Studies on the source of urinary cholesterol in the normal human male

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Abstract The aim of the present study was to obtain information on the source of urinary cholesterol in normal men of various age groups (22–25, 37–42, >63 yrs). Aliquots of 24-hr urine samples were fractionated by ultracentrifugation. Between 10–20% of the total cholesterol in urine (measured by gas-liquid chromatography) separately sedimented after 2×10^4 g-min and after 4×10^5 g-min of centrifugation. However, an average of more than 50% of the total sedimented after 6×10^6 g-min and only 10–20% of total cholesterol remained in this supernatant. These results indicate that most of the cholesterol in urine of normal males is a component of a light particulate fraction. Little difference was seen in the distribution of urinary cholesterol among the various age groups examined. The 4×10^5 g-min supernatant from 24-hour total urine samples was recentrifuged at $10^5 g$ for 60 min. The resulting pellet (100 P) was assayed for protein, total cholesterol and phospholipid. The cholesterol was 12–14% esterified. A molar ratio of total cholesterol to phospholipid of 1 to 0.8 was calculated. Assay of the 100 P fraction for marker enzymes showed an activity pattern characteristic for plasma membranes. Fractionation of the 100 P proteins by electrophoresis and separation of the 100 P phospholipids by thin-layer chromatography revealed patterns clearly different from those of human red cell plasma membrane. Electron micrographs of the 100 P fraction revealed an appearance similar to that of deteriorated membranes. The results suggest that most of the cholesterol in urine of the adult human male is present as a component of membranes derived from endogenous cells of the urogenital system.—**Cenedella, R. J., and J. A. Belis.** Studies on the source of urinary cholesterol in the normal human male. *J. Lipid Res.* 1981. **22:** 122–130.

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Interest in the presence of cholesterol in urine stems from early claims by Bloch and Sobotka (1, 2) that urine from patients with cancer contained greater quantities of cholesterol than that from normal individuals. More recently, Acevedo and co-workers reported that women with carcinomas of the steroid-producing glands and their target tissues (3) and men with prostatic cancer of non-specific stage (4) excreted abnormally large amounts of nonesterified cholesterol (NEC) in their urine. Most of the cholesterol in urine is nonesterified (5–7). Chu et al. (8) confirmed that about 60% of men with prostatic cancer (unstaged) had high levels of urinary cholesterol. Very recently, Belis and Cenedella (9) and Juengst et al. (7) independently reported that about 50% of men with early stage prostatic cancer excreted larger than usual quantities of urinary nonesterified cholesterol (UNEC). Thus, the presence of high levels of UNEC might be of value in the early diagnosis of this cancer.

Little is known about the source of cholesterol in the urine of either normal men or men with prostatic cancer. Urinary cholesterol appears to be of endogenous origin; that is, from the urogenital tract, since Acevedo et al. (4) found no correlation between UNEC excretion and serum levels of cholesterol. The purpose of the present study was to obtain information on the nature and source of UNEC in the normal adult human male. We report that 80–90% of the NEC in urine of normal men sediments upon centrifugation. The results further indicate that most of the NEC in urine of adult men is associated with a light particulate fraction, similar to plasma membranes.

METHODS

Fractionation of urinary cholesterol

Twenty-four-hour urine samples were collected from three different age groups of men (22–25, 37–42, 63–72 yr) and refrigerated until processed

Abbreviations: NEC, nonesterified cholesterol; UNEC, urinary nonesterified cholesterol; 20 P, pellet resulting from recentrifuging the 2×10^4 g-min supernatant of whole urine at 20,000 $g \times 20$ min; 100 P, pellet resulting from recentrifuging the 4×10^5 g-min supernatant of urine at 100,000 $g \times 60$ min; SDS, sodium dodecylsulfate; HDL, high density lipoproteins; GLC, gas–liquid chromatography; EM, electron microscope.

(9). Processing was usually conducted within one day after collection. Urine samples were tested for pH and for the presence of protein, glucose, bilirubin, blood, ketones, nitrate, and urobilinogen with N-Multistix reagent strips (Miles Labs, Elkhart, IN). The pH and specific gravity of urine samples for the 37–42-yr-old group were closely examined and found to range between 5.44–6.23 and 1.0072–1.0176, respectively. Since no abnormal tests were obtained and participants in the study had no history of prostatic disorders, they were considered to be normal.

Approximately 50-ml aliquots of 24-hour urine samples were fractionated by centrifugation. The supernatant, considered as the upper 90% of the volume, from an initial 2×10^4 g-min spin (1400 *g* × 14 min) was collected; a 10-ml aliquot was saved and the remainder was spun for 4×10^5 g-min (20,000 *g* × 20 min in a 42.1 rotor on a Beckman model L ultracentrifuge). After saving an aliquot of this supernatant, the remainder was centrifuged for 6×10^6 g-min (100,000 *g* × 60 min). The nonesterified cholesterol in 10-ml aliquots of the whole urine and of each supernatant fraction was extracted into chloroform and quantitated by gas–liquid chromatography (GLC), essentially as described before in detail (9). Chloroform and other organic solvents were doubly redistilled prior to use. Based upon the concentration of nonesterified cholesterol in whole urine and in each supernatant fraction, the content of cholesterol in the sediments or pellets resulting from the 2×10^4 g-min, 4×10^5 g-min, and the 6×10^6 g-min centrifugations was calculated by difference. Due to potential loss of the minute sediments obtained from centrifugation of individual 10-ml aliquots of the various fractions, this indirect assay was considered to be quantitatively superior to direct assay of these sediments.

