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Research Article

TOTAL NONDIALYZABLE SOLIDS (TNDS) IN HUMAN URINE. I. THE AMOUNT AND COMPOSITION OF TNDS FROM NORMAL SUBJECTS [1]

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A number of high molecular weight compounds have been separated as constituents of normal urine. Regardless of the investigators' intention, in practically all of these studies only one or a few components were isolated. Interpretation and correlation of these numerous data, obtained by various procedures, would be greatly facilitated by the quantitative determination of the amount and composition of the total biocolloid content of normal 24 hour urine specimens. Hamerman, Hatch, Reife, and Bartz (1) have reported the recovery of the total nondialyzable solids (TNDS) from three 24 hour urine specimens by alternate dialysis and vacuum distillation with final lyophilization. The following experiments were designed to expand these data and to study individual variations in TNDS, in order to establish the limits of such variation in normal human urine.

MATERIALS AND METHODS

Subjects for the study were laboratory workers who were in excellent health, had no history or clinical evidence of renal or urinary tract disease, and who were within the average weight and height range for their age group. Each followed his daily routine without special diet, variation in fluid intake, or alteration in physical activity.

Twenty-four hour urine specimens were collected into chemically clean containers, with 10 ml. of phenylmercuric nitrate 1 : 1,000 as an inhibitor of enzymatic and bacterial activity. As soon as the collection was completed, each specimen was measured and the total specimen (or a 1,200 ml. aliquot) divided into 150 ml. portions. Each portion was placed in a 35 cm. Visking cellulose tubing [2] of 2.8 cm. inflated diameter. The sacs were knotted to include a 35 ml. volume of air in each bag. This arrangement kept the sac upright, absorbed mechanical shock, and permitted agitation of the urine during dialysis. The sacs were completely submerged in a closed carboy containing 20 liters of distilled water and dialyzed by brisk stirring at approximately 3° C. The distilled water was changed at daily intervals until the specific conductance of the urine was reduced to less than 9×10^{-5} mhos when measured at 0° C. in a Shedlovsky type closed cell with a constant of 14.3.

The sacs were emptied, with care being taken to flush out any solid which had separated from solution and adhered to the sides or bottom. The 24 hour specimens were then lyophilized in two steps, utilizing a Vertis freeze-drier of 7.8 liters capacity, with Cellosolve and solid carbon dioxide as the freezing mixture, under a pressure of approximately 0.1 mm. Hg. During the first step, 250 ml. portions of dialyzed urine in liter flasks were concentrated until the volume was approximately 25 ml. In the second step, the concentrates and rinsings were combined into a single flask and the lyophilization completed. Recovery of the solids could then be made with a minimum of mechanical loss.

The TNDS, a fluffy tan powder, were finally dried over anhydrous calcium sulfate in a vacuum desiccator to a constant weight. This required three to five days and the nonhygroscopic material thus obtained was utilized in the following studies.

Inorganic constituents. "Bound" water was determined by measurement of weight loss from the air-equilibrated TNDS after heating samples at 105 to 110° C. for two hours. The inorganic ash was calculated after ignition of 100 mg. samples at 500° C. for two to three hours. The calcium (2) and phosphorus (3) contents of the ash were determined by standard methods.

Protides. The nitrogen content of the TNDS was determined by a micro-Kjeldahl method utilizing Nessler's reagent prepared by the method of Koch and McMeekin (4). Samples of the TNDS from each subject were hydrolyzed and the amino acids present identified by two dimensional chromatography in three pairs of solvent systems, all as previously described (5).

Glucides. Total protein-bound hexose was estimated by both the orcinol (6) and anthrone (7) methods, utilizing standards of equal weights of galactose and mannose. Hexosamine was determined by the Schloss modification (8) of the Elson-Morgan reaction. Analyses for "sialic acid" were made by the diphenylamine technique (9), using as a standard either crystalline sialic acid [3]

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[2] A. H. Thomas Co., dialyzer cellophane No. 4465-A2, 4.8 mμ pore size.

[3] Provided by Professor Richard J. Winzler, Department of Biochemistry, University of Illinois College of Medicine, Chicago, Illinois.

derived from human meconium or the sialic acid equivalent of the serum glycoprotein, orosomucoid.[3] All spectrophotometric measurements were made with the Beckman DU spectrophotometer.

