzed against 100 ml 0.3 M phosphate buffer, pH 7.7, twice in a 24-hour period. To the mixture, 30 mg Trypsin (2× crystallized, Nutritional Biochemicals Corp.) and 15 ml of the same phosphate buffer were added. This mixture was incubated at 37° for 16 hours. After the second incubation, the mixture was dialyzed against 100 ml 0.3 M phosphate buffer, pH 6.5, twice within a 24 hour period. Thirty mg of papain (2× crystallized suspension, Nutritional Biochemicals Corp.) and a crystal of $Na_2S_2O_5$ were then added and the mixture incubated at 50° for 18 hours. At the end of this incubation, the mixture was dialyzed against running water overnight and then centrifuged at 5,900 × *g* for 15 minutes in a Servall refrigerated centrifuge, 5°. The small amount of precipitate was discarded. To each 6 ml of supernate a mixture of 0.4 ml of 0.2 M Tris buffer, pH 8.0, 0.5 ml water and 1 ml 1% CTAB were added. The mixture was stored in the refrigerator overnight and centrifuged at 5,900 × *g* for 20 minutes at 5°. The supernate was discarded. The precipitate was washed with 12 ml 95% ETOH saturated with NaCl and then dissolved in 2 ml of water and used as sample for the column.

Chemical analysis. Peptides were determined by Lowry's method(16) with bovine serum albumin as standard. Uronic acid was determined by Dische's method(17); fucose, by that of Dische and Shettles(18); hexose, by the orcinol procedure(19); sialic acid (NANA) by Warren's thiobarbiturate technique(20) and hexosamine, by that of Rondle

* HA and CSH were purchased from Mann Research Laboratories, Inc., New York. Heparin was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Heparitin sulfate, Lot No. Ex-863, was generously supplied by Dr. P. W. O'Connell, Upjohn Co., Kalamazoo, Mich.

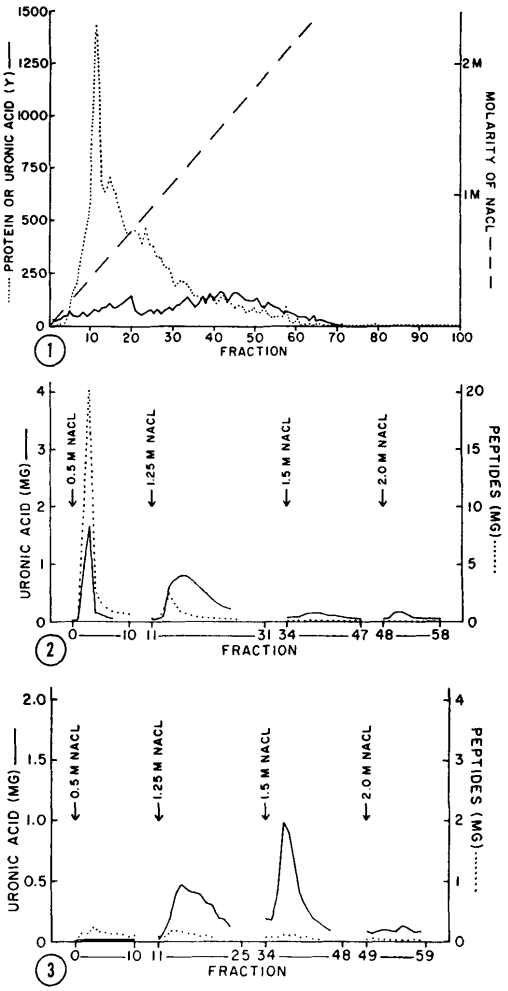

FIG. 1. Linear gradient elution of urinary AMP-GP fraction on Dowex 1-X2 column. Starting solvent - 500 ml water, limiting solvent - 500 ml 4 M NaCl. Ten ml eluates were collected. Solid line represents uronic acid. Dotted line represents peptides.

FIG. 2. Separation of urinary AMP-GP fraction on Dowex 1-X2 column. A pooled sample of AMP-GP fraction from young and middle age groups was used. Solid line represents uronic acid. Dotted line represents peptides.

