THE OCCURRENCE OF AMINES IN HUMAN URINE: DETERMINATION BY COMBINED ION EXCHANGE AND PAPER CHROMATOGRAPHY*

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Certain biogenic amines, such as epinephrine, norepinephrine, serotonin, and histamine, have been known for some time to be of physiological importance. It is reasonable to suppose that some among the many other amines that are excreted in human urine may also have important functions in some tissues of the body.

Biogenic amines are likely candidates for the role of synaptic transmitters within the central nervous system, and the differential distribution of such amines as serotonin, norepinephrine, and dopamine within the brain suggests their possible relationship to specialized functions. Other chemically related amines, such as bufotenin, N,N-dimethyltryptamine, psilocin, and mescaline, are known to have psychotomimetic effects in man. Pharmacological modification of the metabolism of such amines may be associated with altered brain function. For example, a recent study[1] suggests the possible implication of toxic amines in the etiology of schizophrenia, since an exacerbation of mental dysfunction occurred in certain schizophrenic patients on monoamine oxidase blockade after the administration of oral loads of tryptophan or methionine. Thus, a knowledge of the normal pattern of urinary excretion of amines in man, and an identification of the unknown amines regularly present in urine, should be useful not only as a basis for the study of a wide variety of metabolic diseases, but in particular for exploration of the mechanisms of mental dysfunction.

With this view in mind, a preliminary study of the urinary excretion of amines in normal children was carried out. Although more than 40 amines were found to be regularly present in urine, less than half of these could be identified[2]. In the course of the investigation, it became obvious that two-dimensional paper chromatography alone was not sufficient for a complete separation and identification of these amines, even after they had been concentrated and separated from amino acids and the acidic and neutral constituents of urine.

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Several investigators have developed ion exchange chromatographic techniques for purifying individual amines originally present in physiological fluids[3–5]. The experiments reported here were designed to devise a method of separating the components of a complex mixture of amines by the consecutive use of ion exchange column chromatography and paper chromatography and to apply the method to a study of the amines present in the urine of children. The results have confirmed the tentative identification[2] of several amines not previously reported in human urine and have shown that the number of amines regularly present in urine is greater than had been demonstrated previously through the use of paper chromatography alone. The method provides a means for the isolation of unidentified urinary amines in relatively pure form.

MATERIAL AND METHODS

General

Preliminary experiments with authentic amines demonstrated the impracticability of separating both aliphatic and aromatic amines on a single ion exchange resin or with a single developing buffer. Although aliphatic monoamines could be separated on columns of Amberlite CG-120, aliphatic diamines and aromatic amines were so firmly bound to the resin that they could be eluted only by strongly basic buffers, which either destroyed them or made subsequent paper chromatography difficult. The aromatic amines and aliphatic diamines could be separated on columns of Amberlite CG-50, but on this resin many aliphatic monoamines were so readily eluted that little or no separation occurred. Therefore, a procedure was adopted in which both ion exchange resins were employed.

Sources of amines

Most of the authentic amines used in this investigation were obtained from commercial sources. *o*-Tyramine was prepared enzymatically from *o*-tyrosine by incubation with *Streptococcus fecalis* decarboxylase, and *p*-hydroxybenzylamine was prepared from *p*-methoxybenzylamine by demethylation with hydriodic acid. Five amines were obtained as gifts from other investigators*.

Preparation of developers

Because the amine fractions from the ion exchange columns were later to be chromatographed on paper, it was desirable to use volatile developers for the column chromatograms. Four aqueous pyridine–acetic acid buffers were prepared. Their composition and the quantities of reagents used to prepare them are shown in Table I.

Reagent grade glacial acetic acid and redistilled reagent grade pyridine were used. The normality of the pH 6.32 and pH 6.12 buffers refers to their acetic acid content, while the normality of the pH 5.50 and pH 3.50 buffers refers to their pyridine content.

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TABLE I

COMPOSITION OF AQUEOUS VOLATILE BUFFERS (PER LITER)

pH	3.50	5.50	6.12	6.32
Normality	0.2 *N**	0.8 *N**	0.2 *N***	0.1 *N***
Reagents (ml)				
Pyridine	16.1	64.4	175	150
Glacial acetic acid	115	21.0	11.5	5.75

* Normality in terms of pyridine.
** Normality in terms of acetic acid.

Chromatography of aromatic monoamines and aliphatic diamines

Amberlite CG-50, type 2, a weakly acidic carboxylic acid type cation exchange resin, was first purified initially according to the procedure described by HIRS *et al.*[6]. A 100-g portion of resin in the hydrogen form was suspended in 300 ml of pH 6.32 buffer and was stirred for 20 min. Sufficient pyridine was added to raise the pH of the suspension to pH 6.32 ($\pm$ 0.05). After the resin had been filtered off on a coarse sintered glass Büchner funnel and washed 3 times with 100 ml of pH 6.32 buffer, it was suspended in 2 volumes of this buffer and stored at 5° until used.

