Uricosuric Agents in Uremic Sera

IDENTIFICATION OF INDOXYL SULFATE AND HIPPURIC ACID

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ABSTRACT Serum and urine from chronically uremic patients and normal individuals were subjected to gel filtration on Sephadex-G10. The effects of the eluted fractions on the uptake of urate and para-aminohippurate by isolated cortical tubules of rabbit kidney were investigated. According to the origin of the samples, one to three major groups of fractions inhibiting both urate and para-aminohippurate transport were disclosed. The first eluted group occurred for all the samples under study. The second one was demonstrated in both sera and urines from uremic patients but only in urines from normal individuals. The third one was exclusively detected in uremic sera and urines. Among all the compounds identified, only hippuric acid, eluted in the fractions of the second group, was capable of inhibiting the uptake of urate and para-aminohippurate in vitro. The concentration for which this inhibitory effect of hippuric acid occurred was in the range of that existing in uremic sera. Indoxyl sulfate, which accumulates to very high concentrations in uremic serum, could not be disclosed in the above-mentioned fractions. This is explained by the strong adsorption of this indole derivative to Sephadex gel. Potassium indoxyl sulfate, when tested in vitro at the concentration existing in uremic serum, substantially inhibited the uptake of both urate and para-aminohippurate. In normal subjects, ingestion of hippuric acid or potassium indoxyl sulfate significantly increased fractional urinary excretion of uric acid. On the basis of these results, it is suggested that progressive retention of hippuric acid, indoxyl sulfate, and other yet unidentified inhibitors may explain the gradual increase in urinary fractional excretion of urate observed in uremia. The present results may be viewed as an example of a mechanism in which retention of normally excreted end products is responsible for adaptation of tubular transport in uremic subjects.

INTRODUCTION

Maintenance of some degree of plasma urate homeostasis in subjects with chronic renal failure is achieved by an increase in both intestinal uricolysis (1) and absolute urate excretion per residual nephron (2, 3). The role of each component of the tubular bidirectional transport system for urate at different stages of disease has been assessed with the pyrazinamide suppression test (3, 4). However, the mechanism of the increased fractional urinary urate excretion in uremia has not been elucidated. Previous work from this laboratory has shown the existence in uremic sera of one or more dialyzable factors that inhibit urate transport by isolated cortical tubules from rabbit kidney (5). The results suggested that this inhibitor or inhibitors could be involved in the regulation of urinary urate excretion in uremia. The present work was designed to examine if these inhibitors were a normal derivative of metabolism and if they were involved in the regulation of urate excretion. The effects of filtration fractions obtained from normal and uremic sera and urines on the uptake of urate and *para*-aminohippurate (PAH)[1] by isolated cortical tubules from rabbit kidney were therefore investigated. The compounds found to be active in vitro at concentrations in the range of uremic sera were administered to normal individuals to assess their effects on the renal excretion of urate in vivo.

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[1] *Abbreviations used in this paper:* C_{ur}/C_{cr}, ratio of urate clearance to endogenous creatinine clearance; k_{av}, coefficient of distribution; PAH, *para*-aminohippurate; SUN, serum urea nitrogen; T/M ratio, concentration of organic acid in cellular water/concentration in the medium.

The results demonstrate the existence in uremic sera of several substances that inhibit the uptake of both urate and PAH in vitro. Among these substances, two have been identified as hippuric acid and indoxyl sulfate, both of which promote significant increases in urinary urate excretion in normal individuals.

METHODS

Venous blood and 24-h urine samples were obtained from patients with terminal renal failure. Blood was allowed to clot. The separated sera and the urines were stored at −20°C. Mean serum urea nitrogen (SUN) was 212±13 (SEM) mg/100 ml ($n = 20$). The administration of all medications was stopped for at least 5 days before blood and urine samples were taken. None of these patients was being treated with chronic hemodialysis. Control blood and 24-h urine samples were obtained from normal volunteers with SUN below 20 mg/100 ml.

Gel chromatography. All gel filtration experiments were performed at room temperature on a column (2 × 90 cm) of Sephadex-G10 (Pharmacia Fine Chemicals, Uppsala, Sweden). The elution buffer consisted of 0.6 mM Tris-Cl, pH 7.40, containing 15 mM sodium chloride. 6-ml aliquots of serum and urine were lyophilized. The lyophilized 6 ml of serum and urine were reconstituted in 3 ml of elution buffer and applied to the column. Sample volume was about 1% of the column bed volume. The flow rate was maintained at 9 ml/h with a Technicon pump (Technicon Instruments Corp., Tarrytown, N. Y.) and 70-min fractions were collected. Elution volumes were determined by weighing tared tubes. Column void volumes were determined by using blue dextran (Pharmacia Fine Chemicals). For each chromatography, void volume fractions collected before the elution of proteins were used as controls. Control and experimental fractions were lyophilized and the material was redissolved in 3 ml of 0.02 M Tris-Cl, pH 7.40. The concentrations of sodium and potassium were adjusted to 150 and 15 meq/liter, respectively. The final volume of each fraction was 3.65 ml. Fractions were stored at −20°C until tested for effects on renal tubules. 22 chromatographies were performed: 12 on urine and serum obtained from 6 uremic patients and 10 on urine and serum obtained from 5 normal individuals.

