normal birds. The results indicate that there is no defect in the erythropoietic response in birds with genetic muscular dystrophy.

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Urinary Protein and Carbohydrate II. Fractionation of Nondialyzable Ammonium Sulfate Precipitable Glycoproteins in Normal Human Urine. (31344)

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Human urine contains a group of carbohydrate-protein complexes (CPC). Their molecular weights vary from 5,000(1) to several million(2). Some of these CPC are dialyzable(3) through cellophane membrane; some are nondialyzable but ultrafiltrable(1,2-6) and others are nondialyzable and not ultrafiltrable(2,7). The Tamm and Horsfall (T & H) mucoid is the only one which has been isolated in pure form and in substantial amount(2,7). Berggard(1) employed dialyzing membranes of various pore sizes to separate the urinary glycoproteins into 3 fractions of different molecular sizes. Using 'pevikon' block electrophoresis, he further demonstrated the multicomponent nature of each of the 3 fractions of the urinary glycoproteins. Recently, he(8) has reported the isolation of a sialic acid-rich glycoprotein fraction from his fraction B (molecular weight between 10,000 and 40,000). For this purpose, he used zone electrophoresis at pH 8.6 to separate the fast-moving albumin fraction from other CPC. The eluate of this portion of the albumin zone was concentrated and subjected to zone electrophoresis at pH 4.5. Under such treatment, the albumin migrated toward the cathode, and the sialic acid-rich fraction and AMP toward the anode. During the process the latter two substances were almost completely separated, one from the other.

By employing ultrafiltration, 'pevikon' zone electrophoresis and gel filtration, Lundblad isolated nonultrafiltrable(9) and ultrafiltrable(10), nondialyzable fucose-rich glycopeptides from normal human urine. Dische *et al*(4) have precipitated the nondialyzable ultrafiltrable glycan of human urine with increasing concentrations of isopropanol and isopropanol-ether, followed by further separation with continuous flow electrophoresis on glass fiber filter paper. At least 40 glycoprotein fractions were thus identified. Hakomori *et al*(11,12) isolated low molecular weight glycoproteins by treating the supernate from 85-90% ETOH precipitation of concentrated urine with lead acetate and Dowex 1-X2 chromatography and gel filtration. Bourrillon *et at*(13) studied the glycoproteins of urine which were soluble in 50% ETOH. Employing the DEAE-cellulose column technic, they were able to separate at least 4 peaks. The early fractions were rich in neutral sugars and the later peaks were rich in sialic acid. The same authors (14) also fractionated the urinary glycoproteins which had been precipitated by the use of 50% ETOH. Forty peaks were separated from the 50% ETOH precipitate fraction. Recently we(15) reported a simple method for fractionation of the nondialyzable urinary CPC. The method was mild and all urinary constituents retained their natural form. The CPC-II fraction in this procedure contains all urinary glycoproteins except

T & H mucoid. There is no contamination with acid mucopolysaccharides and glycopeptides. This preparation is suitable for further fractionation. The total amount of glycoproteins excreted as CPC-II was approximately 200 mg per day(15). The sialic acid-rich glycoprotein fraction isolated by Berggard(8) was 1 mg per liter of urine. The nonultrafiltrable and nondialyzable fucose-rich glycopeptide fractions A and B reported by Lundblad(9) were 18.2 and 45.4 mg per 34 liters of urine, respectively.

Despite the splendid reports quoted, much work remains to be done before the composition of glycoproteins is well understood. The present study deals with 1) separation of CPC-II into fractions precipitated with addition of $(NH_4)_2SO_4$ to 30%, 60% and 100% saturation; 2) separation of sialic acid-rich glycoproteins from sialic acid-poor glycoproteins in these 3 fractions on diethylaminoethyl (DEAE) cellulose columns; 3) further separation of the column fractions by acrylamide gel electrophoresis; and 4) composition of the glycoprotein fractions as determined by chemical analysis of the DEAE-cellulose column eluate.