Hydrolysis of the TNDS and unidimensional chromatography of the sugars were both performed by the technique of Glegg and Eidinger (10). A 48 hour hydrolysis period was used and double or triple ascensions of 40 cm. were made with each of three solvent systems, as previously described (5).

Lipids. Lipids were extracted from the TNDS with alcohol and alcohol-ether, purified with chloroform, and weighed according to Artom and Fishman (11). Total phospholipids were calculated from the phosphorus content (3) of the chloroform extract (lipid P × 25). Fatty acids were determined acidimetrically (12), either after direct saponification of the TNDS (subjects 2 and 4), or after fractionation of the lipid extract by repeated precipitations with acetone plus $MgCl_2$ and subsequent saponification of the precipitate and extract (pooled subjects 1, 6, 7 and 1, 1, 8). After titration of the fatty acids (total or acetone-soluble), the unsaponifiable matter was extracted and weighed. Total cholesterol was determined on the unsaponifiable matter either colorimetrically (13) or gravimetrically as the digitonin precipitate (14).

TABLE I

Inorganic and nitrogenous constituents of total nondialyzable solids of normal urine

Subject no.	Sex	Electrical conductivity* *mhos* $\times 10^{-6}$	TNDS *mg./24 hrs.*	% of TNDS			% of ash	
				"Bound" H_2O†	N	Ash	Ca	P
1	M	<5.7	413		8.1	6.3		
		40.0	453			6.6		
		91.0	497		6.5	6.3		
		21.0	191		7.1	8.0		
		<5.7	523		9.9	4.8	18.4	7.4
		<5.7	519		9.7	1.1	44.2	31.6
		<5.7	365		8.0	5.9	24.5	10.0
		12.0	304	13.0	8.7	3.9	22.8	7.6
			252					
			276					
		26.0	397					
		47.7	370					
2	M	65.0	508		6.4	4.6		
		36.0	505		8.6	7.1		
		<5.7	398		7.1	6.0	16.7	3.3
		16.0	393	13.0	8.6	4.9	20.1	12.2
		<5.7	364	11.5	9.2	4.1	27.8	4.2
		<5.7	374		8.3	4.9	19.1	10.5
		47.8	429		10.8	8.0	15.4	11.6
		20.0	447		9.5	7.6	6.5	2.8
			427		10.1			
3	M	<5.7	392		7.2	8.8	11.7	
		35.0	516		7.4	7.3	7.5	
4	M	21.0	633		6.8	8.2	7.2	12.0
		27.3	426		6.8	15.7	5.3	1.3
5	M	9.0	419	14.7	10.1	5.0	15.1	3.8
		37.6	626		8.5			
6	F	25.0	427		9.0	10.6		
		26.0	484		8.4	6.3		
		11.0	423		8.8	6.6	16.5	13.6
7	F	10.0	323		8.6	6.1	18.0	7.1
		<5.7	398		9.1	7.8	7.0	10.7
		55.0	617	8.0	10.7	7.2	9.3	15.4
8	F	26.5	330		7.2	15.6	4.5	4.0
		14.3	315		7.2	10.2	5.5	2.9
9	F	<5.7	305		8.9	7.1	22.7	7.8
		<5.7	292	13.1	9.0			

* Closed 1.0 ml. cell with constant of 14.3.

† Loss of water from air equilibrated solids at 105 to 110° C.

TABLE II

Glucide content of total nondialyzable solids of normal urine

Subject no.	TNDS mg./24 hrs.	Per cent of TNDS: Hexose: Anthrone	Hexose: Orcinol	"Sialic acid"	Hexosamine*
1	413	24.9	22.4	10.2	
	453	15.6	15.0	10.0	6.1
	497				6.7
	191	14.3	15.2	14.8	7.2
2	508	15.2	17.0	8.2	5.9
	505	17.6	17.5	9.3	
3	392	19.7	16.8	8.6	6.1
	516	13.9	14.3	8.5	5.9
6	427	16.0	14.8	7.6	
	484	15.7	16.5	9.9	5.5

* Expressed as glucosamine.

Tests for ketosteroids were made by a modification of the Zimmerman reaction (15). As little as 0.5 mg. of neutral 17-ketosteroids occurring in the TNDS of the 24 hour urine specimen would have been detectable by this procedure. An alcohol-chloroform-ether extract of a pooled TNDS sample was tested for 17, 21-dihydroxy-20-ketosteroids by the Porter-Silber test (16).