A certain number of chemical substances dissolved in elution buffer were subjected to chromatography on Sephadex-G10 in exactly the same conditions as described for sera and urines. The elution characteristics of the following compounds were assessed: ammonium chloride, creatinine and urea (Merck, Darmstadt, Germany); tryptophan (ICN Nutritional Biochemicals Div., International Chemical and Nuclear Corp., Cleveland, Ohio); guanidosuccinic acid and anthranilic acid (Mann Research Labs., Inc., New York); methyl guanidine, guanidopropionic acid, *N*-methylnicotinamide, guanidobutyric acid, potassium indoxyl sulfate, guanidoacetic acid, kynurenine, arginine and lactic acid (Sigma Chemical Co., St. Louis, Mo.); [1-^{14}C]hippuric acid, 11–55 mCi/g, (New England Nuclear Corp., Frankfurt, Germany); [1-^{14}C]glycine, 70 mCi/g (C.E.A., Saclay, France); [2-^{14}C]uric acid, 320 mCi/g (The Radiochemical Center, Amersham, Bucks, England). For every experiment described in this paper, the latter compound was recrystallized as described by Sorensen (6) to a specific activity of 10 mCi/g. Elution patterns of [1-^{14}C]hippuric acid dissolved in sera of two uremic patients were also determined.

In vitro bioassay. Suspensions of rabbit renal tubules were prepared according to Burg and Orloff (7) as previously reported (8). The acetate-Ringer solution used for the preparation and incubation of the tubules contained: NaCl, 130 mM; KCl, 5 mM; Na acetate, 10 mM; Mg SO_4, 1 mM; $CaCl_2$, 1 mM; Na phosphate buffer, 5 mM (pH 7.40); glucose, 5 mM; plus 5% vol/vol calf serum. The final suspension contained from 2 to 5% of tissue. Incubations were performed in vessels described by Burg and Orloff (7) for 30 min at 27°C with a gas phase containing 100% O_2. A tracer amount of [2-^{14}C]uric acid or [1-^{14}C]-PAH, 10–25 mCi/g (New England Nuclear Corp.) was added to the bathing medium. Unless stated otherwise, unlabeled uric acid or PAH was added to obtain a final chemical concentration of about 0.5 mM. [Methoxy-^{3}H]Inulin (New England Nuclear Corp.) was also added to achieve a final radioactivity of about 1 μCi/100 ml. Incubation was started by the addition of the tubules suspension to the acetate-Ringer solution containing the isotopes and either the fractions or the investigated chemical compounds. After the completion of incubation, the suspension was transferred to a special centrifuge tube (8) maintained at 0°C. Centrifugation was performed for 5 min at 5,000 *g* in a refrigerated centrifuge. The supernate was decanted and the superficial layer of cells was removed by suction. The weights of tissue plugs were determined before and after dessication in an oven at 70°C. Dessication was carried out until a constant weight was reached. Dried tubules were extracted in 3 ml of 0.18 M trichloracetic acid with a sonifier (Sonifier B 12, Branson Instruments Co., Stamford, Conn.) and the resulting extract was centrifuged for 10 min at 5,000 *g*. For assay, fractions were diluted 1:5 in the incubation medium. As the fractions had been concentrated 1.6-fold after lyophilization, the concentration of the serum and urine constituents in the assay medium was about one third their in vivo levels. This concentration is the highest theoretically achieved, if there is no loss of constituents during the preparative procedure and each constituent is eluted in only one fraction. The experimental fractions were tested by two protocols. Protocol I: the control fraction and 20 experimental fractions yielded by a single chromatography were assayed on the same tubular suspension. This study was performed three times, that is to say, on three different suspensions on three different days. 11 chromatographies were studied on 33 suspensions. Protocol II: three to six consecutive fractions, together with the control fraction, were studied on the same suspension. This operation was repeated for each chromatography until a maximum of 26 fractions was studied. Each fraction was assayed in triplicate. 11 chromatographies were studied. Since no inhibitory effect was detected in fractions beyond 20, only those results yielded by fractions 1–20 are reported. Additional experiments with the above-described in vitro technique were designed to test the capacity of a number of chemical substances to inhibit tubular transport of organic acids. The chemical substances tested were those that were eluted at the same position as the active fractions on the one hand and potassium indoxyl sulfate on the other. Potassium indoxyl sulfate, which is strongly adsorbed to the gel, was tested because it accumulates to very high concentrations in uremic serum (9, 10). Uptake of potassium indoxyl sulfate by isolated renal tubules has also been evaluated with a 0.5 mM concentration of this compound in the medium. Tissue contents of organic acids, water, sodium, and potassium were corrected for extracellular contamination by using inulin space measured for each sample. Organic acid uptake was usually expressed as the T/M ratio,

TABLE I

Effects of Fractions from Uremic and Normal Urines and Sera on Organic Acid Uptake by Isolated Renal Tubules (Absolute Values)

