of potassium in gastric secretory processes is too meager to allow an explanation of these alterations. Certainly, as discussed by Carone and Cooke(2), potassium is essential to the proper operation of energy transferring enzymatic reactions in carbohydrate metabolism and gastric secretory activity requires great energy expenditure.

These functional changes may be likened to those occurring in another secretory gland of high metabolic activity, the renal tubule, when potassium deficiency is induced.

Summary. The concentration of free HCl was found to be significantly decreased in the gastric juice of rats eating a potassium deficient diet. Total 4-hour secretion of free HCl, chloride, potassium, magnesium and pepsin was also significantly decreased but secretion rates of phosphorus, sodium and calcium were not altered.

1. Welt, L. G., Hollander, W., Jr., Blythe, W. B., *J. Chron. Dis.,* 1960, v11, 213.

2. Carone, F. A., Cooke, R. E., *Am. J. Physiol.,* 1953, v172, 684.

3. DeMuro, P., Rowinski, P., Calareau, I., Fraghi, A., *Acta Med. Scand.,* 1961, v170, 403.

4. Simonsen, D. G., Westover, L. M., Wertman, M., *J. Biol. Chem.,* 1947, v169, 39.

5. Zak, B., Hindman, W. M., Fisher, M., *Am. J. Clin. Path.,* 1956, v26, 1081.

6. Anson, M. L., Mirsky, A. R., *J. Gen. Physiol.,* 1932, v16, 59.

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Separation of Sulfated Urinary Acid Mucopolysaccharides Utilizing Cetyltrimethylammonium Bromide and Ethyl Alcohol.* (28190)

GEORGE H. STIDWORTHY, PEGGY JO ELLIS AND RAYMOND G. GOTTSCHALK

Cancer Research Laboratory, Veterans Administration Center, Martinsburg, W. Va., and Department of Pathology, The George Washington University School of Medicine, Washington, D. C.

Since Mörner in 1895 reported(1) the presence of sulfated acid mucopolysaccharides and mucopolysaccharide-protein complexes in human urine, limited information concerning these urinary components had been gained until the nineteen fifties when several different methods of preparation were reported. Fractions containing acid mucopolysaccharides have been isolated from urine by dialysis (2,3), heat treatment(4), ethanol precipitation(5), salting out(6), benzoic acid precipitation(4), or with a cationic detergent(7).

This paper deals with a method of quantitative separation and purification of acid mucopolysaccharides and mucoproteins from urine by precipitation with cetyltrimethylammonium bromide (CTAB) and ethyl alcohol. The procedure used mucopolysaccharide labelled *in vivo* with radioactive sulfate.

Material and methods. Twenty-four hour specimens of urine, collected from human subjects before and after a single intravenous injection with radioactive sulfate (about one mc of $Na_2S^{35}O_4$†), were preserved with thymol and stored at 4°C until analyzed. The procedure is shown in Fig. 1. An aliquot (usually one liter) from the daily collection was adjusted to pH 7 and allowed to stand in the cold at least 2 hours. The resultant precipitate was removed by centrifugation, dialyzed, assayed for uronic acid and radioactivity. The filtered urine was treated with CTAB (6 g/liter), shaken for one hour on a mechanical shaker and allowed to precipitate in the cold 4 to 6 days. After complete flocculation, the precipitate was removed by centrifugation (1000 × *g*). The supernatant was dialyzed, lyophilized and assayed for uronic acid and radioactivity. The precipitated complex was treated with sodium iodide (5% in ethanol) to release the cationic detergent. The freed quaternary ammonium ion is soluble in ethanol while the mucopoly-

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† Abbott Laboratories.

TABLE I. Weight, S^{35} and Hexuronic Acid Distribution in Fractions Expressed as Per Cent of Total.

	Weight	Activity	Uronic acid
pH 7 ppt., fraction "A"	48.7	2.8	3.7
CTAB supern., " "B"	5.6	.4	2.1
H_2O insol., " "C"	35.1	2.7	3.2
66% ppt., " "D"	2.3	47.8	69.7
84% ppt., " "E"	8.2	46.3	21.3
Alcohol supern., " "F"	.1	0	0
Total, "A"—"F"	100%=885.6 mg/24 hr	*	100%=9.87 mg/24 hr

* The total and specific radioactivities change with time. These changes will be presented separately.

saccharide is insoluble. The mucopolysaccharide fraction was washed 2 times with sodium iodide (5% in ethanol) and dissolved in a small portion of distilled water. Ethyl alcohol was added to bring the total alcohol concentration to two-thirds volume; the resultant flocculent precipitate which formed immediately was allowed to stand overnight at 4°C and removed by centrifugation (1000 × *g*, 10 min, 4°C). The alcoholic concentration of the supernatant fraction was increased to 84%. A second precipitate was treated like the first. These 2 fractions, designated 66% and 84% alcohol precipitates, were reprecipitated by dissolving in a small volume of water and adding alcohol to the proper concentration. The fractions were lyophilized, weighed and analyzed. The supernatant alcohol fraction, reduced in volume *in vacuo* in a rotating evaporator and finally dried by lyophilization, was analyzed for uronic acid and radioactivity.

