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Aliphatic Dehydroxylation of Octopamine in the Rat and Rabbit

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The metabolic fate of octopamine has been investigated in the rat and rabbit, using octopamine-2-^{3}H. Urinary metabolites after oral administration were separated and identified by direct comparison with the authentic samples. The principal metabolite of the drug in both animals was the aliphatic dehydroxylated compound, *p*-hydroxyphenylacetic acid. Pretreatment with antibiotics (streptomycin and penicillin G potassium) did not cause to change the metabolic pattern of octopamine, suggesting that gut flora did not participate in the aliphatic dehydroxylation of octopamine.

Octopamine was first identified in the posterior salivary glands of *Octopus vulgaris* by Erspamer, *et al.*[2] It has been detected in mammalian urine after inhibition of monoamine oxidase,[3] and Axelrod, *et al.* have succeeded in the measurement of this compound in the sympathetic and central nervous systems of several species of animals using a sensitive enzymic assay.[4]

Radioactive octopamine is formed *in vivo* by the β-hydroxylation of exogenously administered tyramine-^{3}H.[5] This monophenolamine is hydroxylated at meta-position to convert to noradrenaline *in vivo* and *in vitro*.[6] It is interesting that the sympathomimetic effect of D-octopamine is three times as active as L-isomer which is a naturally occurring substance.[7]

The metabolic fate of octopamine-^{3}H in rat brain has been studied by Breese, *et al.*[8] and more recently Hengstmann, *et al.*[9] have reported gastrointestinal absorption and metabolism of octopamine in man. We will describe in this paper distribution and metabolism of exogenously administered octopamine in the rat and rabbit, especially its new metabolic pathway.

The Distribution of Octopamine in Rat Body

DL-Octopamine-2-^{3}H was subcutaneously injected into rats. Changes with time of the radioactivity in various organs are exhibited in Fig. 1. The radioactivity reached maximum within 30 min after the injection in the serum and all the organs, except kidney in which the maximum was attained at 60 min after the injection. The distribution of radioactivity in rat tissues seems to have a bias toward some tissues involving liver, kidney and heart. In the adrenal, the radioactivity declined more slowly compared with other organs. The radioactivity in the brain was negligible, suggesting that this monophenolamine could not pass through the blood brain barrier.

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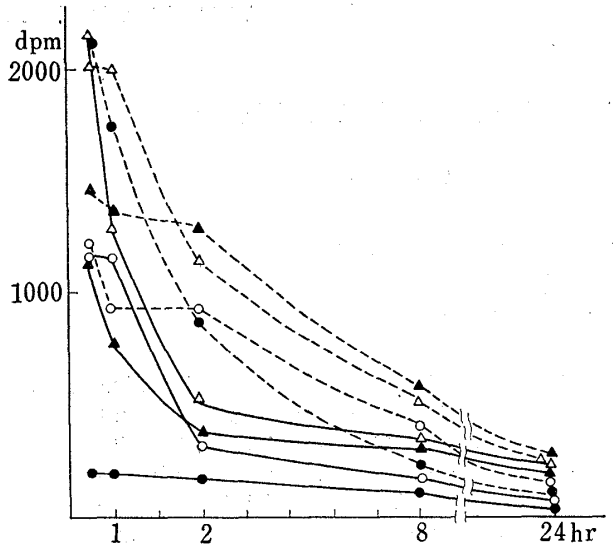

Fig. 1. The Distribution of Octopamine-^{3}H in Rats

DL-Octopamine-2-^{3}H 8μ Ci was injected subcutaneously to each rat. At various times they were killed and their organs were taken out. Each organ was solubilized by Soluene-100 and its radioactivity was determined. Each value represents the mean of three experiments. organ: dpm/30 mg, serum: dpm/0.1 ml

△···△···△ :liver △—△—△ :kidney
▲···▲···▲ :adrenal ▲—▲—▲ :spleen
○···○···○ :serum ○—○—○ :lung
●···●···● :heart ●—●—● :brain

Metabolism of Octopamine in the Rabbit

DL-Octopamine-2-^{3}H was orally given to rabbits. Approximately 25% of the administered radioactivity was excreted in urine and 4% in feces within 48 hr. The low recovery of radioactivity in urine and feces suggested that tritium on the side chain was removed by metabolic action or in part by exchange of hydrogen, and the tritium was circulated in the body fluid of animals in the form of tritiated water.

