

## OBSERVATIONS ON THE FATE OF INDOLALKYLAMINES IN THE ORGANISM

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In our earlier researches on crude enteramine extracts of mammalian gastrointestinal mucosa and octopod posterior salivary glands (Erspamer, 1942, 1943, 1948) we showed that the specific enteramine activity of these materials could be destroyed by an enzyme, enteraminase, apparently indistinguishable from amine oxidase. Later on, Rapport, Green & Page (1948) and Bradley, Buttenworth, Reid & Trautner (1950) found that the lung enzyme, too, which inactivates serum vasoconstrictor (serotonin) had the characteristics of amine oxidase.

The recent availability of pure 5-hydroxytryptamine (enteramine, serotonin) has permitted a closer approach to the problem of the enzymic inactivation of this substance and of related compounds. It was shown that not only tryptamine, as has been known for years, is a substrate of amine oxidase, but also *N*-methyltryptamine, 5-hydroxytryptamine (5-HT), *N*-methyl-5-hydroxytryptamine and, to a much lesser degree, *N,N*-dimethyl-5-hydroxytryptamine or bufotenine (Blaschko, 1952; Blaschko & Philpot, 1953; Govier, Howes & Gibbons, 1953). Enzyme preparations of different origins were tried, and all were active.

As early as 1913 and 1916 Ewins & Laidlaw (1913) and Guggenheim & Löffler (1916) were able to identify indoleacetic acid, both free and conjugated with glycine to form indoleaceturic acid, and small quantities of indolethylalcohol amongst the products originating *in vivo* from the oxidative deamination of tryptamine.

It thus seemed the obvious thing to consider 5-hydroxyindoleacetic acid as a possible important breakdown product of 5-HT and closely related substances, but no experimental evidence on this point has been published.

The present study is designed to investigate, by paper chromatographic methods, the occurrence in urine and blood of 5-hydroxyindoleacetic acid and other possible metabolites of 5-HT and similar indolalkylamines.

## METHODS

*Urine purification.* Immediately after their collection, if necessary under toluene, the urine samples were concentrated under reduced pressure, at 40–50° C, to one-twentieth of the original volume. One volume of 95% ethanol and 4 vol. of acetone were now added, with stirring. After standing in a refrigerator for 8–24 hr the precipitate was filtered off and the filtrate evaporated, under reduced pressure. The residue was taken up, in a warm water-bath, with stirring, in 1 ml. water + 3 ml. ethanol + 25 ml. acetone for every 100 ml. urine. The oily or oily-crystalline precipitate was discarded, after having been washed with a little acetone.

The material was now subjected to three further precipitations. Each time the precipitate was filtered off, the filtrate evaporated to dryness and the residue taken up with the following successive mixtures: (a) 0.5 ml. ethanol + 10 ml. acetone; (b) 0.25 ml. ethanol + 20 ml. acetone; (c) 0.25 ml. ethanol + 2–3 ml. acetone + 5–10 ml. ethyl ether. The quantities of solvents refer to 100 ml. urine.

The final filtrate was evaporated and the residue taken up with enough ethanol + acetone to give a liquid corresponding, per ml., to 10–200 ml. urine. This material, ready for chromatographic analysis, may be stored in a refrigerator for weeks or even months.

The single steps in the purification process were accurately followed by chromatography. It was shown that while indoleacetic, 5-methoxyindoleacetic and 5-hydroxyindoleacetic acids were recovered to about 95% in the final filtrate, other indole compounds, such as indoleacetic acid, appeared in the precipitate, being scarcely soluble in acetone and ether. To enable these compounds to be studied an aliquot of urine samples was taken after the first or the second precipitation.

*Preparation of the blood extracts.* Our observations were made only on rat's blood, obtained by decapitation and directly collected into acetone (4 parts by weight). After 24 hr the extract was filtered, and evaporated to dryness *in vacuo*, and the residue was redissolved in a little water + ethanol (1 ml. = 10–20 ml. of blood). The material was stored in a refrigerator until chromatographic analysis.

*Paper chromatography.* The ascending unidimensional technique on Whatman no. 1 paper was generally employed, and sometimes the bidimensional one.

Solvents: *n*-butanol saturated with *n*-HCl; *n*-butanol + acetic acid + water (4:1:5); *n*-butanol + methylamine 25–30% (8:3); amyl alcohol + pyridine + water (2:2:1).

Developing reagents: (a) 2% alcoholic solution of *p*-dimethylaminobenzaldehyde. After being sprayed with the reagent, the chromatograms were exposed, in large glass chambers, to HCl vapours, until the spots were clearly developed (5–20 min) and then, for some days, to the air.

(b) Heinrich and Schuler's NNCD reagent (4-nitro-2-chloro-1-diazobenzene- $\alpha$ -naphthalene sulphuric acid) freshly dissolved, 1–3 per thousand, in 0.1 *n*-HCl. After spraying the chromatograms were allowed to dry in the air.

(c) Solution of diazotized *p*-nitroaniline, freshly prepared by adding to a cooled acid solution of *p*-nitroaniline the equivalent of sodium nitrite. After spraying, the chromatograms were exposed to ammonia vapours and then left to dry in the air. The disturbing reddish background colour pales or disappears after some hours.