	Uremic urines			Uremic sera			Normal urines			Normal sera	
Fraction	Subject 1	2	3	1	2	3	4*	5	6	4*	5
Control	3.43	8.43	7.50	7.03	8.23	9.10	4.53	5.60	7.33	5.16	7.16
1	3.20	8.13	7.86	7.16	7.03§	9.16	4.43	5.66	6.80	4.26‖	7.53
2	3.20	8.10	6.76	5.90§	5.96‖	8.70	4.23	5.66	7.10	4.30‖	6.40‖
3	3.36	7.90	5.66‡	4.93‖	6.56‖	6.16‖	4.43	5.26	6.33‡	3.66‖	6.46‖
4	2.83§	5.56‖	2.93‖	4.26‖	7.03§	5.83‖	2.90‖	4.16§	5.96§	4.00‖	6.26‖
5	2.56§	5.76‖	4.86§	4.43‖	7.83	5.96‖	3.26‖	2.30‖	7.26	5.00	7.46
5	2.96‡	6.96§	6.26	6.40‡	8.26	7.06‖	3.53§	1.53‖	7.80	4.90	6.93
7	3.16	7.36‡	6.80	6.16‡	8.46	8.40	4.66	2.16‖	7.60	4.96	7.10
8	3.10	8.43	6.70	6.73	8.13	7.80‡	4.20	2.50‖	6.93	4.46‡	6.76‡
9	3.13	6.36§	5.96‡	5.66‖	8.23	8.23	2.53‖	3.67‖	6.10§	2.36‖	4.76‖
10	2.43‖	6.06‖	5.30§	4.83‖	7.23§	7.10§	2.26‖	3.26‖	4.30‖	4.90	7.83
11	1.42‖	3.16‖	5.26§	5.33‖	7.60‡	7.96‡	1.30‖	2.53‖	4.53‖	5.06	7.90
12	3.73	6.76§	6.36	6.83	8.36	7.80‡	3.60§	1.50‖	5.83§	5.46	7.20
13	2.93‡	7.40‡	6.83	6.10§	8.30	8.53	3.93	2.70‖	5.80§	5.20	7.60
14	2.03‖	5.33‖	6.50	5.66‖	7.40‡	8.16¶	4.10	4.76	6.53	5.06	7.50
15	2.50‖	6.36§	5.03§	6.00§	7.23§	8.16¶	3.90‡	5.23	6.80	4.90	7.80
16	3.16	7.03‡	7.20	6.06§	7.70	8.66	3.90‡	5.23	6.73	5.00	7.56
17	3.13	7.56	7.80	6.26‡	8.36	8.96	3.76‡	4.80	6.80	5.00	7.63
18	3.33	7.56	7.10	6.46	8.33	8.10‡	3.80‡	5.33	6.43‡	5.10	7.53
19	3.33	7.76	7.16	6.26‡	8.33	9.13	4.16	5.40	6.83	5.13	7.23
20	3.00	7.56	6.86	6.46	8.20	8.23	4.00	5.93	7.03	5.03	7.23

Each figure is the mean of three T/M ratios, and each fraction was assayed on three different suspensions of tubules.

* Uptake studies performed with [2-^{14}C] uric acid, the others were performed with [1-^{14}C] PAH. Significant change from control:

‡ $P < 0.05$.

§ $P < 0.01$.

‖ $P < 0.005$.

¶ Values close to the 0.05 level of significance (8.14).

where T was the concentration of organic acid in cellular water and M its concentration in the medium.

In all cases, the same tubular suspension has been used for control and experimental studies.

Clearance studies. Four human studies were performed on authors. Every study consisted of two phases: the first included three to four clearance periods 30 min long. After the last control period, either 8 g of hippuric acid or 4 g potassium indoxyl sulfate was ingested orally. Subsequently, 9 to 12 clearance periods of 30–70 min duration were performed. Urine flow of about 3 ml × min^{-1} was maintained by repeated ingestion of water. Blood was sampled at midtime of each control period and at every 30 or 90 min during the experimental phase. The glomerular filtration rate was estimated by measuring endogenous creatinine clearance. Since plasma concentration of creatinine and uric acid were constant in each of the four studies, mean values of 8–10 measurements were used to calculate the clearances of the compounds for each study.

Analytical procedures. Sodium and potassium analyses were performed with a flame photometer (Netheler and Hinz GMBH, Hamburg, Germany). The pH of the fractions was measured by a glass electrode (Radiometer Co., Copenhagen, Denmark). Creatinine in serum, urine, and fractions was determined by the method of Bonsnes and Taussky (11). For the studies involving indoxyl sulfate, a more specific method was used to measure creatinine in urine and serum (12). Indeed, we have found that indoxyl sulfate interferes with the Jaffé reaction and that this interference can be entirely eliminated by using fuller's earth. Urea was measured by the method of Berndt and Bergmeyer (13). Ammonia in fractions was determined by the method of Hutchinson and Labby (14), lactic acid by the method of Hohorst (15), and protein by the method of Lowry, Rosebrough, Farr, and Randall (16). The method used to determine hippuric acid was that of Ellman, Burkhalter, and La Dou (17). Samples of serum, urine, and their fractions were treated by an equal volume of 1.2 M trichloracetic acid and centrifuged at 5,000 *g* for 10 min. Then 10–100 μl of the above supernates were added to 5 ml of 70% sulfuric acid (7 vol of H_2SO_4 [No. 714, Merck], to 3 vol of water) and mixed. The fluorescence intensity of the solutions was measured in a Jobin-Yvon spectrofluorimeter (Jobin-Yvon Instruments, Arcueil, France) at 370 nM with the excitation set at 273 nM. All determinations were carried out in duplicate. To correct for quenching of the hippuric acid-H_2SO_4 fluorescence, the fluorescence intensities were also measured after addition to the samples of 2 μg hippuric acid in 10 μl of water. The method used to estimate indoxyl sulfate was essentially that of Curzon and Walsh (18). Samples of sera, urines, fractions, incubation media, and tissular extracts were diluted 1:2 in 1.5 M trichloracetic acid and centrifuged at 5,000 *g* for 10 min. At the same time, standard aqueous solutions of 0.026–0.15