The pooled urine was fractionated by the method described in Chart 1. The unconjugated metabolites were separated into acidic and basic fractions by solvent extraction at appropriate pH. The aqueous layer was incubated with β-glucuronidase and the deconjugated metabolites were divided into the acidic and basic fractions. The aqueous residue was then submitted to acid hydrolysis. The per cent of radioactivity in each fraction was shown in Table I. Over 70% was present in the unconjugated acidic fraction. The percentage of the radioactivity in the glucuronide and other conjugate fractions were 15% and 4%, respectively.

The acidic fraction (EtOAc extract 1) was applied to paper chromatography. Two peaks of radioactivity appeared as shown in Fig. 2. The chromatographic behaviors of peak A with

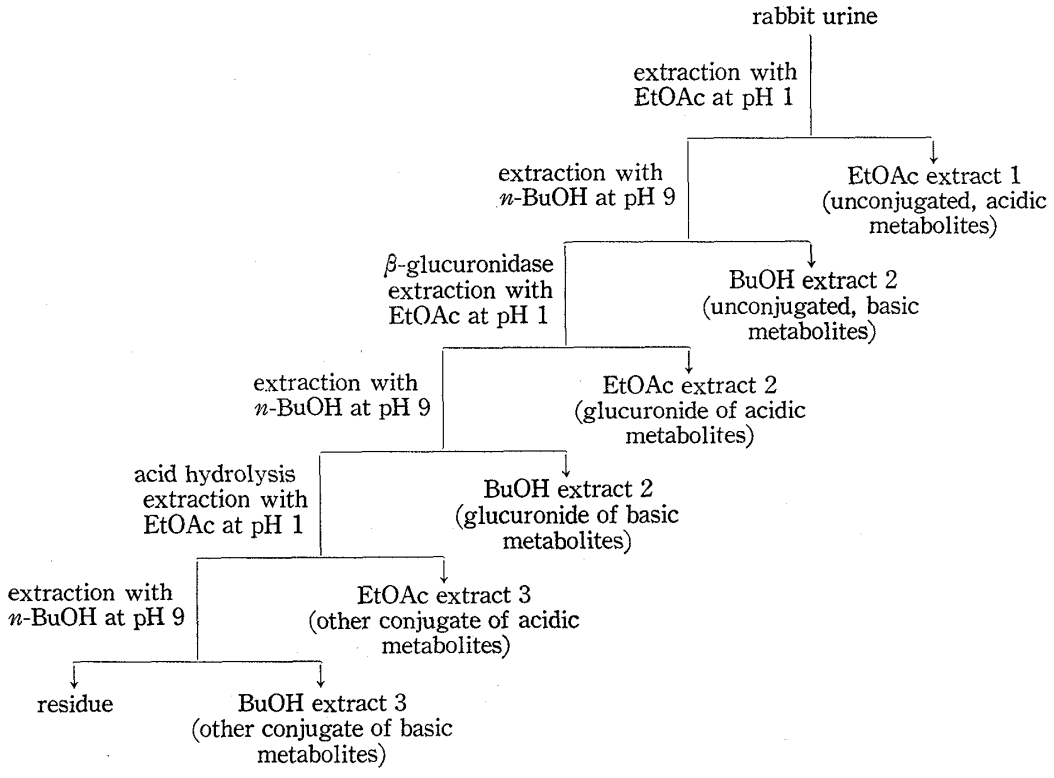

Chart 1

Table I. Urinary Metabolites in Rabbit[a]

Fraction	Per cent of the total urinary radioactivity	
	Acidic	Basic
Unconjugated	73.4	3.6
Glucunonide	13.0	2.0
Conjugates other than glucuronide	3.3	0.7
		unextracted 4.0

a) Octopamine-^{3}H was injected orally. The pooled urine (48 hr) was separated into six fractions by the method described in Experimental.

upper *Rf* values and peak B with lower *Rf* values were similar to those of *p*-hydroxyphenylacetic acid (*p*-HPAA) and *p*-hydroxymandelic acid (*p*-HMA), respectively. The gummy extract of the acidic fraction (EtOAc extract 1) was chromatographed on a column of silica gel and the effluent was separated into three fractions (fraction 1, 2 and 3). Each of them was further purified by preparative thin-layer chromatography (TLC), paper chromatography and recrystallization.