FIG. 3. Separation of the enzymic digest of urinary AMP-GP fraction on Dowex 1-X2 column. A pooled sample of AMP-GP fraction from young and middle age groups was used. Solid line represents uronic acid. Dotted line represents peptides.

and Morgan(21). Glucosamine (Gm) and galactosamine (Galm) were separated on a Technicon amino acid analyzer (Ardsley, N. Y.). Eluates of AMP-GP fraction were exhaustedly dialyzed against tap water and hydrolyzed in 4 N HCl, 100° for 5 hours. Eluates of enzymic digests of AMP-GP were hydrolyzed directly with 4 N HCl, 100° for 5 hours. The hydrolysates were evaporated to dryness under reduced pressure and NaCl was removed by passing through a Dowex 50-X8 column (8 × 320 mm). The column was washed with water and hexosamine was eluted with 2 N HCl and evaporated to dryness and used as sample for the amino acid analyzer.

Results. Linear gradient elution of AMP-GP fraction on Dowex 1-X2 column, chloride form, is shown in Fig. 1. After applying the sample to the column, 160 ml of water was used as the initial eluting solvent. Traces of peptide and uronic acid were recovered. As the gradient elution proceeded, most peptides (73%) were eluted before the NaCl concentration was increased to 1 M. In contrast, the uronic acid was slowly and not completely eluted until the NaCl concentration reached approximately 2.5 M. These facts indicate that some of the AMP were associated with protein. Otherwise, uronic acid should have appeared in greatest concentration in the area where standard AMP preparations show greatest recovery.

Column chromatography (Dowex 1-X2, chloride form) of the urinary AMP-GP fraction is shown in Fig. 2. In this pooled sample, all peptides were eluted in the first two peaks. Eighty percent were eluted with 0.5 M NaCl and 20% with the 1.25 M NaCl. Approximately 85% of the total uronic acid was also present in these eluates, one-third in the 0.5 M and 2/3 in the 1.25 M NaCl collections. In the 1.25 M NaCl eluate, more peptides were eluted in the early fractions than the latter fractions. Ten percent uronic acid was in the 1.5 M NaCl and 5% was in the 2.0 M NaCl collections. The elution pattern of the AMP-GP fraction, with respect to peptides, varied little with age.

Age differences in the percentage distribution of uronic acid in the various NaCl eluates is shown in Table I. A significant increase in the percentage of the total uronic acid in the 0.5 M NaCl peak occurred with increasing age, from 10-15 years to 20-30 years of age. Further increase in age caused no change in the percentage of AMP in this fraction. In

TABLE I. Percentage Distribution of Uronic Acid in AMP-GP Fraction.

	Age group	No. of samples	Molarity of NaCl eluates 0.5	1.25	1.5	2.0
A	10-15	(5)*	14.4 ± 1.2†	54.6 ± 7.7	23.3 ± 6.4	7.8 ± 1.5
		P	A to B <0.001 A to C <0.01			
B	20-30	(6)	35.4 ± 2.8	43.4 ± 3.3	13.4 ± 2.7	7.7 ± 1.8
C	50-80	(3)	31.9 ± 4.6	43.8 ± 1.5	16 ± 3.5	8.2 ± 0.4

* Each sample is a pooled sample from 2-3 subjects.
† Mean ± standard error(22).

contrast, the percentage of AMP eluted with 1.25 M and 1.5 M NaCl decreased as age of the subject increased. There was no change in the percentage of uronic acid in the 2.0 M NaCl fraction with age.

Column chromatography of enzymic digest of AMP-GP fraction on Dowex 1-X2, chloride form, is shown in Fig. 3. Both uronic acid and peptides are decreased in the 0.5 M NaCl eluates. In contrast, there was a great increase in uronic acid in the 1.5 M NaCl eluates. Little change can be seen in the 1.25 M and 2.0 M NaCl eluates. A trace amount of peptide still remained with the AMP in 1.5 M and 2.0 M NaCl eluates.