mM potassium indoxyl sulfate were diluted 1:2 in 1.5 M trichloracetic acid and run with each series of determinations. In incubation media PAH was measured by the method of Smith, Finkelstein, Aliminosa, Crawford, and Graber (19), and uric acid in the incubation media, sera, and urines by differential spectrophotometry (20). For the clearance studies, determination of uric acid in serum and urine was made at least in duplicate. The elution pattern of the mono-substituted guanidine derivatives was obtained by measuring the concentration of these compounds in the fractions by the Sakaguchi reaction as described by Stein, Cohen, and Kornhauser (21). The same method was applied to the fractions from urines of patients D. P., E. C., and F. A. Among these fractions, 20–50 μl of those which contained Sakaguchi-positive material were electrophoresed on Whatman 3-MM paper according to the method of Stein et al. (21). The mono-substituted guanidines were located on the paper with a modified Sakaguchi color reaction (22). Elution pattern of *N*-methylnicotinamide, tryptophan, kynurenine, and anthranilic acid was determined by measuring the ultraviolet absorbances of the fractions. ^{14}C and ^{3}H in the incubation media diluted 1:30 in 0.18 M trichloracetic acid and in tissular extracts were counted in a Mark 1 Nuclear Chicago liquid scintillation counter (Searle Analytic Inc., Des Plaines, Ill.). Aliquots of 0.1 ml were counted in 10 ml of Bray's solution (23). The settings were such that the spillover of ^{14}C into ^{3}H was 8% and ^{3}H into ^{14}C less than 0.05%. Elution patterns of labeled compounds were obtained by measuring the radioactivity of 0.1 ml aliquots from fractions previously diluted 1:5 in 0.18 M trichloracetic acid and centrifuged in 10 ml of Bray's solution. The counting rates were carried out for long enough to achieve a statistical accuracy of at least 3%. The results were expressed as means±SEM. The data from the studies of fractions were subjected to a two-factor analysis of variance (24), one factor being the sample (urine or serum, uremic or normal) and the other the fraction, and the least significant difference was used to compare the experimental fractions to the control ones. Otherwise, Student's *t* test was used for comparison between means.

RESULTS

Effect of the fractions on uric acid and PAH uptake by isolated renal tubules. Results from protocol I are summarized in Table I. Depending on the nature of the sample studied (urine or serum from uremic or normal subjects), from one to three major groups of active fractions were characterized. Those groups are, in most instances, separated by one or several inactive or less active fractions. The first eluted group was common to all the samples studied. It occurred generally earlier for sera than for urines. Its peak of activity is found in fractions 2 to 5. The second group, which was observed for all the samples studied, except for normal sera, consisted essentially of fractions 10 and 11. A third group was detected in fractions 13 to 15 from uremic urines and, although to a lesser degree, from the corresponding sera. These results achieved significance despite (*a*) individual variations from one suspension to another,

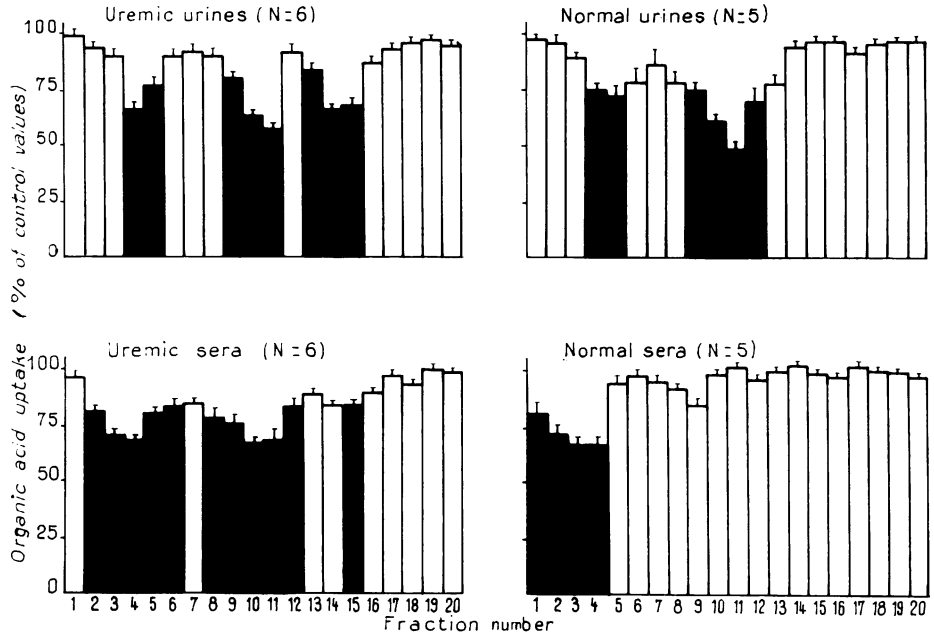

FIGURE 1 Effects of fractions from uremic and normal sera and urines on organic acid uptake by isolated renal tubules (relative values). Mean values are expressed as percent of control values. Each bar represents the mean ±1 SEM of 18 experiments for uremic sera and urines and of 15 experiments for normal sera and urines. Organic acid was either [2-^{14}C]uric acid (sera and urines fractions from two uremic and two normal individuals) or [1-^{14}C]PAH. Black bars indicate an uptake significantly lower ($P < 0.01$) than control.

TABLE II

Chemical Composition of Sera and Urine Fractions and Elution Position of Reference Compounds

Compound	Compound identified in urine and serum chromatograms*			Elution parameters of reference compounds		
	k_{av}‡	n	Fraction number§	k_{av}‡	n	Fraction number§
Protein||	0.10	2	2			
Guanidosuccinic acid¶	0.20	2	4	0.185	2	4
Lactic acid**	0.26±0.005	3	5	0.25	1	5
Glycine				0.36	1	8
Arginine				0.38	1	8
Guanidopropionic acid				0.39	1	8
N-methylnicotinamide				0.39	1	8
Guanidoacetic acid‡‡	0.44	1	8	0.41	1	8
Guanidobutyric acid				0.41	1	8
Ammonium chloride**	0.50±0.011	3	9, 10	0.51	2	10
Nicotinic acid				0.55	1	10
Creatinine	0.55±0.004	6	10, 11			
Methylguanidine				0.59	1	11
Hippuric acid**	0.58±0.017	3	10, 11	0.59±0.016	6	10, 11, 12
Urea	0.74±0.008	6	14, 15	0.73	1	14
Kynurenine				0.87	1	17
Anthranylic acid				1.13	1	22
Uric acid				1.50	1	26
Tryptophan				2.22	1	44
Potassium indoxyl sulfate				7.9	2	130, 135

* Chromatograms from serum and urine of two uremic and one normal individuals from protocol II.