A 45 cm column of Amberlite CG-50 was poured in sections in a jacketed glass tube with an internal diameter of 0.9 to 1.0 cm. The suspension of resin was preheated to 40° before pouring the column, which was maintained constantly at 40° throughout pouring, equilibration, and the running of the chromatogram in order to prevent bubble formation and shrinkage. The column was equilibrated with at least 100 ml of pH 6.32 buffer before commencing the chromatogram. Passage of developer during equilibration and during the subsequent chromatogram was controlled with a Milton Roy Chromatographic Minipump*.

The sample containing from 0.02 to 0.1 mmole of authentic amines was dissolved in 2 ml of pH 6.32 buffer, applied directly to the top of the resin column, and rinsed in with small portions of developer. The chromatogram was first developed with pH 6.32 buffer at a flow rate of 10 ml/h, and 2-ml fractions were collected. After 25 h, or when 250 ml of effluent had been collected, the developing solution above the resin column, in the pump, and in the reservoir was replaced with pH 6.12 buffer. The chromatogram was then continued at the same flow rate for another 25 h, or until a total of 500 ml of effluent had been collected.

Chromatography of aliphatic monoamines

Amberlite CG-120, a strongly acidic sulfonic acid type cation exchange resin, was initially prepared by the hydraulic separation method of MOORE *et al.*[7]. A 100-ml portion of settled resin from Fraction C was washed on a coarse sintered glass Büchner funnel successively with 600 ml of *N* sodium hydroxide, 600 ml of water, 600 ml of 2 *N* hydrochloric acid, 600 ml of water, 400 ml of *N* pyridine, and finally with three 200-ml portions of pH 3.50 buffer. The resin was then suspended in 2 volumes of this buffer and was stored at 5° until used.

A 30 cm column of Amberlite CG-120 was poured in sections in a jacketed glass tube with an internal diameter of 0.9 to 1.0 cm. Packing of the column was done at

* Milton Roy Co., Philadelphia, Pa.

room temperature and facilitated by the use of air pressure at 30 cm of mercury. The resin column was equilibrated at 50° with at least 100 ml of pH 3.50 buffer. The pump was used with this type of chromatogram also.

The sample containing from 0.02 to 0.1 mmole of authentic amines was dissolved in 2 ml of pH 3.50 buffer, the pH was then reduced to approximately 2.2 by the addition of 0.06 ml of 6 *N* hydrochloric acid, and the sample was applied directly to the top of the resin column and rinsed in with small portions of developer. The chromatogram was first developed with pH 3.50 buffer at a flow rate of 30 ml/h and a temperature of 50°; 2-ml fractions were collected. After 600 ml of effluent had been collected, the developing solution above the resin column, in the pump, and in the reservoir was replaced with pH 5.50 buffer. The chromatogram was then continued with this second developer at the same temperature and flow rate for a further 250 ml.

The above conditions do not elute diamines from the chromatogram. It was possible, however, to detect diamines if, after the chromatograms were completed, the columns were eluted with 2 *N* sodium hydroxide. These strongly basic eluates were then neutralized with acetic acid, and after evaporation to dryness, the diamines were extracted with ethanol and acetone.

Examination of effluent fractions

Each fraction of effluent was then examined by reaction with ninhydrin on the Technicon Auto Analyzer* by means of the procedure of SCHROEDER *et al.*[8]. Color produced by ninhydrin-reactive amines was scanned at 570 mμ. When authentic amines which yielded a yellow color with ninhydrin were chromatographed, optical density was also recorded at 440 mμ.

Many of the authentic amines that were chromatographed produce no color with ninhydrin and cannot be located in the effluent in this way. Some, indeed, emerge in mixture with ninhydrin-positive substances. The ninhydrin-negative amines had to be detected by other means. After all fractions had been examined for reaction with ninhydrin as described above, those fractions that contained discrete peaks of ninhydrin-positive material were pooled separately. Of those fractions that contained no ninhydrin-positive material, every three to five were arbitrarily pooled.

Pooled fractions of effluent were then concentrated to dryness by removing the volatile buffer under reduced pressure on a rotary evaporator. The residual material was dissolved in a small volume of methanol and subjected to one- or two-dimensional chromatography on paper. The amines were detected by spraying the paper chromatograms with appropriate reagents for ninhydrin-negative as well as ninhydrin-positive amines. The solvents and spray reagents for the paper chromatography of amines have been described in detail elsewhere[2].

Subjects and patients

Urine specimens were obtained from 7 normal children and from 2 juvenile psychotics. The free and conjugated amines were separated from other urinary constituents and were concentrated approximately 1000-fold by a technique previously described[2]. Aliquot portions of these concentrates equivalent to volumes of original urine that contained 250 to 350 mg of creatinine were chromatographed on ion exchange columns of the same size in the same manner as has been described above.

* Technicon Instruments Corporation, Chauncey, N. Y.

