Table 1

The Incorporation of [^{14}C] Orotic into Amino Acids and Macromolecules in the Dogfish, *Squalus Acanthias, In Vivo*

	Incorporation into 4 Carbon Dicarboxylic Amino Acids (pCi/g or ml)				Incorporation into Macromolecules (pCi/g or ml)	
	L-Aspartic Acid		L-Asparagine			
Tissue	1 hr	24 hr	1 hr	24 hr	1 hr	24 hr
Plasma	<280.0	<280.0	<260.0	<260.0	2476.6	30231.6
Erythrocytes	---	---	---	---	17121.0	16506.5
CSF	---	---	---	---	<430.0	<430.0
Brain	<280.0	<280.0	<260.0	<260.0	725.4	<430.0
Bile	---	---	<260.0	<260.0	<430.0	<430.0
Liver	<280.0	<284.4	<260.0	<260.0	548.7	4464.0
Pancreas	<280.0	<280.0	<260.0	<260.0	2055.3	1683.3
Spleen	<280.0	<280.0	<260.0	<260.0	1227.6	2352.9
Rectal Gland	<280.0	<280.0	<260.0	<260.0	9476.7	5412.6
Kidney	3544.5	1263.5	519.6	<260.0	1776.1	36297.9
Duodenum	---	<280.0	---	<260.0	948.6	1971.6
Heart	<280.0	<280.0	<260.0	<260.0	2771.4	1618.2
Muscle	<280.0	<280.0	<260.0	<260.0	<430.0	<430.0
Gill	<280.0	<280.0	<260.0	<260.0	14889.3	4398.9
Gastric mucosa	<280.0	<280.0	<260.0	<260.0	1255.5	827.7
Intestinal mucosa	722.0	---	<260.0	---	2325.0	<430.0
Testes	317.2	<280.0	<260.0	<260.0	<430.0	781.2

tration of free L-[4-^{14}C] aspartic acid and L[4-^{14}C] asparagine in the body fluids and proteins of representative viscera. For this purpose, a highly sensitive and selective enzymic radiometric assay was utilized (MDIBL Bulletin 12: 68, 1972). From the data presented in Table 1, it can be appreciated that considerable net radioactivity from [6-^{14}C] orotic acid persists in the viscera of the dogfish after intravenous administration of the precursor. This very likely represents incorporation of label into nucleic acids. However, the case with the dicarboxylic amino acids was quite different: L-[4-^{14}C] aspartic acid was found in relative abundance only in kidney; free L-[4-^{14}C] asparagine also was present only in that organ. In pronase-hydolyzed proteins from the principal organs of the dogfish, no significant labélling of L-aspartyl and L-asparginyl residues was observed. Moreover, high speed (12,000 g) supernatants of dogfish and skate kidney failed to degrade [6-^{14}C] orotate to L-[4-^{14}C] aspartic acid or L-[4-^{14}C] asparagine in vitro. These findings warrant the conclusion that while orotic acid can be degraded to L-aspartic acid or to L-asparagine in *Squalus acanthias*, quantitatively the route appears to be of minor importance.

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Inhibition of Mammalian L-Asparagine Sysnthetase by Material(s) Present in Homogenates of Fish Tissues: Possible Role of Mineral Cations.