‡ Calculated from the equation $k_{av} = (V_e - V_o)/(V_t - V_o)$, were V_e = elution volume of the substance of interest, V_o = void volume, and V_t = total volume of the gel bed (25). k_{av} are means±SEM when $n > 2$.

§ Only the fractions containing maximal concentration of compounds for each chromatography are indicated.

|| Measured only in the serum fractions from two uremic patients.

¶ Detected only in the urine fractions from two uremic patients.

** Measured only in the urine fractions from two uremic patients and from a normal individual.

‡‡ Detected only in the urine fractions from a normal individual.

and (*b*) the scatter of the responses of three repetitive studies of the same fraction on three different suspensions. This allowed us to pool together the results obtained from both protocol I and protocol II, expressed as percentage of control values. Since in either protocol three determinations were obtained for one fraction, the three relative values of each fraction were considered as repetitions in the variance analysis. The results, expressed in Fig. 1, groups data obtained with uric acid with that of PAH. It can be seen that the elution pattern of inhibitory fractions is essentially similar to that yielded by protocol I. Indeed, three inhibitory groups clearly separated by one or several inactive fractions appear both in urines and sera from uremic patients. The first two groups are also observed in normal urines, and only the former in normal sera.

Elution pattern of serum and urine constituents. Table II shows the chemical composition of filtration fractions of sera and urines previously tested for their effects on urate uptake (Fig. 1). The table also shows the elution positions of reference compounds chromatographed in the same conditions. The recovery of creatinine, urea, and hippuric acid of the biological samples and of all the reference compounds except indoxyl sulfate averaged 48±2% in fraction 1, and 75±2% and 87±1.6% in consecutive fractions 2 and 3, respectively. Elution position of compounds identified in the fractions of biological samples, i.e., guanidosuccinic acid, lactic acid, guanidoacetic acid, ammonium ion, creatinine, urea, and hippuric acid, were the same as those of the reference compounds. Co-chromatography of [1-^{14}C]hippuric acid with sera of two uremic patients was eluted with maximal concentration in fraction 10. Hence, these results were put together in the table with those obtained by chromatography of an aqueous solution of the labeled compound. However, due to its protein bind-

ing, [1-^{14}C]hippuric acid was eluted from fraction 3 onwards, whereas it appeared only with fractions 8 or 9 when it was in aqueous solution.

The effects of serum and urine fractions on the uptake of organic acids were tested until the coefficient of distribution, k_{av}, for the effluent volume was approximately 1. Hence, the compounds adsorbed to the gel escaped this experimental approach. This was especially the case of indoxyl sulfate, which was eluted from fraction 125 to 142. Mean k_{av} for this compound averaged 7.9 in two experiments.

Nature of the inhibitors of the organic acid transport disclosed in sera and urines of normal and uremic patients. The effect on urate uptake by isolated tubules of chemicals with an elution position similar to that of active fractions, and of indoxyl sulfate are shown in Table III. Urea, creatinine, and ammonium ion were assayed at the highest concentrations achieved when fractions from three uremic and two normal urines were tested. Urea and creatinine did not inhibit uric acid uptake, whereas it was slightly decreased by ammonium ion. This small inhibition of 4.2±1.3% caused by ammonium ion could not explain the major depression induced by fractions of the second group. No significant inhibition of urate transport was induced by guanidosuccinic acid, methylguanidine, and nicotinic acid. In contrast, hippuric acid at a concentration as small as 0.05 mM caused a mean change in urate uptake of −7.26±1.06%. At a concentration of 0.1 and 0.3 mM of this agent in the medium, mean decrease of urate uptake was 33.3±8.40% and 51.1±3.40%, respectively. Hippuric acid concentrations in urine fractions previously tested for their effects on urate transport are shown in Table IV. Since fractions have been used diluted 1:5, the concentration of hippuric acid achieved in the test system with fractions 9–11 from these three urines must average 0.24 mM. Under these conditions, the inhibition induced by these fractions averaged 34%. Since 0.3 mM hippuric acid inhibits urate transport by 50%, the concentration of this agent found in the urine fractions appeared sufficiently high to explain entirely their activity. Hippuric acid has not been measured with sufficient precision in uremic sera fractions. Hence, its concentration has been measured directly in sera of six undialyzed patients and five normal subjects. In uremic patients with mean SUN of 189±8 mg/100 ml; mean hippuric acid concentration was 0.28±0.051 mM (range 0.09 − 0.40). In the normal subjects' sera this compound could not be detected. These data should be considered approximate due to the high quenching of H_2SO_4

TABLE III

Effect of Retained Substances in Uremia on Uric Acid Uptake and Electrolyte Content of Isolated Renal Tubules

Exp. no	Compound	Initial concentration	Uric acid uptake T/M ratio	K^+	Na^+
		mM		*meq/kg dry wt*	
1	None, control (8)*		4.45±0.14	256±2.7	163+11.9
	Hippuric acid (5)	0.10	2.96±0.37‡	247±11	146±10
	Hippuric acid (5)	0.30	2.17±0.16§	259±3	166±7
2	None, control (3)		3.91±0.16		
	Guanidosuccinic acid (3)	0.017	4.45±0.42		
	Guanidosuccinic acid (3)	0.17	4.16±0.03		
3	None, control (5)		3.48±0.13		
	Creatinine (4)	5.3	3.85±0.18		
	Urea (5)	60	3.82±0.20		
4	None, control (5)		6.88±0.21	264±3	120±5
	Potassium indoxyl sulfate (5)	0.15	3.87±0.15§	255±8	127±10
	Methylguanidine (5)	0.50	6.35±0.19	264±4	111±5
5	None, control (5)		4.90±0.03		
	Hippuric acid (5)	0.05	4.54±0.05‡		
	Potassium indoxyl sulfate (5)	0.05	4.48±0.02‡		
6	None, control (5)		5.49±0.03		
	Ammonium chloride (5)	1.2	5.27±0.08‖		
7	None, control (5)		4.74±0.18		
	Nicotonic acid (5)	0.1	4.59±0.12		

Values are means ±SEM.