TABLE II

ELUTION VOLUMES OF AMINES CHROMATOGRAPHED ON AMBERLITE CG-50*

Compound	*Range of elution (ml)*	*Elution peak*** *(ml)*	*Compound*	*Range of elution (ml)*	*Elution peak*** *(ml)*
Histidine	25–31	28	3-Hydroxy-4-methoxyphenylethylamine	145–163	
1-Methylhistidine	25–31	28	Putrescine	140–171	159
Arginine	31–42	35	*p*-Hydroxybenzylamine	151–171	162
Ethanolamine	32–40	36	Cadaverine	163–183	
Ammonia	33–42	38	Dopamine	163–190	
Ethylamine	38–46	42	Benzylamine	169–189	178
Pyrrolidine	49–58	54	*p*-Tyramine	170–196	186
N-Acetylhistamine	49–65		3-Ethoxy-4-hydroxybenzylamine	177–193	
Pyridoxamine	68–79	74	*m*-Tyramine	183–202	
N-Methylmetanephrine	78–89		*p*-Methoxybenzylamine	192–215	203
Methanephrine	102–115	109	Histamine	202–225	214
Epinephrine	101–124		Bufotenin	205–235	
Normetanephrine	112–130	121	Phenylethylamine	228–254	240
1-Methylhistamine	112–130	121	*o*-Tyramine	218–262	241
Norepinephrine	116–132		*p*-Methoxyphenylethylamine	228–261	244
Synephrine	118–135		Kynuramine	229–261	244
Isoamylamine	119–136	128	2,2'-Dithiobis-(ethylamine)	277–297	289
Mescaline	124–142		Serotonin	283–303	
3,4-Dimethoxybenzylamine	127–142		Agmatine	305–321	312
Octopamine	132–147		N,N-Dimethyltryptamine	310–335	
3-Methoxy-4-hydroxybenzylamine	134–150		5-Methoxytryptamine	328–352	339
Epinine	135–151		Tryptamine	380–415	397
3,4-Dimethoxyphenylethylamine	140–154		Spermidine	408–466	438
3-Methoxytyramine	141–158		5-Methyltryptamine	465–505	483

* Authentic compounds were chromatographed in mixtures on Amberlite CG-50 columns 45 cm in length and 0.9 to 1.0 cm in diameter, at a flow rate of 10 ml/h and a temperature of 40°. Chromatograms were developed with pH 6.32—0.1 *N* pyridine *acetate* buffer for the first 250 ml, and thereafter with pH 6.12—0.2 *N* pyridin *acetate* buffer.

** Elution peaks were not obtained for a number of amines giving no color or weak colors with ninhydrin.

The aliphatic monoamines in the urines of 4 normal children and 1 psychotic child were separated on Amberlite CG-120 columns. One of the normal children had been maintained on a plant-free diet and had been given neomycin orally for 5 days prior to urine collection in order to eliminate amines of exogenous plant origin from the urine and to minimize those formed by the bacterial flora of the intestinal tract.

Aromatic amines and aliphatic diamines in the urines of 3 normal children and one psychotic child were separated on Amberlite CG-50 columns. Two of the normal children so studied were administered monoamine oxidase inhibitors (nialamide or pheniprazine) prior to and during urine collections.

RESULTS

Chromatography of authentic amines

The elution volumes of a number of authentic aromatic or heterocyclic amines and of several aliphatic polyamines from Amberlite CG-50 are listed in Table II. Also shown are the emergence points of several basic amino acids and aliphatic monoamines commonly present in urinary amine concentrates. These compounds are always eluted from Amberlite CG-50 at the same effluent volumes when the columns are operated under the conditions described.

The following observations were made concerning the effect of chemical structure on the elution volume from Amberlite CG-50. Methylation of the amino group hastens elution from the resin, and tertiary amines are eluted more rapidly than secondary amines. Acetylation of the amino group accelerates elution very markedly. Hydroxylation of the β carbon atom of the side chain of phenylethylamine derivatives speeds elution from the resin. Hydroxylation of the benzene ring of aromatic amines hastens their emergence from the resin, but this effect is greatest when the ring is hydroxylated in the *para* position and least in the *ortho* position. Methylation of a single hydroxyl group in the ring of an aromatic amine generally causes the emergence point of the compound to revert to that of the original unhydroxylated analogue. If two or more ring hydroxyl groups are methylated, however, the opposite holds true, and the compound is eluted more rapidly. The longer the carbon chain of aliphatic amines or the side chain of aromatic amines, the more elution from Amberlite CG-50 is delayed. The presence of the indole nucleus slows elution. Increase in the number of amino groups in polyamines delays elution, and when as many as four are present, as in spermine, elution from the resin cannot be effected with the developing buffers used.

Table III presents the elution volumes of a group of authentic aliphatic monoamines from Amberlite CG-120 columns that were operated as described above. Included are the elution volumes of ammonia and the basic amino acids to be found in urinary amine concentrates.