In some cases the free and esterified cholesterol present in the total lipids extracted from 10-ml aliquots of whole urine were separated by thin-layer chromatography on silica gel G (10). The silica gel zones corresponding to cholesterol and cholesteryl esters were separately extracted with diethyl ether. The residue recovered from the silica gel zone corresponding to cholesteryl esters was saponified in 0.12 ml of 33% KOH plus 2.0 ml of 95% ethanol for 1 hr at 70°C. Sterols were extracted into hexane, recovered, and quantitated by the GLC analysis. Blanks consisting of 10 ml of saline were carried through the chloroform extraction, thin-layer chromatography, saponification, and GLC analyses. Corrections were made for the presence of cholesterol and cholesteryl ester-like material in the solvent blanks.

Collection of urine sediment fractions

Total 24-hr urine samples were fractioned as described in **Fig. 1.** The combined pellet recovered from the 20,000 *g* × 20 min spin of urine was termed the 20 P fraction and the combined pellet obtained from centrifuging the 20,000 *g* supernatant (4×10^5 g-min supernatant) for 60 min at 100,000 *g* was termed the 100 P fraction (Fig. 1). The recovered 20 P pellet was yellowish in color and gelatinous in texture. The 100 P pellet was yellowish-white and flocculent. The pellets were resuspended and washed in isotonic saline and finally suspended in 1–2 ml of saline by repeated uptake and explusion through a 22 gauge needle. Throughout the processing procedure all fractions were kept at 0 to 5°C. The 20 P and 100 P fractions were saved for chemical analyses and the 100 P fraction was also saved for measurement of marker enzyme activities.

Chemical assays

Lipids. Between 60 to 80% of the resuspended pellet fractions was laked in 20 volumes of chloroform-methanol 2:1 (v/v) and the lipids were extracted according to Folch, Lees, and Sloane Stanley (11). Generally, 10% aliquots of the recovered lipid were separately assayed by colorimetric methods for total cholesterol and total phospholipid (12). The phospholipids in the remaining lipid extracts were fractionated by two-dimensional thin-layer chromatography using silica gel H (13). The separated phospholipids were individually recovered and assayed for phosphorus content (12). The concentration of esterified cholesterol in the lipids of selected 100 P fractions was estimated by measurement of cholesterol (by GLC assay) before and after saponification. Total lipids from human erythrocyte ghosts, prepared according to Bond and Hudgins (14), were identically extracted and analyzed.

Proteins. Small aliquots of the 20 P and 100 P fractions were diluted (1/20 to 1/60) with isotonic saline and the protein content measured (15). Aliquots of the fractions containing 200–400 μg of protein were solubilized in 2% sodium dodecylsulfate and reduced with 5% mercaptoethanol. Proteins were separated by polyacrylamide gel electrophoresis using 10% gel rods (8 cm) with a 4% stacking gel (1.5 cm) according to Laemmle (16) and using a Bio-Rad electrophoresis cell and apparatus run at 3 mA/gel. After fixing the gels in 12.5% (w/v) trichloroacetic acid, the proteins were stained with either Coomassie brilliant blue for total protein or with periodic acid-Schiff reagent for glycoproteins (17). Marker proteins of bovine serum albumin (68,000 daltons), ovalbumin