*Quantitative estimation of the indoleacetic acids in extracts of urine and blood.* After the indoleacetic acids under investigation were localized separately on the chromatograms, the problem of their quantitative evaluation arose. Two procedures were tried.

The first one is based on the simple visual comparison, with respect to area and density of colour, of the spots given by a series of dilutions of the 'unknown' and the spots obtained with a series of drops containing known concentrations of the corresponding pure substance.

In our case, comparison gave particularly reliable results when 3–10  $\mu$ g of pure indolic compounds were put on the paper. After repeated analyses the error does not exceed 10–15%.

The second method, which is now being worked out in detail, is based on the elution of one or more paper strips containing the spot under investigation and on the successive reading in a colorimeter of the intensity of the colour reaction produced by *p*-dimethylaminobenzaldehyde in strongly acid medium. This more precise method has allowed us to control some of the most

important data obtained by the visual comparison and to demonstrate their reasonable accuracy. It is, however, obvious that elution will be practicable only when spots are sufficiently intense and absolutely pure.

## RESULTS

*Chromatographic characteristics of some indolalkylamines and of their corresponding indoleacetic acids*

Table 1 shows the  $R_F$  values obtained with our four different solvent systems. Pure indoleacetic acid was not available: its characteristics, as shown in Tables 1 and 2, are inferred from the study of chromatograms obtained with urine extracts of rats given tryptamine.

TABLE 1.  $R_F$  values of several indolalkylamines and indoleacetic acids in four solvent systems

Compound (10 $\mu$ g)	<i>n</i> -Butanol saturated with <i>n</i> -HCl	<i>n</i> -Butanol + acetic acid + water	<i>n</i> -Butanol + methylamine	Amyl alcohol + pyridine + water
Tryptamine	0.42-0.46	.	0.87-0.92	.
*Indoleacetic acid	0.84	0.90	0.52	0.50
Indoleacetic acid	0.58	0.83	0.46	0.45
<i>N</i> -Methyltryptamine	0.46-0.50	.	.	.
<i>N, N</i> -Dimethyltryptamine	0.46-0.50	.	.	.
*5-Methoxytryptamine	0.32-0.36	.	0.78-0.82	.
*5-Methoxyindoleacetic acid	0.76-0.80	0.87-0.91	0.41-0.46	0.42-0.46
*5-Hydroxytryptamine	0.18-0.22	0.43-0.45	0.58-0.66	0.59-0.63
*5-Hydroxyindoleacetic acid	0.58-0.63	0.78-0.84	0.14-0.18	0.34-0.38

\* Synthesized by B. Asero and V. Colò, Farmitalia S.p.A. Research Laboratories, Milan.

TABLE 2. Colour reactions produced by the three reagents used for developing the chromatograms

Compound (10 $\mu$ g)	<i>p</i> -Dimethylamino-benzaldehyde + HCl vapours	NNCD reagent in 0.1 <i>N</i> -HCl	Diazotized <i>p</i> -nitroaniline + NH <sub>3</sub> vapour
Tryptamine	+ + violet	+ orange	(+) yellow
Indoleacetic acid	+ + blue-violet	+ orange	+ lemon-yellow
Indoleacetic acid	+ + + blue	+ orange	+ orange-yellow
<i>N</i> -Methyltryptamine	+ + violet	+ orange	(+) yellow
<i>N, N</i> -Dimethyltryptamine	+ + violet	+ orange	(+) yellow
5-Methoxytryptamine	+ + blue-violet	+ orange-red	+ orange-yellow
5-Methoxyindoleacetic acid	+ + + blue	+ + peach-red	+ orange-yellow
5-Hydroxytryptamine	+ + blue-violet	+ + peach-red	+ + cherry-red
5-Hydroxyindoleacetic acid	+ + + blue	+ + violet-red	+ + cherry-red

(+) indicates a very weak reaction.

Table 2 illustrates the intensity and shade of colour reactions produced by the three reagents we have employed.

It should be noted that the development of colour reactions is generally rapid for the indolalkylamines, whereas it is rather slow and gradual for the indoleacetic acids, for which it requires 12-24 hr to complete. An exception is the coupling reaction with diazotized *p*-nitroaniline, whose development is always immediate.

Table 2 shows that the *p*-dimethylaminobenzaldehyde reaction is the most sensitive, and the coupling reaction with *p*-nitroaniline the least sensitive colour reaction for the majority of the substances examined. The diazonium

salt of *p*-nitroaniline can only be used with advantage for hydroxyindole derivatives. The NNCD reagent is very sensitive for 5-hydroxyindole derivatives, and less for compounds lacking the phenolic hydroxy group, owing to the lighter colour tones.

For the chromatographic identification of a given urinary or haematic spot the fulfilment of the following conditions was required: (a) perfect superimposition, on mixed chromatograms, of the spot under investigation and of the spot of the corresponding pure substance, whatever the solvent system employed; (b) identity of the three colour reactions, in respect both of their rapidity of development and of their tone, for the unknown spot and for that given by the pure compound. The use of one single solvent, especially if this is an acid one, may cause big errors.

In the quantitative estimation of the indoleacetic acids the *p*-dimethylaminobenzaldehyde reaction was generally used. Heinrich and Schuler's reagent was very useful for 5-hydroxyindoleacetic acid.

