ACTA MED OKAYAMA 2000; 54(1): 1-8

Improved HPLC Determination of Acidic Opines by Phenylisothiocyanate Derivatization and Its Application to Marine Animals

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We present here a reliable and sensitive method for the determination of acidic opines such as *meso*-alanopine, *β*-alanopine, tauropine and strombine in biological samples. Interfering primary amino acids were eliminated by reaction with o-phthalaldehyde, and the derivatized compounds were passed through Sep-Pak Plus PS-1 cartridges. The acidic opines were recovered by flushing the cartridges with water, then determined by high performance liquid chromatography after a second derivatization with phenylisothiocyanate. All 4 acidic opines were detected within 30 min. This method ensured good separation and guaranteed almost full recovery of all acidic opines. This method was applied to analyze opines in marine animals and to test whether opines are metabolized in the livers of the rat and fish.

Key words: opines, high-performance liquid chromatography determination, marine animals, phenylisothiocyanate, Sep-Pak cartridge

Opines are anaerobic metabolites found in a wide range of marine invertebrates and mollusks (1-5), and include imino acids such as octopine, *meso*-alanopine, strombine, tauropine and *β*-alanopine (Fig. 1). Plants also produce octopine and lysopine, which are now established as marker of plant tumors (6). Except for their roles in balancing of redox potential (7, 8) and as attractants and cryoprotectants (9, 10) in marine animals, little is known about the biochemistry or physiological significance of opines in invertebrates and mammals. Dietary octopine was reported to significantly decrease the serum total, very low-density lipoprotein and low-density lipoprotein cholesterol levels in rats fed a cholesterol-enriched diet (11). To investigate the functions of each opine in detail, we present here a sensitive, reliable, fast and efficient quantitative determination method.

Materials and Methods

Chemicals. Amino acids, sodium bromide, sodium acetate, acetic acid, phenylisothiocyanate (PITC), *o*-phthalaldehyde (OPA) were purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan). Sep-Pak Plus PS-1 cartridges (265 mg resin/cartridge) were obtained from Nihon Waters K.K. (Japan). The strongly acidic cation exchanger Diaion SK was purchased from Mitsubishi Chemical Ind. Ltd. (Tokyo, Japan). Amberlite IRA-93 was obtained from Organo Japan Co. (Tokyo, Japan). Dowex 1-X2 was obtained from Bio Rad Laboratories (CA, USA).

Synthesis of L-α-bromopropionic acid. L-*α*-bromopropionic acid was synthesized from L-alanine. Briefly, L-alanine (8 g) and NaBr (32 g) were dissolved in 100 ml of 2N HBr, and the solution was brought to 0 °C with vigorous stirring. Sodium nitrite (11 g) was added in small portions and at such a rate that the temperature of the solution remained between 0 and 5 °C. The reaction was conducted for 4 h. The reaction mixture was then extracted with ether using an auto-extractor, the ether extract was dried over anhydrous Na_2SO_4 and the solvent was removed by evaporation. The yield was 43.63%. This L-*α*-bromopropionic acid was used for the synthesis of opines.

Synthesis of meso-alanopine. L-Alanine (1 g) and L-*α*-bromopropionic acid (4 g) were dissolved in *ca.* 60 ml of 1N NaOH. The reaction was conducted for about 3 h keeping the solution at a temperature between

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90–100 °C and alkaline pH. At the end of the reaction, the solution was brought to room temperature and acidified with acetic acid. This solution was applied to a Diaion SK column (18.5 × 4.5 cm, H^+ form). The column was washed thoroughly with water and eluted with 1 l of 2N NH_3. The eluate was concentrated and applied to an Amberlite IRA-93 column (20.4 × 3.3 cm, free form). The column was washed with water and then eluted with 1 l of 0.2N acetic acid and then with 1 l of 1N acetic acid by which *meso*-alanopine was eluted. The eluate was concentrated, and the residue was crystallized from water and ethanol. The compound was recrystallized several times from the same solvent and dried in a dessicator. The yield was 49.20% and m.p. 231–235 °C (decomp.). Analysis: Calculated for $C_6H_{11}NO_4$, H 6.88, C 44.71, N 8.69; found H 6.64, C 44.59, N 8.73.

