Urinary Protein and Carbohydrate I. Fractionation of Nondialyzable Components of Human Urine. (30134)

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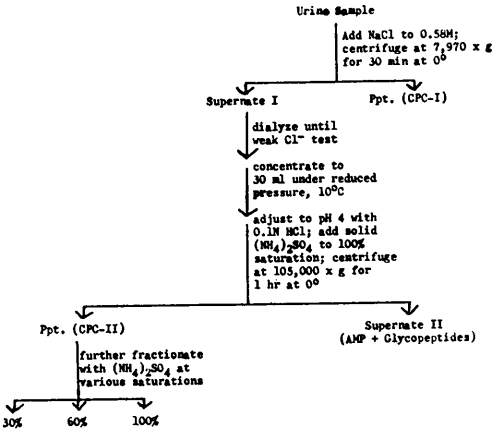
Although the presence of carbohydrate-protein complexes in human urine was reported as early as 1895(1), this field did not become active until 1950, when Tamm and Horsfall(2,3) isolated one of the urinary mucoproteins (T & H mucoids) capable of inhibiting viral hemagglutination. Gottschalk (4), Odin(5), Boyce(6) and King(7) found this purified protein to be carbohydrate-rich, containing galactose, mannose, glucosamine, sialic acid and fucose. Waldron(8), Hamerman(9), Boyce *et al*(6,10), King(11), Berggard(12) and Anderson(13) observed that, in addition to the T & H mucoids, there was a mixture of carbohydrate-rich proteins present in the nondialyzable fraction of human urine. More recently a mixture of acid mucopolysaccharides (AMP), in which chondroitin sulfate is a principal constituent, has been recognized in the nondialyzable fraction of human urine(14-16). This urinary AMP has been precipitated with lead acetate(17), benzoic acid(18,19) or cationic detergent(20-21). To date, no method has been reported for quantitative fractionation of the carbohydrate-protein complexes and AMP in their natural form. Such a method will enable us further to fractionate these materials into their individual components and correlate them one with another within the framework of the individual specimen.

The present study deals with (1) separation of the nondialyzable portion of human urine into 3 major fractions (a) carbohydrate-protein complex I (CPC-I, *i.e.*, T & H mucoids), (b) carbohydrate-complex II (CPC-II, mixture of carbohydrate-rich proteins) and (c) AMP plus glycopeptides; (2) characterization of these 3 fractions; (3) determination of normal values for each fraction in normal male subjects of several ages.

Method of fractionation. Twenty-four hour specimens of urine were collected from 63 normal male subjects, aged 4-82 years. The specimens were collected in sterile bottles, each containing 0.1 g sodium azide as preservative. All samples were tested for protein with sulfosalicylic acid and those giving a positive reaction were eliminated. If the specific gravity of the urine was greater than 1.020, the specimen was diluted with water. One-half of each sample was used for fractionation as indicated in Schema I. The other half was discarded. In the precipitation steps, the subfractions were stored in the refrigerator overnight and then centrifuged. After removal of supernate I the CPC-I was suspended in water in cellulose tubing* under toluene and dialyzed at 5° against distilled water until completely dissolved and free of chloride ions. The solution was cleared by centrifuging in a Spinco Model L ultracentrifuge at 78,410 × *g* for 1 hour. The CPC-II was suspended in water, and it and supernate II were each dialyzed against tap water overnight at room temperature and

SCHEMA I
Method of Fractionation



* Purchased from Arthur H. Thomas Co., Philadelphia, Pa. Pore diameter 48 angstrom units.

then against distilled water at 5°, until free of ammonium ion.

A few samples of CPC-II were further fractionated by adding solid $(NH_4)_2SO_4$ to 30%, 60% and 100% saturation. The precipitates were removed by centrifugation at 105,000 $\times$ *g* for 1 hour.

Samples for the determination of chemical composition were further purified as follows: CPC-I was reprecipitated once with saturated $(NH_4)_2SO_4$ and once with 0.58 M NaCl. CPC-II was washed with saturated $(NH_4)_2SO_4$ at pH 4.0 in order to remove the occluded AMP and glycopeptides.