Hexuronic acid was determined by a modification of the carbazole method(8) and compared to a glucuronic acid lactone standard. Hexosamine was measured by the Elson Morgan reaction(8) and micro-estimation of nitrogen was carried out by the Kjeldahl method using the apparatus of Jenden and Taylor(8). Radioactivity was detected using the micromil window in the D-47 Geiger counting apparatus.‡

Results. In Fig. 1 the acid mucopolysaccharide rich fractions are indicated by larger, italicized letters. Representative values of distribution of weight, radioactivity and uronic acid of the fractions of the purification procedure are shown in Table I. These are the average values obtained from 9 daily collections (8 following S^{35} injection) of urine of a patient having cancer of the colon. The 66% and 84% alcohol precipitates account together for 94% of the radioactivity and 91% of the uronic acid while they represent only 10% of total weight.

Results of the analysis of mucopolysaccharide content of the CTAB precipitated fractions are presented in Table II. In the total CTAB precipitable fraction, uronic acid and hexosamine are in approximately equivalent ratio. AMPS calculated as the sodium salt

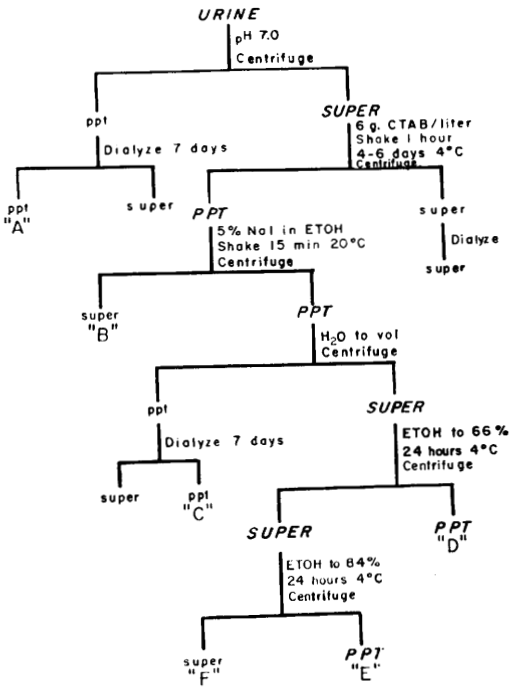

FIG. 1

‡ Nuclear-Chicago.

TABLE II. Per Cent Composition of Mucopolysaccharide Containing Urine Fractions.

	Total CTAB precipitate	66% alcohol precipitate	84% alcohol precipitate	84% precipitate papain treated*	chondroitin SO_4 ($C_{14}H_{19}NSO_{14}Na_2$)
Uronic acid	17.5 ± 1.9†	32.8 ± 1.9	2.9 ± .4	28.8 ± 4.0	37.5
Chondroitin sulfate (U.A./0.375)	46.6	87.5	7.7	76.9	100
Hexosamine	17.1 ± 1.6	32.2 ± 1.4	2.4 ± .2	27.0 ± .9	34.5
Chondroitin sulfate ($HxNH_2$/0.345)	49.7	93.5	6.8	78.4	100
Total nitrogen	10.6 ± .4	4.1 ± .4	14.4 ± .4	6.4 ± .4	2.8
Protein nitrogen (Total N—calculated $HxNH_2$(N))	7.9	1.5	14.2	4.3	0
% protein (6.13 × protein N)‡	48.3	9.4	87.2	26.1	0
Protein + AMPS (Expressed as chondroitin sulfate sodium salt), calculated from					
Uronic acid	94.9	96.9	94.9	103.0	
Hexosamine	98.0	102.9	94.0	104.5	

* Based on 4 preparations. † Mean ± standard deviation.
‡ Based on pure mucoprotein having a nitrogen content of 16.3%(15).

of chondroitin sulfate accounts for about half of the precipitated material. Protein calculated as mucoprotein accounts for the remaining 50%.

Analysis of the 66% alcohol insoluble fraction indicates that this fraction is richest in uronic acid and hexosamine in approximately equimolar proportions. This fraction contains approximately 90% AMPS, and less than 10% protein.

The 84% alcohol insoluble fraction contains a smaller proportion of uronic acid and hexosamine in equimolar amounts and contains 87% protein. This fraction was subjected to papain digestion(8) which resulted in a weight loss of 55 to 70%. This loss was accounted for by digestion of the protein moiety since total uronic acid and hexosamine content remained essentially unchanged before and after treatment. The total protein of this nondialyzable fraction was reduced from 87 to 26%. Concentrations of uronic acid, hexosamine and nitrogen of this hydrolyzed mucoprotein are shown in Table II.

The compositions of the 66 and 84% fractions were about constant from day to day while their proportion and the total amounts excreted varied. Similar results were obtained by this method in a series of cancer patients and normal subjects.