RESULTS

In Tables I, II, and III are presented the values for inorganic constituents, total N, glucides and lipids, determined on the TNDS of 37 24 hour urine specimens from nine subjects. These values represent the averages of duplicate or triplicate determinations and are expressed as dry weight percentage of lyophilized TNDS. In Table IV are recorded the average excretions of the various constituents, together with the standard deviations of these averages. Also in Table IV, a comparison has been made between the variability of the amounts for the whole group and that for two individual subjects.

Inorganic constituents

Calcium and phosphorus make up a variable percentage of the total ash of the TNDS (4.5 to 44.2 per cent and 1.3 to 31.6 per cent, respectively). However, the 24 hour rate of excretion of these two constituents is much more constant, both for the group of subjects and for subject No. 2 (Table IV). Tests for inorganic phosphorus were negative prior to ashing, although calcium could be measured equally well on the unashed material. There was no consistent calcium to total phosphorus ratio for the TNDS. Also, the lipid P : N ratio was inconsistent, but the lipid P : ash P ratio was about 1 : 25. It will be seen in Table IV that the average excretion of ash for subjects Nos. 1 and 2 was significantly lower than in the group as a whole. No obvious explanation is apparent for this finding. In all other respects studied, there was no significant difference between the values obtained from these two subjects and those for the group as a whole.

Protides

The nitrogen content of the TNDS averaged 8.4 per cent, corresponding to the excretion of 36.2 ± 11.5 mg. in 24 hours. When the nitrogen content of the TNDS is corrected for the nitrogen

TABLE III

Lipids of total nondialyzable solids of normal urine

Subject no.	TNDS mg./24 hrs.	Per cent of TNDS: Total lipids	Total phospholipids*	Fatty acids: Total	Fatty acids: Phospholipid	Unsaponifiable: Total	Unsaponifiable: Cholesterol
2	505			1.13		1.46	0.72†
2	398	2.24	0.22				
4	633	4.26	1.04				0.67†
4	426	2.89	0.16				
6	427	4.27	0.98				
1, 6, 7‡	Pooled	3.52	0.95	1.57§	0.74	1.49	0.98‖
1, 1, 8‡	Pooled	2.51	0.76	0.81§	0.42	1.36	

* Lipid P × 25.
† Colorimetric method (13).
‡ Pooled samples.
§ Phospholipid fatty acids, plus fatty acids. Soluble in acetone.
‖ Gravimetric method (14).

TABLE IV

Average excretion of inorganic and organic constituents of total nondialyzable solids of normal urine *

	TNDS	Ash	Calcium	Phosphorus	Nitrogen	Hexose (orcinol)	Sialic acid	Hexosamine	Total lipids
Number of subjects	7	7	8	7	7	4	4	4	6
Number of determinations	16	14	16	14	16	9	9	7	6
Mean values	433	36.7	3.5	2.4	36.2	72.6	40.7	26.5	15.6
Standard deviation	114.6	13.2	1.1	2.0	11.5	19.1	7.5	6.1	8.1
				Subject number 1					
Number of determinations	12	8			8				
Mean values	380	20.9†			35.0				
Standard deviation	108.4	9.0			12.8				
				Subject number 2					
Number of determinations	9	8	6	6	8				
Mean values	427	25.4‡	3.8	1.8	36.5				
Standard deviation	52.2	8.2	1.0	1.2	6.7				

* Expressed as mg. per 24 hours.

† The difference between this value and that for the group was tested by the "t" test. The probability is $<0.01 > 0.001$.

‡ The difference between this value and that for the group was tested by the "t" test. The probability is $<0.05 > 0.02$.

content of amino sugars (7.8 per cent) and sialic acid (3.79 per cent) and then multiplied by the factor 6.25, an estimate of 47 per cent protein content is obtained for the TNDS, equivalent to 204 mg. of protein per 24 hour sample.

The following amino acids were identified by chromatography, arranged in decreasing order of color intensity after development with ninhydrin: aspartic and glutamic acids, alanine, leucine, isoleucine, valine, phenylalanine, glycine, serine, threonine, lysine, arginine, proline, tryptophan, and tyrosine. The last two were very weak and not consistently seen in all chromatograms. Histidine, cystine, cysteine, methionine, taurine, tryptamine, ornithine, hydroxyproline, and hexosamine were not identified by chromatography. This is not conclusive evidence for their absence, since hydrolytic destruction or alteration of certain compounds may have occurred, especially in the presence of carbohydrates.