Age differences in distribution of uronic acid in the enzymic digest of AMP-GP fractions, is shown in Table II. With increasing age, less total amount of uronic acid was excreted in the urine. The percentage distribution of uronic acid was 60-90% in 1.25 M and 1.5 M NaCl eluates. With increasing age, higher percentage of uronic acid was present in the 0.5 M NaCl and a lower percentage of uronic acid occurred in the 1.25 M and 1.5 M NaCl eluates. No age differences can be detected in the 2.0 M NaCl fractions.

TABLE II. % Distribution of Uronic Acid in Enzymic Digests of AMP-GP Fractions.

Subject No.	Age (yr)	Total (mg)	Percentage distribution Molarity of NaCl eluates 0.50	1.25	1.50	2.00
1	6	11.0	8.2	53.6	34.0	4.3
2	7	9.0	5.0	45.8	43.7	5.4
3	7	11.0	8.1	43.0	40.8	8.3
4	14	11.0	5.5	48.2	41.1	5.4
5	14	12.0	3.3	59.0	22.4	15.2
6	29	12.0	8.3	45.9	42.6	0.6
7	31	9.0	13.2	43.3	34.9	8.5
8	37	5.5	14.1	37.5	41.9	6.6
9*	53	14.0	6.5	43.5	39.8	10.9
10	70	4.5	31.7	38.8	22.5	7.1
11	70	4.6	28.0	32.3	31.6	8.3
	Average		9.7	44.6	35.9	7.3

* A schizophrenic patient.

Relative composition of individual peaks of AMP-GP fraction and its enzymic digest is shown in Table III. As compared with uronic acid, the 0.5 M NaCl eluate contains a similar amount of sialic acid; 3 times as much hexosamine and peptides and 5 times as much hexose. The Gm/Galm ratio is 7.1. After enzymic digestion, the uronic acid:hexose:peptide ratio becomes 1:1:1 and the Gm/Galm ratio decreased to 2.9. This indicates that more than one half of the glucosamine was released from the AMP during digestion. Some of the chondroitin sulfate bound to or associated with peptide has resisted digestion and remains in the 0.5 M NaCl eluates. The 1.25 M NaCl fraction contains a uronic acid:hexosamine:hexose:peptide ratio of 1:0.9:0.7:0.5. After enzymic digestion, 2/3 of the peptides were removed and the Gm/Galm ratio reduced from 1:1 to 0.25:1. This is further evidence for the presence of both enzyme-labile and -resistant AMP-GP in this fraction. The 1.5 M and 2.0 M NaCl fractions were little changed by enzymic treatment, except that the peptide moiety was reduced to a negligible figure.

After digestion, galactosamine comprises all but a trace of the hexosamine present in the 1.5 M NaCl fraction, which indicates that CSA and/or CSC are the only acid mucopolysaccharides in this fraction. The Gm/Galm ratio of 0.1 in the 2.0 M NaCl fraction indicates a ratio of heparin:CSB of 0.1. The 0.25 ratio of Gm/Galm in the 1.25 M NaCl fraction points to the possibility of a ratio of 0.2 of HS:CSH in this fraction. The 3 to 1 ratio of Gm/Galm in the 0.5 M NaCl fraction

TABLE III. Relative Composition (Wt Base) of Individual Peaks of AMP-GP in Human Urine and Its Enzymic Digests, Dowex 1-X2, Cl^- (Assume Uronic Acid = 1).

Molarity of NaCl	No. of samples	Uronic acid	Hexosamine	(Gm/Galm)*	Hexose	Fucose	Sialic acid	Peptides
				AMP-GP fraction†				
0.50	9	1	3.07	7.12	5.17	0.53	1.29	3.18
1.25	9	1	0.86	1.06	0.71	0.38	0.18	0.52
1.50	8	1	0.78	0.07	0.44	0.04	0.01	0.50
2.00	7	1	0.73	0.11	0.73	0.11	0.01	0.70
				Enzymic digest of AMP-GP fraction‡				
0.50	4	1	3.30	2.94	1.00	—	—	1.04
1.25	4	1	1.06	0.25	0.58	—	—	0.12
1.50	4	1	1.04	Trace	0.61	—	—	0.05
2.00	4	1	0.51	0.08	1.25	—	—	0.06

* Gm/Galm = glucosamine/galactosamine. Data were obtained from 2 sets of different samples.