* Number of experiments.

‡ Significant change compared to control studies ($P < 0.005$).

§ $P < 0.001$.

‖ $P < 0.02$. The other changes are not significant, $P > 0.05$.

TABLE IV

Hippuric Acid Levels in Uremic and Normal Urine Fractions

Subject	Urine	Fraction number 8	9	10	11	12	13	Recovery
	mmol/liter			*mmol/liter*				%
Uremic patients								
D. P.	1.91	0	1.18	1.21	0.83	0.28	0	111
E. C.	2.73	0	0.56	1.84	1.87	0.25	0	101
Normal subject								
F. A.	2.04	0	0.11	0.95	2.28	0.11	0	103

fluorescence by sera. Potassium indoxyl sulfate was as effective as hippuric acid in inhibiting the transport of urate. Mean decrease in urate uptake was 8.5±0.44% and 43.7±2.19% with 0.05 and 0.15 mM of potassium indoxyl sulfate in the medium, respectively. Indoxyl sulfate concentration in the sera of eight undialyzed patients with mean SUN of 210±16 mg/100 ml was 0.22± 0.078 mM (range 0.11–0.34). In sera from five normal persons it was 0.004±0.002 mM (range 0.004—0.008).

Table III also shows that neither hippuric acid nor potassium indoxyl sulfate at the highest concentrations assayed induced any significant modification of the tissular contents of sodium and potassium.

The effects of the compounds previously found to inhibit urate uptake were also tested on the transport of PAH by isolated renal tubules (Table V). Potassium indoxyl sulfate, hippuric acid, and ammonium chloride caused a depression of PAH uptake, the magnitude of which was comparable to that disclosed for uric acid.

Mechanism of inhibitory effect of potassium indoxyl sulfate on PAH transport. The effects of potassium indoxyl sulfate on the uptake of PAH were studied at concentrations of PAH in the medium varying from 0.1 to 2.7 mM. PAH was choosen since its uptake by renal tissue follows the pattern of Michaelis-Menten

TABLE V

Effect of Hippuric Acid, Potassium Indoxyl Sulfate, and Ammonium Chloride on PAH Uptake by Separated Renal Tubules

Exp. no	Compound	Initial concentration	PAH uptake T/M ratio
		mM	
1	None, control (5)*		5.38±0.09
	Hippuric acid (5)	0.10	3.78±0.09‡
	Hippuric acid (4)	0.30	2.77±0.20‡
	Potassium indoxyl sulfate (5)	0.05	4.89±0.03§
	Potassium indoxyl sulfate (5)	0.10	4.37±0.07‡
2	None, control (5)		8.93±0.24
	Ammonium chloride (5)	1.2	8.24±0.14‖

* Number of experiments.
‡ Significant change from control ($P < 0.001$).
§ $P < 0.005$.
‖ $P < 0.05$.

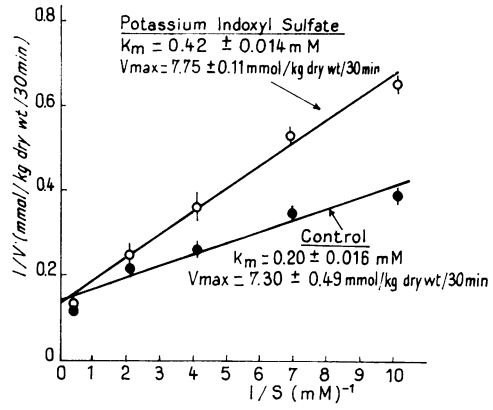

FIGURE 2 Lineweaver-Burk plots of the inhibition of PAH uptake in isolated renal tubules by indoxyl sulfate. The concentration of potassium indoxyl sulfate was 0.2 mM. Vertical bars represent ±1 SEM, whereas the number of experiments for each mean is three. Lines were drawn by the least squares method.

kinetics (26), whereas uptake of urate is a process difficult to saturate (27). Fig. 2 represents the reciprocal of reaction rate versus the reciprocal of substrate concentration along with data for K_m and V_{max}. The results indicate that potassium indoxyl sulfate inhibits PAH uptake by increasing K_m ($P < 0.01$) whereas V_{max} is not significantly affected ($P > 0.05$).

Accumulation of potassium indoxyl sulfate by isolated renal tubules. Table VI summaries the studies of potassium indoxyl sulfate uptake by separated renal tubules at a medium concentration of this agent of 0.5 mM. The results shows that this compound is accumulated against a concentration gradient. This process is temperature-dependent.

Effect of potassium indoxyl sulfate and hippuric acid on urinary urate excretion in man. In two normal persons, potassium indoxyl sulfate and hippuric acid induced a mean increase of urate clearance of 36±4.47% and 39±7.54% during a period of 6 h for the former and of 2 h for the latter compound. These changes were

TABLE VI

Uptake of Potassium Indoxyl Sulfate in Isolated Renal Tubules

Temperature	Indoxyl sulfate uptake T/M ratio
°C	
0	1.34±0.13*
27	3.21±0.17

Potassium indoxyl sulfate concentration in the medium was 0.5 mM. Incubation time was 60 min. Data are means±SEM of six experiments in each group.
* Significant change from the 27°C studies ($P < 0.001$).