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Thus far, attempts to detect mammalian-type L-asparagine synthetase (L-glutamine hydrolyzing, EC 6.3.5.4) in the organs of the dogfish, (*Squalus acanthias*) and skate (*Raja ocellata*) have been unsuccessful (MDIBL Bull. 12: 68, 1972; ibid 13: 27, 1973). Likewise, th enzymes for the condensation of cyanide and L-cyste to yield the L-asparagine precursor, -cyano-L-alan appear to be absent from these marine organisms (MDI Bull. 13: 27, 1973). Nevertheless, L-asparagine abundant in the body fluids and proteins of *Squalus* mo notably in the pancreas (19.48 nmole/mg protein) a spleen (20.30 nmole/mg protein). To resolve th apparent discrepancy, we hypothesized that mater present in the homogenates so far examined might b capable of depressing and even masking the biosynthe of L-asparagine. Several such inhibitors are known to b present in the tissues of mammals. These fall into thre classes; 1) low molecular or metallic inhibitors; 2 intermediary metabolites, and 3) large molecular o polymeric inhibitors. Calcium ions are the principal repre sentatives of the first catagory, and L-asparagine an S-adenosyl-L-methionine of the second catagory, an L-asparaginase, proteases and hyaluronic acid of the thir catagory. In previous communication (MDIBL Bull. 1 68, 1972) we have demonstrated that most organs of th skate, with the exception of plasma and muscl contained a considerable concentration of one of thes so-called inhibitors — [i.e., L-asparaginase (EC 3.5.1.1) It is the purpose of the present report to document th existence of other such endrogenous inhbitors in th tissues of these two species of fish.

Dogfish and skate were caught off the Maine coa and were killed by exsanguination. Twenty percent (w/ homogenates of the principal viscera were prepared 50% (v/v) ethanol or in 0.05 M Tris HCL buffer, pH 8. The homogenates were centrifufed at 12000 g for minutes and a 5 l aliquot of the resultant supernata was added to the reaction mixture previously describe

Table 1

Extracts of Tissue from Dogfish (*Squalus acanthias*) and Skate (*Raja ocellata*): Concentration of Minerals and Percent Inhibition of L-Asparagine Synthetase from L5178Y/AR

	Dogfish (*Squalus acanthias*)						Skate (*Raja ocellata*)				
	% Inhibition L-Asparagine Synthetase (%)		Mineral concentrations * (mmoles/Kg wet wt.)				% Inhibition L-Asparagine Synthetase (%)		Mineral Concentrations * (mmoles/Kg wet wt.)		
Tissue Extract	Ethanolic Extract	Aqueous Extract	Ca	Cu	Zn	Mg	Aqueous Extract	Ca	Cu	Zn	Mg
Brain	0	14	2.14±0.38	1.70±0.48	3.75±0.94	41.66±14.59	57	2.63±0.55	2.92±0.77	2.99±0.86	40.03±2.94
Liver	92	7	1.47±0.26	1.20±0.10	2.13±0.11	28.56±4.46	51	2.36±0.19	2.65±0.55	3.67±0.76	40.33±3.24
Kidney	0	24	1.79±0.15	1.14±0.11	2.13±0.11	31.19±3.30	62	2.58±0.24	2.59±0.57	3.61±0.71	42.20±4.28
Pancreas	66	30	2.30±0.11	1.19±0.08	2.09±0.34	33.12±3.29	92	2.52±0.24	2.45±0.51	3.64±0.68	46.90±4.40
Spleen	19	4	1.54±0.13	2.56±0.65	1.87±0.14	35.68±3.85	38	2.41±0.28	1.15±0.08	3.72±0.69	45.95±4.25
Uterus	0	16	2.01±0.13	1.13±0.08	1.82±0.21	30.12±3.66	47	3.14±0.28	2.88±0.52	4.64±0.82	45.02±4.36
Muscle	13	0	1.58±0.11	1.09±0.09	4.87±2.95	34.56±4.02	17	3.22±0.81	2.84±0.44	4.84±0.84	45.95±4.11
Plasma	–	22	3.11±0.11	1.27±0.14	2.18±0.03	33.75±0.10	80	2.20±0.25	2.50±0.50	3.81±0.53	43.12±4.13

*Mean ± S.E.

MDIBL Bull. 12: 68, 1972) for the measurement of L-asparagine synthetase. L-asparagine synthetase, patially purified from nodules of Leukemia 5178Y/AR (L-asparaginase resistant), served as the source of enzyme. Controls received 5 l of o.05M Tris HCL buffer, pH 8.4 or 0% (v/v) ethanol in liew of fish extract. Inhibition was computed by comparing the activity of the enzyme seen in the presence of extract to that seen in its absence. Analyses were conducted in quadruplicate on the homogenates of 2-4 fish. The remaining volume of each homogenate was diluted to 2 ml with concentrated HCL. The samples were analyzed individually for total concentrations of Ca, Cu, Mg and Zn using atomic absorption spectrophotometry.