Discussion. Most of the information published on the acid mucopolysaccharides of human urine is based on studies of the nondialyzable fraction. This technique of preparation yields a mixture of materials. The presence of plasma proteins(9,10), of blood group substances, of the high molecular weight glycoprotein of Tamm and Horsfall, and of PAS positive components similar to those of the α and β glucoproteins of human serum have been demonstrated(11). The nondialyzable fraction may not include all of the urinary AMPS. Miettinen(11) has shown that 45-75% of total daily output of urinary hexosamine is dialyzable. Since free hexosamine is not detectable in the urine(12), this fraction must be composed of lower molecular weight mucosubstances.

The aliphatic ammonium salt CTAB was first used by Dutta, Jones and Stacey(13) to precipitate polyanions in the fractionation of nucleic acids. Its use was soon extended to the polysaccharide field(14) and to isolation of AMPS(7).

This method uses CTAB to precipitate the carbohydrate rich polyanions of human urine, sodium iodide to release the quaternary ammonium complex and graded alcohol concentrations to separate 2 mucoprotein fractions. Di Ferrante and Rich(7) measured urinary

mucopolysaccharide excretion by the uronic acid content of a total CTAB precipitate but did not purify this material. The purity and quantitative yield of the mucoprotein fractions obtained in this study are indicated by the equimolar ratios of hexuronic acid and hexosamine, by the nitrogen content and by the radioactivity. The fractions appear free of serum proteins, glycoproteins and other large molecules found in urine.

Summary. A method for isolation of sulfated acid mucopolysaccharides (AMPS) and corresponding mucoproteins from human urine by absorption on and precipitation with cetyltrimethylammonium bromide is described. After *in vivo* labelling with radiosulfur nearly all of the bound radioactivity was found in 2 fractions, one insoluble in 66% alcohol and the other soluble in 66% alcohol, but insoluble in 84% alcohol. The first is essentially free AMPS containing less than 10% protein while the second is a mucopolysaccharide-protein complex containing about 87% protein. Digestion of the second fraction with proteolytic enzymes yields a nondialyzable AMPS with about 26% protein. Chemical analysis indicates no other high molecular weight urinary substances are present in these preparations.

1. Mörner, K. A. H., *Skand. Arch. Physiol.*, 1895, v6, 332.

2. Hammerman, D., Hatch, F. T., Reife, A., Bartz, K. W., *J. Lab. Clin. Med.*, 1955, v46, 848.

3. King, J. S., Jr., Boyce, W. H., Little, J. M., Artom, C., *J. Clin. Invest.*, 1958, v37, 315.

4. Anderson, A. J., McClagan, N. F., *Biochem. J.*, 1955, v59, 638.

5. Tamm, I., Horsfall, F. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 108.

6. ———, *J. Exp. Med.*, 1952, v95, 71.

7. Di Ferrante, N., Rich, C., *J. Lab. Clin. Med.*, 1956, v48, 491.

8. Stidworthy, G. H., Ph. D. Thesis, 1961, Univ. of Okla.

9. Rigas, D. A., Heller, C. G., *J. Clin. Invest.*, 1951, v30, 853.

10. Berggärd, I., *Clin. Chem. Acta*, 1961, v6, 413.

11. Miettinen. T., *Scand. J. Clin. Lab. Invest.*, 1961, v13, Sup. 61.

12. Björnesjö, K. B., Werner, I., Odin, L., *ibid.*, 1959, v11, 238.

13. Dutta, S. K., Jones, A. S., Stacey, M., *Biochem. Biophys. Acta*, 1953, v10, 613.

14. Scott, J. E., in *Methods of Biochemical Analysis*, D. Glick, Editor, Interscience, New York - London, 1960, vVIII, p145.

15. Eastoe, J. E., in *Biochemist Handbook*, C. Long, Editor, Van Nostrand, Toronto, New York, London, 1961, p726.

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Relation Between Sensitivity to Lactogenic Hormones and Tumorigenesis in Hyperplastic Mammary Nodules in C3H/Crgl Mice.* (28191)

HOWARD A. BERN

Department of Zoology and Cancer Research Genetics Laboratory, University of California, Berkeley

The hyperplastic alveolar nodule is the recognizable preneoplastic lesion in the mouse mammary gland, and tumors arise with high frequency when such nodules are transplanted into mammary fat pads which have been cleared of their normal parenchyma(1). Nodules, in general, appear to be more responsive to hormones than comparable normal tissues (2); their very existence as lobuloalveolar structures in an inactive gland is based upon this increased sensitivity. However, there is variation in sensitivity among nodules *in situ* (3,4) and among outgrowths that develop when these nodules are transplanted(5). We have previously raised the question as to whether the neoplastic change is more likely to occur in hyperplastic alveolar nodules showing greater hormone sensitivity than in nodules showing lower hormone sensitivity, or *vice versa*(6). To examine this question, intact mice have been subjected to various

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