significant ($P < 0.05$). In these experiments, creatinine clearances were not significantly altered by these two compounds: control periods for indoxyl sulfate, 102±3.5 ml/min (E. B.-P., $n = 4$); 121±4 ml/min R. A. P, $n = 4$); experimental periods, 105±2.5 ml/min (E. B.-P., $n = 10$); 120±4 ml/min (R. A. P., $n = 12$), control periods for hippuric acid, 82±2.3 ml/min (E. B.-P., $n = 3$); 100±1.7 ml/min (R. A. P., $n = 3$), experimental periods, 83±2.4 ml/min (E. B.-P., $n = 11$) 108±4 ml/min (R. A. P., $n = 9$). Consequently, the ration of urate clearance to endogenous creatinine clearance (C_{ur}/C_{cr}) significantly increased after the ingestion of either potassium indoxyl sulfate or hippuric acid (Fig. 3). After potassium indoxyl sulfate ingestion, maximal effect was disclosed between the 4th and the 5th h. C_{ur}/C_{cr} increased from a mean control value of 0.081±0.003 to a peak value of 0.127 (E. B.-P.) and from 0.059±0.003 to 0.104 (R. A. P.). In both individuals this rise was apparent within 120 min of ingestion of the drug. Significant elevations of these ratios were sustained during the whole period of observation ($P < 0.02$). The uricosuric effect of potassium indoxyl sulfate in E. B.-P. paralleled its increase in urinary excretion. Two peaks of urinary indoxyl sulfate excretion were observed for R. A. P., due presumably to a delayed intestinal absorption of this compound. In the latter, changes of urinary excretion of indoxyl sulfate were followed, after a constant delay of 60 min, by comparable changes in C_{ur}/C_{cr} ratios. The concentration of indoxyl sulfate reached in the serum averaged 0.18±0.018 mM in E. B.-P. ($n = 4$, range 0.13 — 0.21) and 0.15±0.01 mM in R. A. P. ($n = 6$, range 0.12 — 0.18).

Hippuric acid at a dosage twice that of potassium indoxyl sulfate (2.8:1 on a molar basis) induced a smaller and briefer uricosuric effect. Maximal effect on the ratio C_{ur}/C_{cr} was disclosed between the 4th and the 6th h; in E. B.-P., it rose from a mean control ratio of 0.089±0.005 to 0.115; in R. A. P., from 0.079±0.009 to 0.110. Significant elevations of these ratios persisted for 120 min in E. B.-P. ($P < 0.02$) and for 90 min in R. A. P. ($P < 0.05$).

DISCUSSION

The present work supports the hypothesis that increased urinary fractional excretion of urate in chronic uremic patients (2, 3) is induced by the accumulation of uricosuric agents in the sera of these patients. Several substances in uremic serum promote the depression of urate and PAH transport by isolated tubules from kidney cortex of rabbits. Two of these compounds, identified as indoxyl sulfate and hippuric acid, caused a significant increase in urinary fractional excretion of urate in two healthy human subjects.

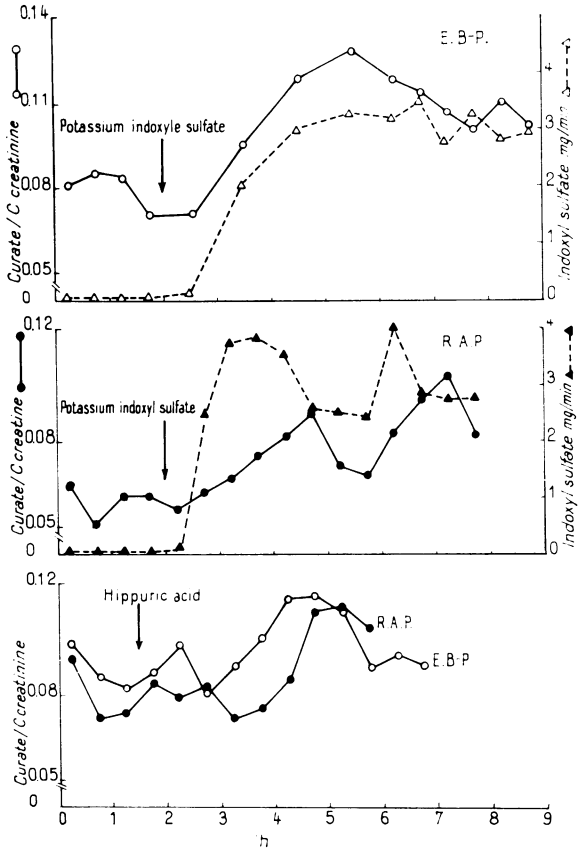

FIGURE 3 Effect of a single oral dose of 4 g potassium indoxyl sulfate (arrow) and 8 g hippuric acid (arrow) on urate excretion in normal subjects. The solid lines represent the C_{ur}/C_{cr} ratios, the interrupted lines the urinary excretion of indoxyl sulfate.

Several characteristics of the inhibiting material were obtained by study of the activity of gel filtration fractions from uremic and normal sera and from urines on the transport of urate and PAH by separated renal tubules. The first major group of active fractions to be eluted occurred in all samples of uremic and normal sera and urines. The substance responsible for the inhibitory effect of these fractions has not been identified. However, two observations support the hypothesis that this inhibitor could be a low molecular-weight compound, having presumably a carboxylic group. First, this inhibitor was eluted at the same time as guanidosuccinic acid and just before lactic acid. This early elution of carboxylic acids, occurring just after the protein peak, was caused by the presence of carboxylic groups in the Sephadex gel (28). Second, the elution of this inhibitor in the fractions of the 11 sera studied began before those of the corresponding urines. This different elution pattern suggests that this inhibitor is a low molecular-weight, protein-bound substance progressively separated during the gel elution process.

