As shown in Table 1 the specific activity (dpm/mg) in the liver was higher than for any other tissue at all time periods. Furthermore specific activity actually increased in the liver from six to 24 hours and remained at or above the activity at six hours throughout the course of the study (20.275 dpm/mg at 12 days). In most of the other tissues there was a significant decrease in activity between six and 24 hours. There were only trace levels of radioactivity in CSF at all times. Urine was collected continuously in a few fish after cannulation of the urinary papillae. Only small amounts of radioactivity were present in the urine (0.103 dpm/µl at six hours to 0.069 dpm/µl at 96 hours). However in bile there was a consistent increase in activity with time, peak levels being attained at seven days. By 12 days biliary radioactivity had declined to 2.854 dpm/µl. The delayed pattern of biliary excretion of radioactivity in dogfish is in contrast to that in a mammalian species, the rat, where ^{14}C-PCB and its metabolites were rapidly excreted in the bile after an IV injection. At all times (Table 2) most of the recovered radioactivity was found in the liver; there were only trace amounts (<1%) in the other tissues at six hours and the percentages found were significantly lower at all times after six hours (except in the brain at 24 hours). Serial blood samples were drawn in three fish at times ranging from 2.5 to 300 minutes and the decline in radioactivity (dpm/mg) was plotted semilogarithmically versus time. As shown in Figure 1, blood disappearance of radioactivity appears to follow a multiple exponential decay with time; the initial phase of which could correlate with the rapid uptake by the liver over this early time period (Table 2).

In an attempt to characterize the radioactivity present, aliquots of liver sampled at various time points were homogenized and extracted four times with benzene. Thin-layer chromatographic (TLC) analysis of the extracted radioactivity on precoated 250 µ silica gel GF plates (activated at 110°C for one hour and developed in hexane) demonstrated that most of this radioactivity co-chromatographed with authentic 2,4,5,2^{1},5^{1}-pentachlorobiphenyl (R_f 0.7-0.8) at all time periods studied. However the percent of total sample radioactivity extracted into the benzene decreased with increasing time after injection of ^{14}C-PCB (from 100 percent at six hours to 56 percent at seven days).

In conclusion ^{14}C-PCB is rapidly cleared from the blood after intravascular injection in dogfish and taken up almost exclusively by the liver. Due to the high lipid content of dogfish liver (~50 percent by weight), this organ appears to function both as the major site of rapid uptake and the major site of storage for PCB (and perhaps other highly lipid soluble compounds). The delayed excretion into bile of PCB-derived products may be due to a poor partitioning of the compound from hepatic lipid into the parenchymal cell where metabolism and excretion can occur. Further the low hydroxylating activity of dogfish liver (Bend *et al.*, Bull. MDIBL, 12, 12, 1973) may also contribute to the delayed excretion of PCB.

1973 #24

STUDIES OF THE FATE OF PHENYLACETIC ACID IN SOME FISH

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A large number of nonnutrient carboxylic acids are detoxified in mammalian species by com-

bination of the carboxylic acid group with the $-NH_2$ group of an amino acid or peptide prior to excretion in the urine (Metabolic Conjugation and Metabolic Hydrolysis, Academic Press, New York, 1: 1, 1970). The amino acid used in this process is most commonly glycine but varies with species and with the molecular structure of the nonnutrient carboxylic acid. We are studying the fate of nonnutrient carboxylic acids in certain marine animals using phenylacetic acid as a model compound. ^{14}C-Phenylacetylcoenzyme A, the active intermediate in amino acid conjugation of phenylacetic acid (Biochim. Biophys. Acta, 24: 654, 1957) was used to study metabolism *in vitro*. Species studied were the flounder *(Pseudopleuronectes americanus)*, little skate *(Raja erinacea)*, and dogfish shark *(Squalus acanthias)*. Washed mitochondria, washed microsomes, and soluble fraction (microsomal supernatant) were prepared from the 600 × g supernatant of liver and kidney homogenates (using the conventional method established for mammals). Succinate cytochrome c reductase activity was assayed by standard methods (Methods of Biochemical Analysis, 4: 307, 1957) in each subcellular fraction as a mitochondrial marker enzyme. Measurable activity was found only in the fraction referred to as "mitochondria." Glycine and glutathione N-acyltransferase activities were assayed by incubating aqueous solutions of ^{14}C-phenylacetylcoenzyme A and glycine or glutathione with buffered mitochondrial suspension or soluble fraction, pH 8.3 at 30° for 12 to 60 minutes. The reaction was stopped by addition of 40 percent aqueous trichloroacetic acid and samples were then extracted with ethyl acetate. Phenylacetic acid, phenylacetylglycine, and enzymic reaction product of glutathione and phenacetylcoenzyme A were extracted into the ethyl acetate leaving unchanged phenylacetylcoenzyme A in the aqueous phase. The ethyl acetate extracts were dried over anhydrous sodium sulphate and aliquots chromatographed on Whatman No. 1 paper developed in solvent I (Methylethylketone : Water : glacial acetic acid = 200 : 95 : 0.4, v/v, organic phase). Chromatography showed that no phenylacetylglycine was synthesized from phenylacetylcoenzyme A and glycine in the presence of liver, kidney mitochondria or soluble fraction for any of the three species tested. In the absence of added glutathione, phenylacetylcoenzyme A was metabolized by marine kidney mitochondria to products which could be extracted into ethyl acetate (Table 1). In the presence of glutathione and dogfish kidney mitochondria, phenylacetylcoenzyme A was metabolized rapidly to ethyl acetate-soluble products, the major metabolite having $R_f = 0.05$ in solvent I. After incubation of phenylacetylcoenzyme A with liver mitochondria or liver or kidney soluble fraction the major component of the ethyl acetate phase was phenylacetic acid (>80 percent) in all three species.