Radioactive metabolite A was obtained from fraction 2 as colorless needles, mp 147—149°, which showed positive reaction with Folin-Ciocalteu's reagent. On the nuclear magnetic resonance (NMR) spectrum signals were observed at 3.40 ppm as a singlet due to CH_2 proton and at 6.66 and 7.02 as a doublet (J=8 cps) due to aromatic proton. The mass spectrum of metabolite A exhibited the molecular ion peak at *m/e* 152. From these results it seemed very likely to be *p*-HPAA. Actually by direct comparison with the authentic sample the structure of metabolite A was definitely established as *p*-HPAA. Moreover the evidence of the structure of metabolite A was obtained by diverse isotope dilution method (Table II).

Metabolite B was obtained as a crystalline mass, mp 80—82°, by further purification of fraction 3 of the column chromatography. This radioactive metabolite showed positive results with Folin-Ciocalteu's and periodate-benzidine reaction. The direct comparison with authentic *p*-HMA by mixed melting point and infrared (IR) spectrum established the structure of metabolite B. $[\alpha]_D$ of metabolite B showed its negativity in the optical specificity.

The ratio of *p*-HPAA/*p*-HMA in the unconjugated fraction (EtOAc extract 1) was 9:5. The values in the glucuronide and other conjugate fractions (EtOAc extract 2 and 3) were almost the same as in the unconjugated fraction.

Further, purification of fraction 1 by preparative thin-layer chromatography (TLC) and recrystallization gave nonradioactive compound as colorless needles, mp 208°. This substance showed a positive result with Folin-Ciocalteu's reagent. On the NMR spectrum signals due to aromatic proton were observed at 6.72 and 7.78 ppm as a doublet (J=8 cps). The mass spectrum exhibited the moleculer ion at *m/e* 138. Finally it proved to be identi-

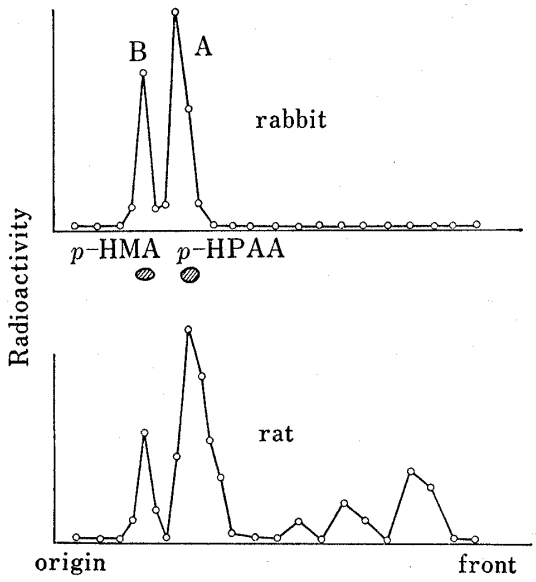

Fig. 2. A Representative PPC Patterns of Acidic Metabolites in Rabbit and Rat

A portion of acidic fractions from rabbit and rat urines were submitted to PPC (*n*-BuOH–iso-PrOH–ammonia–water, 3:1:1:1) and the radiochromatogram was determined.

Table II. Identification of Metabolite A with *p*-Hydroxyphenylacetic Acid by Diverse Isotope Dilution Method[a)]

No.	Crystallized from	Specific activity (dpm/mM)
1	MeOH–benzene	141000
2	MeOH–benzene	158000
3	MeOH–benzene	158000

a) Metabolite A (about 1 mg) and *p*-HPAA (34 mg) were mixed and recrystallized from MeOH–benzene three times.

cal with authentic *p*-hydroxybenzoic acid by means of mixed melting point and IR spectrum.