The second group of active fractions was disclosed in uremic sera and urines and in normal urines. No activity could be detected in comparable fractions of normal sera. Among the compounds with an elution position corresponding to this group of fractions, i.e., creatinine, methylguanidine, nicotinic acid, ammonium ion, and hippuric acid, only the last two were able to inhibit urate transport in vitro. However, it was observed that only hippuric acid at concentrations found in the fractions from normal and uremic urines could account for the inhibitory effect of these fractions. Since it was difficult to measure with precision the concentration of hippuric acid in the fractions of uremic sera, it was impossible to know if this compound was also responsible for the inhibitory effect of these fractions. However, hippuric acid may at least partially contribute to the depressor effect of these fractions. Indeed, it has been observed that this compound substantially inhibits the transport of both urate and PAH at concentrations comparable to those found in the sera of undialyzed patients. The third group of active fractions eluted was only detected with uremic urine and serum. Comparing the activity of urine fractions to the activity of sera fractions showed that the concentrations of this inhibitor would be approximately six times less in uremic sera than in the corresponding urines. The elution position of this inhibitor suggested a low molecular weight, presumably less than 500.

On the basis of these chromatographic studies, it cannot be concluded that the depressor effect of uremic human sera and urines on urate and PAH transport was solely caused by the three active substances found in the eluates. Indeed, a certain number of active substances could have been eliminated during lyophilization or adsorbed by Sephadex gel. This latter possibility was confirmed by the observation that indoxyl sulfate, which was strongly adsorbed to the gel, was able to inhibit the in vitro transport of urate and PAH. This inhibitory effect of indoxyl sulfate occurred at concentrations comparable to those previously disclosed in uremic sera by Pasternack, Kuhlbäck, and Tallgren (9) and by Mütting (10), as well as by the present study.

Kinetic studies showed that indoxyl sulfate competitively inhibited the entry of PAH into the renal tubules. This inhibitor was accumulated in the tubules against a concentration gradient by a temperature-dependent process. These results support the conclusion that the renal tubules actively transport indoxyl sulfate by the carrier-mediated system involved in the transport of PAH, uric acid, and hippuric acid (26). Indoxyl sulfate and hippuric acid did not change significantly the electrolyte content of renal tubules, indicating that their depressor effect on the uptake of organic acid was not secondary to an inhibition of the sodium-potassium pump.

The depressor effect of uremic sera on the transport of organic acid by kidney slices and isolated tubules has already been documented (5, 29–33). However, the compounds responsible for the inhibitory effect of uremic sera have not been identified. Nevertheless, Bourke et al. (32) observed that hippuric acid inhibited the uptake of PAH by separated renal cortex tubules from rabbits, albeit at concentrations greatly in excess of 0.08 mM encountered in sera of uremic rats. These authors also observed that the depressor effect of sera of uremic rats was partially decreased after chronic treatment of the animals with neomycin. In light of the data presented in this work, these results are readily explained by the reduction of formation of indoxyl sulfate and hippuric acid under the action of neomycin. Indeed, the formation of the cyclic moiety of these two compounds is carried out by the gut flora.

The present studies demonstrate that indoxyl sulfate and, to a lesser degree, hippuric acid significantly increased urinary fractional excretion of uric acid in two normal individuals. In the indoxyl sulfate studies it should be stressed that the uricosuric effect of this compound occurred at a serum concentration comparable to those found in the sera of eight undialyzed patients. Furthermore, the uricosuric effect paralleled the increase of urinary excretion of indoxyl sulfate. These observations suggest that the competition between indoxyl sulfate and PAH demonstrated in vitro was also operative in vivo between indoxyl sulfate and uric acid. In the present study only the net effects of indoxyl sulfate and hippuric acid on the tubular transport of uric acid are indicated. The relative contributions of tubular reabsorption and tubular secretion to uric acid excretion in patients with far advanced renal failure with the pyrazinamide suppression test have yielded contradictory results. Steele and Rieselbach (3) have observed that both tubular reabsorption and secretion were decreased, whereas Danovitch, Weinberger, and Berlyne (4) have reported a striking increase in tubular secretion of urate. The results of Steele and Rieselbach agree with those of the present work. Indeed, indoxyl sulfate and hippuric acid should be regarded as competitive inhibitors of uric acid. Consequently, they probably exert their uricosuric effect by inhibiting either the reabsorption of urate or both the reabsorption and secretion of this compound. However, the possibility of an increase in the proximal tubular secretion of urate induced by indoxyl sulfate and hippuric acid cannot be discarded. Indeed, recently Grantham, Irwin, Qualizza, Tucker, and Whittier (34) have demonstrated that human uremic serum promoted an important secretion of fluid, and presumably solute, into the lumen of proximal straight tubules from rabbit

kidneys in vitro. This hypothesis is further substantiated by the fact that the substance(s) responsible for the secretory effect of human sera have characteristics similar to those of indoxyl sulfate and hippuric acid (34).

It is difficult to ascertain whether or not the unidentified in vitro inhibitors of organic acid transport detected in uremic sera and urines are involved in the increased fractional urate excretion in uremia. Indeed, it is well established that inhibitors of urate uptake by rabbit kidney cortex slices can either increase or decrease excretion of urate in man (27). Although the uricosuric effect of indoxyl sulfate and hippuric acid disclosed in the normal individuals was relatively slight, the high retention of these inhibitors in patients with reduced number of functional nephrons should have an important influence on the tubular transport of urate. Furthermore, the possibility exists that indoxyl sulfate and hippuric acid are accumulated by the tubules in vivo, as suggested by the behavior of these compounds in vitro. Finally, on the basis of these results, we would suggest that the progressive retention of uricosuric substances, like indoxyl sulfate and hippuric acid, during the evolution of chronic renal disease may explain the gradual fractional increase of urate excretion reported in this state (2, 3). However, retention of uricosuric agents might not be the only mechanism responsible for the increased fractional excretion of urate in chronic renal failure. Diminished secretion of urate and persistence of incomplete reabsorption has been reported in uremic patients after a reduction in the filtered load of urate (3). This suggests a decreased tubular permeability to urate.

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