Metabolic Alterations of Catecholamines and Other Compounds In Elasmobranch Tissues. (32062)

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It has been suggested that many of the biodegradation reactions which inactivate drugs and foreign compounds are essentially absent in fishes(1), since the fish and its surrounding medium represented in essence an infinitely large dialysing system. More recent evidence, however, indicates that a number of the detoxication reactions observed in mammals also are operative in fishes(2). In this communication we report studies on the metabolic alteration of catecholamines. For purposes of comparison, the presence of a number of microsomal detoxication enzymes was determined in some of the tissues studied.

Materials and methods. The tissues were excised from sharks anaethesized with tricane methane sulfonate (Sandoz MS 222) and excised from sharks anaesthetized with tricane or were frozen (in dry ice) for later use. Unless otherwise noted, preparation of the enzymes was as follows: the tissue was homogenized first in a blender with 3 volumes of 1.15% KCl and then re-homogenized in a tight fitting glass-Teflon Potter-Elvehjem homogenizer. In the case of the liver, the homogenates were centrifuged for 10 minutes at 2,000 rpm and the large fatty layer was removed. Subsequently, the homogenates were centrifuged at 9,000 rpm for 10 minutes to obtain the so-called microsomal and soluble supernatant fractions (9,000 $\times$ *g* fraction). Microsomes were sedimented from this fraction by centrifugation at 30,000 rpm for 60 minutes, resuspended in 1.15% KCl and sedimented again. *Catechol-O-methyl transferase* was estimated by the method of Axelrod *et al*(3) using S-adenosylmethionine-C^{14} (New England Nuclear, 52.6 mc/mM) as the methyl donor. The reaction mixture was adjusted to pH 7.9-8.0 with either phosphate or Tris buffer and incubated in a final volume of 0.5 ml for 60 minutes at 25°C. The reaction was stopped by the addition of $Na_2B_4O_7$ (pH 10.0) and the methylated products were extracted with toluene : isoamyl alcohol (3:2;v/v). Aliquots of the organic extract were assayed for radioactivity by liquid scintillation counting. *Assay of monoamine oxidase* activity was performed according to Wurtman and Axelrod(4) using tryptamine-2-C^{14} (2 mc/mM) as a substrate.

Azo-reductase was measured in the 9,000 $\times$ *g* fraction or with microsomes at pH 7.4 using a TPNH generating system. Substrates used were 4-sulfamyl-2,4-diamino-azobenzene hydrochloride (Prontosil rubrum) and N,N-paradimethyl aminoazobenzene (Butter yellow). The formation of sulfanilamide from Prontosil rubrum was measured with the Bratton-Marshall reagent(5). The disappearance of Butter yellow was measured according to Mueller and Miller(6). *Nitroreductase* activity was estimated according to Fouts and Brodie(7) measuring the conversion of p-nitrobenzoic acid to p-aminophenol. *O-demethylation,* using p-nitrophenyl methyl ether (p-nitroanisole) as a substrate, was measured by the increased absorbance at 410 mμ caused by the formation of p-nitrophenol(8). *Hydroxylation* of 3,4 benzpyrene was measured by the method of Gelboin and Blackburn(9). Oxidation of antipyrine (2,3-dimethyl-1-phenyl-pyrazolin-5-one) to 4-hydroxyantipyrine was determined according to Brodie and Axelrod(10).

Results. Catechol - O - methyltransferase (COMT) activity was found to be present at various levels in all the shark tissues studied here. The highest levels of this enzyme were observed in the kidneys (mesonephros) of adult sharks. Levels of COMT in the kidney of a brown shark fetus (45 cm; assumed one month pre-partum) were quite low in contrast. In the elasmobranch it appears that this enzyme is principally associated with the microsomal fraction although some activity

TABLE I. Catechol-O-Methyltransferase Activity in Shark Tissue. (Values are expressed as moles of l-norepinephrine methylated per gram tissue per hour $\times 10^9$.)