TABLE 3. Concentration of 5-hydroxyindoleacetic acid (5-HIAA) in the urine of various animals

Animal species	5-HIAA ( $\mu\text{g/ml. urine}$ )	Animal species	5-HIAA ( $\mu\text{g/ml. urine}$ )
Man I (1)	2	Ox (2)	? (<0.3)
Man II (1)	2	Calf (6)	5
Man III (1)	2.5	Horse (2)	? (<0.3)
Man IV (1)	3.5	Kid (6)	1.25
Man V (8)	3.8	Lamb (6)	2.25
Man suffering from diabetes insipidus	0.2	Hog (5)	1.5
Dog I (1)	4	Rabbit (2)	? (<0.3)
Dog II (3)	2.5	Guinea-pig (4)	? (<0.3)
Rat I (100)	1.5	Toad (30)*	0.27
Rat II (55)	1.25		

In parentheses is the number of specimens from which the examined urine or mixture of urines was obtained.

\* Toad urine was collected after the animals had been kept for 12–16 hr in water.

#### *Breakdown products of the indolalkylamines in urine*

##### (1) *Metabolites of endogenous and exogenous 5-HT.*

The most important and hitherto the only identified metabolic derivative of 5-HT is 5-hydroxyindoleacetic acid (5-HIAA). Table 3 shows the average content of this acid in normal urine of some animal species.

On the chromatograms of normal urine several indolic spots of unknown constitution are always present, with or even without the 5-HIAA spot. The number of these spots varies according to the animal species and the solvent used; their colour, after spraying with developing reagents, may be very similar to that given by the 5-HIAA spot. It should be added that the  $R_F$  value of 5-HIAA may coincide with that of other unknown indole compounds, causing the formation of large composite spots. This has been observed on chromato-

grams of hog's urine following acid solvents, and on chromatograms of rat's urine after *n*-butanol saturated with *N*-HCl.

It is therefore necessary to use several solvent systems even in the qualitative identification of 5-HIAA. Only experiment will show, in each case, the most suitable solvent for the quantitative estimation of urinary 5-HIAA. For human urine, for example, good results were obtained with the butanol-acetic-acid-water mixture; for dog's, rat's and hog's urine with alkaline solvents.

Table 4 shows the 5-HIAA content of human, dog, rat, rabbit and guinea-pig urine after the administration of 5-HT by various routes and in different doses. In the last column the percentage recovery of 5-HT, as 5-HIAA, is given (1 mg 5-HIAA is equivalent to 0.92 mg 5-HT).

TABLE 4. Recovery of 5-hydroxytryptamine as urinary 5-HIAA after administration by various routes

	Amount of administered 5-HT (mg)		Administra- tion route	Urine volume (ml.)	Excess urinary output of 5-HIAA ( $\mu$ g)		Percentage recovery of 5-HT as 5-HIAA
	Per kg	Total			Per ml.	Total	
Man V (8)*	0.13	65.6	i.m.	6359 (4)**	2.2	14290	20
Insipidus diabetic (1)	0.2	16.4	i.m.	3050 (7)	3.0	9150	52
Dog (1)	1.2	9.3	s.c.	325 (18)	8.0	2600	25
Rabbit (2)	2.5	9.8	s.c.	650 (17)	0.3 (?)	130 (?)	1-1.5 (?)
Guinea-pig (4)	8.0	19.6	s.c.	506 (18)	0.3 (?)	150 (?)	0.5-1 (?)
Rat (12)	6.0	13.3	os	158 (11)	5.1	804	5.5
Rat (12)	6.0	12.0	s.c.	116 (11)	31.6	3660	28
Rat (12)	6.0	12.8	i.p.	144 (11)	28.9	4160	33

In parentheses is the number of specimens from which the examined urine or mixture of urines was obtained (\*), and the urine collection period, in hours (\*\*). i.m. = intramuscularly; s.c. = subcutaneously; os = *per os*; i.p. = intraperitoneally.

In order to obtain a more abundant and regular flow of urine, a moderate amount of water was administered to some of the experimental animals by mouth immediately before the introduction of 5-HT: 1 l. in humans (of course, with the exception of the diabetic patient), 30 ml./kg in the dog, and 5 ml./100 g in rats.

The tabulated values for 5-HIAA were obtained by subtracting the amount of this acid normally present from the total. It is clear that this calculation is indispensable only when relatively small doses of 5-HT are injected and the normal urine contains large quantities of 5-HIAA (e.g. humans, dogs).

The following conclusions may be drawn from Tables 3 and 4:

(a) 5-HIAA must be considered a normal constituent of mammalian urine and probably also of the urine of other vertebrate groups. This does not imply, however, that it is present in detectable amounts in all animal species. In this connexion it is noteworthy that whereas the urine of lambs, kids and calves contains large amounts of 5-HIAA, this substance does not occur in detectable quantities in the urine of adult herbivores. It may be inferred that

with the transition from milky to vegetarian diet the fate of indole compounds in the organism may undergo profound changes, possibly involving a more radical breakdown of the indole ring. This point deserves further investigation.

(b) After administration of 5-HT there is a conspicuous increase in the urinary output of 5-HIAA only in those animal species, whose urine contains this acid as a normal constituent. Otherwise, as in the case of the rabbit and the guinea-pig, the parenteral introduction even of high doses of 5-HT provokes the appearance of only insignificant amounts of 5-HIAA.