Table 1 documents that plasma, as well as 20% pancreatic homogenates of the skate, inhibited murine L-aspargine synthetase by 80% and 92% respectively. Brain, liver, kidney and uterus extracts from this species produced approximately 50% inhibition. On the other hand, aqueous dogfish extracts were uniformly weaker as sources of the inhibitory principle(s) (0-30%). Suprisingly, though, ethanolic extracts of the liver and pancreas of *Squalus* did produce strong inhibition (92% and 66% respectively); indeed, in the case of the hepatic homogenates, the synthesis of L-asparagine was brought to a virtual standstill. In this case, it is noteworthy that the inhibitor did not partition into the lipid layer, i.e. with the liver oils.

In view, of the known potency of calcium ion as an inhibitor of L-asparagine synthetase (50% inhibition at 25 mM), the influence of the calcium-selective chelator, EGTA (ethylenglycol-bis-(-amino-ehtyl ether) N,N'-tetraacetic acid) on the inhibition produced by fish organ homogentates was examined (Table 2). At a concentration of 1Mm this counteragent only modestly relieved the inhibition produced by skate plasma and pancreas (19% and 9%, respectively). By contract, EGTA totally nullifies the inhibitory activity of mouse plasma; thus in this case, the inhibition can be wholly ascribed to calcium ions.

Copper and zinc also are strongly inhibitory to the mammaliam enzyme (more than 50½ inhibition at 0.25 mM). We therefore determined the concentration of these metals as well as of magnesium (the ordinary metal cofactor in the synthesis of L-asparagine) in the tissues sampled (Table 1). It is interesting to note that the concentration of copper and zinc in skate tissues were consistently greater (30%) than those found in dogfish tissues. Magnesium concentrations in all tissues were significantly greater than the other minerals analyzed. Sea water is known to be rich in magnesium compared to calcium. (Biology of Marine Animals: Hazele, Watson and Viney Ltd. England, p. 9 1967). There appeared to be no significant correlation between percent inhibition and concentration of individual metallic elements (Table 1).

Because the title enzyme is a so-called sulfhydryl protein, attempts also were made to inhibit any putative sulhydryl ligands present in the extract by the use of the reducing agent, dithiothreitol. At 4 mM, reversal by this dithiol of the inhibition produced by skate serum and be extracts of skate pancreas and shark liver was negligible (1%, 9% and 11% respectively).

Attempts next were made to nullify the action of the putative proteases and of macromolecular polysaccharides in the most inhibitory fish organs by the addition of diisopropylfluorophosphate (a serine protease inhbitor) and cetylypyridinium chloride (a polysaccharide-complexing agent) respectively. These counteragents failed, however, to relieve inhibition produced by skate pancreas or plasma to a significant degree. Ammonium, on the other hand, which produced 50% inhibition of skate pancreatic L-asparaginase at 0.2 M, proved to be the most potent counteragent examined in the case of the skate pancreatic extracts (which are rich in L-asparaginase), but was inert in the case of skate plasma (which is devoid of amidohydrolase).

It can be concluded that skate and dogfish organ extracts can, in fact, inhibit mammalian L-asparagine synthetase. In the case of the skate pancreas, L-asparagine will account for a third of the inhibition seen. Calcium appears to be responsible for 20% of the inhibition exerted by plasma. The factors responsible for the remaining inhibition were not successfully identified by means of counteragents.