The following observations were made concerning the effect of varying chemical structure on the elution volumes of aliphatic monoamines on Amberlite CG-120. Substitution of a methyl group on the amino group hastens elution from the resin. Hydroxylation of the aliphatic chain speeds elution, and the greater the number of hydroxyl groups the more rapidly the compound is eluted. Increase in the number of carbon atoms in the aliphatic chain delays emergence from the resin. Whereas diamines fail completely to be eluted from Amberlite CG-120, acetylation of one of the two amino groups results in their early elution.

TABLE III

ELUTION VOLUMES OF AMINES CHROMATOGRAPHED ON AMBERLITE CG-120*

Compound	*Range of elution (ml)*	*Elution peak (ml)*	*Compound*	*Range of elution (ml)*	*Elution peak (ml)*
Glucosamine	115–132	121	γ-Methylmercaptopropylamine sulfoxide	290–300	295
Galactosamine	115–132	121	Ammonia	296–345	308
N-Acetylethylenediamine	145–160	152	*n*-Propylamine	312–332	322
β-Methoxyethylamine	150–165	157	Isobutylamine	316–345	328
N-Methylethanolamine	156–171	163	Hydroxylamine	334–371	347
3-Amino-1-propanol	175–191	183	1-Methylhistidine	340–375	361
Serinol	175–191	183	Cyclopropylamine	350–382	364
Dimethylamine	187–200	193	3-Methylhistidine	353–388	370
β-Hydroxypropylamine	195–209	201	*n*-Butylamine	390–420	405
N-Methylethylamine	198–215	206	Isoamylamine	430–465	445
Diethylamine	212–224	218	γ-Methylmercaptopropylamine	447–490	467
Ethanolamine	210–230	219	*n*-Amylamine	504–542	520
2-Aminobutanol	220–236	228	Histidine	591–629	600
Pyrrolidine	250–270	260	Ornithine	646–656	650
Piperidine	250–272	261	Lysine	653–662	656
Methylamine	262–280	273	Carnosine	657–666	662
Ethylamine	272–294	282	Arginine	820–855	832

* Authentic compounds were chromatographed in mixtures on Amberlite CG-120 columns 30 cm in length and 0.9 to 1.0 cm in diameter, at a flow rate of 30 ml/h and a temperature of 50°. Chromatograms were developed with pH 3.50—0.2 *N pyridine* acetate buffer for the first 600 ml, and thereafter with pH 5.50—0.8 *N pyridine* acetate buffer. The breakthrough of the second developer occurred at 636 ml.

Although the detection of ninhydrin-reactive amines in the effluent fractions is much facilitated by the use of the Technicon Auto Analyzer, the older manual methods for detecting ninhydrin-reactive materials in the effluent of chromatographic columns are equally applicable[9]. Regardless of which ninhydrin procedure is used, it is adequate to determine the absorbance of the reaction mixture at 570 mμ only, because pyrrolidine is the only amine likely to occur in sufficient amount in urine to produce a measurable absorbance at 440 mμ. In our hands, the absolute elution volumes of amines have been very reproducible. In the hands of other investigators, they would no doubt vary somewhat from the figures given in Tables II and III because of differences in the dimensions of the columns, but the relative emergence points of the various amines should not vary.

Ion exchange chromatography as described is not suitable for the detection of small amounts of catecholamines, because they appear to be unstable at the pH of the developing buffer used. Some of the more volatile aliphatic amines, such as methylamine, dimethylamine, ethylamine and piperidine, may partly be lost when effluent fractions are taken to dryness prior to paper chromatography. When γ-methylmercaptopropylamine, the decarboxylation product of methionine, is chromatographed on Amberlite CG-120, part is converted to the sulfoxide. This is not disadvantageous, however, because detection of the sulfoxide in a chromatogram strengthens identification of the parent amine.

Chromatography of amines in urine

Table IV lists the amines regularly present in urine which were detected by ion exchange chromatography on Amberlite CG-50 and subsequent paper chromatography. The unidentified compounds are numbered for the sake of simplicity according to the system of PERRY *et al.*[2], and the solvents and spray reagents used in paper chromatography are those described by these authors. For each compound, Table IV presents its elution volume, its R_F values on paper in four different solvents, and the colors produced by various spray reagents.

Table V lists the amines regularly found in urine amine concentrates and separated on columns of Amberlite CG-120. The numbering of unidentified compounds corresponds to the system previously reported[2]. R_F values in four different solvents are recorded, as well as appropriate color reactions.

In both Tables IV and V, amines have been listed in the order of their emergence from the ion exchange columns. Unidentified compounds have been presumed to be amines because of their chromatographic behavior on resin columns and on paper, as well as their color reactions.

Although the number of urines studied by this technique has been small, the aromatic amines listed are assumed to be present regularly in urine, because they were observed routinely in a much larger group of children's urines that had been studied by paper chromatography alone[2,10]. The aliphatic amines listed in Table V were present in the 5 urines that were chromatographed on Amberlite CG-120. The regular occurrence of some of them, however, is more questionable, because two-dimensional paper chromatographic examination on a larger number of urines had failed to detect their presence. Several additional compounds, presumably amines, that were detected in only one or two of the urines are not listed in Tables IV and V.