(c) After the administration of 5-HT by the mouth, the urinary recovery of the substance as 5-HIAA is much less than after parenteral administration.

The subcutaneous or intramuscular injection of 5-HT caused in all the animals tested, including man, an evident reduction in the urine flow. In agreement with previous observations (Erspamer & Ottolenghi, 1953), the antidiuretic action was slight after intraperitoneal administration, and lacking after oral administration.

### (2) *Metabolites of 5-methoxytryptamine (5-MT)*

Fifty-eight rats weighing 9.8 kg were given by mouth 1 g of 5-MT hydrochloride (= 100 mg/kg); to a further twelve rats (2.1 kg) 10 mg/kg of the same product was injected subcutaneously, immediately after a water load. In both cases a complete blockade of diuresis, lasting 4-5 hr, was observed, as well as the appearance of an intense cutaneous vasodilatation, accompanied by itching and the evacuation of abundant formed faeces.

The urine chromatograms showed three unusual indolic spots, of which one was very intense and the other two rather faint. The first spot appeared to be due to 5-methoxyindoleacetic acid (5-MIAA).

A total of 377 mg of 5-MIAA was found in the 410 ml. of urine excreted, over an 11 hr period, by the rats given orally 1000 mg of 5-MT hydrochloride (= 840 mg 5-MT base); 12.6 mg of 5-MIAA was found in the 113 ml. of urine excreted, over the same period, by the rats injected subcutaneously with 21 mg of 5-MT hydrochloride (= 17.6 mg 5-MT base). The percentages recovered were thus 40-42 and 71, respectively (1 mg of 5-MIAA is equivalent to 0.93 mg of 5-MT base).

The two minor indolic spots ( $R_F$  values after *n*-butanol saturated with *N*-HCl: 0.49 and 0.55) have not been further investigated. We know only that the corresponding substances are largely precipitated in the last stages of purification of the urine extracts.

### (3) *Metabolites of 5-hydroxyindolalkylamines other than 5-HT*

*Bufotenine.* Since pure bufotenine was not available, numerous unidimensional chromatograms of concentrated extracts from the parotid glands of *Bufo vulgaris* were run, and the bufotenine spots eluted. It was thus possible

to inject subcutaneously into ten rats (2050 g) not less than 1.2–1.5 mg/kg of practically pure bufotenine.

On the urine chromatograms four unusual indolic spots made their appearance. One could be identified as 5-HIAA, another as unchanged bufotenine. Of the two remaining unknown spots, the more important showed, after *n*-butanol saturated with *N*-HCl, an  $R_F$  value of 0.04–0.05 and developed an orange colour when sprayed with the NNCD reagent.

*Bufotenidine*. The product available appeared on chromatographic analysis as a mixture consisting of 90% bufotenidine and 10% 5-HT + *N*-methyl-5-HT + bufotenine. A quantity of this product corresponding to about 4 mg/kg of indolalkylamine bases was injected subcutaneously into twelve rats weighing 2305 g.

Of the three unusual indolic spots appearing on urine chromatograms one, rather faint, could be identified as 5-HIAA. The complexity of the administered product does not allow us to identify the parent substance of the small amount of 5-HIAA present in urine. Its derivation from bufotenidine seems, however, highly improbable. Of the two other unknown spots, the more intense one shows, like the analogous spot observed after administration of bufotenine, an  $R_F$  value of 0.04–0.05 following *n*-butanol saturated with *N*-HCl, and is orange in colour when developed with the NNCD reagent. Owing to its intensity it may be considered as being composed of some metabolic derivative of bufotenidine.

#### (4) *Metabolites of tryptamine and N-methyltryptamines*

Three groups of twelve rats each were injected subcutaneously, immediately after an oral water load, with 20 mg/kg tryptamine hydrochloride (= 16.2 mg/kg tryptamine base), 40 mg/kg *N*-methyltryptamine picrate (= 17.4 mg/kg free base) and 40 mg/kg *N,N*-dimethyltryptamine picrate (= 18.2 mg/kg free base) respectively. In all instances a moderate reduction of urine flow was observed.

The chromatographic analysis of the urines collected over an 11 hr period showed the presence of three to five new indolic compounds, two of which could be identified as *indoleacetic acid* (IAA) and *indoleaceturic acid* (IAUA).

The main results are illustrated in Table 5.

Indoleaceturic acid was identified in the urine of rats given tryptamine. The concentrated extract corresponding to 30 ml. of this urine (= 7.4 mg of tryptamine hydrochloride, subcutaneously) was chromatographed using as a solvent the butanol–acetic acid–water mixture. Eighty chromatograms were thus obtained. The transverse paper strips containing the spots which were thought to be due to indoleaceturic acid were cut out and eluted, at 60° C, with a mixture of 50% ethanol–water, the eluate taken to dryness and the residue redissolved in 10 ml. of water. Five ml. of conc. HCl was added and the material

was then gently boiled for 4 hr under a reflux condenser. The liquid, which had become at first pink and then brownish, was now evaporated to dryness and the residue redissolved in 1 ml. water + 1 ml. ethanol.