Sodium ^{14}C-phenylacetate in distilled water (50 mg/kg + 5 μCi ^{14}C) was injected IV into two flounder and one dogfish shark which had their ureters catheterized for collection of urine. Urine was collected for 72 hours after injection of the phenylacetate and counted for radioactive content. A postmortem sample of flounder bile was counted for ^{14}C and bile from the dogfish shark was collected continuously for 72 hours by biliary cannulation and counted for ^{14}C content.

Most of the ^{14}C recovered was excreted in urine in the 0-48 hour period by both species. Less than five percent of the dose of ^{14}C-phenylacetate was found in the postmortem sample of flounder bile or in the total collection of dogfish bile. Paper chromatograms of flounder and dogfish shark urine in solvent I showed one metabolit at $R_f = 0.08$ and flounder urine showed an additional small peak at $R_f = 0.95$, which is the R_f of standard phenylacetic acid. Chromatograms of flounder and dogfish shark urine developed in solvent II (n-butanol : acetic acid : water - 2: 1 : 1, v/v) had one metabolite peak at $R_f = 0.57$ and flounder urine also had a small peak at $R_f = 0.90$ which is the R_f of phenylacetic acid. The metabolite, with an $R_f = 0.08$ in solvent I and $R_f = 0.57$ in solvent II,

TABLE 1

METABOLISM OF PHENYLACETYL COENZYME A BY KIDNEY MITOCHONDRIA

Species	Glycine present	Glutathione present	Incubation time (mins)	nmoles extracted into ethyl acetate/ min/mg protein	% ^{14}C in ethyl acetate phase as		
					Phenylacetic acid	Metabolite I	Metabolite II
Pseudopleuronectes americanus	+	-	30	2.4	60	20	12
	-	-	30	2.4	55	23	20
Raja erinacea	+	-	15	3.4	35	3	60
	-	-	15	4.0	15	25	60
Squalus acanthias	+	-	30	2	65	10	25
	-	-	30	2	65	10	25
	-	+	12	8	45	53	2

R_f values in solvent I are as follows: Phenylacetic acid = 0.95, Phenylacetylglycine = 0.89, Metabolite I = 0.08, Metabolite II = 0.80.

was not phenylacetylglycine and has not yet been identified.

These initial studies indicate that certain marine animals handle phenylacetic acid differently from most mammalian species. Parenterally administered phenylacetic acid is excreted slowly by fish in urine and to a lesser extent in bile as an unidentified metabolite. Metabolism of phenylacetyl-coenzyme A as measured by the formation of ethyl acetate soluble products occurs most readily in kidney mitochondria in the presence of exogenous glutathione. We hope to identify the unknown compounds which are presumably amino acid or peptide conjugates, and to study further peptide conjugation in marine species.

1973 #25

THE EVOLUTION OF A FACILITATED DIFFUSION PATHWAY FOR AMIDES IN THE VERTEBRATE ERYTHROCYTE

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There is increasing evidence that urea movement across cell membranes may involve more than simple diffusion. This applies not only to urea reabsorption by the kidney and toad bladder but to urea movement across the erythrocyte membrane. Macey and Farmer have shown for example that phloretin blocks the entry of urea into the human erythrocyte but has no effect on osmotic water flow or the entry of a number of other solutes (Biochim. Biophys. Acta, 211, 104-106, 1970). They concluded that urea movement across the human erythrocyte membrane was by facilitated or carrier-mediated diffusion.

We have surveyed the erythrocytes of representative vertebrates, from hagfish to man, to determine whether amide movement takes place by simple diffusion or facilitated diffusion. Two criteria were used in this study: 1) the relative rates of entry of urea and its more lipophilic analogue, acetamide; 2) the presence or absence of an inhibitory effect of 6×10^{-4} M phloretin. A more rapid rate of entry of acetamide and absence of an inhibitory effect of phloretin would be consistent with simple diffusion, while more rapid entry of urea and inhibition of entry by phloretin would suggest facilitated diffusion.

Osmotic hemolysis of erythrocytes at 5°C was used to determine the rate of amide entry in the presence and absence of phloretin. 0.02 to 0.1 ml of an erythrocyte pellet was introduced into a solution containing urea or acetamide at concentrations of 0.15 to 2.0 M and after a time interval of 0.5 to 40 minutes the suspension was centrifuged and the extent of hemolysis of the supernatant was read in a spectrophotometer at 510 mμ. Under the conditions of these experiments the rate of hemolysis is determined by the rate of entry of urea or acetamide (Am. J. Physiol., 224, 1109-1115, 1973).

Our findings are summarized in Table 1. We would conclude from this study that amide movement across the erythrocyte membrane occurs by simple diffusion in representatives of the Agnatha, Osteichthyes, Chondrichthyes, and Aves. Facilitated diffusion develops at the level of the Amphibia