SEROTONIN AND ITS METHYLATED DERIVATIVES IN HUMAN URINE*

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Substances with pharmacological properties similar to those of serotonin (5-hydroxytryptamine) have been found in human and dog urine and in rat, rabbit, and dog brain by Twarog and Page (1). Eluates of chromatograms of urine showed greatest activity from areas having the same R_F value as serotonin. Since then, these eluates have been rechromatographed and other positive indole spots have appeared, which suggest the presence of methylated derivatives of serotonin. These are now characterized tentatively as *N*-methylserotonin and bufotenine.

Methods

Preparation of Urine Extracts

24 hour samples of urine from healthy adults were collected in 10 ml. portions of 6 N HCl and the HCl concentration was adjusted to approximately 0.05 N at the end of the collection period. After concentration under reduced pressure at 40–50° to one-tenth the original volume, the samples were incubated with urease[1] (approximately 50 gm. of urease per liter of original urine) for 2 to 4 hours at 46°, the pH being maintained at 5.0 to 6.5. 10 volumes of acetone were added at the end of the incubation and the samples stored for 24 to 48 hours at 7°. After filtration, the acetone extract was evaporated to approximately one-fiftieth the original urine volume; acetone precipitation was then repeated. After removal of acetone by evaporation, the solution was adjusted to pH 5.0, and methanol was added to a concentration of 30 per cent. This solution was mixed, small portions at a time, in a mortar with 20 volumes of acetone; a yellow precipitate and a light amber filtrate resulted. After storage for 24 hours at −15°, the filtrate was decanted through glass wool, concentrated *in vacuo* (40–50°) to 1/200 the original urine volume, and subsequently fractionated on aluminum oxide columns.

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[1] 25 mg. tablets obtained from Hynson, Westcott and Dunning, Inc., Baltimore, Maryland.

Preparation of Aluminum Oxide Columns

Reagent aluminum oxide (Merck) suitable for chromatographic adsorption was used for all columns. Benzene was drawn into a 2.3 cm. × 80 cm. tube with a sintered glass disk at the bottom. A 1 cm. layer of absorbent cotton on the disk was covered with a 1 cm. layer of acid-washed reagent grade sea sand poured in as a slurry in benzene. A 21 cm. column of aluminum oxide was layered on top of the sand by the addition of a slurry of aluminum oxide in benzene during tapping of the outside of the column to facilitate even packing of the adsorbent. Another 1 cm. layer of sand and a 1 cm. layer of absorbent cotton were then added to the top of the column. The column, always covered with solvent, was washed with benzene diluted with increasing quantities of methanol until a final washing with 100 ml. of absolute methanol.

Paper Chromatography

Ascending unidimensional paper chromatograms on Whatman No. 1 filter paper were run for 18 hours at 23° in butanol saturated with 14 per cent ammonium hydroxide. A 2 per cent solution of *p*-dimethylaminobenzaldehyde in 1.2 N HCl was used as the color-developing reagent to produce the "indole spots."

Bioassays

Pharmacological assays were done by the method of Twarog and Page (1), as suggested by Welsh and Taub (2), with the heart of *Venus mercenaria*.

Samples of *N*-methylserotonin creatinine sulfate and bufotenine (*N*,*N*-dimethylserotonin) creatinine sulfate (monohydrate) were supplied by Dr. Merrill E. Speeter of The Upjohn Company. The *Venus* heart was 5- to 10-fold more sensitive to bufotenine and twice as sensitive to the *N*-methyl derivative as to serotonin itself. Earlier, the *Venus* heart had been found (1) to be 20 to 100 times less responsive to cinobufotenine than to serotonin. At that time, cinobufotenine was mistakenly believed to be bufotenine; however, chromatography of synthetic bufotenine and cinobufotenine has subsequently shown them to be different. Erspamer (personal communication) showed that cinobufotenine is, in fact, bufotenidine, the quaternary form of bufotenine.

Comparison of the pressor effects of equimolar solutions (10^{-3}) of serotonin, *N*-methylserotonin, and bufotenine on the arterial pressure of dogs anesthetized with pentobarbital showed serotonin to be the most active, *N*-methylserotonin less active, and bufotenine least active, an order of activity just the reverse of that observed with the clam heart.

Separation of Serotonin, N-Methylserotonin, and Bufotenine on Alumina Columns

5 ml. of a 90 per cent methanol solution containing 866 γ of bufotenine creatinine sulfate, 640 γ of *N*-methylserotonin creatinine sulfate, and 810 γ of serotonin creatinine sulfate (Abbott) were pipetted onto an aluminum oxide column. The adsorbates were then eluted with methanol diluted with increasing amounts of water as follows: 95 ml. of absolute methanol, 100 ml. of 80 per cent methanol, 100 ml. of 60 per cent methanol, 25 ml. of 40 per cent methanol, 25 ml. of 20 per cent methanol, and 50 ml. of water. Eighty fractions of 5 ml. each were collected.