Paper electrophoresis was done in a Spinco Model R cell with Spinco/Beckman No. 300-028 paper strips, barbital buffer pH 8.6, ionic strength 0.075 and a current of 2.5 milliamperes for 16 hrs. The samples were applied at the middle of the strips. In each run, a serum and a chondroitin sulfate standard were included for comparison. Each strip was cut lengthwise into 3, for 3 different stainings. One strip was stained with 0.25% toluidine blue in 50% ETOH for 15 minutes and washed 3 times for 5 minutes with 5% acetic acid in 50% ETOH. AMP appeared as a purple spot. Another strip was stained

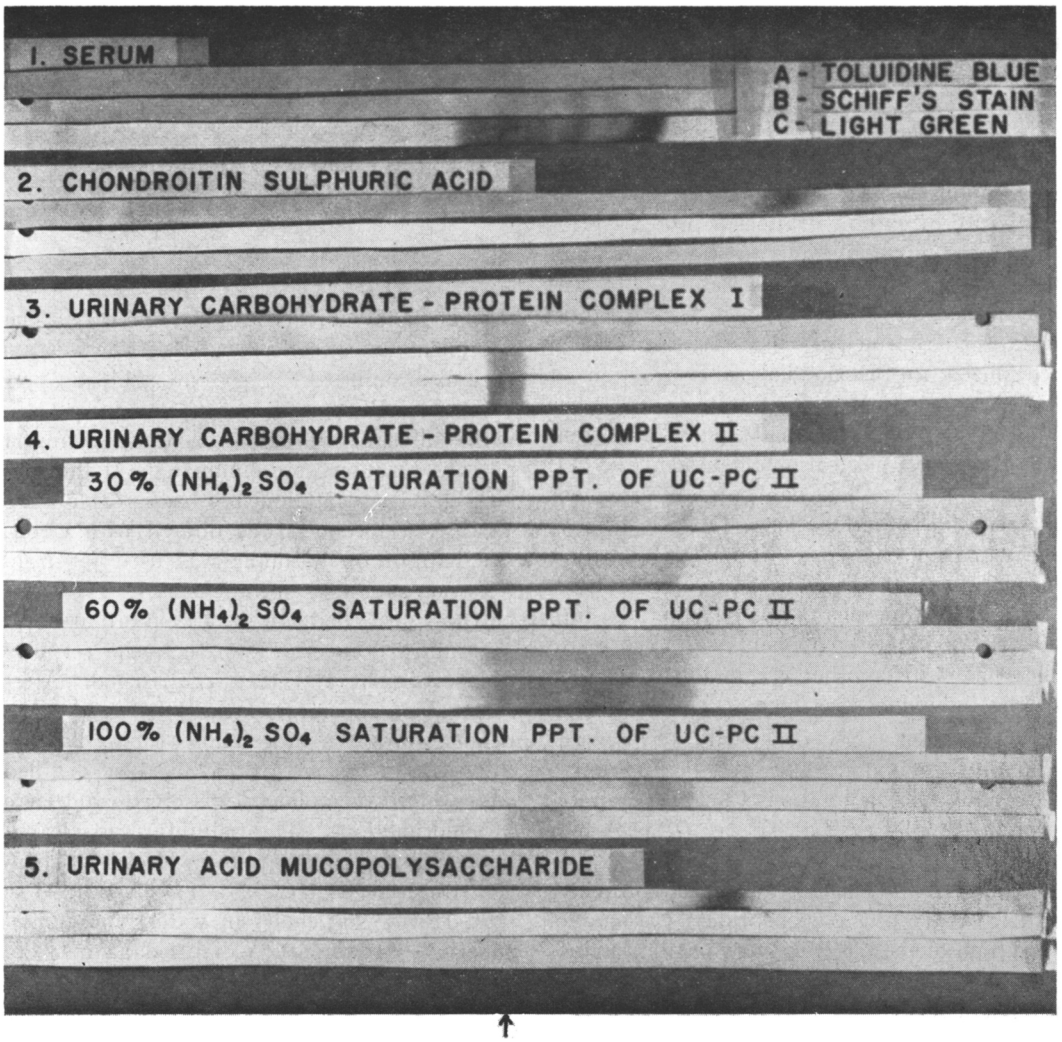


FIG. 1. Paper electrophoretic patterns of the 3 components of the nondialyzable fraction of human urine. Location of the origin on all the strips is indicated by arrow.

with Schiff's periodate reagent(22). Carbohydrates were indicated by the development of a purplish pink color. The third strip was stained with an aqueous solution of 1% Light Green for 10 minutes and rinsed twice with 1% acetic acid for 5 minutes. Protein gave a green colored spot.