Methods. CPC-II was prepared as described previously(15). Solid $(NH_4)_2SO_4$ was added to 30% saturation (0.234 g/ml). The mixture was stirred thoroughly, set in the refrigerator overnight, and centrifuged at 27,000 $\times$ *g* for 1 hr at 5°. The precipitate was washed with 0.234 g/ml $(NH_4)_2SO_4$ solution, then dialyzed* overnight againts running water at room temperature followed by dialysis against distilled water at 5°C until free of $(NH_4)^+$. To the supernate, $(NH_4)_2SO_4$ was added to 60% saturation (0.468 g/ml). The precipitate thus obtained was separated in a manner similar to that employed for the 30% precipitate. A further precipitate was obtained from the supernate by 100% saturation with $(NH_4)_2SO_4$ (0.78 g/ml). The precipitates obtained from 60% and 100% saturation with $(NH_4)_2SO_4$ were washed and freed of $NH4^+$ as described above.

Column chromatography on DEAE-cellulose. DEAE-cellulose was equilibrated with 0.01 M phosphate buffer, pH 8, in a water jacketed column (2.2 cm diameter and 18 cm height) at 5°C. Samples of the $(NH_4)_2SO_4$ precipitates were concentrated to 5 ml and applied to the column. Linear gradient elution with decreasing pH and increasing molarity was made by addition of 0.3 M NaH_2PO_4 to the 0.01 M phosphate buffer with a 2-chamber constant-level gradient device. The starting volumes of both solutions were 800 ml. The rate of elution was 20 ml/hr. The eluate was scanned at 280 mμ with a Vanguard† UV scanning device. Ten ml fractions were collected. The sialic acid-poor glycoproteins were completely eluted within the first 40 fractions. Sodium chloride was added to the 0.3 M NaH_2PO_4 bottle to give a 1 M concentration. Elution was continued until UV absorbed material was eluted, which required collection of approximately 80 fractions. The recovery of protein applied to this column varied from 85 to 107%.

Chemical analysis. Proteins were determined by the method of Warburg and Christian(16). Hexose was determined by the orcinol method of Weimer(17), sialic acid by Warren's thiobarbiturate method(18), and fucose by Dische's method(19).

Acrylamide gel electrophoresis. A 12-tube Canalco‡ electrophoresis apparatus was used. Column fractions were concentrated under reduced pressure at 10°C and dialyzed against the Tris-glycine buffer at pH 8.3 used for electrophoresis. Electrophoresis was performed according to the method of Davis (20). For samples of low concentration, the samples were used in place of the water in the sample gel. For very dilute samples, the samples themselves were used in place of the sample gel with one drop excess on the top of the column. A 2 cm^2 cellulose sheet made of dialyzing tubing was carefully laid on without trapping any air bubbles. With the Canalco top bath upside down, the rubber adapters were placed in the reversed position. The

* The cellulose dialyzing tubing was purchased from Arthur H. Thomas Co., Philadelphia, Pa., pore diameter 48 angstrom units. This tubing will retain molecules up to a molecular weight of approximately 12,000.

† Vanguard Instrument Co., LaGrange, Ill.

‡ Canal Industrial Corp., Bethesda, Md.

TABLE I. Chemical Composition of Eluate of 30% $(NH_4)_2SO_4$ Precipitate (DEAE-30) from Urinary CPC-II.

Fraction No.	Total mg recovered			% Distribution			Ratios*		
	Protein	Hexose	Sialic acid	Protein	Hexose	Sialic acid	H/P	S.A./P	H/S.A.
4–10	17.96	1.48	.15	9.29	8.68	7.32	.082	.008	9.87
11–15	11.92	1.24	.21	6.17	7.27	10.24	.104	.018	5.90
16–20	13.22	1.33	.22	6.84	7.80	10.73	.101	.016	6.05
21–28	17.55	2.08	.10	9.18	12.19	4.88	.119	.006	20.80
29–32	11.82	1.43	.05	6.11	8.38	2.44	.120	.004	28.60
33–54	24.89	4.20	.26	12.88	24.62	12.68	.169	.010	12.54
NaCl added									
55–65	50.80	3.06	.85	26.88	17.94	41.46	.060	.017	3.60
66–80	45.15	2.24	.21	23.36	13.13	10.24	.050	.005	10.67
Total	193.31	17.06	2.05	100.01	100.01	99.99			

* H is hexose; P is protein; S.A. is sialic acid.

cellulose covered columns were also turned upside down and slipped into the adapters. The top bath was then returned to normal position. During electrophoresis, air bubbles often formed in the Tris-glycine buffer. To prevent the disturbance caused by the air bubbles, several holes were punched in each sheet with a #20 needle fiitted to a 1 ml syringe immediately after starting the current. Carbohydrates were stained as follows: After electrophoresis, the gels were immersed in 7% acetic acid for 30 minutes at room temperature, then placed in 0.2% periodic acid and set in the refrigerator for 30 minutes. The excess periodic acid was removed electrophoretically with 7% acetic acid, 8 milliamperes per gel for 30 minutes. Finally the gel was placed in Schiff's reagent§ in the refrigerator. Carbohydrates stained pink. The stained gel column could be kept for several months in the refrigerator.