The material was chromatographed using as solvents phenol saturated with water and the butanol-acetic acid-water mixture. On the paper strips sprayed with ninhydrin a large, intense purple spot, which was chromatographically indistinguishable from control spots given by pure glycine, made its appearance. Glycine was absent on chromatograms run before acid hydrolysis, as well as on chromatograms of the acid hydrolysate of the material obtained by extracting with water Whatman no. 1 paper. The search for indoleacetic acid, the other breakdown product of indoleacetic acid, was unsuccessful, as expected; the indole ring was ruptured following the acid treatment.

TABLE 5. Recovery of subcutaneous tryptamine, *N*-methyltryptamine and *N,N*-dimethyltryptamine as urinary indoleacetic (IAA) and indoleacetic (IAUA) acids. Each substance injected into twelve rats

	Total amount of administered substance (mg)	Urine volume (ml.)	Total content, in mg, of		Percentage recovery of the tryptamines as	
			IAA	IAUA	IAA	IAUA
Tryptamine	33.2	135	9.27	28.5	25.6	59.0
<i>N</i> -Methyltryptamine	36.5	162	4.1	12.4	12.2	23.5
<i>N,N</i> -Dimethyltryptamine	38.7	176	1.0	8.3	2.7	14.7

It has been found, after elution of the glycine spot and estimation after treatment with ninhydrin, that the total urine excreted by the rats given tryptamine would contain, if subjected to acid hydrolysis, approximately 9.3 mg of glycine. This corresponds to 28.5 mg of indoleacetic acid or to 21.4 mg of indoleacetic acid. It may thus be calculated that out of the 33.2 mg of tryptamine base administered to the rats, 28.1 mg (= 84.6%) was present in the urine as free or conjugated indoleacetic acid.

The urine chromatograms of rats given *N*-methyltryptamine do not differ from those of rats given tryptamine. The chromatograms of rats given *N,N*-dimethyltryptamine show, on the contrary, two other interesting indole spots. When *n*-butanol saturated with *N*-HCl is used as a solvent, their  $R_F$  values are respectively 0.7 and 0.05. The nature of these spots is unknown. It may, however, be observed that the spot with  $R_F$  0.05, or a similar one, can be also detected on urine chromatograms of rats given bufotenine or bufotenidine.

It is worth remembering that in the process of urine purification, indoleacetic acid always remains in the solvent, unlike indoleacetic acid which is largely precipitated by acetone and acetone-ether.

##### (5) Fate of indoleacetic acid (*heteroauxin*)

Forty rats weighing 7.95 kg were given by mouth 1.2 g of indoleacetic acid (150 mg/kg). The substance had no pharmacological effects and the volume of the urine was unchanged.



Urine collected over a 7 hr period contained 245 mg (=20.5%) of unaltered indoleacetic acid and an approximately equal amount of a substance chromatographically indistinguishable from indoleacetic acid, giving origin to glycine following acid hydrolysis.

On the whole, it may be calculated that about one-third of the indoleacetic acid given by mouth was recovered from the urine either free or conjugated; for the remaining two-thirds probably underwent rupture of the indole ring.

*Metabolic derivatives of indolalkylamines in blood*

5-HT (8 mg/kg, in terms of free base), 5-MT (16 mg/kg) and tryptamine (16 mg/kg) were injected subcutaneously, each into three groups of three rats each. The single groups of animals were killed by bleeding 30 min, 2 hr and 6 hr after the injection. Control blood was drawn from six rats.

The results are shown in Table 6.

TABLE 6. Appearance of derivatives of indolalkylamines in blood after subcutaneous injection into rats

	Quantity of blood obtained by decapitation (ml./kg)	IAA ( $\mu$ g/ml.)	IAUA	5-MIAA ( $\mu$ g/ml.)	5-HIAA ( $\mu$ g/ml.)
Control blood	33.8	N.D.	N.D.	-	N.D.
5-HT blood					
after 30 min	23.1	N.D.	N.D.	-	N.D.
after 2 hr	28.3	N.D.	N.D.	-	0.4-0.6
after 6 hr	38.3	N.D.	N.D.	-	N.D.
5-MT blood					
after 30 min	22.5	N.D.	N.D.	2-3	N.D.
after 2 hr	28.2	N.D.	N.D.	10	N.D.
after 6 hr	39.4	N.D.	N.D.	2	N.D.
Tryptamine blood					
after 30 min	33.3	5	++	-	N.D.
after 2 hr	34.4	2	+	-	N.D.
after 6 hr	36.6	0.3-0.5	?	-	N.D.

N.D. = not detectable.

It appears that normal blood does not contain detectable amounts of 5-hydroxyindoleacetic, indoleacetic or indoleacetic acid. These products, however, as well as 5-methoxyindoleacetic acid, may be found in blood after parental administration of high doses of their parent indolalkylamines. The maximum level is attained more rapidly for indoleacetic acid and indoleacetic acid, more slowly for 5-methoxyindoleacetic and 5-hydroxyindoleacetic acid.

An interesting fact is shown in the second column of Table 6: the quantity of blood obtained by decapitation of rats given 5-HT or 5-MT is, after 30 min, and, to a lesser degree, even after 2 hr, definitely smaller than that obtained in control rats or in rats given tryptamine. This phenomenon is probably due to the intense systemic vasodilatation produced by high doses of 5-HT and 5-MT and to the consequent stagnation of blood in the hypotonic vascular bed.