Three unidentified ninhydrin-positive spots were found repeatedly. The approximate R_F values in each direction of two of the unidentified spots were, respectively, 0.13 and 0.06, and 0.15 and 0.72, in the lutidine-phenol system (5), 0.50 and 0.07 for the single unknown spot seen in the butanol: acetic acid-butanol: ethanol system (5), and 0.24 and 0.03, 0.20 and 0.12, and 0.01 and 0.08 for three unidentified spots seen in the propanol: ethylacetate-isobutyric acid system (5). The hydrolysis was considered to be strong enough to eliminate the possibility of these being peptides.

Glucides

Values for total protein-bound hexose obtained by the anthrone and orcinol techniques were in excellent agreement (Table II). When examined by chromatography, the resin-hydrolyzed (10) material consistently gave five spots which migrated on all chromatograms identically with galactose, mannose, glucose, rhamnose, and fucose, in respective order of decreasing color intensity. A "specific" test (17) indicated that less than 0.05 per cent ketohexose was present in TNDS, and no maximum absorption at 560 mμ was noted when the method of Dische and Borenfreund (18) was applied to the TNDS for the detection of ketoses.

The average value of 9.7 per cent for sialic acid content of the TNDS is considered to be reliable. When the absorption curves for the products of the reactions with TNDS, orosomucoid, and crystalline sialic acid were plotted over the range of 300 to 900 mμ, identical maxima at 525 and 712 mμ and minima at 450 and 577 mμ were obtained for all these substances.

Lipids

The presence of small amounts of lipids in normal human urine was first noted by Mörner (19)

and has been reported subsequently by many investigators [see literature in Bloor (20)]. The content of total lipids, total phospholipids, fatty acids, and unsaponifiable material in the TNDS is rather variable (Table III). The values for total lipids and cholesterol, calculated as mg. per 24 hours, found in the various specimens are within the range of values for undialyzed urine given by previous authors (20). The chloroform soluble phosphorus of the TNDS exhibited much greater individual variations than did the total lipids. It appears that the chloroform soluble phosphorus represents chiefly the glycerolphospholipids, which contain approximately 4 per cent phosphorus and 67 per cent fatty acids. Indeed, if these percentages are used as factors, similar values are obtained for the total phospholipids calculated either from the lipid phosphorus or from the fatty acid content of the acetone precipitate. Thus, in the pooled samples from subjects 1, 6, and 7, the total phospholipids are calculated as 9.5 mg. per Gm. on the basis of phosphorus content and as 11.2 mg. per Gm. on the basis of fatty acid content. Likewise, in the pooled samples from subjects 1, 1, and 8 the corresponding values are 7.6 and 6.2 mg. per Gm., respectively.

Steroid and other analyses

Tests for neutral 17-ketosteroids and 17,21-dihydroxy-20-ketosteroids were negative. The quantitative Aschheim-Zondek test revealed the presence of gonadotrophic hormone when TNDS were administered intravenously to female rabbits. The diuretic factor of Little (21) is present in this material. Type specific sera have demonstrated the presence of blood group specific substances A, B, and O in the TNDS (1, 22).

DISCUSSION

On the basis of these data, it appears that the average composition of TNDS of normal urine is approximately: 47 per cent protein, 16.6 per cent protein-bound hexose, 9.7 per cent sialic acid, 6.2 per cent hexosamine, 3.3 per cent lipid, 12.2 per cent water, and 8.5 per cent ash. These average figures account for 103.5 per cent of the TNDS. Both the relative composition and amounts of the TNDS excreted are rather constant in the normal individual, when one considers the vast number of metabolic processes that may be represented by this material. The present data are too few to permit unequivocal conclusions, but no marked differences in TNDS have been observed between male and female or between white and Negro subjects. The pathways by which these materials reach the urine are probably a combination of glomerular filtration, urinary epithelial secretion, and possibly renal tubular secretion. The contribution of transitional epithelial cells and blood cells to the TNDS remains unknown. Microscopic examination of the residue after dialysis and subsequent ultrafiltration revealed no evidence of any formed cell, cast or other morphological element. Dialysis thus resulted in the lysis of the cellular constituents so that their total contribution to the TNDS could be only the nondialyzable portion of the cells. This quantity cannot be determined at the present time, since there is no obvious way of separating these materials from the TNDS. Centrifugation at the speed required for an Addis count will also remove a variable quantity of mucoprotein from normal urine (23). Mucoprotein so removed may vary from 5 to 50 mg. per 24 hour specimen, depending upon the specific gravity, salt content, and pH of the urine. Presumably, the error introduced by such a procedure in the final weight of the TNDS would be much greater than any error which could be expected to result from the presence of nondialyzable cellular residue in normal urine.