	Whole homogenate	9,000 × *g* supernatant	Soluble fraction	Microsomal fraction
	Spiny dogfish (*Squalus acanthias*)			
Liver	19.40	14.45	1.94	21.4
Kidney	32.4	28.5	14.7	30.7
Sympathetic nerve	9.7	—	—	—
Interrenal body	29.4	—	—	—
Muscle	3.8	—	—	—
	Brown shark (*Carcharhinus milberti*)			
Liver	11.51	—	—	4.13
Kidney	17.74	7.08	—	12.21
Heart	1.02	0.73	—	—
Spleen	6.10	1.27	—	—
Brain	—	0.32	—	—

was observed in soluble fractions prepared from kidney homogenates (Table I). Magnesium ions were not required as they are for the mammalian enzyme. Between pH 5.0 and 10.0, optimum activity was obtained with Tris buffer (0.1 M) at pH 7.9 to 8.0 and with phosphate buffer (0.1 M) above pH 9.0.

Optimum activity with *Squalus acanthias* COMT was obtained at 37°C (Table II) but an incubation temperature of 25°C was chosen for most of the experiments since this was thought to be more representative of the temperature of the natural environment. With norepinephrine as substrate the methyl transferase activity of both kidney and liver was strongly inhibited by equimolar concentrations of pyrogallol or tropolone. Incubation of liver or kidney microsomes with a number of other substrates led to the conclusion that N-methyltransferase and monophenol-O-methyltransferase activities were insignificant (Table III). These data indicated that the methyltransferase system consisted principally of COMT. Comparison of COMT activity to monoamine oxidase activity indicated that the latter enzyme was considerably more active on a basis of the amount of substrate inactivated *in vitro* (Table IV). In the absence of substrate, considerable methyltransferase activity was observed especially in the inter-renal body. The nature of the endogenous substrate has not been elucidated. However, epinephrine and norepinephrine are present in shark tissues, and details of these measurements will be reported elsewhere. The isolation of phospholipids indicated that C^{14}-methyl transfer to these compounds was in-

TABLE II. Effect of Temperature on COMT Activity in Microsomal Preparations of Liver and Kidney of *Squalus acanthias*. (Moles $\times 10^9$ of norepinephrine methylated per hour per gram of fresh tissue.)

Temperature (°C)	Liver	Kidney
+4	3.06	8.93
25	21.1	30.9
37	27.6	32.2

TABLE III. O-Methyltransferase Activity in *Squalus acanthias* Liver and Kidney Microsomal Preparations. (Substrates* methylated per gram tissue per hour in moles $\times 10^9$.)

	Liver	Kidney
Tyramine	.44	.65
Phenylethylamine	.33	.36
Phenylethanolamine	.24	.26
Octopamine	.41	.45
l-norepinephrine	21.1	30.9
l-epinephrine	43.6†	—

* Substrate concentration 10^{-3} M.
† Tiger shark.

TABLE IV. Monoamine Oxidase and COMT Activity in *Squalus acanthias* Tissues.

	Tryptamine-2-C^{14} oxidized, moles/g/hr $\times 10^6$	l-norepinephrine methylated, moles/g/hr $\times 10^9$
Liver	10.53	19.40 (2.37)*
Kidney	9.0	32.4 (7.9)
Muscle	9.77	3.88 (0.28)
Nerve	1.47	9.7 (3.02)
Interrenal body	9.19	29.4 (28.7)

* Figures in parentheses indicate endogenous activity in absence of substrate.

TABLE V. Azo-Reductase and Ether Cleavage Activity in Shark Liver Microsomal Enzymes.*

Enzyme	Source	Substrate	μmoles of drug metabolized/g/hr at 25°C
Azo-reductase	Dusky shark	Butter yellow	.0048
"	Brown shark (adult)	" "	.0038
"	Brown shark (fetus)	" "	.0120
O-demethylation	Tiger shark	p-nitroanisole	.004
"	Brown shark	"	.002
"	Sprague-Dawley rat	"	.21 (37°C)

* These figures are averaged from 3 experiments. Variation was less than 10%. Variation among individuals of a species was not determined.

significant. The methylated product(s) of endogenous substrate(s) from liver and kidney was found to be water soluble and amphoteric.

Using shark liver microsomal preparations for the study of a number of common detoxication reactions, only very low levels of these enzymes were measured. Reductive cleavage of the azo bond of either Prontosil or Butter yellow was not observed with a tiger shark *(Galeocerdo cuvieri)* liver enzyme preparation. Similarly, nitro reductase activity could not be demonstrated in the liver of this shark. Hydroxylation reactions, using 3,4-benzpyrene or antipyrine as substrates, were absent in a tiger shark, a dusky shark *(Carcharhinus obscurus)* and a brown shark *(Carcharhinus milberti)*. Some of the drug metabolizing enzymes which were found in active shark liver preparations are shown in Table V.