The radioactivity of the basic fraction (BuOH extract 1, 2 and 3) was about 6% in the urine. The paper chromatographic pattern of the fraction was represented in Fig. 3. The peak closest to the origin corresponded to octopamine and the middle peak corresponded to synephrine or tyramine. These peaks showed positive results with both Folin-Ciocalteu's and ninhydrin reagents. In order to characterize the second peak, the basic fraction was applied to periodate oxidation by the method of Kakimoto, *et al.*[3] The first and second peaks of radioactivity were oxidized and converted to the corresponding aldehyde, presumably *p*-hydroxybenzaldehyde, and only one peak nearest to the solvent front (Rf=0.91) remained. Authentic octopamine and synephrine were oxidized by periodate in the same manner, whereas tyramine was not. From these findings it was suggested that the first peak was octopamine and the second peak was synephrine. The third peak moved near to the solvent front has remained unknown.

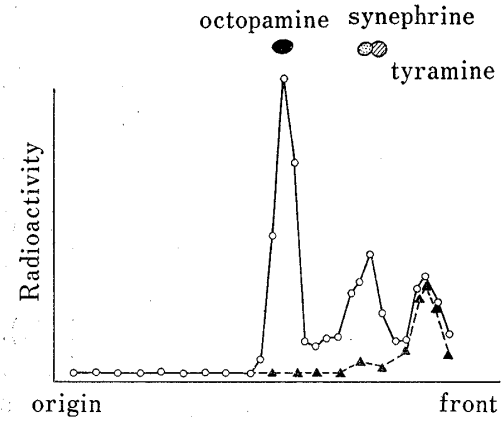

Fig. 3. A Representative PPC Pattern of Basic Metabolites in Rabbit

A portion of basic fraction and its periodate oxidized sample were submitted to PPC(*n*-BuOH–iso-PrOH–ammonia–water, 3:1:1:1) and the radiochromatogram was determined.
○—○—○ :basic fraction,
▲···▲···▲ :its periodate oxidized sample

Metabolism of Octopamine in the Rat

DL-Octopamine-2-^{3}H was administered orally to rats. The unconjugated, acidic fraction obtained from the urine was submitted to paper chromatography. The representative pattern was shown in Fig. 2. The peak closest to the origin corresponded to *p*-HMA and the second corresponded to *p*-HPAA. Three peaks nearer to the solvent front remained unknown. The ratio of *p*-HPAA/*p*-HMA was 3:1. It was proved that β-dehydroxylation on the side chain of octopamine occurred not only in the rabbit but also in the rat.

Effect of Feeding Antibiotics

Daily administration of antibiotics (streptomycin and penicillin) to the rabbit used in the previous experiment for four days had no effect on the metabolic pattern of octopamine-2-^{3}H. The ratio of *p*-HPAA/*p*-HMA in the urine of rabbit treated with the antibiotics was 7:5. This value did not greatly differ from the data obtained in the untreated experiment. This fact suggests that aliphatic β-dehydroxylation of octopamine may be independent of the participation of gut flora.

Discussion

From the data on the distribution of injected octopamine in the rat, it is evident that this monophenolamine can not pass through the blood brain barrier as well as other biogenic amines, such as noradrenaline and dopamine.

When octopamine-2-^{3}H was given orally to rabbits and the urine collected for 48 hr was fractionated into acidic and basic fractions, 89.7% and 6.3% of total radioactivity in the urine were present in acidic and basic fractions, respectively (Table I). Almost of the radioactive, acidic metabolites was excreted as unconjugated form and the remaining part as conjugated form such as glucuronide and others. Two main metabolites of the drug were isolated from the acidic fraction, purified and identified as *p*-HPAA (radioactive), *p*-HMA (radioactive).

p-Hydroxybenzoic acid is a natural substance excreted in animal urine, so there is no evidence to suggest that *p*-HBA isolated from the rabbit urine is derived from octopamine. However, several papers reported the same metabolic modification; catecholamine-^{14}C was metabolized to vanillic acid[10a] and 3,4-dihydroxymandelic acid-^{14}C was oxidized to vanillic acid and 3,4-dihydroxybenzoic acid in human.[11] Based on these results, we assumed that a portion of *p*-HBA isolated from the rabbit urine was derived from octopamine.