Ultracentrifugation was performed by the sedimentation velocity method at 25° employing Schlieren optics in a Beckman Spinco Model E ultracentrifuge. Ten mg of CPC-I was dissolved in 2.5 ml 0.02 M phosphate buffer, pH 6.8. The soluble fraction was used. 1.1 mg of CPC-II was dissolved in 1 ml 0.1 M NaCl. Both samples were run in 12 mm standard cells at a speed of 59,780 rpm, bar angle 70°.

Chemical determinations. Proteins and peptides were determined by Lowry's method (23) with bovine serum albumin as standard. Uronic acid was determined by Dische's method(24); hexosamine, by that of Rondle and Morgan(25); fucose, by that of Dische and Shettles(26); hexose, by the orcinol procedure(27); and NANA (sialic acid) by Warren's thiobarbiturate technic(28). The amount of CPC-I and CPC-II was calculated from the chemical composition (Table II). Mg of CPC-I = mg of protein/0.772 and mg of CPC-II = mg protein/0.6828. Glycopeptide is reported as mg protein in glycopeptide. AMP was calculated as uronic acid $\times$ 2.59 ($C_{14}H_{19}NSO_{14}Na_2/C_6H_{10}O_7$ = 2.59).

Results. The electrophoretic patterns of the 3 urinary fractions are shown in Fig. 1. CPC-I gave a single sharp band staining for both carbohydrate and protein but not with toluidine blue. It did not move from the origin under the present experimental conditions. CPC-II was in a broad band extending from the origin to the albumin area. It takes the Schiff periodate and Light Green stains, but not the toluidine blue reagent. After sub-fractionation, the 30% $(NH_4)_2SO_4$ precipitate consists principally of protein and a small amount of carbohydrate. The 60% $(NH_4)_2SO_4$ precipitate contains at least 2 glycoproteins. The 100% $(NH_4)_2SO_4$ precipitate separated into a protein and a carbohydrate. It is clear that the CPC-II is multicomponent. In contrast, the AMP and glycopeptide fraction showed a single band, stains only with toluidine blue and possesses an electrophoretic mobility greater than that of albumin and similar to that of the chondroitin sulfate standard. The glycopeptides could not be detected by Schiff's staining. This is probably due to loss in the staining and washing procedures. The glycopeptides could be shown only when the strips were sprayed with ninhydrin. The glycopeptides had less mobility than the AMP and spread between origin and albumin regions.

TABLE I. Effect of NaCl Concentration Upon Precipitation of CPC-I from Human Urine.*

NaCl (molarity)	Total protein precipitated (mg)	Co-precipitation of CPC-II	
		Amt (mg)	Identification by electrophoresis
.029	17.0	Not tested	Not tested
.145	17.3	" "	" "
.580	17.6	" "	" "
.580	15.8	.105	—
1.160	20.2	.081	—
3.077	22.7	.201	+
6.154	21.0	.775	+

* A sample from four 24-hr pooled specimens of urine was used. Aliquots of 500 ml were taken for precipitation with various amounts of NaCl. Solutions of CPC-I were made and analyzed as described in text. In the last 4 samples sodium chloride was added to reprecipitate the CPC-I. The co-precipitation of CPC-II from the supernate was checked by chemical determination and electrophoresis.

The ultracentrifuge pattern of CPC-I is shown in Fig. 2. It gave a single peak with a high sedimentation rate. In contrast, the ultracentrifuge pattern of CPC-II is shown in Fig. 3 and starts with a single peak. However, it did not migrate appreciably during the 144-minute period of centrifugation.

The effect of NaCl concentration upon the precipitation of CPC-I is shown in Table I. It is clear that NaCl concentration as low as 0.029 M/liter of urine is capable of precipitating CPC-I completely from urine. When the NaCl concentration exceeded 3 M some of CPC-II was co-precipitated from the urine. Therefore, 0.58 M NaCl was chosen for precipitation of CPC-I.