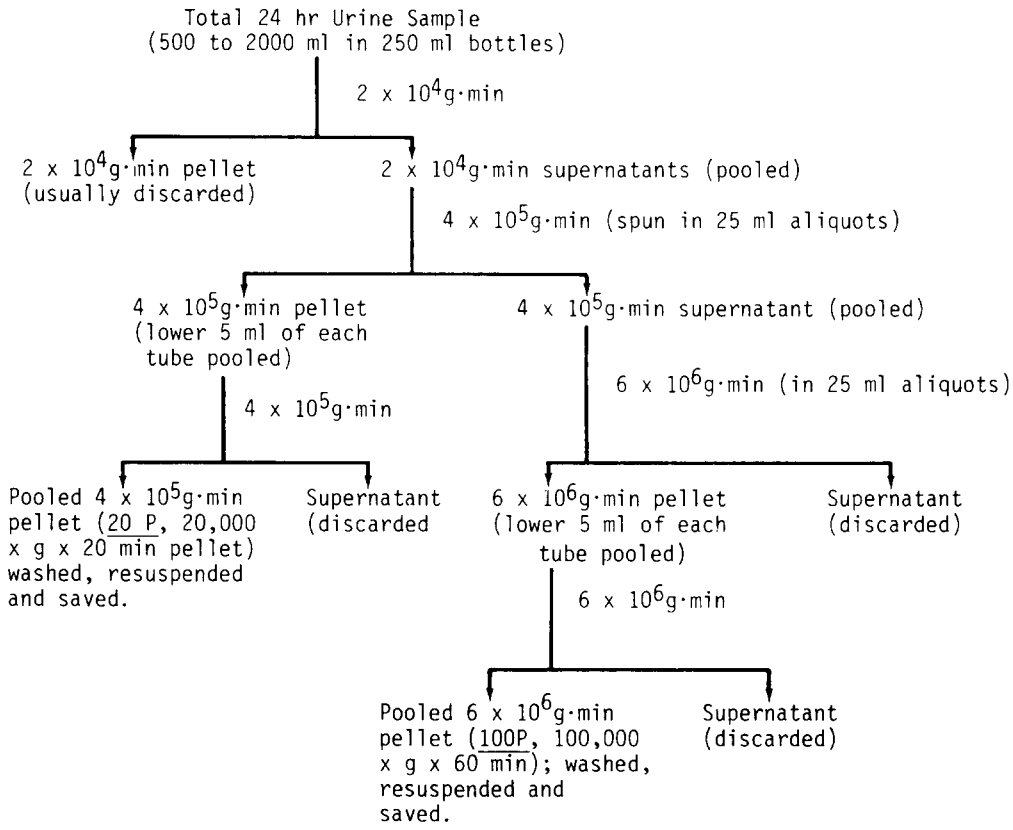

Fig. 1. Flow diagram of the fractionation of urine by ultracentrifugation. Method used for separation and recovery of pellet fractions from 24-hr total urine samples.

(43,000 daltons), and carboxypeptidase (34,600 daltons) were used to calibrate the gels.

Assay of marker enzyme activities

Aliquots of hepatic microsomes and plasma membranes, prepared and purified according to Touster et al. (18), and aliquots of the 100 P fractions were assayed for marker enzyme activities. Rat liver was used only because it was convenient and a well known tissue with regard to the subcellular distribution of marker enzymes. 5′-Nucleotidase was measured as a marker for plasma membranes (18). Glucose-6-phosphatase (19), NADPH-cytochrome c reductase (20), and non-specific esterase activities (21) were measured to assess the presence of microsomes. Activities were expressed as μg of substrate converted/min per mg protein and compared relative to 5′-nucleotidase activity. Although NADPH-cytochrome c reductase and non-specific esterase activities paralleled one another, non-specific esterase activity was preferentially examined due to the simplicity of the assay and the smaller specific activity values obtained. The 100 P fractions were assayed for these enzyme activities immediately after their preparation.

Electron microscopy

Aliquots of the 100 P fractions were centrifuged in small heavy-walled polyethylene cups. The pellets were fixed in 4% glutaraldehyde in Milonigs buffer with secondary fixation in 2% osmium tetraoxide. The samples were dehydrated in graded alcohols and embedded in Spurs and epon. Samples were cut on a LKB ultramicrotome and stained with lead citrate. The sections were viewed and photographed on a RCA EM-U4.

RESULTS

Aliquots of 24-hour urine samples from three groups of normal males of varying ages (22–25, 37–42, >63 yr) were separated by centrifugation into four fractions: a 2×10^4 g-min pellet, a 4×10^5 g-min pellet (obtained by centrifuging the 2×10^4 g-min supernatant at 20,000 $g \times 20$ min), a 6×10^6 g-min pellet (obtained by centrifuging the 4×10^5 g-min supernatant at 100,000 $g \times 60$ min) and the supernatant from the 6×10^6 g-min spin. No statistically significant differences between groups were

TABLE 1. Fractionation by centrifugation of urinary non-esterified cholesterol (UNEC) in normal men

Group and Individual	24-hr Total UNEC (mg)[b]	% Distribution of Urinary NEC[a]			
		2×10^4 g-min pellet	4×10^5 g-min pellet	6×10^6 g-min pellet	6×10^6 g-min supernatant
22–25 yr					
R.B.	0.77	1.4	10.3	53.8	15.0
D.S.	0.71	3.2	29.8	77.4	9.1
W.C.	1.05	16.0	23.1	47.3	13.7
S.R.	0.72	4.8	17.3	46.4	31.5
J.P.	0.73	7.6	3.5	76.4	12.6
D.M.	0.86	0	18.6	45.0	36.5
R.M.	0.83	20.6	21.0	39.3	19.1
	0.81 ± 0.05	7.7 ± 2.9	17.7 ± 3.2	55.1 ± 5.9	19.6 ± 3.9
37–42 yr					
R.C.	0.46	14.3	10.5	67.9	7.2
R.O.	1.44	16.5	24.8	51.0	7.7
E.M.	0.74	12.4	6.0	60.5	21.1
A.W.	0.99	30.2	16.0	42.2	11.7
A.A.	0.98	8.0	22.9	61.0	8.1
	0.92 ± 0.16	16.3 ± 3.7	16.0 ± 3.6	56.5 ± 4.5	11.2 ± 2.6[d]
63–72 yr[c]					
P.C.	0.54	6.1	19.8	56.7	17.4
S.D.	1.57	25.4	19.1	45.7	9.2
C.G.	1.82	8.1	18.3	68.6	5.0
C.K.	4.04	15.8	34.6	39.8	9.8
	1.99 ± 0.74	13.9 ± 4.4	23.0 ± 3.9	52.7 ± 6.4	10.4 ± 2.6[d]
Overall averages	1.14 ± 0.21	11.9 ± 2.2	18.5 ± 2.0	54.9 ± 3.1	14.7 ± 2.2

[a] Non-esterified cholesterol content of each pellet was determined by the differences in the NEC content (measured by GLC) of the supernatant fractions before and after each centrifugation. Values are means ± one standard error.