Octopamine-2-^{3}H was given orally to rats and the acidic metabolites in the urine were isotopically determined by paper chromatography. Main metabolites were *p*-HPAA and *p*-HMA as same as that in the rabbit. In both animals *p*-HPAA was excreted more than *p*-HMA and the ratio of *p*-HPAA/*p*-HMA in the urine of rabbit and rat were 9: 5 and 3: 1, respectively. Hengstmann *et al.* have recently found that the urinary metabolite excreted after administration of octopamine in man was only *p*-HMA and its conjugate.[9] Breese *et al.* reported that the major metabolite formed after intracisternal injection of octopamine-^{3}H is *p*-hydroxy phenylglycol and its conjugate.[8] The study of Breese indicates that an intermediate aldehyde produced from octopamine by deamination with monoamine oxidase is predominantly reduced to the glycol in brain, while it is oxidized to the carboxylic acid in liver.

In our study, the principal metabolite formed from octopamine in the rabbit and rat is particularly interesting, because it is the aliphatic dehydroxylated compound, *p*-HPAA. Several papers described that same aliphatic dehydroxylation was observed in less than a few percent on metabolism of catecholamine.[10] However, such intense aliphatic β-dehydroxylation in normal animal observed in our experiment has not heretofore been described in literature to the best of our knowledge. In guinea-pig pretreated with tolubutamide, noradrenaline-^{3}H was transformed mostly to *m*-hydroxyphenylacetic acid by both ring and aliphatic dehydroxylation,[12] and this observation differed from that in the animal without the pretreatment.

The question of what intermediates participate in the transformation of octopamine to *p*-HPAA has remained unresolved. We speculated that octopamine might be metabolized to *p*-HPAA *via* tyramine, but this speculation may be improbable, for tyramine has not been identified in our investigation. Smith and his co-worker also described that in guinea-pig pretreated with an inhibitor of monoamine oxidase in addition to tolubutamide, the majority of noradrenaline administered was converted into normetanephrine in contrast to *m*-hydroxyphenylacetic acid in the animal treated with tolubutamide alone, and assumed that deamination might be requisite to aliphatic and aromatic dehydroxylation.[12]

It is unlikely that gut flora participates in the aliphatic dehydroxylation of octopamine, while some evidences of the aromatic dehydroxylation by gut flora have been reported.[13]

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Experimental

Materials——DL-Octopamine HCl was presented by Morishita Pharmaceutical Co., Ltd. DL-Octopamine-2-^{3}H (2.89 Ci/mmole) was purchased from New England Nuclear Corp. and was checked for purity by paper chromatography (*n*-butanol–iso-propanol–ammonia–water, 3:1:1:1 by volume). The other samples were obtained commercially as followed: *p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid and tyramine (Tokyo Kasei Kogyo Co., Ltd); *p*-hydroxymandelic acid and synephrine (Sigma Chemical Co.), soluene-100 (Packard Instrument Co., Inc.) and beef liver β-glucuronidase (Tokyo Zoki Kagaku Co., Ltd.).

Instruments——Melting points were taken on a Yanagimoto melting point apparatus and were uncorrected. IR spectra were recorded on a Jasco IRA-1 spectrometer and NMR spectra on a Hitachi Model 20A apparatus. Mass spectral measurements were run by direct insertion technique on a Hitachi Model RMV-6E spectrometer. $[\alpha]_D$ value was determined on a Yanagimoto OR-50.

Radioactive Measurement——Two radioactive scintillators were used: (1) toluene scintillator contained 4 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis-2-(5-phenyloxazole)benzene (POPOP) per liter of toluene. (2) dioxane scintillator contained 7 g of PPO, 300 mg of POPOP and 100 g of naphthalene per liter of dioxane. Counting was carried out on a Aloka LSC 502 liquid scintillation spectrometer. The quenching was corrected by the channel ratio and external standard methods.

Thin-layer Chromatography (TLC)——TLC on Silica gel HF_{254} (E. Merck) was carried out by the following solvent system, benzene–ethyl acetate–acetic acid (70:30:1).

Paper Chromatography (PPC)——PPC on Whatman No. 3 was carried out with the solvent system, *n*-butanol–isopropanol–ammonia–water (3:1:1:1) for about 18 hr by descending method. The radiochromatogram was obtained by the method which the sheet was cutted into sections (about 1 cm) and each of them was individually placed into counting vials and the radioactivity was determined in dioxane scintillator (10 ml).