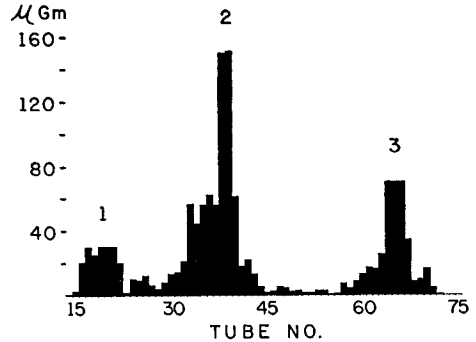

FIG. 1. Aluminum oxide chromatography of bufotenine, *N*-methylserotonin, and serotonin. Peak 1 assayed against bufotenine, Peak 2 against *N*-methylserotonin, and Peak 3 against serotonin and plotted as micrograms of the corresponding compound.

After removal of the methanol at reduced pressure (50°), assays (*V. mercenaria*) of the fractions revealed three peaks of serotonin-like activity as shown in Fig. 1. Paper chromatography of Tube 19, from Peak 1, revealed a positive indole spot with the same R_F as bufotenine. The contents of Tube 37 from Peak 2 showed two positive indole spots, one with the R_F of *N*-methylserotonin and a second, fainter spot with the R_F of bufotenine. Tube 65 from Peak 3 produced an indole-positive reaction at the same R_F as that of serotonin. Thus, though some overlapping occurred, separation into three peaks warranted use of this method on the urine samples. That some bufotenine occurred in Peak 2 and that this is several times as active on *Venus* heart as *N*-methylserotonin account for the apparent 150 per cent recovery.

Fractionation of Urine on Aluminum Oxide Columns

Urine extracts prepared as described were added to aluminum oxide columns in 5 ml. portions of methanol solution and were eluted in the same

manner as the serotonin and methylated derivatives. Bioassay revealed three peaks of activity which corresponded to those of bufotenine, *N*-methylserotonin, and serotonin. A 24 hour specimen from another person showed less activity in the first two peaks and none in the third peak, although there was a shoulder of activity extending from the second peak to Tube 60.

TABLE I

Paper Chromatograms of Eluates from Aluminum Oxide Column

Peak No. (Al_2O_3 column)	Eluate, 1st paper chromatogram	Indole spots on rechromatography				
		Bufotenidine, 0.14–0.16; hydroxyindole-acetic acid, 0.08–0.10	Unidentified	Serotonin, 0.55–0.64	*N*-Methyl-serotonin, 0.75–0.81	Bufotenine, 0.85–0.90
	R_F	R_F	R_F	R_F	R_F	R_F
1, A	0 –0.17					
1, B	0.17–0.32		0.49		0.78	
1, C	0.32–0.46		0.55	0.65	0.75	
1, D	0.46–0.58			0.61	0.74	0.85
1, E	0.58–0.67	0.16		0.69	0.78	0.85
1, F	0.67–0.87	0.15		0.62	0.81	0.91
1, G	0.87–1.0					
2, A	0 –0.16					
2, B	0.16–0.31		0.41			
2, C	0.31–0.41	0.13	0.44			
2, D	0.41–0.60	0.14	0.46			
2, E	0.60–0.85				0.74	0.83
2, F	0.85–1.0					0.80
3, A						
3, B						
3, C						
3, D						
3, E					0.77	
3, F			0.31			
			0.42	0.55	0.76	
3, G				0.55	0.76	

To obtain enough material for paper chromatography, four 24 hour samples of urine were pooled from four subjects and extracted as described. After chromatography on an aluminum oxide column, three definite peaks of activity were found. The third, however, reached a maximum in the tenth tube preceding that which, from the data in Fig. 1, would have contained the peak of serotonin activity.

The active fractions in each peak were combined and treated as follows: Peak 1, Tubes 11 to 32 were combined and concentrated to 6.5 ml. 5 ml. of the supernatant fluid were stirred in a mortar with 350 ml. of acetone,