Synthesis of D-tauropine. Tauropine was synthesized in the same manner as *meso*-alanopine by reacting taurine (1.25 g) with L-α-bromopropionic acid (5.0 g), keeping the solution alkaline with 1N NaOH. After acidification with acetic acid, the reaction mixture was applied to a Dowex 1-X2 column (6.7 × 4.7 cm, OH^- form). The column was washed with water and then with 1 l of 1N acetic acid. Tauropine was eluted with 1.5 l of 0.1N HCl, concentrated and crystallized as described above. The yield was 37.06% and m.p. 264–266 °C (decomp.). Analysis: Calculated for $C_5H_{11}NSO_5$, H 5.62, C 30.45, N 7.10; found, H 5.38, C 30.15, N 7.14.

Synthesis of D-strombine. D-Strombine was synthesized from glycine (1.5 g) and L-α-bromopropionic acid (6.12 g) in the same way. The reaction mixture was acidified with acetic acid and then applied to a Diaion SK column (18.5 × 4.5 cm, H^+ form). The absorbate was eluted with 800 ml of 2N NH_3. The eluate was concentrated and applied to the Dowex 1-X2 column (6.4 × 4.7 cm, OH^- form). The column was washed with water, then eluted with 1.5 l of 0.2N acetic acid and 1 l of 1N acetic acid. D-strombine was crystallized from the 1N acetic acid fractions as described above. The yield was 13.22% and m.p. 207–209 °C (decomp.). Analysis: Calculated for $C_5H_9NO_4$, H 6.17, C 40.81, N 9.52; found, H 6.40, C 40.62, N 9.64.

Synthesis of β-alanopine. β-Alanopine was synthesized by reacting D-alanine (1.78 g) with acrylic acid (4.32 g) keeping the solution alkaline by adding 110 ml of 5% $NaHCO_3$. The reaction mixture was applied to the Diaion SK column (18.0 × 4.5 cm, H^+ form) and washed with 50% MeOH. The absorbate was eluted with 800 ml of 2N NH_3 and concentrated. The concentrate was applied to an Amberlite IRA-93 column (20.4 × 3.3 cm, free form) and washed with water. The column was eluted with 1 l of 0.1N acetic acid and then 1 l of 1N acetic acid. β-alanopine was crystallized from the 1N acetic acid fraction as described above. The yield was 37.58% and m.p. 230–232 °C (decomp.). Analysis: Calculated for $C_6H_{11}NO_4$, H 6.88, C 44.71, N 8.69; found H 7.11, C 44.32, N 8.73.

High-performance liquid chromatography (HPLC). A Shimadzu LC 6A liquid chromatograph was used with a Shimadzu SPD 6A spectrophotometric detector. The separation of PITC-derivatized opines were performed on Cosmosil 5C-18 AR-II (150 × 4.6 mm I. D.) (Nacalai tesque, Kyoto, Japan) with gradient elution. The linear gradient program for the separation consisted of 0% B traversing to 50% B in 30 min. This was followed by a washing step programmed to 100% B for 10 min. The column was then equilibrated with 100% A for 5 min. The flow rate was 1 ml/min. The elution solvent consisted of 100 mM sodium acetate in 10% acetonitrile (pH 3.43) (solvent A) and 60% acetonitrile in water (solvent B). The PITC-derivatized opines were detected by UV absorption at 254 nm.

Determination of acidic opines. PITC derivatization of the acidic opines *meso*-alanopine, D-tauropine, D-strombine and β-alanopine was performed according to the method described by Sato *et al.* (12). Briefly aliquots of acidic opine solutions at various concentrations were taken into 2 ml Eppendorf tubes and dried with a Savant Speed Vac concentrator (NY, USA). After drying, the samples were dissolved in 20 μl of ethanol-triethylamine-water (2:2:1, by volume) and again dried. The dried samples were redissolved in 50 μl of ethanol-triethylamine-water (7:1:1, by volume) and 5 μl of PITC reagent was added. The reaction mixture was shaken and kept at room temperature for 20 min. The reagents were removed under vacuum. PITC-derivatized opines were dissolved in 100 μl of acetonitrile-water (2:7, by volume), and 10 μl aliquots of the solution were injected into the column for analysis.