[b] In previous work (Belis and Cenedella. 1979 *Cancer* **43:** 1840), values above 1.2 mg UNEC/24 hr were taken to be abnormally high.

[c] Assumed to be normal older men; however, status of prostate was not evaluated in these individuals and one individual possessed a very high UNEC (4.04 mg).

[d] $P(t)$ of difference from 22–25-yr-old group ≤0.10 (Student "t" test).

observed in the distribution of nonesterified cholesterol among the various pellet fractions. An average of between 10–20% of the total UNEC separately sedimented at both 2×10^4 g-min and 4×10^5 g-min (**Table 1**). However, an average of more than 50% of the total UNEC sedimented at 6×10^6 g-min. In every sample, this pellet fraction contained the single largest percentage of the total UNEC. Only 10–20% of the total UNEC remained in the supernatant of the 6×10^6 g-min centrifugation. Thus, only a minor fraction of the total NEC in urine appeared to be "soluble". Urine from the younger males seemed to possess relatively more NEC in this "soluble" fraction.

Between about 1 to 14% of the total cholesterol in whole urine and 12–14% in the 6×10^6 g-min pellet (100 P fraction) was found to be esterified (**Table 2**). This value range is less than the 17–21% of total urinary cholesterol reported as esterified by Vela and Acevedo (6) and Juengst et al. (7). How-

TABLE 2. Esterified cholesterol in whole urine and the 6×10^6 g-min pellet (100 P fraction)

Individual	Age	Esterified Cholesterol (% Total)	
		Whole Urine[a]	6×10^6 g-min pellet (100 P)[b]
R.C.	41	1.3	11.6
R.O.	39	6.6	Not determined
E.M.	43	9.3	11.8
A.W.	38	5.3	13.9
A.A.	40	14.6	12.1

[a] Cholesterol and cholesteryl esters in the total lipids extracted from 10-ml aliquots of whole urine were separated by TLC and recovered. The cholesteryl ester fractions were saponified. Free cholesterol and that recovered after saponification were quantitated by GLC analysis. Corrections were made for the presence of trace levels of cholesterol and cholesteryl ester-like materials in solvent blanks carried through the procedures.

[b] Twenty percent aliquots of the total lipid recovered from the 6×10^6 g-min pellet (100 P fraction) were either directly assayed for nonesterified cholesterol by GLC assay or saponified and then assayed (total cholesterol). Esterified cholesterol was calculated by difference.

TABLE 3. Ratios of marker enzyme activities in the 100,000 *g* pellet (100 P) to 5′-nucleotidase

Enzyme Source	Relative Enzyme Activities[a]		
	Esterase	5′-Nucleotidase	Glucose-6-Phosphatase
Microsomes (liver)	4.88	1.00	3.75
Plasma membranes (liver)	0.479	1.00	0.307
R.C. (100 P fraction)	0.241	1.00	0.315
R.B. (100 P fraction)	0.181	1.00	0.283
D.S. (100 P fraction)	0.109	1.00	0.204

[a] The 10^5 *g* pellets from three males (ages 22, R.B.; 25, D.S.; and 40 yr, R.C.) along with purified rat liver microsomes and plasma membranes were assayed for 5′-nucleotidase (plasma membrane marker), non-specific esterase activity (microsomal marker enzyme), and glucose-6-phosphatase (microsomal marker enzyme). Enzyme activity was calculated as μM of substrate reacted/min per mg protein. 5′-Nucleotidase activities were as follows: microsomes = 0.094; plasma membranes = 0.708; and 100 P fractions, RC = 0.054, RB = 0.157, DS = 0.090.

ever, the possible contribution of trace amounts of cholesterol and cholesteryl ester-like material in the solvents used for cholesterol extraction was apparently not evaluated in their studies. If the blanks were ignored in our studies, cholesteryl esters would have constituted between 9–17% of the total cholesterol in whole urine and the 100 P fraction.

Sedimentation of most of the nonesterified cholesterol in urine at 100,000 *g* × 60 min suggested that cholesterol in urine was present mainly as a component of a light particulate fraction, perhaps of a fraction analogous to microsomes, plasma membranes, or lipoproteins. In order to further examine these possibilities, the 4×10^5 g-min supernatant from total 24-hour urine samples was recentrifuged at 100,000 *g* × 60 min. The resulting pellet (100 P) was recovered, washed, resuspended, and assayed for marker enzyme activities and chemical composition.

The 5′-nucleotidase, α-napthyl acetate esterase and glucose-6-phosphatase activities of the 100 P fraction from three males (22, 25, and 40 yr of age) were measured and compared to those of purified hepatic microsomes and plasma membranes. Enzyme activities were expressed relative to 5′-nucleotidase, a marker enzyme for plasma membranes (18). The 100 P fraction possessed a high 5′-nucleotidase activity relative to the esterase and glucose-6-phosphatase activities (**Table 3**). The overall activity profile of the 100 P fraction was similar to a representative plasma membrane fraction.