(Text continued p. 369)

TABLE IV

URINARY AMINES CHROMATOGRAPHED ON AMBERLITE CG-50*

Compound No.	Identification	Elution zone (ml)	R_F values				Color reactions with spray reagents				
			BuAc	MBF	ANF	IpAm	DPNA	DSA	DQCI	DMCA	Ninhydrin-lutidine
9	Unidentified	46–58	0.44	0.59	0.39	0.46	Orange	Orange-red			
8	N-Acetylhistamine	49–65	0.47	0.71	0.33	0.73	Orange	Orange-red			
42	Unidentified	67–77	0.35	0.43	0.28	0.17					Pink
10	Unidentified	67–83	0.58	0.69	0.63	0.79	Orange	Orange-red			
25	Unidentified	93–103	0.71	0.73	0.70	0.54	Lavender	Orange	Green		
45	Unidentified	99–114	0.52	0.62	0.52	0.50	Pink	Yellow	Blue		
19	Metanephrine	102–115	0.58	0.66	0.57	0.64	Purple	Orange	Blue		Purple (70°)
44	Unidentified	110–120	0.86	0.87	0.92	0.64					Purple
46	Unidentified	113–122	0.63	0.67	0.67	0.61	Blue				
18	Normetanephrine	112–130	0.54	0.56	0.45	0.47	Purple	Orange	Blue		Purple
37	1-Methylhistamine	112–130	0.35**	0.51	0.18	0.57					Purple
15	Synephrine	118–135	0.60	0.65	0.57	0.64	Pink	Yellow	Blue		Purple (70°)
14	Octopamine	132–147	0.57	0.57	0.49	0.50	Pink	Yellow	Blue		Purple
17	3-Methoxy-4-hydroxy-benzylamine	134–150	0.52	0.58	0.62	0.51	Purple	Orange	Blue		Yellow→ yellow-brown
26	Unidentified	139–157	0.63	0.67	0.66	0.65	Purple	Orange	Grey-blue		
24	Unidentified	140–162	0.49	0.57	0.38	0.27	Red-purple	Brown	Red-purple (f.)		

16	3-Methoxytyramine	141–150	0.04	0.04	0.01	0.07	Blue-grey	Orange	Grey		
47	Putrescine	140–171	0.30**	0.32	0.12	0.47					Purple
13	*p*-Hydroxybenzylamine	151–171	0.58	0.62	0.61	0.60	Pink	Yellow	Blue		Yellow→ yellow-brown
48	Cadaverine	163–183	0.30**	0.37	0.13	0.55					Purple
49	Unidentified	173–186	0.31	0.46	0.13	0.76					Purple
11	*p*-Tyramine	170–196	0.62	0.67	0.59	0.72	Lavender	Orange	Grey		Purple
20	Unidentified	174–191	0.53	0.63	0.49	0.58	Yellow	Blue (f.)	Yellow		
12	*m*-Tyramine	183–202	0.67	0.70	0.58	0.69	Purple-pink	Orange-yellow	Blue		Red-purple
7	Histamine	202–225	0.44**	0.27	0.07	0.57	Orange	Orange-red			Purple
3	Bufotenin	205–235	0.61	0.68	0.71	0.86	Red-purple	Brown	Grey	Blue	
4	Kynuramine	229–261	0.66	0.74	0.66	0.78				Purple	Purple
21	Unidentified	230–270	0.75	0.80	0.80	0.85	Pink→ red-purple	Yellow	Blue		
50	2,2′-Dithiobis(ethyl-amine)	277–297	0.30**	0.36	0.15	0.63					Purple
2	Serotonin	283–303	0.51	0.50	0.51	0.63	Red-purple	Brown	Grey	Blue	Yellow-brown
1	Tryptamine	380–415	0.75	0.70	0.72	0.81				Purple	Purple

* Solvents used in paper chromatography were: BuAc = *n*-butanol–acetic acid–water (12:3:5); MBF = 2-methyl-3-butyn-2-ol–formic acid–water (75:5:20); ANF = acetonitrile–formic acid–water (80:2:18); and IpAm = isopropanol–ammonium hydroxide–water (8:1:1). Color reactions are listed for the following reagents[4]: DPNA = diazotized *p*-nitroaniline; DSA = diazotized sulfanilic acid; DQCI = dichloroquinone chloroimide; DMCA = dimethylaminocinnamaldehyde; and ninhydrin–lutidine. Blank spaces indicate failure to give color with a reagent. The symbol (f.) indicates that color fades rapidly. Temperatures shown in parentheses indicate color develops only if sheets are heated.