Determination of acidic opines in biological samples. Fresh muscles of 4 marine molluscs were cut into small pieces and homogenized with 10 volumes of water. The homogenates were boiled for 10 min and then centrifuged at 10,000 × g for 20 min. The supernatant (corresponding to 10 mg wet weight) was

derivatized with 100 μl of *o*-phthalaldehyde solution. *o*-Phthalaldehyde (0.02 g) was dissolved in a mixture of 80 μl of methanol and 300 μl of pyridine, which was adjusted to pH 8 with HCl. After 5 min, the reaction mixture was passed through a Sep-Pak Plus PS-1 cartridge, and acidic opines were eluted with 1.5 ml of water. The water eluate was dried with a concentrator and derivatized with PITC described for acidic opine determination.

Metabolism of acidic opines in fish and rat liver homogenates. Fresh livers of fish, *Pagrus major*, and male Wistar rats (180 g) were homogenized in 9 volumes of 50 mM potassium phosphate buffer (pH 7.4) using a Potter-Elvejhem homogenizer with a Teflon pestle keeping the samples on ice. The homogenate was centrifuged at 700 × g for 10 min, and then various amounts of opines were added to 900 μl of the supernatant. The reaction mixtures were made up to 1 ml with water, incubated at 37 °C for 1 h and boiled for 10 min at the end of the incubation period. After deproteinization, the mixtures were centrifuged at 10,000 × g for 20 min, and the supernatant was used for analysis of acidic opines. As a control, reaction mixtures were kept on ice.

Results

HPLC chromatograms of opines. Fig. 2 shows the results of HPLC of authentic opines (a) and those in the extracts of fresh muscles of the marine mollusk, *Ruditapes philippinarum* (b). They showed good separation and little interference. Analysis of *meso*-alanopine gave two peaks, consistent with the findings reported by Sato *et al.* (12). They explained their observations as being due to the inflexibility of rotation around the N-group of the derivative.

Calibration curves. Various amounts of *meso*-alanopine, strombine, tauropine and β-alanopine were derivatized with PITC and analyzed by HPLC. The calibration curves were linear from 50 pmol to 2 nmol as shown in Fig. 3. The relationships between the peak area (y, Volt. sec) and the amounts of opines were as follows: $y = 0.165X$ ($r^2 = 0.998$) for *meso*-alanopine, $y = 0.217X$ ($r^2 = 0.995$) for strombine, $y = 0.208X$ ($r^2 = 0.999$) for tauropine, $y = 0.185X$ ($r^2 = 0.998$) for β-alanopine. The minimum level of detection was 10 pmol for strombine, tauropine and β-alanopine, and 40 pmol for *meso*-alanopine.

Recovery test. Various amounts (0.0, 0.5, 1.0,

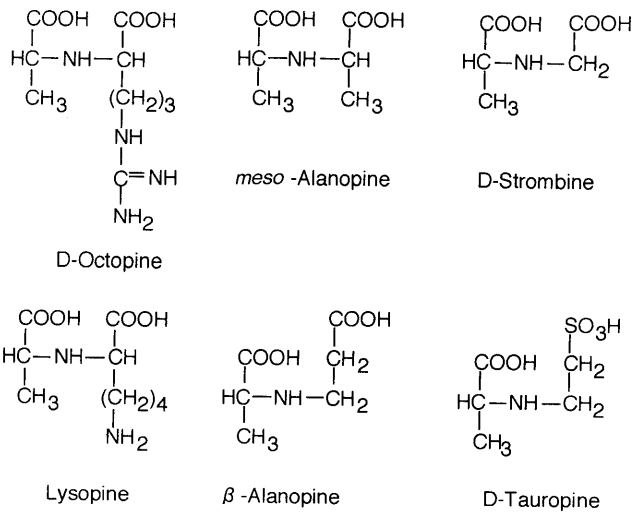

Fig. 1 Chemical structures of opines.