The dialysis was intended to achieve a maximal separation of the diffusible materials with minimal decomposition of the TNDS. The duration of the dialysis adopted for these studies appeared to be adequate, since there was no relationship between the observed conductance and the quantity of TNDS (Table I). Also, the fact that the amount of ash in the 24 hour TNDS is rather constant (Table IV) is an indication of the reproducibility of the conductance-controlled dialysis and of its efficiency, since the theoretical ratio of dialyzable to nondialyzable material in normal urine is approximately 150 : 1. It seems necessary to adopt some arbitrary criterion such as a definite value of electrical conductance as an end point for the dialysis. This is an asymptotic process which causes a continual disturbance of the equilibria

between bound and unbound molecules in the urine. Thus, whereas calcium may be bound in normal urine to some poorly diffusible molecules (24, 25), in the present study the excretion of nondialyzable calcium is relatively constant (Table IV). This was possible because a definite, although arbitrary, criterion was adopted to terminate dialysis. If other criteria had been selected, other values for the excretion of nondialyzable calcium might have been obtained.

Similarly, hexuronic acid (26, 27) and hexosamine (28, 29) exist in urine in both dialyzable and nondialyzable [4] forms; accordingly, the detection and estimation of these compounds in the TNDS will depend to some extent upon the conditions of dialysis. It is impossible to determine by conventional techniques the exact amount of hexuronic acid in the TNDS since it is relatively small, particularly susceptible to hydrolytic destruction, and is also progressively removed by dialysis. Hydrolysis of the TNDS with resin (10) or by the procedure of DeFrates and Boyd (30) resulted in a clear, pale yellow hydrolysate which gave a clearly positive naphthoresorcinol test for hexuronic acid. However, we were unable to detect hexuronic acid after the more severe hydrolysis described by Hamerman, Hatch, Reife, and Bartz (1) nor did the characteristic absorption peak appear with this material after reaction with thioglycolic acid (31), although both chondroitin sulfate and material isolated from urine by the procedure of Di Ferrante and Rich (27) gave positive tests for hexuronic acid under the same conditions. Therefore, no attempt was made to estimate the quantity of hexuronic acid in TNDS, since the result could have been only a reflection of the reproducibility of the hydrolytic conditions. On the other hand, the separation of hexosamine as an integral part of the molecule from that which is free or loosely bound to the TNDS appears to have been clearly accomplished. A positive test for hexosamine was observed only on the hydrolyzed material. Comparison of the results for hexosamine with those of other investigators (1, 28, 29) suggests that the hexosamine content of TNDS of normal urine falls within reasonably reproducible limits.

[4] The term "bound" is inappropriate since molecules having molecular weights up to 20,000 can pass Visking cellophane membranes of the kind used here, and some of these compounds probably contain hexuronic acid and/or hexosamine.

SUMMARY

The total nondialyzable solids (TNDS) of normal human urine specimens have been recovered by lyophilization and determined to be 433 mg. per 24 hours, with a standard deviation of 114.6. The TNDS were analyzed for inorganic ash, bound water, calcium, phosphorus, nitrogen, hexose, sialic acid, hexosamine, total lipids, total phospholipids, fatty acids, and unsaponifiable matter. The approximate composition of the TNDS is: 47 per cent protein, 16.6 per cent protein-bound hexose, 9.7 per cent sialic acid, 6.2 per cent hexosamine, 3.3 per cent lipids, 12.2 per cent "bound" water, and 8.5 per cent ash. With respect to these constituents, there was no significant variation among normal adult subjects. The amino acids and sugars were studied by paper chromatography. The excretion for some of the nondialyzable constituents, expressed as mg. per 24 hours, follows: inorganic ash, 36.7 ± 13.2; calcium, 3.5 ± 1.1; phosphorus, 2.4 ± 2.0; nitrogen, 36.2 ± 11.5; hexose, 72.6 ± 19.1; sialic acid, 40.7 ± 7.5; hexosamine, 26.5 ± 6.1; and total lipids, 15.6 ± 8.1. Each of these values was obtained from four to eight subjects who submitted a total of 7 to 16 24 hour urine specimens.

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