Chemical analyses of the 3 nondialyzable fractions of human urine are shown in Table II. CPC-I and CPC-II are both carbohy-

TABLE II. Chemical Analysis of the 3 Nondialyzable Fractions of Human Urine.

Fraction	No. of samples	Composition (per cent)					
		Uronic acid	Hexosamine	Hexose	NANA	Fucose	Protein
CPC-I	5	.85 ± .11*	7.58 ± .80	14.25 ± .53	4.73 ± .34	1.01 ± .10	77.20 ± 1.29
CPC-II	8	1.01 ± .15	10.02 ± 1.08	13.45 ± .81	6.47 ± .91	1.09 ± .15	68.28 ± 9.30
AMP and glycopeptides	9	7.23 ± .46	22.14 ± 2.22	37.21 ± 2.30	9.31 ± .62	3.79 ± .42	22.94 ± 1.07

* Mean ± standard error(30).

drate-rich proteins. Total carbohydrate content is about 30%. This agrees well with reported data(4-7). Hexose represents approximately 50% of the total carbohydrate. Hexosamine and NANA are present in high concentration. Fucose and uronic acid each occur in approximately 4% concentration.

The AMP and glycopeptide fraction was approximately 80% carbohydrate. Among the carbohydrates present, hexose comprises approximately 50% and hexosamine an additional 25%. Preliminary fractionation on Dowex 1-X2 column(29) with increasing concentration of NaCl gave 4 sharply separated peaks. The protein/uronic acid ratios were 4:1 and 1:4 for the 0.5 M NaCl and 1.25 M NaCl fractions, respectively. The peaks eluted with 1.5 M and 2.0 M NaCl contained almost no protein. From this, it is clear that urinary AMP are partly bound with peptides and partly exist in free form. A detailed study of the AMP plus glycopeptide fraction will be reported elsewhere.

The relative composition of the sub-fractions of CPC-II in reference to hexosamine and the % distribution of total protein is shown in Table III. Approximately two-thirds of the total protein was found in the 60% $(NH_4)_2SO_4$ precipitate. One fifth was found in the 100% $(NH_4)_2SO_4$ precipitate. Only 1/10 is present in the 30% precipitate. The ratio of carbohydrate/protein was highest in the 100% $(NH_4)_2SO_4$ precipitate. This fraction also has the highest NANA content. In the 30% $(NH_4)_2SO_4$ precipitate the principal carbohydrate component was hexose. The ratio of hexose/NANA decreased as the solu-

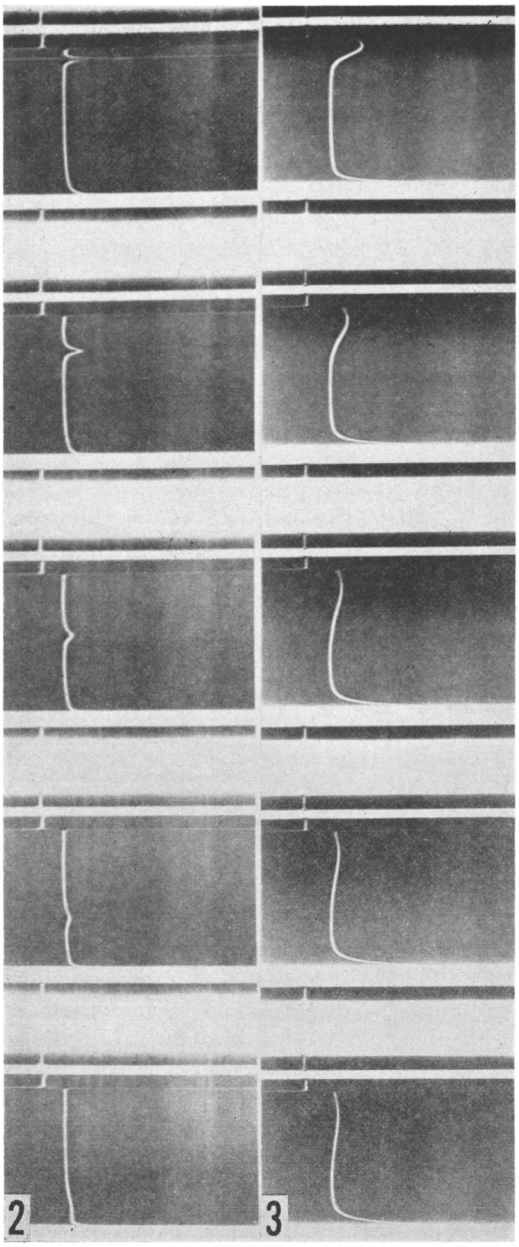


FIG. 2. Sedimentation velocity patterns of CPC-I. Pictures were taken at 8 min intervals.