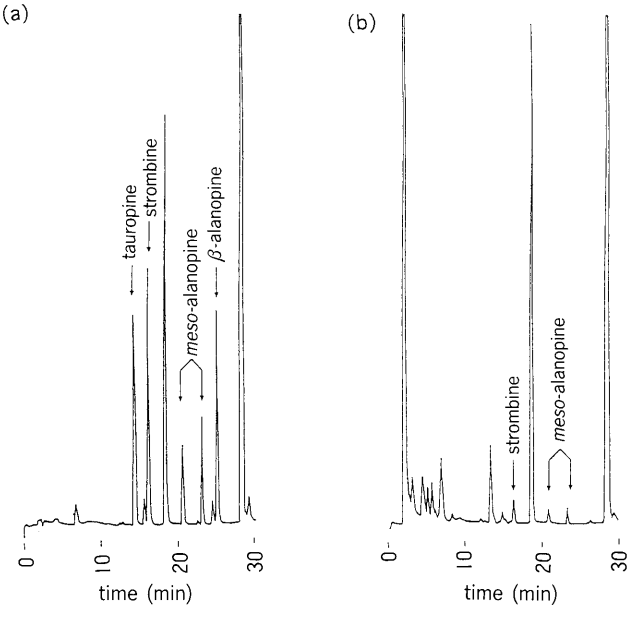

Fig. 2 Chromatograms of authentic opines (**a**) and that obtained from muscles of *Ruditapes philippinarum* (**b**).
(**a**) were written in determination of acidic opines.
(**b**) Fresh muscles of *Ruditapes philippinarum* was boiled. The extract was reacted with *o*-phthalaldehyde. The reaction mixture was passed through a Sep-Pak Plus PS-I cartridge. The passed through eluate was derivatized with phenylisothiocyanate and analyzed by HPLC as (**a**).

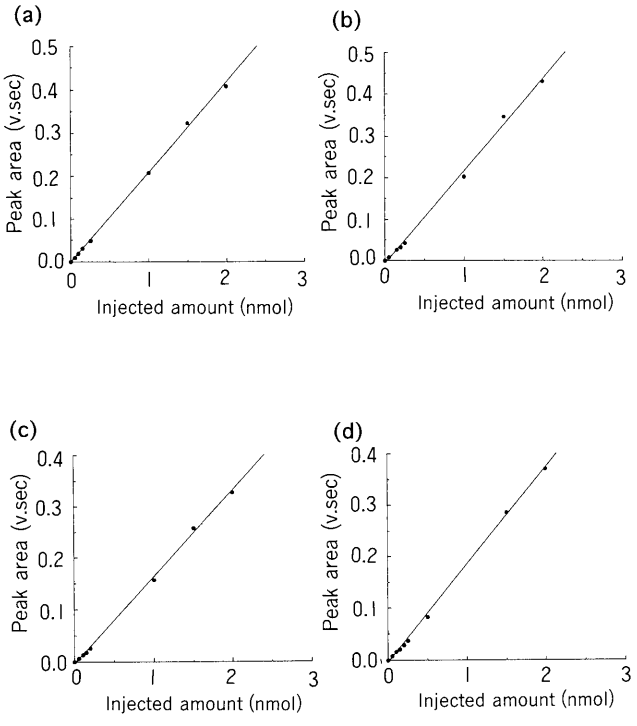

Fig. 3 Calibration curves of (**a**) tauropine (**b**) strombine (**c**) *meso*-alanopine (**d**) β-alanopine. Various amounts (0.5 to 20 nmol) of aqueous solutions of opines were treated as described under Fig. 2.