† Samples were dialyzed free of NaCl and dried under reduced pressure at 10°.

‡ Color tests were done directly on column eluates.

— Not done.

after enzymic digestion leads to the suggestion that there is glycopeptide bound CSH in this fraction.

Discussion. Since the excretion of urinary AMP was reported to be increased in Hurler's syndrome(4,5), the amount of urinary AMP has been found to vary in other connective tissue diseases(10,23,24,25), as well as in such conditions as diabetes(26,27), malignancy(28), mongolism(29), urticaria pigmentosa(30), rickets(31), and arteriosclerosis(32). Because urinary AMP is a mixture of AMP's, we must first know the normal composition of urinary AMP before attempting to assign significance to both qualitative and quantitative variations. In Schiller's method(15), the sequence for the elution was: 0.5 M NaCl for HA; 1.25 M NaCl for HS; 1.5 M for CSA or CSC and 2.0 M NaCl for heparin. In a recent publication by Matalon and Dorfman(22), CSB was eluted in the 1.7 M NaCl eluate.

The present study supports previous conclusions(2,6,9) that chondroitin sulfate A or C is the principal AMP in the urine of human beings and further demonstrates that urinary AMP are bound with various types of glycopeptides. Approximately 1/4 of the AMP is excreted in a practically peptide-free form and eluted in the 1.5 M and 2.0 M NaCl fraction. Our hexosamine data indicate the probability that 1/5 of the 1.25 M NaCl fraction is HS and 1/10 of the 2.0 M NaCl fraction is heparin. In this case, the remainder of the 2.0 M NaCl fraction should be CSB. The present report does not exclude the possibility that HA may also be present in the 0.5 M NaCl fraction. Hexose tests were done on several 3 M NaCl eluates. No evidence could be found for the presence of KS in this fraction.

The present report is in agreement with earlier findings(7,9) that hexose is tightly bound to all urinary AMP. Finally, this report demonstrates that with increasing age, more peptide-bound and more enzyme-resistant peptide-bound AMP is excreted in human urine.

Summary. 1. Urinary acid mucopolysaccharide-glycopeptide fraction and its enzymic digest were fractionated on Dowex 1-X2 column, chloride form. The chemical composition of the eluates was determined. 2. The acid mucopolysaccharide-glycopeptide fraction of normal urine contains a mixture of acid mucopolysaccharide-glycopeptide complexes (70-80%) with a small amount of free acid mucopolysaccharide (20-30%). With increasing age, there were higher percentages of bound acid mucopolysaccharides and less free acid mucopolysaccharides in urine. 3. After enzymic digestion, there was an increase of chondroitin sulfate A or chondroitin sulfate C and a decrease in bound acid mucopolysaccharides in 0.5 M and 1.25 M NaCl eluates. With increasing age, a smaller proportion of the glycopeptides could be digested with a mixture of pronase, trypsin and papain. 4. In

normal subjects, 60-90% of acid mucopolysaccharides are equally distributed between the 1.25 M and 1.5 M NaCl fraction. With increasing age, less acid mucopolysaccharides are distributed in these two fractions. 5. The present report suggests the presence of heparitin sulfate, heparin and chondroitin sulfate B as minor components of AMP in normal urine. 6. There was no evidence for the presence of keratosulfate in the normal urines examined. The possible presence of hyaluronic acid in normal urine is not excluded by the results of the present study.

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A New Micro-Neutralization Test for Antibody Determination and Typing of Parainfluenza and Influenza Viruses. (32272)

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The conventional tube method for determining the neutralizing antibody titer of sera for the parainfluenza viruses is relatively time consuming. Recently, Schmidt *et al*(1) reported a micromethod which utilizes the hemadsorption technique for end point determination. The results are read with a standard light microscope. This report describes a new