** Hydrochlorides of these diamines have a lower R_F in BuAc than do the amines after elution from the resin.

TABLE V

URINARY AMINES CHROMATOGRAPHED ON AMBERLITE CG-120*

Compound No.	Indentification	Elution zone (ml)	R_F values				Color with ninhydrin-lutidine	Color after nickel sulfate
			BuAc	MBF	ANF	IpAm		
51	Unidentified	105–118	0.57	0.80	0.79	0.57	Purple (70°)	Pink
52	Unidentified	119–133	0.47	0.66	0.39	0.66	Purple	Pink
53	Unidentified	163–182	0.59	0.73	0.69	(d.)	Grey-purple (100°)	Grey-pink
54	Dimethylamine	187–200	0.48	0.63	0.59	(d.)	Purple (70°)	Pink
32	β-Hydroxypropylamine	195–209	0.48	0.59	0.40	0.65	Purple	Pink
40	Unidentified	195–209	0.41	0.48	0.31	0.53	Yellow	Yellow
30	Ethanolamine	210–230	0.40	0.54	0.28	0.56	Purple	Pink
55	Unidentified	210–230	0.65	0.83	0.75	0.80	Grey (100°)	Pink-grey
56	Unidentified	227–245	0.12	0.38	0.12	0.04	Purple	Pink
57	Unidentified	230–246	0.50	0.61	0.41	0.57	Purple (70°)	Pink
33	Pyrrolidine	250–270	0.53	0.75	0.62	(d.)	Yellow (70°)	Yellow
59	Piperidine	250–272	0.58	0.78	0.65	(d.)	Blue-purple (100°)	Purple
58	Methylamine	262–280	0.41	0.53	0.25	(d.)	Purple	Pink
31	Ethylamine	272–294	0.51	0.65	0.58	(d.)	Purple	Pink
41	Unidentified	247–294	0.31	0.37	(d.)	(d.)	Pink	Pink
44	Unidentified	670–685	0.86	0.87	0.92	0.64	Purple	Grey-pink
42	Unidentified	670–685	0.35	0.42	0.28	0.17	Pink	Salmon

* Solvents used in paper chromatography were: BuAc = *n*-butanol–acetic acid–water (12:3:5); MBF = 2-methyl-3-butyn-2-ol–formic acid–water (75:5:20); ANF = acetonitrile–formic acid–water (80:2:18); and IpAm = isopropanol–ammonium hydroxide–water (8:1:1). The symbol (d.) in the vertical columns listing R_F values indicates the compound is destroyed or volatilizes in that solvent. The final two vertical columns show colors given by compounds when paper chromatograms are sprayed with ninhydrin–lutidine[2] and then countersprayed with nickel sulfate. Temperatures shown in parentheses indicate color develops only if sheets are heated.

The urines of the two psychotic children studied showed all of the compounds listed in Tables IV and V. The identity of an unusual amine detected in the urine of one of these patients and not in that of normal children will be reported elsewhere[11].

Figs. 1 and 2 present graphically the results of chromatograms on Amberlite CG-50 and Amberlite CG-120 respectively of urinary amine concentrates. In these

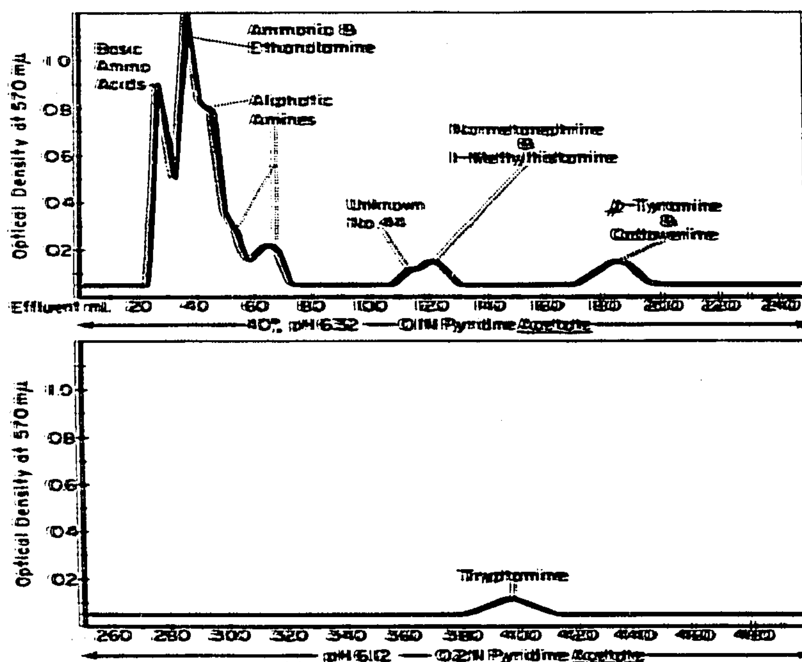

Fig. 1. Chromatographic analysis of ninhydrin-reactive amines and related compounds in normal urine amine extract on a 1 × 45-cm column of Amberlite CG-50.