Table I Recovery of added acidic opines from *M. edulis* muscles supernatant

Opines	Opines added (pmol)	Found (pmol)	Recovery %	Average (± SD) %
Tauropine	0	0	0	
	500	442.70	88.54	
	1000	990.90	99.09	94.15 (± 4.86)
	1500	1457.85	97.19	
	2000	1835.60	91.78	
Strombine	0	265.45	0	
	500	720.55	91.02	
	1000	1274.65	100.92	96.40 (± 4.11)
	1500	1708.15	96.18	
	2000	2215.05	97.48	
meso-Alanopine	0	315.95	0	
	500	789.40	94.69	
	1000	1342.45	102.65	101.79 (± 5.13)
	1500	1858.95	102.87	
	2000	2455.20	106.96	
β-Alanopine	0	0	0	
	500	454.10	90.82	
	1000	905.35	90.54	89.97 (± 0.91)
	1500	1345.50	89.70	
	2000	1776.10	88.81	

Various amounts of opines were added to 50 μl of the *M. edulis* muscles supernatant, then reacted with *o*-phthalaldehyde and passed through Sep-Pak Plus PS-I cartridges. The water eluate after concentration derivatized with phenylisothiocyanate and measured by HPLC as described in "Materials and Methods". SD, standard deviation.

1.5 and 2.0 nmol) of *meso*-alanopine, tauropine, β-alanopine and strombine were added to 50 μl of the supernatant of *M. edulis* muscle homogenate and mixed well. The supernatant containing the opine mixture was derivatized with 100 μl of *o*-phthalaldehyde solution and applied to the cartridge. The acidic opines were recovered from the cartridges and derivatized with PITC as described above. Results are shown in Table 1.

Efficiency of cartridges to remove OPA derivatized primary amino acids. The efficiency of separation of acidic opines from other amino acids was evaluated in samples containing acidic opines and 23 different amino acids (alanine, arginine, aspartic acid, asparagine, cysteine, cystine, glutamic acid, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, valine, proline, hydroxyproline, β-alanine and taurine). Amino acid solutions (containing 0.2 μmol of each amino acid) were used for this purpose. Three sets of experiments were conducted and the results are shown in Fig. 4. In the first, second and third sets, amino acid solutions were derivatized only with PITC, with OPA and then PITC, or were mixed with 20 nmol opines and derivatized as described above respectively. No interfering peaks were observed on the chromatograms (Figs. 4b and 4c).

Distribution of acidic opines in some marine animals. We analyzed 4 marine molluscs for acidic opines and the results are shown in Table 2. In *Mytilus edulis* and *Ruditapes philippinarum*, we found higher concentrations of *meso*-alanopine than strombine, while tauropine and β-alanopine were not detected. In *Cellana nigrolineata*, only tauropine and β-alanopine were detected, whereas no opines were detected in *Mya arenaria*.

Discussion

meso-Alanopine was prepared by Karrer and Appenzeller (13) and Abderhalden and Haase (14). These groups both used complicated methods: the former