Compared to most mammalian systems, azo-reductase and O-demethylation activities were low. Yield of microsomal protein from shark liver was also quite low (3.7 mg/g) in comparison to the rat (26 mg/g). The activity of these shark enzymes, if related to microsomal protein, is about 10% that of the rat enzymes. The contribution of such low levels of activity, however, may not be insignificant in the overall homeostatic mechanism of this species of fish because potential substrates presumably are encountered at low concentrations only.

Discussion. The enzymes for the inactivation of neurohumoral catecholamines apparently are present in most elasmobranch tissues. The finding that the catechol-O-methyltransferase system in sharks is a particulate rather than a soluble enzyme is of interest. Microsomal enzymes have been considered to be comparatively primitive (undifferentiated) from a phylogenetic point of view. A study of the development of this enzyme through a number of species which represent different stages of evolution might further our understanding of the evolution of biochemical processes and their macromolecular catalysts. The interrelationship of catecholamines and lipid metabolism might be pursued to advantage in the elasmobranch liver since lipid metabolism in this animal may be largely concentrated in this organ.

The presence of low levels of some drug metabolizing enzymes in the tissues of several elasmobranch species has been demonstrated. Confirming the observations of Adamson *et al* (11), nitroreductase activity in shark liver was not observed. Similarly, the hydroxylation of benzpyrene and antipyrine could not be detected. Azo-reductase activity was demonstrated in the liver of the brown shark but not in the tiger shark. In the former, it appeared that this enzyme was relatively more active in the fetal than in the adult fish. It is conceivable that in the tiger shark the azo-reductase has been deleted in the evolutionary process. Since it is possible that this enzyme is identical with cytochrome-C reductase (of which low levels were present), further studies on this and related species should be undertaken.

Our observations and those of Adamson *et al*(11) and Buhler(2) indicate that the detoxication of *foreign* compounds in fishes occurs as well as disposal through the gills by dialysis.

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Direct Localization and Visualization of Hyaluronate Lyase Activity by Agar Gel Electrophoresis.* (32063)

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Procedures for localization, visualization and identification of enzymatically active substances following electrophoresis have been described for several enzyme systems, *i.e.*, xanthine oxidase, lactic dehydrogenase, phosphatase, esterase, and glutamic oxaloacetic transaminase(1-7). All of these enzymes were derived from mammalian species. Electrophoretic fractionations of enzyme-containing material from these studies were performed with a variety of supporting matrices, such as paper strips, starch blocks, starch-agar gel, or cellulose acetate strips. Such electrophoretic preparations, exhibiting visualized enzyme activities, have been termed zymograms(2). In most cases, preparation of zymograms has required a complex and often time-consuming series of histochemical steps to localize the enzyme activity.

Electrophoretic localization and visualization studies with enzyme systems of microbial origin have only recently received attention in the literature. Some of these studies include analysis of bacterial coagulases(8), hemolysins(9), and deoxyribonucleases(10). This report is concerned with description of a rapid enzymophoretic technique for direct localization and visualization of hyaluronate lyase (hyaluronidase) derived from bacteria, as compared to hyaluronidase derived from mammalian testes.

Material and methods. Enzyme source. Staphylococcal hyaluronidase-rich concentrates were derived from a strain of *Staphylococcus aureus* (AEMC-1801) isolated in 1960 from an infected hospitalized patient (11). The organism was cultivated in brain heart infusion broth dialysate medium(11,12). Enzyme-rich concentrates were prepared by ammonium sulphate precipitation of 18-hour culture supernatants, essentially as described by Harris and Harris for preparation of streptococcal hyaluronidase(12). Concentrated staphylococcal supernatants contained 12 to 15 antigens as demonstrated by agar gel diffusion analysis with hyperimmune rabbit antiserum(11).

Testicular hyaluronidase samples were kindly supplied by Dr. George Warren, Wyeth Institute for Medical Research, Radnor, Pa. Streptococcal hyaluronidase-rich concentrates were prepared from a Group A hemolytic *Streptococcus pyogenes,* Type H-44, origi-

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