*Absence of 5-HIAA from amniotic liquid and faeces*

No detectable amounts of 5-HIAA could be demonstrated by chromatographing the concentrated extract corresponding to 10–20 ml. of human amniotic liquid.

In order to rule out the possibility of an intestinal origin of the 5-HIAA found in urine, 152 g of human faeces and the entire intestinal content (320 g) of a dog were separately extracted with 3 vol. of acetone and the filtrates treated as the urine samples. On the chromatograms of the purified extracts no trace of 5-HIAA could be detected, even when quantities corresponding to 3–5 g of fresh material were put on paper.

*Formation of 5-HIAA in vitro*

The following tissue homogenates were tried: guinea-pig's small intestine and liver, hepatopancreas of *Octopus vulgaris* (all three notoriously rich in amine oxidase), dog's duodenal mucosa and liver.

To 5 samples of 10 ml. of 0.07 M phosphate buffer at pH 7.4, each containing 10 mg of 5-HT creatinine sulphate 10 ml. of the homogenate, diluted to one-third with phosphate buffer, was added. The liquids were kept for 1 hr in a water-bath at 38° C and oxygen was bubbled through. 60 ml. acetone was then added to each vessel. After standing in a refrigerator overnight the liquids were filtered and concentrated to 2 ml. by distillation under reduced pressure.

On chromatographic analysis the 5-HT samples treated with the dog's homogenates were found to contain large amounts of unaltered 5-HT and only traces of 5-HIAA. This acid was, on the other hand, present in fairly large quantities on the chromatograms of the 5-HT samples incubated with guinea-pig's and octopod homogenates. The sample incubated with the homogenate of guinea-pig's liver showed a total content of at least 1 mg of 5-HIAA and only traces of unaltered 5-HT.

We had previously observed (Erspamer & Boretti, 1951), on bidimensional chromatograms of acetone extracts prepared from non-fresh posterior salivary glands of *Eledone*, the occurrence of an intense indolic spot (spot XIV), which was considered to be due to a substance chemically near to 5-HT. We have now shown that we had to deal with 5-HIAA, produced post-mortem by the action of amine oxidase on 5-HT, large amounts of both enzyme and substrate being present in the salivary tissue.

## DISCUSSION

(1) The first fact established in the present investigation is that 5-hydroxy-indoleacetic acid must be considered a normal constituent of urine, at least in several animal species. In this respect our observations agree perfectly with the simultaneous, independent observations of Titus & Udenfriend (1954).

It is highly probable that the 5-HIAA of normal urine originates from endogenous 5-HT. In fact: (a) no other substance is known, at least in mammals, to

be capable of producing 5-HIAA; (b) the quantity of 5-HT present in the organism is always more than sufficient to account for the amount of 5-HIAA detected in the urine; (c) the urinary output of 5-HIAA may be increased even up to 50 times and more, following the parenteral administration of 5-HT; (d) the administration of exogenous 5-HT to animal species, e.g. guinea-pigs or rabbits, whose normal urine is lacking or extremely poor in 5-HIAA does not appreciably increase the urinary output of this acid; (e) a production of 5-HIAA by the normal intestinal flora may be ruled out.

The occurrence of indoleacetic acid (never that of indoleaceturic acid) in normal human and mammalian urine has repeatedly been reported (Kögl, Haagen-Smit & Erxleben, 1933; Dietrich & Müller, 1951; Wieland, De Ropp & Avenier, 1954). The values obtained differ according to the research workers. Although we have not paid much attention to the problem, we can say that on our chromatograms of normal urine this substance was never present in detectable amounts.

It seems probable that the high values found by some older investigators should be revised, owing to the possible confusion of indoleacetic acid with 5-hydroxyindoleacetic acid, whose existence was then unknown. We should expect the error to be particularly serious when colorimetric methods have been used in the titration of heteroauxin, but it is certainly remarkable even in the case of bioassay, since 5-HIAA shows 6% of the activity of heteroauxin in the pea slit-internode test (Ek & Witkop, 1953).

(2) It is quite certain that the enzyme which catalyses the transformation of 5-HT into 5-HIAA, probably via 5-hydroxyindoleacetaldehyde, is amine oxidase, whose widespread distribution in vertebrate and invertebrate tissues has been well established for several years (cf. Blaschko, 1952). Both the writer and Titus & Udenfriend (1954) were able to demonstrate that 5-HIAA is formed *in vitro* following incubation of 5-HT with homogenates of mammalian intestine, liver and kidney and of octopod hepatopancreas.

In confirmation of previous observations *in vitro* (Blaschko & Philpot, 1953; Govier *et al.* 1953) it has been demonstrated that amine oxidase also attacks *in vivo* not only indolalkylamines with a primary or secondary amino group in the side chain, but also those with a tertiary amino group. In the last case, however, the oxidative deamination progresses more slowly and is only partial, as shown by the smaller quantity of free and conjugated indoleacetic acid detectable in urine after administration of *N, N*-dimethyltryptamine and by the regular occurrence in urine of unchanged bufotenine.

So far, 5-HIAA and 5-HMAA have been found in urine only in a free form, unlike indoleacetic acid which was preferably conjugated with glycine to form indoleaceturic acid. In the present investigation this acid, described by Ewins & Laidlaw in 1913, has been identified for the first time by paper chromatography.