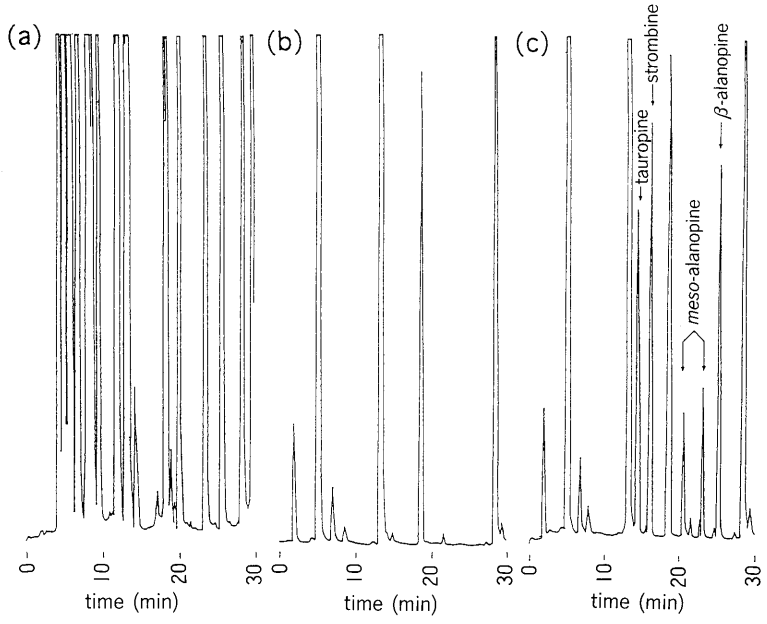

Fig. 4 Chromatograms of usual amino acids or opines dervatized by phenylisothiocyanate (PITC) with or without treatment of *o*-phthalaldehyde (OPA) and cartridges.
(**a**): A mixture of 23 different usual amino acids (0.2 μmol of each amino acid) was derivatized with PITC as in Fig. 2 (a)
(**b**): The above amino acids mixture was reacted with OPA solution. The reaction mixture was passed through a Sep-Pak Plus PS-I cartridge. The cartridge was flushed with water and the water eluate was concentrated. The residue was then derivatized with PITC and analyzed by HPLC.
(**c**): The above amino acids mixture containing 20 nmol of each opines was treated in a similar manner as in (**b**).

Table 2 Distribution of acidic opines in whole muscles of marine molluscs

Marine animals	*meso*-alanopine	strombine	tauropine	β-alanopine
Mytilus edulis	14.35 ± 3.35	7.35 ± 1.84	ND	ND
R. philippinarum	19.32 ± 0.81	19.39 ± 2.65	ND	ND
*C. nigrolineata**	ND	ND	571.98	227.83
*Mya arenaria**	ND	ND	ND	ND

Muscles were excised immediately after collecting the animals and analyzed as described in "Materials and Methods". Concentrations are expressed as μmole/100 gm of wet weight. Values are means ± SD of 4 animals. *One sample used for the analysis.
ND, not detected.

synthesized this opine through barium and silver salt formation, while the latter distilled its ester.

Different determination methods have been used for the quantification of opines, including enzymatic analysis (15), high voltage paper electrophoresis (16), isotachophoresis (17), gas liquid chromatography (15) and HPLC (12, 18–20). Enzymatic analysis cannot discriminate between *meso*-alanopine and strombine. Moreover, purified opine dehydrogenase is required for analysis, and this makes the process time consuming. The requirement for partial purification of the sample by ion exchange chromatography makes the high voltage paper electrophoresis method a lengthy procedure, and in addition this method shows low sensitivity. The isotachophoresis method also shows low sensitivity and resolution. Gas liquid chromatographic determination of opines requires time-consuming pretreatment of extracts and a long analysis time.

Several HPLC determination methods are available for opine analysis. Among these, the PITC derivatization method developed by Sato *et al.* (12) seems to be the most sensitive. Four acidic opines can be determined simultaneously with this method. However, this method also requires time-consuming sample preparation procedures including column chromatography.

Many researchers have analyzed proline and hydroxyproline and used *o*-phthalaldehyde to eliminate the primary amino acids present in biological samples to determine secondary amino acids (21). To determine opines, we also used OPA for the first eliminating reaction and then PITC for the second derivatization. In this study, we used Sep-Pak Plus PS-1 cartridges. Acidic opines were

recovered by flushing these cartridges with water, and the water eluate containing opines were derivatized with PITC. In this way, we reduced the sample preparation time and thereby analysis time; in our procedure, only 10 min was required to eliminate amino acids from the sample to obtain opine sample solution. The second advantage of the present method was that the standard acidic opines added to marine animal supernatant were almost fully recovered.

The basic imino acid octopine could not be determined by this method as 4 peaks appeared when this compound was derivatized with PITC. Moreover, when the pH of the mobile phase, sodium acetate buffer, was 5.0, octopine was co-eluted with derivatives of strombine and *meso*-alanopine. Also, *o*-phthalaldehyde does not react with the guanidino amino group of octopine, and as a result it also appears along with acidic opine solution. To overcome these drawbacks, we analyzed the opines using sodium acetate at pH 3.43 as the mobile phase. At this pH, the retention times of tauropine, strombine, *meso*-alanopine and β-alanopine were 14.23, 15.98, 20.51 and 23.01, and 25.01 min, respectively, where the peaks of octopine appeared before tauropine. The retention times of 2 other imino acids, hydroxyproline and proline, were 5.16 and 13.39 min, respectively.