Results. The chemical compositions of DEAE-cellulose fractionation of 30% $(NH_4)_2SO_4$ precipitate (DEAE-30) from CPC-II are shown in Table I. Before addition of NaCl, proteins in these fractions had a hexose/protein ratio 0.10-0.15. After addition of NaCl, this ratio decreased to 0.05. Proteins in this fraction contained very little sialic acid (NANA) with a sialic acid/protein ratio between 0.005-0.02. The hexose/sialic acid ratio was high. Fifty per cent of the total protein and of sialic acid and 40% of hexose in DEAE-30 was eluted following addition of NaCl.

Chemical composition of eluates from DEAE-cellulose fractionation of 60% $(NH_4)_2SO_4$ precipitate (DEAE-60) is shown in Table II. Proteins in the early fractions had a hexose/protein ratio ranging between 0.11 to 0.09. Following addition of NaCl, this ratio decreased slightly to 0.08-0.09 and the sialic acid/protein ratio increased from 0.030 to 0.062. The hexose/sialic acid ratios ranged from 3 to 5. Following addition of NaCl, this ratio was decreased to 1.50. Forty per cent of the protein and hexose and 65% of the NANA were eluted after addition of NaCl. The NANA/protein ratios in all fractions were higher than those in the comparable fractions from DEAE-30 and varied from 0.15 to 0.34.

Chemical compositions of the eluate of DEAE-cellulose fractionation of 100% $(NH_4)_2SO_4$ precipitate (DEAE-100) are shown in Table III. The hexose/protein ratios varied from 0.07 to 0.20. Following addition of NaCl, this ratio decreased and showed variations between 0.046 and 0.087. The sialic acid/protein ratios ranged from 0.025 to 0.114. After addition of NaCl, this ratio increased to 0.20. The hexose/sialic acid ratios were 1.4-3.4 and 0.43-0.8, respectively, before and after addition of NaCl.

§ Schiff's reagent: To 0.5 g of basic fuchsin in 100 ml water, 1 g potassium metabisulfite and 10 ml of 1 N HCl are added. Shake occasionally until straw colored (about 3 hr); 0.25-0.5 g activated charcoal is added. The solution is shaken, filtered and stored in tightly stoppered bottles in the refrigerator. Reagent should be water clear. If it is yellow, more charcoal is added and the solution refiltered.