Direct chemical analysis of the 100 P fraction revealed a protein to total cholesterol to phospholipid weight ratio of about 5.6 to 1 to 1.6 (**Table 4**). As presented earlier, about 12–14% of the total cholesterol in this fraction appears to be esterified (Table 2). Most of the protein consisted of a high molecular weight glycoprotein (as judged by positive staining with Schiff's reagent) that probably was carried over from the 20 P fraction (pellet which sedimented at 20,000 *g* × 20 min). Sphingomyelin was the main phospholipid in the 100 P fraction, comprising over 40% of the total (**Fig. 2** and **Table 5**). The 100 P fraction, unlike the red cell membrane and other plasma membranes (22, 23), contained a relatively low percentage of phosphatidylcholine (about 10%). Based upon the relative percent composition of the phospholipids and an estimation of their molecular weights, a molar ratio of total cholesterol to phospholipid in the 100 P fraction was estimated to be 1 to 0.81. The high percent content of sphingomyelin and the low percent of phosphatidylcholine in the 100 P fraction is unusual for membranes. Sphingomyelins are high in plasma membranes and even more so in lysosomal membranes (23), but not to the level of 40%. Further, the relative concentration of phosphatidylcholine is high in both of these membranes. Only the ocular lens possesses a phospholipid profile similar to that of the 100 P fraction. Sphingomyelins comprise 40–60% and phosphatidylcholine 5–10% of the total phospholipids in the human lens (24). The unusual phospholipid profile of the 100 P fraction from urine might represent a particular membrane of certain cells of the male urogenital tract or might reflect changes in phospholipids resulting from their passage through the urogenital system.

The proteins present in the 100 P fraction and in purified human red cell membranes were separated by SDS electrophoresis using 10% polyacrylamide gel rods. The 20 P fraction, which contained 4–10 times

TABLE 4. Major components of the 100,000 *g* pellet (100 P) from urine[a]

Male	Age	Protein/Total Cholesterol/Phospholipid in 100 P Fraction (w/w/w)
R.B.	22	4.71/1/1.50
D.S.	25	7.07/1/1.42
D.J.S.	25	5.94/1/1.68
R.C.	40	4.54/1/1.89
	Mean ± S.E.	5.57 ± 0.59/1/1.62 ± 0.10

[a] The 20,000 *g* supernatant from 24-hr total urine samples was recentrifuged at 10^5 *g* for 60 min. The recovered pellet (100 P) was directly assayed for protein. Lipids were extracted into chloroform–methanol 2:1, recovered and assayed for total cholesterol and phospholipids by colorimetric assay. The concentration of total cholesterol in the 100 P fraction of RB = 352 μg, DS = 332 μg, DJS = 274 μg, and RC = 333 μg. Approximately 12–14% of the total cholesterol in the 100 P fraction was esterified (see Table 2).

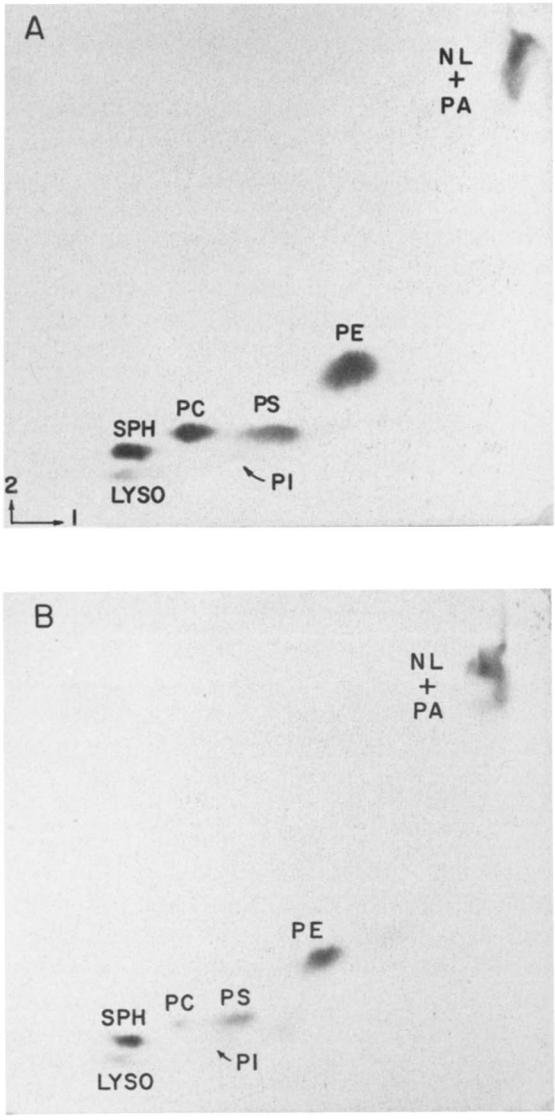

Fig. 2. Separation of RBC, (A) and 100 P, (B) phospholipids by two-dimensional TLC on silica gel H. The direction one solvent was $CHCl_3$–CH_3OH–CH_3COOH–0.9% NaCl 100:50:16:8 (v/v/v/v). For direction two, the same reagents were used at a volume ratio of 100:16:16:4, respectively. SPH, sphingomyelins; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; LYSO, lysophosphatides; NL + PA, neutral lipids + phosphatidic acid and polyglycerol phosphatides. Lipids were visualized for photography by exposure to iodine vapor.