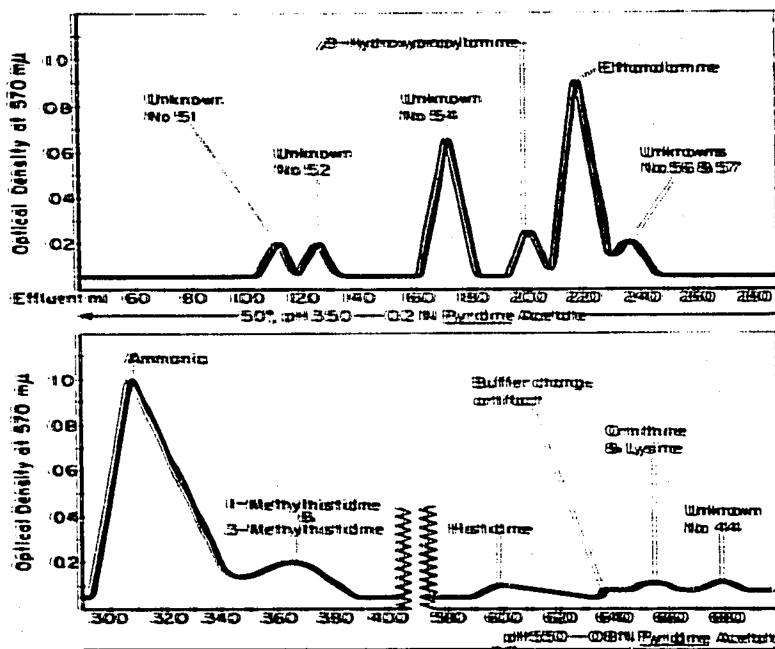

Fig. 2. Chromatographic analysis of ninhydrin-reactive amines and related compounds in normal urine amine extract on a 1 × 30-cm column of Amberlite CG-120.

instances, the urinary amines in 300 mg creatinine equivalents of urine were applied to the resin columns, and the effluent fractions were examined by reaction with ninhydrin on the Technicon Auto Analyzer, recording optical density of the color produced at 570 mμ. The chromatogram depicted in Fig. 1 was that of a child who had been administrated pheniprazine as a monoamine oxidase inhibitor, and the peaks for *p*-tyramine and tryptamine are therefore more prominent than would ordinarily be expected. The wide variety of amines present in urine is not apparent in these graphs, because many aromatic amines, and all the secondary aliphatic amines, fail to produce sufficient color with ninhydrin to register on the recorder. The location of these ninhydrin-negative amines in the column effluent is not difficult to calculate, however, if based upon the emergence points of certain ninhydrin-positive amines, such as those shown for normetanephrine, *p*-tyramine, and tryptamine in Fig. 1.

The present technique has advantages if an amine is present in urine in such low concentration that it cannot easily be detected during routine paper chromatography. By subjecting to paper chromatography the material in the entire effluent zone in which the suspected amine emerges, trace amounts of it may be recognized. In the present investigation, 2,2′-dithiobis(ethylamine), the amine derived from cystine, was identified in this way. The appropriate effluent zones from columns of Amberlite CG-50 were chromatographed on paper, and the sheets were sprayed with a nitroprusside reagent[12] which is specific for certain sulfur-containing amino acids and amines. *o*-Tyramine was similarly identified in the urine of one normal child on monoamine oxidase blockade, although it could not be detected in the urines of 21 children on monoamine oxidase blockade studied by paper chromatography alone[10].

DISCUSSION

Sixteen aromatic and heterocyclic amines which are usually present in the urines of normal children have been identified in this investigation. Fifteen of these had been detected in a previous study[2] by means of paper chromatography. The present methods have identified 1-methylhistamine, which before had been listed as an unidentified amine. The occurrence of this amine in human urine has been reported by SCHAYER[13]. The tentative identification of kynuramine in urine[2] has been strengthened by the present investigation. The amine present in urine is eluted from Amberlite CG-50 at the same volume as authentic kynuramine, has R_F values on paper that are identical with those of the authentic compound in 4 different solvents, and gives the same color reactions with dimethylaminocinnamaldehyde and with the Ekman spray reagent[12]. The identification of *p*-hydroxybenzylamine and of 3-methoxy-4-hydroxybenzylamine, first reported by KAKIMOTO AND ARMSTRONG[5], has been confirmed.

Of the 16 identified aromatic and heterocyclic amines found in children's urine, synephrine, 3-methoxy-4-hydroxybenzylamine, and *p*-methoxybenzylamine appear to be of exogenous origin, and are eliminated from the urine when children are fed a plant-free diet. Bufotenin and kynuramine are usually detectable only after the administration of monoamine oxidase inhibitors. In the urines examined during this study, *o*-tyramine was detected in one urine, and benzylamine in another. The latter is present in all amine extracts of urines from subjects receiving nialamide. This drug is excreted in the urine; and when urine is hydrolyzed during preparation of amine extracts, the benzylamine moiety of nialamide is freed. In addition to the 16 identified

aromatic amines found in children's urine, another 10 unidentified bases, presumably aromatic amines, were present in the effluents from columns.