small portions at a time. This method of extraction was necessary because the addition of acetone produced a brown, tarry precipitate which was difficult to extract. After 48 hours at −15°, the supernatant fluid was decanted and evaporated to 2 ml. The solution was streaked on Whatman No. 3 filter paper and chromatographed in the butanol-ammonia solvent system. After drying, the chromatogram was cut into seven strips and eluted with water. Each of the eluates was saturated with sodium chloride, adjusted to pH 2.0 to 2.5, and extracted three times with equal volumes of butanol. Petroleum ether (b.p. 38–42°), equal in volume to the butanol, and 0.1 ml. of 0.1 N HCl were added to each butanol extract. The aqueous layer was again chromatographed in the butanol-ammonia system; the indole derivatives were identified by treatment with *p*-dimethylaminobenzaldehyde in 1.2 N HCl. Peak 2, Tubes 33 to 47 were combined and concentrated to 1.8 ml. A butanol extract of this solution, saturated with sodium chloride, pH 2.0 to 2.5, was extracted into HCl as were the eluates from Peak 1. This aqueous phase was chromatographed and eluted in six fractions. These eluates were then rechromatographed and the color was developed as before. Peak 3, Tubes 48 to 80 were combined and concentrated to 1 ml. The solution was chromatographed, eluted in seven fractions, and rechromatographed for indole derivatives.

The R_F values of all the areas which gave a blue color with *p*-dimethylaminobenzaldehyde reagent are given in Table I.

DISCUSSION

The serotonin-like activity of normal human urine depends upon more than one material. Paper chromatograms of the active peak from the aluminum oxide column corresponding to bufotenine show positive indole spots with R_F values similar to those of serotonin, *N*-methylserotonin, and bufotenine. The appearance of all three compounds in one peak may be due to the fact that the extracts were added in aqueous methanol rather than absolute methanol solution. The second active peak, corresponding to the *N*-methylserotonin, also gave positive indole spots with R_F values similar to those of bufotenine and *N*-methylserotonin. The third peak of activity gave positive indole reactions which corresponded to serotonin and *N*-methylserotonin.

It should be pointed out that tryptamine could account for some of the activity. It gives a positive indole spot and has on paper an R_F value nearly the same as that of bufotenine in this solvent system. However, it was not studied on the aluminum oxide column.

In addition to those identified above, positive indole spots with the R_F of bufotenidine were found in urine eluates from Peaks 1 and 2 from the aluminum oxide column. These spots could be hydroxyindoleacetic acid (R_F 0.08 to 0.10). It has been demonstrated previously (3, 4) that sero-

tonin (enteramine) is metabolized to the latter compound. Neither bufotenidine nor hydroxyindoleacetic acid was run as a standard on the alumimum oxide column.

It has been shown previously that the heart of *V. mercenaria* is at least 1000 times more sensitive to serotonin than to other known pressor substances, such as adrenaline and tyramine (1). Furthermore, we have shown in this work that the mono and dimethyl derivatives of serotonin are more active by this means of testing than is serotonin itself. The fact that the quantities of the active substance in the eluates from the paper chromatograms were in the order of micrograms rather than mg. is further evidence that the substances are indoles. However, since the quantities of the active substances that were isolated were too small for determinations of chemical or physical properties other than R_F values, the evidence of structure is indicative rather than conclusive.

The fact that bufotenine, a normal constituent of toad skin, appears in the urine of healthy humans emphasizes the wide distribution of indole derivatives. Among these, serotonin and bufotenine may bear on central nervous function (1, 5, 6) and disease. Further support for this hypothesis comes from the isolation (7) of bufotenine from a narcotic snuff (cahoba, *Piptadenis peregrina*) used to facilitate communications with "unseen powers" by participants in Haitian voodoo. This is a far cry from the vasoactive properties which originally led to the isolation of serum vasoconstrictor (8).

SUMMARY

Three peaks of activity as assayed on the heart of *Venus mercenaria* were eluted from aluminum oxide columns when extracts of normal human urine were passed through them. The biological activities and relative positions of these peaks correspond to those of bufotenine, *N*-methylserotonin, and serotonin (5-hydroxytryptamine). Eluates from paper chromatograms of the peaks from the aluminum oxide column when rechromatographed revealed spots with R_F values corresponding to those of bufotenine, *N*-methylserotonin, serotonin, and unidentified indoles. No further identification was practicable.

BIBLIOGRAPHY

1. Twarog, B. M., and Page, I. H., *Am. J. Physiol.*, **175**, 157 (1953).
2. Welsh, J. H., and Taub, R., *Biol. Bull.*, **95**, 346 (1948).
3. Titus, E., and Udenfriend, S., *Federation Proc.*, **13**, 411 (1954).
4. Erspamer, V., *Rend. sc. farmital*, **1**, 1 (1954).
5. Gaddum, J. H., in Wolstenholme, G. E. W., and Cameron, M. P., Ciba conference symposium on hypertension, Boston (1954).
6. Woolley, D. W., and Shaw, E., *Federation Proc.*, **13**, 325 (1954).
7. Stromberg, V. L., *J. Am. Chem. Soc.*, **76**, 1707 (1954).
8. Rapport, M. M., Green, A. A., and Page, I. H., *Science*, **108**, 329 (1948).