Gas-liquid Chromatograpay (GLC)——The apparatus used was a Hitachi Gas Chromatograph Model K-53 equipped with hydrogen flame ionization detector and stainless steel column (1.0 m × 3 mm i.d.) packed with 3% OV-17 on Gas Chrom Q (60—80 mesh). The temperatures of column and injection chamber were kept at 140° and 200° (condition 1) or 155° and 210° (condition 2), respectively. N_2 was used as carrier gas at flow rate 60 ml/min. All samples were trimethylsilylated with N,O-bis(trimethylsilyl)acetamide before injection.[14]

Distribution of Octopamine in Rats——Wistar male rats weighing 150—200 g and one group consisted of three rats were used. Octopamine-2-^{3}H 8 μCi (3.77 mCi/mmole) was subcutaneously injected into the back of rat. At various time after injection animals were killed by bleeding. Their organs, liver, heart, lung, adrenal, spleen, kidney, and brain were quickly taken out and washed with saline. A portion of each organ (30 mg) was weighed and placed into a counting vial containing 0.5 ml of Soluene 100. After they were allowed to stand at room temprature (25°) overnight, toluene scintillator (10 ml) was added to each vial and the radioactivity was counted. The radioactivity of serum (0.2 ml) was counted in dioxane scintillator.

Fractionation of Metabolites in Rabbit Urine——Two male albino rabbits weighing 2.5 kg were used. Octopamine-2-^{3}H (4.04 μCi/mmole) was administered orally in a dose of 160 mg/kg as the HCl salt. After 8 hr the same dose of octopamine was given again and the urine was collected for 48 hr after the last administration. The pooled urine was adjusted to pH 1 with diluted HCl and extracted with ethyl acetate (EtOAc), and the extract was washed with small amount of water, dried over Na_2SO_4 and evaporated under diminished pressure (EtOAc extract 1). The aqueous layer was adjusted to pH 9 with 2 N NaOH and extracted with *n*-BuOH (BuOH extract 1). Conjugated metabolites in the aqueous layer after the extraction with organic solvent were hydrolyzed with beef liver β-glucuronidase (300 units/ml) at pH 4.8 and 37° for 24 hr. Deconjugated acid metabolites were extracted with EtOAc at pH 1 (EtOAc extract 2) and basic metabolites were extracted with *n*-BuOH at pH 9 (BuOH extract 2). For acid hydrolysis of the remaining aqueous layer 1/20 volume of 2 N H_2SO_4 was added and the solution was allowed to stand at 37° for 48 hr. EtOAc extract 3 and BuOH extract 3 were obtained by the method described above. The tritium in each organic phase was counted in dioxane scintillator.

Separation of Acidic Metabolites——Gummy extract (EtOAc extract 1) was submitted to column chromatography of silica gel (100 mesh, Kanto Chemical Co., Inc.) (2.5 × 40 cm) using benzene and EtOAc. Each 20 ml of the effluent was collected into fractions, carried out TLC analysis with authentic *p*-HPAA, *p*-HMA and *p*-HBA and combined into 3 fractions which were further purified as described in the following sections.

Solvent system			Fraction
benzene		(300 ml)	
benzene – EtOAc	8 : 2	(160 ml)	1
benzene – EtOAc	8 : 3	(200 ml)	2
benzene – EtOAc	5 : 5	(300 ml)	3

14) M.G. Horing, E.A. Boucher, and A.M. Moss, *J. Gas. Chromatog.*, **5**, 297 (1967).

Identification of Acidic Metabolites

***p*-Hydroxyphenylacetic Acid (Metabolite A)**——Purification of fraction 2 by preparatic TLC and crystallization from benzene–MeOH gave colorless needles (40 mg), mp 147—149°. The metabolite showed positive reaction with Folin-Ciocalteu's reagent. IR ν_{max}^{KBr} cm^{-1}: 1710 (C=O). NMR (4% $(CD_3)_2SO$ solution) δ: 3.40 (2H, s, CH_2), 6.66 (2H, d, J=8 cps), 7.02 (2H, d, J=8 cps). Mass Spectrum *m/e*: 152 (M^+), 107 (M^+–COOH). GLC (TMS derivative) condition 1, t_R: 6.3 min. Mixed melting point on admixture with the specimen showed no depression and IR spectra comparison proved identity of two samples. The identification of radioactive metabolite 2 with authentic *p*-HPAA was carried out by diverse isotope dilution method. Metabolite 2 (about 1 mg) and *p*-HPAA (34 mg) were mixed and recrystallized from MeOH–benzene three times. The radioactivity of crystals was counted after every crystallization and the specific activity was determined.