Recently, we reported a method for the determination of naturally occurring imino acids in plants. This method was comprised of the formation of dinitrophenyl derivatives, extraction of the derivatives by ethyl acetate, and measurement by HPLC (22). This method could not be used, however, for analysis of opines in marine animals because *meso*-alanopine cannot react with 2,4-dinitroflurobenzene due to steric hindrance.

In adductor muscles of *M. edulis*, the concentration of strombine was reported to be higher than that of *meso*-alanopine (16, 17, 20). In these studies, strombine and *meso*-alanopine were determined after anoxia or recovery from anoxia. The accumulation of strombine in higher levels than alanopine was not in agreement with the relative activities of the corresponding synthesizing enzymes (17). They demonstrated that the higher concentration of strombine in this animal was due to higher steady-state concentration of glycine than alanine during recovery. Our analytical results clearly agreed with the values of the enzymatic activity of the respective synthesizing enzymes (23). In *Mya arenaria*, enzyme activities for production of *meso*-alanopine and strombine were not detected (2).

Since this method is applicable to determination of opines, we studied the metabolism of acidic opines in fish

Table 3 Metabolism of opines in fish liver homogenate

Opines	Added to liver homogenate (nmol)	Found after incubation at 0 °C (nmol)	Found after incubation at 37 °C (nmol)
Tauropine	0	0	0
	25	21.88	25.52
	50	48.44	50.52
	75	70.31	72.39
Strombine	0	0	0
	25	23.12	23.47
	50	50.31	48.22
	75	72.28	72.63
meso-Alanopine	0	0	0
	25	24.44	23.20
	50	51.49	49.94
	75	76.68	76.06
β-Alanopine	0	0	0
	25	23.08	26.28
	50	50.96	51.28
	75	74.04	75.64

The complete incubation mixture consisted of 0.9 ml of the 700 × g supernatant of fish liver homogenate as an enzyme source, 25, 50, 75 nmol of each opines and water in a total volume of 1 ml. The reaction mixtures were incubated at 37 °C for 1 h with shaking. For control, the reaction mixtures were kept on ice. After incubation, the opines were measured by the procedure described in "Materials and Methods". Values are means of 2 to 3 determinations.

Table 4 Metabolism of opines in rat liver homogenate

Opines	Added to liver homogenate (nmol)	Found after incubation at 0 °C (nmol)	Found after incubation at 37 °C (nmol)	Remaining %
Tauropine	0	0	0	
	25	23.94	20.63	86.17
	50	47.75	38.93	81.53
	75	71.56	54.58	76.27
Strombine	0	0	0	
	25	19.33	15.68	81.12
	50	41.73	32.43	77.71
	75	66.86	46.76	69.94
meso-Alanopine	0	0	0	
	25	23.08	19.37	83.93
	50	47.19	36.06	76.41
	75	75.47	52.29	69.29
β-Alanopine	0	0	0	
	25	22.81	17.87	78.34
	50	45.49	34.91	76.74
	75	72.28	51.12	70.72

The complete incubation mixture consisted of 0.9 ml of the 700 × g supernatant of rat liver homogenate as an enzyme source, 25, 50, 75 nmol of each opines and water in a total volume of 1 ml. The reaction mixtures were incubated at 37 °C for 1 h with shaking. For control, the reaction mixtures were kept on ice. After incubation, the opines were measured by the procedure described in "Materials and Methods". Values are means of 2 to 3 determinations. Remaining percentages of the compounds were obtained comparing the experimental groups with their corresponding control groups.

and rat liver homogenates. As shown in Tables 3 and 4, fish liver showed no metabolizing activity, although fish are habituated to eat marine animals. In the rat liver, however, tauropine, strombine, *meso*-alanopine and β-alanopine were found to be metabolized by 18.68, 23.74, 23.46 and 24.73%, respectively, in comparison to controls. Therefore, further studies are required to clarify the metabolic fate of these compounds in rat liver.

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Received January 7, 1999; accepted January 11, 1999.