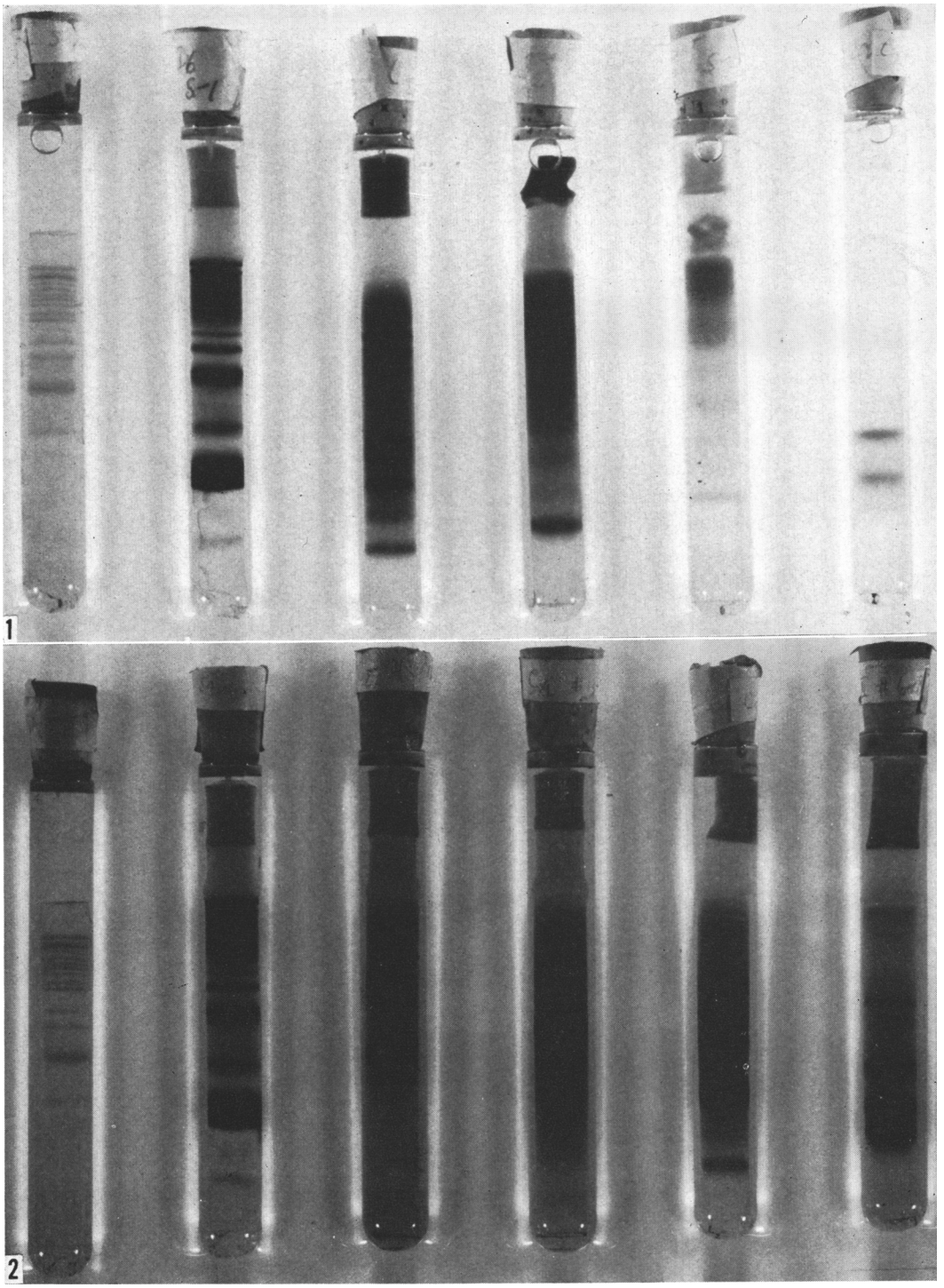

FIG. 1. Disc electrophoresis of eluates from DEAE-cellulose fractionation of 30% $(NH_4)_2SO_4$ ppt. of urinary CPC II (Table I). Tube No. 1—Serum, Schiff stain; Tube No. 2—Serum, protein stain; Tube No. 3—Fr. 3-9, protein stain; Tube No. 4—Fr. 10-21, protein stain; Tube No. 5—Fr. 26-34, protein stain; Tube No. 6—Fr. 54-74, protein stain.

FIG. 2. Disc electrophoresis of eluate from DEAE-cellulose fractionation of 60% $(NH_4)_2SO_4$ ppt. of urinary CPC II (Table II). Tube No. 1—Serum, Schiff stain; Tube No. 2—Serum, protein stain; Tube No. 3—Fr. 3-12, protein stain; Tube No. 4—Fr. 13-22, protein stain; Tube No. 5—Fr. 23-35, protein stain; Tube No. 6—Fr. 52-70, protein stain.

Fifty per cent protein, 40% hexose and 70% sialic acid were present in the eluate following addition of NaCl.

Fucose was determined in one set of experiments. None of the peaks reported here was very rich in fucose. Our previous data (15) indicated that CPC-III fraction which contained AMP and glycopeptides had the highest content of fucose. It is logical to believe that the fucose-rich, low molecular weight, glycoproteins reported by Lundblad (10,11) were present in our CPC-III fraction.

Disc electrophoretic patterns of DEAE-30 are shown in Fig. 1. Ten or more components are present in each of the 3 gels (tubes 3-5) containing eluates from DEAE-columns before addition of NaCl. Most of them had mobilities similar to serum proteins. However, 2-3 components had mobilities faster than albumin. Two major and 2 minor components are present in the last tube which contained fractions after addition of NaCl. Two of them had a mobility greater than albumin.