FIG. 3. Sedimentation velocity patterns of CPC-II. Pictures were taken at 32 min intervals.

TABLE III. Relative Composition of Subfractions of CPC-II of Human Urine.*

% saturation with $(NH_4)_2SO_4$	Proportionate distribution (hexosamine = 1.0)						% distribution of protein
	Hexosamine	Uronic acid	Hexose	NANA	Fucose	Protein	
30	1	.19	1.83	.54	.24	10.24	13.3
60	1	.12	1.49	.92	.15	13.28	64.8
100	1	.12	1.19	1.26	.14	4.06	21.8

* Averaged results from 3 determinations. In all determinations, the value for hexosamine has been arbitrarily fixed at 1.0.

TABLE IV. Daily Excretion of the 4 Nondialyzable Components in Human Urine for Several Age Groups (mg).

Age	No. of samples	CPC-I	CPC-II	Glycopeptide	AMP
A. 0-10	7	35 ± 11* A~C $<.05$	108 ± 22 A~B or D $<.02$ A~C $<.001$	14 ± 3	13.1 ± 1.9 A~C $<.01$
B. 11-20	15	53 ± 8	217 ± 25	18 ± 3	17.8 ± 2.0 B~C $<.001$ B~D $<.01$
C. 21-40	23	56 ± 4	208 ± 15	14 ± 1	$9.2 \pm .6$
D. 41-80	18	45 ± 4	189 ± 20	13 ± 1	$11.1 \pm .9$

* Mean $\pm$ standard error(30).

bility in $(NH_4)_2SO_4$ increased (*i.e.*, approximately 4:1 in the 30% precipitate and 1:1 in the 100% precipitate).

The satisfactory reproducibility of the present method on 5 equal aliquots of a pooled urine sample is shown by the mean and standard error of each fraction, *i.e.*, CPC-I 16.98 $\pm$ 0.37; CPC-II 40.60 $\pm$ 1.96; glycopeptides 9.34 $\pm$ 0.35 and AMP 7.10 $\pm$ 0.10.

The excretion of the 4 nondialyzable components in human urine at different ages is shown in Table IV. The daily excretion of CPC-I was approximately 50 mg. No age changes in level of excretion were observed. This value agrees well with that reported by Boyce *et al*(6). Excretion of CPC-II was 100 mg/day in children younger than 10 years of age and 200 mg/day for adolescents and adults. The latter is more than double that reported by Boyce(31). This difference may be due to a loss of material in the ultrafiltrate. Recently, Dische *et al*(32) separated about 40 glycoproteins from the nondialyzable ultrafiltrable fraction of urine. Excretion was greater in adults than in children. Daily excretion of the peptide in glycopeptide was 15-20 mg. No age difference was observed. Excretion of AMP was highest between 10-20 years of age; it decreased after 20 years of age.

Consecutive 24-hour samples were collected from 2 laboratory workers. The daily variation in excretion of these 2 subjects is shown in Table V.

Discussion. The present method separates the urinary carbohydrate-containing substances in reference to differences in the solubility of the natural forms and involves only very mild treatment. Each fraction has specific characteristics. This method enables investigators to study simultaneously all 3 fractions and correlate them. Since both

TABLE V. Daily Variation in Excretion of the 4 Nondialyzable Components in Human Urine (mg).

Subject	CPC-I	CPC-II	Glycopeptides	AMP
A	81	341	19	11.3
A	34	255	10	7.9
A	40	359	17	14.5
A	45	290	15	9.9
B	75	217	18	8.9
B	56	132	11	6.9
B	53	145	12	13.0

CPC-II and AMP fractions are multicomponents, further fractionation of these two moieties is essential.