Phospholipid	Distribution of Phospholipid-Pi	
	RBC (A)	100 P (B)
	% total	
SPH	28.1	41.4
PC	30.8	8.2
PE	23.6	16.3
PS	14.9	27.7
PI	2.0	3.1
LYSO	1.7	3.4
PA		

TABLE 5. Relative composition of phospholipids in human erythrocyte plasma membrane and in the 100 P fraction from urine

Phospholipid	% Total Phospholipid-Phosphorus[a]	
	Human RBC (N = 2)	100 P Fraction (N = 3)
Sphingomyelin	28.1, 30.3 (24)[b]	43.4 ± 4.7
Phosphatidylcholine	30.8, 28.1 (31)	9.8 ± 2.8
Phosphatidylethanolamine	23.6, 17.8 (27)	19.3 ± 4.9
Phosphatidylserine	14.9, 12.2 (10)	18.5 ± 5.8
Phosphatidylinositol	2.0, 4.4 (5.1)	5.0 ± 0.7
Lysophosphatides	0.7, 4.0 (2.4)	1.8 ± 0.4
Phosphatidic acid plus polyglycerol phosphatides	0, 0 (0)	0.7 ± 0.3

[a] Total phospholipid extracted from two samples of human red cell ghosts (one from blood bank cells and one from fresh cells) and from three separate $10^5 g \times 60$ min pellets (100 P) of urine were separated by two-dimensional thin-layer chromatography. The results are expressed as the mean percent of the total phospholipid-phosphorus ± one standard error.

[b] Values in parentheses are from Van Deenen and DeGier (22, average of values presented in Table X).

more total protein than the 100 P fraction, was also examined. Many proteins in the 30,000–70,000 molecular weight range were present in the 100 P fraction (**Fig. 3**); however, a high molecular weight glycoprotein (stained positively with Schiff reagent, band *a* in Fig. 3) constituted the major protein in this fraction. An identically migrating glycoprotein comprised essentially all of the protein of the 20 P fraction. In view of the much higher content of protein in the 20 P than 100 P fraction, it seems likely that the high molecular weight glycoprotein recognized in the 100 P fraction represents carry-over from the 20 P fraction. The electrophoretic profile of red cell membrane proteins was sufficiently different from that of the urine pellets to exclude the possibility that the 100 P fraction contained significant amounts of erythrocyte membrane. For example, the red cell membrane contained major amounts of very high molecular weight proteins which poorly penetrated the gels; these proteins were not found in the urine pellets. Among several other differences, the red cell membrane contained a major protein at about 45,000 daltons that was absent from the urine fractions.

The possibility was examined that once in urine erythrocyte membranes might lose or gain proteins resulting in their resembling the 100 P fraction. Freshly prepared human red cell ghosts were added to a 6×10^6 g-min supernatant of a 24-hr urine sample (i.e., minus the sediment fractions) and incubated for several hours at 37°C followed by overnight incubation at room temperature. The urine-

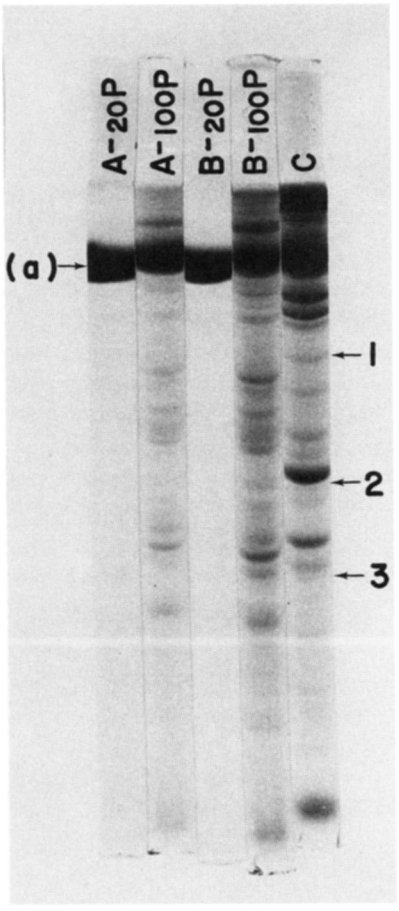

Fig. 3. Electrophoretic profile of proteins recovered from the urine pellets and from human red cell ghosts. Aliquots of protein (20–40 μg) from the 20,000 *g* × 20 min pellet (20 P) and the 100,000 *g* × 60 min pellet (100 P) from 24-hour urine samples from two normal males (A, 22 yr; B, 25 yr) and from human red cell ghosts (C) were separated by SDS-polyacrylamide gel electrophoresis using 10% gel rods with a 4% stacking gel. Gels were stained with Coomassie brilliant blue. The band in all five gels marked as (a) also stained with periodic acid-Shiff reagent (glycoproteins). The numbers 1, 2, and 3 indicate the approximate location of marker proteins; 1, bovine serum albumin (mol wt 68,000); 2, ovalbumin (mol wt 43,000); and 3, carboxypeptidase (mol wt 34,600).

treated red cell membranes were recovered by centrifugation. The electrophoretic profile indicated that the urine-treated erythrocyte membranes lost some lower molecular weight components; however, they retained the high molecular weight proteins characteristic of red cell membranes and absent from the 100 P fraction.