Disc electrophoretic patterns of DEAE-60 are shown in Fig. 2. The patterns were similar to those of DEAE-30 in that many components (tubes 3-5) are present in the eluates before addition of NaCl. Several of the components had mobilities greater than albumin. Following addition of NaCl, 5 major and many small components were present in the

TABLE II. Chemical Composition of Eluate of 60% $(NH_4)_2SO_4$ Precipitate (DEAE-60) from Urinary CPC-II.

Fraction No.	Total mg recovered			% Distribution			Ratios*		
	Protein	Hexose	Sialic acid	Protein	Hexose	Sialic acid	H/P	S.A./P	H/S.A.
5–11	22.48	2.44	.66	8.35	9.67	6.09	.109	.029	3.70
12–19	24.04	2.50	.82	8.93	9.91	7.57	.104	.034	3.05
20–30	39.68	3.95	1.13	14.73	15.66	10.43	.110	.028	3.50
31–35	28.66	2.57	.44	10.64	10.19	4.06	.090	.015	5.84
36–49	32.41	3.11	.77	12.03	12.33	7.11	.095	.024	4.04
NaCl added									
50–57	58.65	4.66	3.10	21.78	18.47	28.62	.079	.053	1.50
58–68	63.40	6.00	3.91	23.54	23.78	36.10	.095	.062	1.53
Total	269.32	25.23	10.83	100.00	100.01	99.98			

* Same as Table I.

TABLE III. Chemical Composition of Eluate of 100% $(NH_4)_2SO_4$ Precipitate (DEAE-100) from Urinary CPC-II.

Fraction No.	Total mg recovered			% Distribution			Ratios*		
	Protein	Hexose	Sialic acid	Protein	Hexose	Sialic acid	H/P	S.A./P	H/S.A.
5– 7	7.56	.86	.46	4.42	6.01	2.76	.114	.061	1.87
8–15	21.49	1.80	1.28	12.56	12.65	7.69	.084	.060	1.41
16–25	27.75	2.01	1.47	16.22	14.13	8.83	.072	.053	1.37
26–31	14.79	1.10	.48	8.64	7.73	2.88	.074	.032	2.29
32–35	11.20	.96	.28	6.55	6.75	1.68	.086	.025	3.43
36–46	7.65	1.56	.87	4.47	10.96	5.22	.203	.114	1.79
NaCl added									
47–52	25.25	2.15	4.30	14.76	15.11	25.83	.085	.170	.50
53–60	30.13	2.63	6.06	17.60	18.48	36.40	.087	.201	.43
61–70	25.30	1.16	1.45	14.78	8.15	8.71	.046	.057	.80
Total	171.12	14.23	16.65	100.00	99.97	100.00			

* Same as Table I.