This method has several advantages. The CPC-I separated by the present method is electrophoretically and ultracentrifugally a single component. Its chemical analysis and the level of daily excretion agree well with reported data(4-7). Its low solubility in buffer salt solution and its high sedimenting rate are the same as for T & H mucoids.

Boyce *et al*(31) used barbiturate to separate the nondialyzable solids of urine into barbiturate-soluble (RS-1) and -insoluble fractions (R-1). The latter is identical with T & H mucoids. The former should be equal to the combination of our CPC-II and AMP and glycopeptides. Our data on excretion of CPC-II are higher than RS-1 reported by Boyce. Our method has an advantage over that of Boyce in the separation of AMP and glycopeptides from CPC-II.

Berggard(12) reported the fractionation of the nondialyzable material of normal urine in terms of differences in molecular weight by ultrafiltration through membranes of different pore sizes. Under those conditions, varied groups of substances could appear in a single fraction. Moreover, the zone electrophoretic data of Berggard(12) revealed a uronic acid-rich peak in all 3 fractions. We believe that our method has the advantage of sharply separating components with different chemical and physical properties.

Previously Melo *et al*(33) reported a method for the fractionation of urinary nondialyzable material into 3 fractions: (1) nonprotein bound carbohydrate, which was tentatively identified as MPS, (2) carbohydrate bound to protein, soluble in 6% perchloric acid and precipitated by 5% phosphotungstic acid in 2 N HCl, and (3) carbohydrate bound to protein, insoluble in 6% perchloric acid. The latter was obtained by the difference of total tungstic acid precipitable protein—protein soluble in 6% perchloric acid. The authors did not actually identify the material in any of their fractions. The only chemical analyses reported were for hexosamine, hexose and protein. Hexosamine is present in both glycoprotein and AMP. Therefore, a determination of hexosamine does not differentiate glycoprotein and AMP.

Melo *et al* and also Berggard(12) filtered the urine before fractionation. Filtration twice through Whatman #1 filter paper has been tested in our laboratory and it was found that 90% of T & H mucoids were removed by the filtration.

Concerning the AMP and glycopeptide fraction of urine, our data suggest that urinary AMP are partially bound to a peptide material and partially exist in a free form.

Previous workers used different precipitating agents such as lead acetate, benzoic acid, cetyl trimethyl ammonium bromide (CTAB), etc., to precipitate carbohydrate-protein complexes and AMP, which causes considerable difficulty in later purification. Furthermore, it was reported(34,35) that cationic detergent depolymerizes T & H protein. It was not known to what extent the native form of bound AMP is destroyed by the cationic detergent. However, it is difficult to believe that they escape such treatment unchanged. The total excretion of AMP obtained with the present method agrees well with that reported by Di Ferrante and Rich (20).

Summary. 1. A simple preliminary fractionation method for the nondialyzable urinary carbohydrate containing solids is reported. Sodium chloride is added to urine to 0.58 M to precipitate CPC-I (Tamm and Horsfall mucoid). After dialysis and concentration, CPC-II is separated from AMP and glycopeptide with addition of $(NH_4)_2SO_4$ to 100% saturation. 2. The present method enables (a) simultaneous quantitative evaluation of 3 carbohydrate-containing fractions in urine, (b) determination of the glycopeptide and AMP content of the AMP fraction, and (c) further fractionation of these into individual components. 3. The mean and standard error of the daily excretion of each component for all samples was: CPC-I—50 $\pm$ 6 mg; CPC-II—193 $\pm$ 23 mg; AMP—13 $\pm$ 2 mg; and glycopeptide—15 $\pm$ 2 mg. With increasing age, the excretion of CPC-II is increased. The excretion of AMP is highest between 10-20 yrs of age. It decreases thereafter.

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Selection and Intake of Carbohydrates by Wild and Domesticated Rats.* (30135)

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The extensive use of the Norway rat as a laboratory animal can in part be explained by observations that suggest that this animal has nutritional requirements similar to those of man. The literature on taste and nutrition contain many studies using one of the "tame" varieties of the Norway rat as an experimental subject. These animals are usually obtained from commercial breeders and are the product of selective pressures, substantially different from those occurring in their natural habitat. For commercial animals an acute sense of taste would have no apparent survival value.

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