Electron micrographs of the 100 P fraction revealed a variety of structures. These included circular vesicles which were both intact and fragmented and a background of amorphous and granular material. These structures appear similar to membranes that have undergone varying degrees of degeneration (25). Some degenerative changes in 100 P components might be expected during the 24-hr urine collection period and the several days of processing required to isolate the 100 P fraction. Further, at the site of its origin, the 100 P fraction might represent degenerated cellular components. In addition to the vesicular structures mentioned above, the 100 P fraction contains many dense bodies of about 50 to 100 nm in diameter.

DISCUSSION

Between 80 to 90% of the nonesterified cholesterol in urine of normal men is associated with particulate fractions. The small amount of cholesterol which sedimented upon low speed centrifugation of urine presumably reflects the presence of intact cells, such as erythrocytes, and of larger cellular debris. However, it is clear that most of the cholesterol in urine is associated with a light particulate fraction, one which sedimented at $10^5 \times g$. This observation might appear to disagree with the findings of Rosenthal, Sevin, and Marino (26) that indicated that cholesterol in human urine is bound to specific soluble proteins: one of 3.6×10^5 daltons and a group of glycoproteins of varying molecular weights that could be aggregates of a single 2.5×10^4 dalton monomer. However, the quantitative significance of these cholesterol-binding proteins in accounting for total urinary cholesterol is unclear from their data and, perhaps more importantly, their findings are for females with cancer. The nature and source of urinary cholesterol in normal males could be very different from that in females with cancer.

The cholesterol-rich pellet (100 P fraction) isolated from normal men by ultracentrifugation of urine possessed an enzyme activity profile characteristic of plasma membranes and a molar ratio of total cholesterol to phospholipid of 1 to 0.8, a ratio similar to that found in various isolated plasma membranes (23). The percent of total cholesterol as cholesteryl ester (12–14%) in this fraction is also similar to that of plasma membranes (11–12%) (23). Further electron micrographs indicated that the 100 P fraction at least partially resembled membranes which had undergone varying degrees of deterioration (25). It seems unlikely that this fraction contains significant amounts of erythrocyte membrane, since the protein and phospholipid profiles of the fraction were clearly different from those of the erythrocyte membrane. Based upon these observations, we suggest that cholesterol in urine of normal adult males, regardless of age, could be present as a component of

non-erythrocytic plasma membranes, endogenously derived, i.e., from cells of the urogenital tract. This conclusion does not preclude the possibility of cholesterol also being present in urine in other macromolecular associations, perhaps as a component of lipoproteins.

Segal and co-workers (27) recently reported that the urine of normal humans contained HDL apoproteins which were assembled into a macromolecular particle containing cholesterol and phospholipid. The particle resembled plasma HDL. However, the particle differed from plasma HDL species in that its density (>1.24 g/ml) was greater than that of plasma HDL. The authors speculate that these HDL-like particles might be excreted by the kidney and then undergo changes in its passage through the urinary tract. Apoproteins of very low density and low density lipoproteins were not present in the urine.

HDL-like particles might account for some of the urinary cholesterol present in the 100 P fraction. Particles typical in size for HDL (10–20 nm) were not recognized since they were beyond the resolving power of our EM photos. The dense circular bodies which were seen are apparently too large (50–100 nm) to represent the HDL-like particles. It seems unlikely, however, that HDL-like particles account for most of the total cholesterol in the 100 P fraction, since most of the cholesterol in plasma HDL is esterified (28), but only 12–14% of the total cholesterol in 100 P was esterified. Rather, we conclude that most of the cholesterol present in the urine of the adult human male is a component of a light-particulate fraction which possesses many characteristics common to plasma membranes, but yet distinct from the red blood cell membrane. We suggest that these urinary membranes are derived from endogenous cells of the male urogenital system.