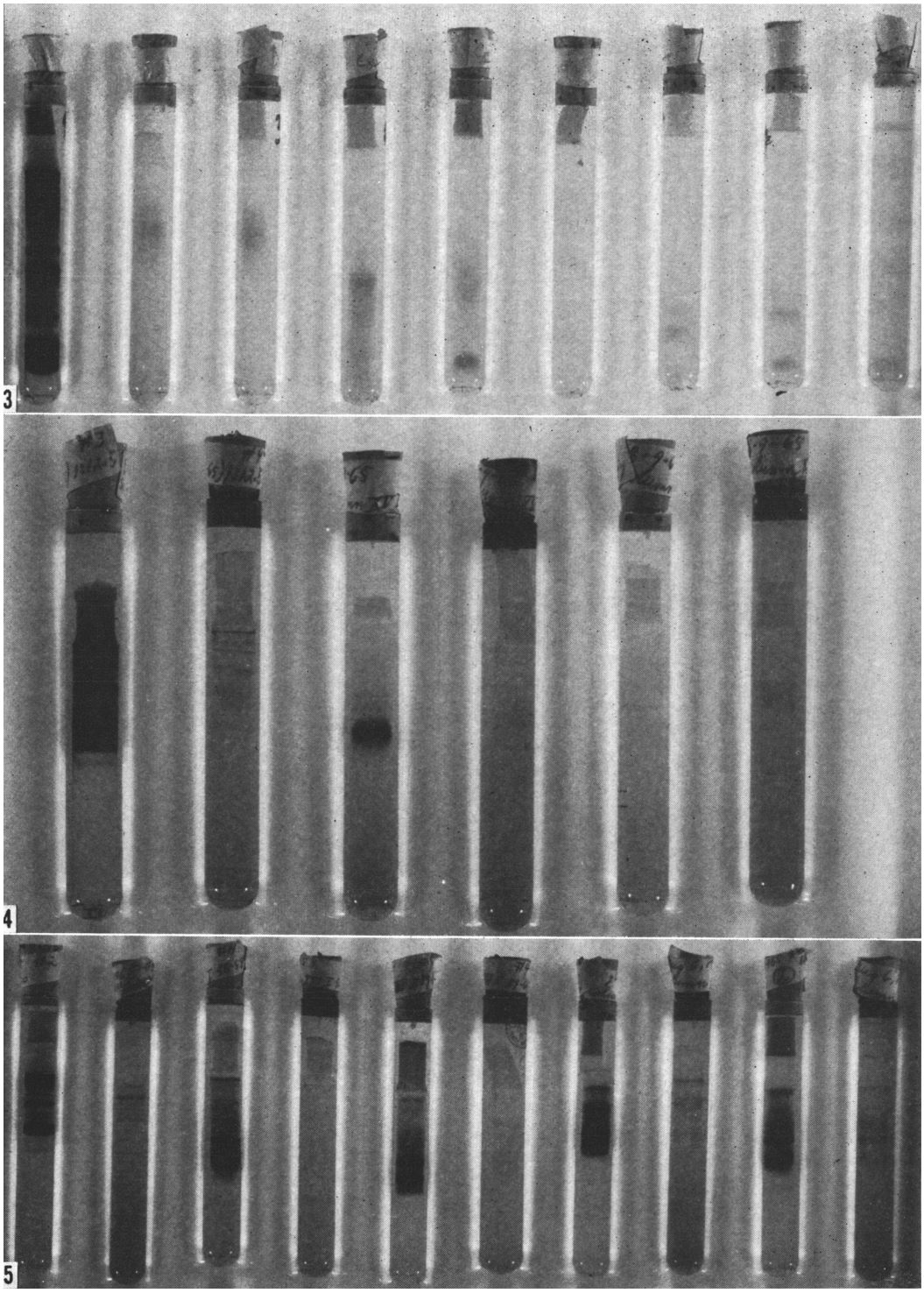

FIG. 3. Disc electrophoresis of eluate from DEAE-cellulose fractionation of 100% $(NH_4)_2SO_4$ ppt. of urinary CPC II (Table III). Tube No. 1—Serum, protein stain; Tube No. 2—Fr. 2-8, protein stain; Tube No. 3—Fr. 9-12, protein stain; Tube No. 4—Fr. 13-20, protein stain; Tube

No. 5—Fr. 20-31, protein stain; Tube No. 6—Fr. 32-36, protein stain; Tube No. 7—Fr. 48-52, protein stain; Tube No. 8—Fr. 53-56, protein stain; Tube No. 9—Fr. 57-70, protein stain.

FIG. 4. Disc electrophoresis of eluates from Table I and Table II. Tube No. 1—Serum, protein stain; Tube No. 2—Serum, carbohydrate stain; Tube No. 3—Table I, Fr. 54-59, protein stain; Tube No. 4—Table I, Fr. 54-59, carbohydrate stain; Tube No. 5—Table I, Fr. 60-74, protein stain; Tube No. 6—Table I, Fr. 60-74, carbohydrate stain; Tube No. 7—Table II, Fr. 52-55, protein stain; Tube No. 8—Table II, Fr. 52-55, carbohydrate stain; Tube No. 9—Table II, Fr. 56-70, protein stain; Tube No. 10—Table II, Fr. 56-70, carbohydrate stain.

FIG. 5. Disc electrophoresis of eluate from Table III. Tube No. 1—Serum, protein stain; Tube No. 2—Serum, carbohydrate stain; Tube No. 3—Table III, Fr. 48-52, protein stain; Tube No. 4—Table III, Fr. 48-52, carbohydrate stain; Tube No. 5—Table III, Fr. 53-70, protein stain; Tube No. 6—Table III, Fr. 53-70, carbohydrate stain.

eluate (tube 6). The major components were all relatively fast moving electrophoretically.