Ten aliphatic amines were identified in most or all of the urines studied, as well as an additional 10 bases which could not be identified, but which are probably aliphatic amines. The latter 10 unidentified amines were present in the urine of a normal child who had been placed on a plant-free diet and given neomycin by mouth, and it is reasonable to assume that most or all of them are of endogenous origin.

In an earlier study in which paper chromatography alone had been used[2], it was possible to identify only ethanolamine, pyrrolidine, ethylamine, and β-hydroxypropylamine of the aliphatic group. In the present investigation, methylamine, dimethylamine, and piperidine have also been demonstrated in urine. Their excretion in human urine has been established by other investigators[4,14-16]. The earlier tentative identification[2] of β-hydroxypropylamine has been more solidly established in the present study. Not only does the compound from urine migrate on paper chromatograms at the same rate as the authentic amine in 4 different solvents, but it emerges from columns of Amberlite CG-120 at the same effluent volume.

Putrescine, cadaverine, and 2,2'-dithiobis(ethylamine) have not been reported as constituents of normal urine, although the first two diamines are known to be excreted by cystinurics[17]. The identification of these three diamines in the present investigation is based on identical migration of the urinary compounds and the authentic amines on paper in 4 different solvents, as well as elution from columns of Amberlite CG-50 at the same effluent volumes. In addition, the 2,2'-dithiobis-(ethylamine) from urine gave the expected color reaction with nitroprusside.

Efforts to identify the 10 unknown compounds listed in Table V have been unsuccessful. Their elution volumes from Amberlite CG-120 and their migration rates on paper fail to match those of any of a large number of authentic aliphatic amines. It was felt that some of them might be N-acetylated diamines, or the decarboxylation products of the amino acids, glutamine and asparagine, for which authentic samples were unavailable. For this reason, 6 of these unidentified amines (Compounds 51, 52, 53, 55, 56, and 57) were hydrolyzed in 2 N hydrochloric acid at 110° for 5 h. Under these conditions, N-acetylhistamine and N-acetylethylenediamine were almost completely hydrolyzed to histamine and ethylenediamine respectively. No change was produced in Compounds 53, 55 and 56 by acid hydrolysis. New ninhydrin-reactive substances were produced by the hydrolysis of Compounds 51, 52 and 57. These were neither the identifiable diamines which might have been expected had the parent compounds been N-acetylated diamines, nor were they β-alanine or γ-aminobutyric acid, as might have been expected had the parent compounds been the amines derived from asparagine or glutamine. It is possible that some of the unidentified ninhydrin-reactive bases found in urine may be decarboxylated simple peptides.

No attempt has been made in this investigation to assess the physiological significance of the wide variety of amines found in human urine. Since most of these continue to be excreted even after reduction of the bacterial flora of the intestine and elimination of plant foods from the diet, it seems likely that they represent intermediate or end points in important metabolic processes. The identity of the unknown amines constantly present in urines should be determined, and the physiological role of these, as well as of many of the identified amines, should be worked out. The techniques here described should be valuable in achieving this end. Not only

may clues to the chemical nature of an unidentified substance be obtained from its point of emergence from the ion exchange column, but the procedure no doubt may also be scaled up to permit isolation in sufficient quantity for chemical identification. In the meantime, knowledge about the normal pattern of excretion of amines in human urine can serve as a useful standard against which to compare the situation encountered in various metabolic diseases.

The methods described here for the identification of amines in urine should be applicable to the study of other physiological fluids and tissue extracts. It is likely that these will be found to contain only a fraction of the number of amines listed in Tables II and III. In such a case, ion exchange column chromatography may accomplish complete separation of most of the amines present.

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SUMMARY

A technique has been developed by which complex mixtures of amines can be separated by column chromatography on the ion exchange resins Amberlite CG-50 and Amberlite CG-120. Identification of amines is made on the basis of the speed of their elution from the resin column, as well as by subsequent paper chromatography of effluent fractions. The method can be applied to amines derived from various physiological fluids, and offers the possibility of isolating unidentified compounds for chemical characterization.

Using the combined technique of ion exchange column chromatography followed by paper chromatography, 26 amines were identified in human urine. These were: methylamine, dimethylamine, ethylamine, ethanolamine, β-hydroxypropylamine, pyrrolidine, piperidine, putrescine, cadaverine, 2,2'-dithiobis(ethylamine), histamine, N-acetylhistamine, 1-methylhistamine, *p*-hydroxybenzylamine, 3-methoxy-4-hydroxybenzylamine, *p*-tyramine, *m*-tyramine, octopamine, synephrine, 3-methoxytyramine, normetanephrine, metanephrine, tryptamine, serotonin, bufotenin, and kynuramine. An additional 20 unidentified bases, presumably amines, were found to be regularly excreted in urine.

Evidence is presented supporting the identification of kynuramine, β-hydroxypropylamine, putrescine, cadaverine, and 2,2'-dithiobis-(ethylamine). These 5 amines have not previously been considered to be constituents of normal human urine.

It is suggested that study of the urinary excretion of amines may provide useful clues as to the mechanisms involved in various metabolic disorders, including those characterized by mental dysfunction.

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