Table 2

Reversal of the Inhibition of L-asparagine Synthetase from L5178Y/AR by Extracts of Dogfish, *Squalus acanthias* and Skate, *Raja ocellata* with Various Counter Agents

Counter Agent	% Reversal of the Inhibition of L-Asparagine Synthetase of L5178Y/AR			
	Dogfish Extracts		Skate Extracts	
[M]	Aqueous Liver Extract	Ethanolic Liver Extract	Plasma	Pancreas
	(78)		(72)	(68)
EGTA [4 mM]	0	0	21	9
NH_4Cl [1 M]	0	0	2	30
Cetylpyridium chloride [140 mM]	0	0	0	4
Dithiothreitol [4 mM]	0	11	1	9
Diisopropylfluorphosphate [7 mM]	0	0	8	1

Legend: Numbers in parenthesis are the % inhibition of L-asparagine synthetase of L5178Y/AR by tissue extracts in the absence of counter agents.

The possibility also was considered that fish L-asparagine synthetase was a thermolabile enzyme so that the 37° incubation temperature was giving rise to spurious underestimation of its activity. Precedent for such a pitfall exists (Drug Metab. Disposition 2: 546, 1974). Incubations therefore were carried out at 12°, 25°, and 37° for standard (30 min.) as well as protracted (3 hr) periods. Under these conditions, the specific activity of L-asparagine synthetase in dogfish uterus, pancreas, spleen, kidney and liver was not significantly different than previously reported (MDIBL Bull. 12: 68, 1972).

In view of the potency of the inhibition produced by skate tissue extracts, it is possible that the unidentified factors present in these extracts could mask or moderate the synthesis of L-asparagine *in vivo* — provided that the putative piscine synthetase is functionaaly analogous to the mammalian enzyme. Perhaps the generalized increase in inhibitory metallic elements in skate tissues compared to dogfish tissues could account for the greater net potency of inhibition of L-asparagine synthetase in the former case. Studies to examine these points are in progress.

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Effects of Xenobiotic Compounds on Development of the Embryo of *Fundulus heteroclitus*

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Having demonstrated that embryos of the teleost *Fundulus heteroclitus* serve as good test systems for the effects of DDT on one aspect of the ecosystem (Crawford, R.B. and Guarino, A.M. Arch. Environ. Contam. Tox., in press) we determined that other compounds should be tested. Therefore nine xenobiotic compounds were analyzed for their effects on development in *Fundulus* embryos. The compounds selected were pesticides and other enviromental contaminants.

Eggs were incubated in Stender dishes at 16° Incubation in xenobiotic-containing media was begun min. post-fertilization, using 10 ml of media per dish an 30 to 40 eggs per dish. Solutions were changed daily fo the first 10 days and every 3 days thereafter. Observation of development were made with the dissecting micro scope with staging performed according to criteria o Oppenheimer.

All compounds tested were in 50% filtered sea wate at concentrations of 10, 1, 0.1 and 0.01 ppm. Solution were prepared according to the solubility of th xenobiotic. For example, diquat and paraquat, being quit water soluble, were dissolved directly in 50% sea water A group of less soluble compounds (malathion, aroclor parathion, pentachlorophenal, and 2,3,4-T) were dis solved in acetone. Ten μl of each stock acetone solutio was added to 100 ml of 50% sea water to make the tes solutions. In the cases of the very insoluble compounds (aldrin and sevin), they were first dissolved in acetone Emulphor (polyethoxylated castor oil), an emulsifying agent, was added at the ratio of 1:9. Then 10 μl of this stock solution was added to 100 ml of 50% sea water.

The results of these egg incubations are summarized in Table 1. All were found to cause abnormal develop ment. Malathion, parathion, and pentachlorophenal at 1(ppm prevented normal development beyond the blastula In the case of malathion and parathion, approximately 50% formed a keel which developed and differentiated abnormally and then died. Even in the cases where keel did not form, cell differentiation occurred. Melanophores xanthophores, and guanophores were formed, blood islands appeared and masses of contracting muscle could be seen. These effects were much like those reported previously when specific inhibitors of RNA or protein synthesis were introduced to the incubation media a various times after fertilization. Pentachlorophenol (which can uncouple oxidative phosphorylation) mimiced the effect of anaerobic media or cyanide by completely inhibiting gastrulation.