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REFERENCES

1. Bloch, E., and H. Sobotka. 1938. Urinary cholesterol in cancer. *J. Biol. Chem.* **124:** 567–572.
2. Sobotka, H., and E. Bloch. 1939. Urine extractives in cancer. *Am. J. Cancer.* **35:** 50–54.
3. Acevedo, H. F., E. A. Campbell, D. W. Hayeslip, J. Gilmore, L. P. Merkow, J. C. Frich, Jr., and R. C. Grauer. 1971. Urinary cholesterol. IV. Its excretion in women with neoplasms of the genital system. *Obstet. Gynecol.* **37:** 425–436.
4. Acevedo, H. F., E. A. Campbell, E. L. Saier, J. C. Frich, Jr., L. P. Merkow, D. W. Hayeslip, S. P. Bartok, R. C. Grauer, and J. L. Hamilton. 1973. Urinary cholesterol. V. Its excretion in men with testicular and prostatic neoplasms. *Cancer.* **32:** 196–205.
5. Vela, B. A., and H. F. Acevedo. 1969. Determination of urinary cholesterol by gas–liquid chromatography. *Steroids.* **14:** 499–517.
6. Vela, B. A., and H. F. Acevedo. 1969. Urinary cholesterol. I. Isolation and characterization of "free" cholesterol in normal and pregnant subjects. *J. Clin. Endocrinol.* **29:** 1251–1258.
7. Juengst, D., A. Pickel, E. Elsaesser, F. J. Marx, and H. J. Karl. 1979. Urinary cholesterol excretion in men with benign prostatic hyperplasia and carcinoma of the prostate. *Cancer.* **43:** 353–359.
8. Chu, T. M., S. K. Shukla, A. Mittleman, and G. P. Murphy, 1975. Comparative evaluation of serum acid phosphatase, urinary cholesterol and androgens in diagnosis of prostatic cancer. *Urology.* **6:** 291–294.
9. Belis, J. A., and R. J. Cenedella. 1979. Urinary nonesterified cholesterol excretion in adenocarcinoma of the prostate. *Cancer.* **43:** 1840–1846.
10. Cenedella, R. J. 1980. Concentration-dependent effects of AY-9944 and U18666A upon sterol synthesis in brain: variable sensitivities of metabolic steps. *Biochem. Pharmacol.* **29:** 2751–2754.
11. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipid from animal tissues. *J. Biol. Chem.* **226:** 497–509.
12. Pollet, S., S. Ermidou, F. Le Saux, M. Monge, and N. Baumann. 1978. Microanalysis of brain lipids: multiple two-dimensional thin-layer chromatography. *J. Lipid Res.* **19:** 916–921.
13. Zelenka, P. 1978. Phospholipid composition and metabolism in the embryonic chick lens. *Exp. Eye Res.* **26:** 267–274.
14. Bond, G. H., and P. M. Hudgins. 1979. Kinetics of inhibition of NaK-ATPase by Mg^{2+}, K^+, and vanadate. *Biochemistry.* **18:** 325–331.
15. Lees, M. B., and S. Paxman. 1972. Modification of the Lowry procedure for the analysis of proteolipid protein. *Anal. Biochem.* **47:** 184–192.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London).* **227:** 680–685.
17. Segrest, J. P., and R. L. Jackson. 1972. Molecular weight determinations of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. *In* Methods in Enzymology. V. Ginsberg, S. P. Colowick, and N. O. Kaplan, editors. Academic Press, NY. Vol. 28, Part B, 54–63.
18. Touster, O., N. N. Aronson, Jr., J. T. Dulaney, and H. Hendrickson. 1970. Isolation of rat liver plasma membranes; use of nucleotide pyrophosphatase and phosphodiesterase I as marker enzymes. *J. Cell Biol.* **47:** 604–618.
19. DeDuve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Applemans. 1955. Tissue fractionation studies.

6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* **60:** 604–617.
20. Sottocasa, G. L., B. Kuylenstierna, L. Ernster, and A. Bergstrand. 1967. An electron-transport system associated with the outer membrane of liver mitochondria. *J. Cell Biol.* **32:** 415–438.
21. LaBadie, J. H., and N. N. Aronson, Jr. 1973. Lysosomal β-D-mannosidase of rat liver. *Biochim. Biophys. Acta.* **321:** 603–614.
22. Van Deenen, L. L. M., and J. de Gier. 1964. Chemical composition and metabolism of lipids in red cells of various animal species. *In* The Red Blood Cell. C. W. Bishop and D. M. Surgenor, editors. Academic Press, NY. 243–307 (see Table X).
23. Emmelot, P. 1977. The organization of the plasma membrane of mammalian cells: structure in relationship to function. *In* Mammalian Cell Membranes. G. A. Jamieson and D. M. Robinson, editors. Butterworths, Boston. Vol. 2, 1–54 (See Tables 1.1 and 1.2).
24. Broekhuyse, R. M. 1969. Phospholipids in tissues of the eye. III. Composition and metabolism of phospholipids in human lens in relation to age and cataract formation. *Biochim. Biophys. Acta.* **187:** 354–365.
25. Bradford, H. F., D. G. Jones, H. K. Ward, and J. Booher. 1975. Biochemical and morphological studies of the short and long term survival of isolated nerve-endings. *Brain Res.* **90:** 245–259.
26. Rosenthal, A., I. F. Sevin, and L. Marino. 1978. Characterization of the nonesterified cholesterol binding protein from human urine. *In* Progress of the Third International Symposium on the Detection and Prevention of Cancer. H. E. Nieburg, editor. Dekker, NY. 249–253.
27. Segal, P., L. I. Gidez, G. L. Vega, D. Edelstein, H. A. Eder, and P. S. Roheim. 1979. Apoproteins of high density lipoproteins in the urine of normal subjects. *J. Lipid Res.* **20:** 784–788.
28. Scanu, A. M., and C. Wisdom. 1972. Serum lipoproteins: structure and function. *Ann. Rev. Biochem.* **41:** 703–730.