In view of the high recovery of indoleacetic, 5-HIAA and 5-MIAA from urine following administration of the corresponding primary or secondary indolalkylamines, there is no doubt that amine oxidase is the most important enzyme involved in the inactivation of these compounds, as postulated by Blaschko and his collaborators (Blaschko, 1952; Blaschko & Philpot, 1953; Blaschko & Hellmann, 1953; Blaschko & Himms, 1954).

In the case of tertiary amines and, still more, of quaternary ammonium bases, other enzymes must come into play. This is shown by the presence, on urine chromatograms, of peculiar indolic spots which are lacking after administration of primary or secondary amines (e.g. the one with an  $R_F$  value of 0.04–0.05 after *n*-butanol saturated with *n*-HCl), and by the fact that bufotenidine is rapidly and completely destroyed, in spite of its resistance to amine oxidase, both *in vitro* and *in vivo*.

(3) All indoleacetic acids detected in urine are also found in blood, following parenteral administration of adequate amounts of the parent indolalkylamines. The maximum blood level is attained early for indoleacetic acid, and later for 5-MIAA and 5-HIAA. This may depend on the fact that afferent vasospasm and the ensuing reduction of renal excretion are much more intense and prolonged after 5-HT and 5-MT than after equal doses of tryptamine. The metabolites of the first two substances are consequently retained in the blood longer than indoleacetic acid.

(4) The presence of 5-HIAA in normal urine provides unambiguous evidence of the occurrence of free 5-HT in plasma. Plasma, indeed, is the necessary intermediate between the platelets, which carry 5-HT and have no inactivating enzymes, and the cells of parenchymatous organs (liver, kidney, intestine, etc.) which are rich in amine oxidase. The oxidative deamination of 5-HT to 5-HIAA can take place only in these cells.

Failure to demonstrate 5-HT in plasma is surely due to the inadequacy of our biological and chemical assay methods.

(5) The quantitative results obtained in the present investigation and the data on the 5-HT content in the organism of various animal species reported in previous papers (Erspamer, 1954*a-c*), give us for the first time reliable quantitative information on the metabolism of endogenous 5-HT and, in our opinion, strongly substantiate the 'physiological' nature of the action of 5-HT on renal haemodynamics and function.

The data concerning human beings, dogs and rats deserve particular attention.

The organism of a dog weighing 10 kg contains altogether 1.58 mg of 5-HT, of which as much as 1.42 is in the gastro-intestinal mucosa, 0.086 in the spleen and only 0.073 in the blood. In normal dog urine approximately 3  $\mu$ g of 5-HIAA per ml. are detectable, i.e. about 450  $\mu$ g in the 150 ml. of urine excreted in a 24 hr period by a dog weighing 10 kg. These 450  $\mu$ g must originate

from the oxidative deamination of  $410\mu\text{g}$  of 5-HT. From our experiments on the fate of exogenous 5-HT we have learned that in reality less than 50% of the metabolized 5-HT appears in the urine as 5-HIAA. Therefore, to get a more exact idea of the amount of endogenous 5-HT metabolized every day we must at least double the above values. This signifies that at least  $800\mu\text{g}$  of 5-HT are released into the plasma daily in a free, active form and then inactivated. A quantity of 5-HT corresponding to the 5-HT content of the entire organism is metabolized every 48 hr, and a quantity corresponding to the whole blood 5-HT every 2 hr.

It may be of interest to recall here that an abrupt liberation of platelet 5-HT, such as occurs for example in the defibrination of the blood, imparts to the dog's blood a powerful constrictor action on the renal vessels, which disappears only after the defibrinated blood has circulated for 10–20 min through the lung or the liver (Starling & Verney, 1925; Herrick & Markowitz, 1929). It should be stressed that the quantity of 5-HT which provokes this powerful vasoconstriction could be renewed every 2 hr, if the daily urinary output is  $450\mu\text{g}$  HIAA/dog, or at least every 20 min if the higher estimates ( $3000\mu\text{g}/\text{dog}/24\text{ hr}$ ) given by Titus & Udenfriend (1954) are correct.

The total blood of a human being contains about  $400\mu\text{g}$  of 5-HT ( $0.06\mu\text{g}/\text{ml}$ ). Human urine shows an average content of  $3\mu\text{g}/\text{ml}$  of 5-HIAA, viz.  $4500\mu\text{g}$  in the 1500 ml. of a 24 hr period. This corresponds to  $4100\mu\text{g}$  of 5-HT, a figure which, again, must be multiplied by three or at least by two to get the amount of 5-HT which is really released into the plasma and destroyed every 24 hr. Experiments with parenteral exogenous 5-HT have, indeed, shown that in human beings, too, 5-HT is only in part recoverable from urine as 5-HIAA. If therefore  $8000$ – $12,000\mu\text{g}$  of the 5-HT are metabolized every day, a quantity of the substance corresponding to the total blood 5-HT is renewed every hour.

The rat organism contains  $125\mu\text{g}$  of 5-HT per kg of body weight:  $80\mu\text{g}$  in the gastro-intestinal mucosa,  $11\mu\text{g}$  in the spleen and  $34\mu\text{g}$  in the blood.