***p*-Hydroxymandelic Acid (Metabolite B)**——Preparative PPC of fraction 3 gave metabolite B as a crystalline mass (20 mg), mp 80—82°. This compound showed positive reaction with Folin-Ciocalteu's reagent and periodate reagent. IR ν_{max}^{KBr} cm^{-1}: 1690 (C=O). GLC (TMS derivative) condition 1, t_R: 12.0 min. $[\alpha]_D^{20}$ =0 (c=0.5, EtOH). Mixed melting point and IR spectra in comparison with the authentic sample showed identity of two samples.

***p*-Hydroxybenzoic Acid**——Purification of fraction 1 by preparative TLC and crystallization from EtOAc–benzene gave metabolite 1 as colorless needles (20 mg), mp 208°. This compound showed positive reaction with Folin–Ciocalteu's reagent. UV $\lambda_{max}^{H_2O(pH\ 2,\ pH\ 7)}$ 256 nm, $\lambda_{max}^{H_2O(pH\ 10)}$ 279 nm. IR ν_{max}^{KBr} cm^{-1}: 1675 (C=O). NMR (4% CD_3OD solution) δ: 6.72 (2H, d, J=8 cps), 7.78 (2H, d, J=8 cps). Mass Spectrum *m/e*: 138 (M^+), 121 (M^+–OH), 93 (M^+–COOH). GLC (TMS derivative) condition 1, (t_R): 5.3 min. Mixed melting point on admixture with the authentic sample and IR spectra comparison showed identity of two samples.

Basic Metabolites——The mixture of BuOH extract 1, 2, and 3 was dissolved in water and the solution was adjusted to pH 10 with borate buffer and reextracted with toluene–isoamylalcohol (3:2). An aliquot of the extract was applied to PPC and the radiochromatogram was determined. Three peaks of radioactivity appeared at *Rf* 0.55, 0.77, and 0.91. Authentic octopamine, synephrine and tyramine were located at *Rf* 0.54, 0.75, and 0.80, respectively. These peaks showed positive results with Folin–Ciocalteu's reagent and ninhydrin reagent. A portion of the solvent extract was concentrated to dryness *in vacuo*. The residue was dissolved in AcOH (0.2 ml) and 0.5% KIO_4 (0.8 ml) was added to the solution. After the solution was allowed to stand at room temperature for 20 hr, it was adjusted to pH 10 with borate buffer and extracted with toluene–isoamylalcohol. The extract was submitted to PPC. The two peaks of radioactivity at *Rf* 0.55 and 0.77 disappeared on the paper chromatogram and one peak at *Rf* 0.91 remained. Authentic octopamine and synephrine were oxidized by periodate in the same manner, while tyramine was not. Each band of the two peaks (*Rf* 0.55 and 0.77) was eluted by 0.1 N HCl and the evaporated samples were submitted to GLC. GLC (TMS derivative) condition 2; peak 1 t_R: 4.2 min, Peak 2 t_R: 5.0 min. The mixtures of peak 1 and octopamine and of peak 2 and synephrine exhibited a signle peak.

Administration of Octopamine to Rats——DL-Octopamine-2-^{3}H (186 μCi/mmole) was given to five rats by oral administration (70 mg/kg). The urine was pooled for 48 hr and extracted with EtOAc at pH 1. A portion of the acid metabolite fraction was applied to PPC, and the radiochromatogram was determined.

Administration of Antibiotics to Rabbits——Daily oral administration of streptomycin (350 mg) and penicillin G potassium (100000 units) was carried out to each rabbit used in the previous experiment for four days. On the final day of the antibiotics administration, octopamine-2-^{3}H 14 μCi (186 μCi/mmole) was given by oral administration at the same time. The urine was collected for 48 hr and extracted with EtOAc at pH 1. The acidic metabolites were applied to PPC and the radiochromatogram was determined. The amounts of metabolites, *p*-HPAA and *p*-HMA, were estimated.