The disc electrophoretic pattern of DEAE-100 is shown in Fig. 3. The protein bands were lightly stained due either to their relatively small molecular weights or to the small amount of protein and the patterns were relatively simple as compared to the corresponding fractions of DEAE-30 and DEAE-60. Mobility increased as buffer concentration was increased and pH was decreased. Four to 5 components were present in the eluates following addition of NaCl, all of which were fast moving electrophoretically under the experimental conditions used (tubes 7-9).

Paired samples of disc electrophoretic patterns of DEAE-30 and DEAE-60 following addition of NaCl were stained for carbohydrate and protein and shown in Fig. 4. Very few components containing Schiff positive material were seen in DEAE-30 samples (tubes 4 & 6). Three strong Schiff positive bands with slow mobility similar to serum glycoproteins were present in tube #8, representing fractions 52-55 of DEAE-60. One slow moving and 3 fast moving carbohydrate-rich proteins were present in fractions 56-70 of DEAE-60.

Paired samples of disc electrophoretic patterns of DEAE-100 eluate following addition of NaCl were stained for carbohydrate and protein and shown in Fig. 5. Fast moving components found in fractions 48-52 and fractions 53-70, respectively, were rich in carbohydrate. These had a mobility greater than albumin.

Discussion. Recently several investigators (1,4,6,8-10) have used dialysis and ultrafiltration for separating the glycoproteins in urine. The principles involved are concerned with the separation of groups of compounds by the differences in their molecular sizes. A major disadvantage of the method lies in its failure to separate compounds of differing chemical compositions. The uronic acid containing compounds are distributed in each subfraction(1,4). The present method of precipitating urinary glycoproteins with increasing concentration of $(NH_4)_2SO_4$ permitted us to separate the glycoproteins into groups of decreasing molecular size and to leave the glycopeptides and acid mucopolysaccharide-containing compounds in the supernate.

Boyce *et al*(21) have found nondialyzable, barbiturate-soluble glycoproteins in urine which correspond to our CPC-II and CPC-III fractions(15). They made an electrophoretic study on the Cohn fractionation precipitates of Boyce's preparation(22), which revealed its complexity. Later King noted ultrafiltrable(5) and nonultrafiltrable(6) materials in this fraction. The ultrafiltrable material was electrophoretically heterogeneous. However, one fraction of the latter, after elution from DEAE-cellulose, was found to be homogeneous electrophoretically. Also, King reported that both ultrafiltrable and nonultrafiltrable fractions had blood group substance activity.

The present report includes the 3 fractions of nondialyzable $(NH_4)_2SO_4$ precipitable urinary glycoproteins. The protein distribution was 13.3%, 64.8% and 21.8% in 30%, 60% and 100% $(NH_4)_2SO_4$ precipitates respectively(15). The multicomponent pictures are shown in Tables I-III and amplified with the disc electrophoretic patterns. This study further demonstrated that 1) NaCl can be used to elute the tightly-bound sialic acid-rich glycoprotein from DEAE-cellulose column; 2) the sialic-rich glycoproteins occurred in vari-

ous molecular sizes; 3) many components were present in the eluates of the 30% and 60% $(NH_4)_2SO_4$ precipitate before addition of NaCl; 4) relatively simple pictures are shown in eluates of 100% $(NH_4)_2SO_4$ precipitate and sialic acid-rich fraction of 30% and 60% $(NH_4)_2SO_4$ precipitates; 5) approximately 50% of the glycoproteins in the $(NH_4)_2SO_4$ precipitates were contained in the sialic acid-rich fraction.

Because the separations achieved have been more sharply definitive than those seen as a result of methods previously employed in our own work or in that of others, the present procedures are to be used in determining variations in urinary carbohydrate protein complexes in several diseases, in which disturbances of connective tissue are known or suspected to exist. Pilot studies have been encouraging.

Summary. After the removal of Tamm and Horsfall mucoid, urinary glycoprotein was precipitated with $(NH_4)_2SO_4$ at 30%, 60% and 100% saturation. The precipitates were further fractionated on DEAE-cellulose columns with phosphate buffer of increasing concentration and decreasing pH. Sialic acid-rich glycoproteins were later eluted by addition of NaCl. Disc electrophoresis was used for demonstration of the components present. Many components were shown in the $(NH_4)_2SO_4$ precipitates. Relatively few components were present in the sialic acid-rich fractions.

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