In the 775 ml. urine excreted over a 12 hr period by 155 rats weighing  $26.5\text{ kg}$  ( $59\text{ ml. urine}/\text{kg}/24\text{ hr}$ ),  $1070\mu\text{g}$  of 5-HIAA were found, viz.  $81.4\mu\text{g}/\text{kg}/24\text{ hr}$ . This corresponds to  $75\mu\text{g}$  5-HT/kg/24 hr. This figure has to be multiplied by three, on the basis of our experiments on the fate of exogenous 5-HT in the rat, and this gives  $225\mu\text{g}$  of 5-HT/kg/24 hr, viz.  $9$ – $10\mu\text{g}/\text{kg}/\text{hr}$ . It follows that a quantity of 5-HT corresponding to the entire blood 5-HT is metabolized every 3–4 hr and a quantity corresponding to the 5-HT of the entire organism is metabolized every 12–14 hr.

Now, in hydrated rats a subcutaneous injection of  $4\mu\text{g}/\text{kg}$  of 5-HT is sufficient to produce a significant reduction of diuresis, over a 60–90 min period (Erspamer & Ottolenghi, 1953). This dose corresponds to the amount of the substance which is released into the plasma and metabolized by the rat organism every 25–30 min.

The above quantitative considerations concerning the rat and, with some reservations, the dog, seem to support our hypothesis that 5-HT is concerned in the regulation of the function of the kidney (Erspamer & Ottolenghi, 1953; Erspamer, 1954*a-c*). In this connexion we have also to keep in mind that no other biological action of the substance, besides the renal one, has so far been provoked by doses of 5-HT such as are present in and metabolized by the organism.

(6) In our opinion 5-HT originates in the enterochromaffin cells of the gastro-intestinal mucosa from which, when needed, it is released into the circulation. We consider platelet 5-HT as mere transport 5-HT (Erspamer, 1954*b, c*).

This view does not seem to be shared by Udenfriend & Weissbach (1954), who believe that 5-HT 'is synthesized and incorporated at the site of platelet formation', being metabolically inert until the platelet disintegrates, and that 'the high concentration (of 5-HT) in platelets is not due to a concentrating mechanism for 5-HT nor do the platelets form 5-HT from its precursor 5-OH-tryptophan'.

The quantitative data on 5-HT metabolism reported in the present paper do not support the hypothesis of Udenfriend & Weissbach. It is sufficient to keep in mind that the average life span of platelets is 4-6 days, whereas a quantity of 5-HT corresponding to the total blood 5-HT is metabolized every 1-3 hr. It should be added that Humphrey & Toh (1954) showed that platelets are capable of concentrating 5-HT from their suspending medium.

(7) The quantitative estimation of urinary 5-HIAA, in those animal species in which the acid is detectable, will probably represent the method of preference for a comprehensive evaluation of the rate of metabolism of endogenous 5-HT under normal and pathological conditions, as well as a valuable source of information for a better understanding of the biological significance of 5-HT. Indeed, it is obvious that changes in the urinary output of 5-HIAA must follow changes in the turnover of 5-HT, no matter whether these changes are due to some modification in the activity of amine oxidase, or much more probably, to some modification in the amount of 5-HT exposed to amine oxidase.

#### SUMMARY

1. 5-Hydroxyindoleacetic acid (5-HIAA) is a normal constituent of the urine of carnivorous and omnivorous mammals, and probably of other groups of vertebrates. This acid is present in large amounts also in the urine of herbivorous mammals, but only during suckling; it disappears with the transition to vegetable food.

2. 5-HIAA could not be detected in human amniotic liquid, nor in human or dog faeces. This last observation rules out the possibility that urinary 5-HIAA has a bacterial intestinal origin.

3. Administration of 5-HT provokes a remarkable increase in the urinary output of 5-HIAA, but only in those animal species whose urine contains the acid as a normal constituent. 5-60% of the 5-HT administered may be recovered from urine as 5-HIAA.

4. Administration of 5-methoxytryptamine is followed by the appearance in urine of great amounts of 5-methoxyindoleacetic acid, that of tryptamine and *N*-methyltryptamines by the appearance of varying amounts of indoleacetic acid, both free and conjugated with glycine (indoleacetic acid).

5. The indoleacetic acids found in urine could be detected also in rat's blood following parenteral administration of adequate doses of the corresponding indolalkylamines.

6. These results emphasize the importance of amine oxidase in the inactivation of 5-HT and related indolalkylamines in the living organism. Amine oxidase acts not only on indolalkylamines with a primary amino group in the side chain, but also on those with a secondary, or even a tertiary amino group. The quaternary ammonium bases, e.g. bufotenidine, seem on the contrary not to be attacked by the enzyme.

7. The quantitative data on the urinary output of 5-HIAA under normal conditions and after administration of exogenous 5-HT as illustrated in this paper, combined with the quantitative data on the occurrence and distribution of 5-HT in the organism of the common laboratory animals, as reported in previous papers, have allowed us to obtain, for the dog and the rat, reliable information not only on the absolute amount of 5-HT which is daily metabolized in the body, but also on the relation between the rate of metabolism of 5-HT and the content of this substance in the blood, the gastro-intestinal mucosa and the entire organism of the experimental animal. The importance of these data in regard to the problem of the biological significance of 5-HT is discussed.

8. The quantitative estimation of 5-HIAA in urine is suggested as the method of preference in the study of the metabolism of endogenous and exogenous 5-HT under normal, experimental and pathological conditions.

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