it is not clear at present what rate the Na^+-dependent Ca^{2+} influx plays in activation of tension. Therefore, if the counter-transporter is to play a physiological role in Ca^{2+} homiostasis, it has to be in sequestration of Ca^{2+}. However, radio isotropic studies (C. Van Breemann et al., in this issue of Bull.) suggests that sequestration of Ca^{2+} is primarily handled by the SR rather than the Na-dependent counter transport of Ca^{2+} across the surface membrane.

CHANGES IN THE HEPATIC CYTOCHROME P-450 DEPENDENT MONOOXYGENASE SYSTEM OF WINTER FLOUNDER FOLLOWING TREATMENT WITH POLYCYCLIC AROMATIC HYDROCARBONS

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Marked variability in hepatic monooxygenase activities of winter flounder caught in the area of Mt. Desert Island was first reported by this lab (Bend et al., MDIBL Bull., 17: 47, 1977). We have now characterized 2 subpopulations of flounder based on individual hepatic levels of 7-ethoxyresorufin deethylase activity (7-ERF), aryl hydrocarbon hydroxylase activity (AHH), and response of hepatic AHH to in vitro 7,8-benzoflavone (ANF). One subpopulation has high hepatic 7-ERF and AHH activities, the latter which are inhibited by ANF; the other subpopulation has low hepatic 7-ERF and AHH activities, their AHH activity being stimulated by ANF. In mammals, studies have shown that these elevated activities (AHH and 7-ERF) are correlated with the presence of a certain cytochrome species (cytochrome $P\text{-}450_1$ or cytochrome P-448) whose formation is a response to exposure to a specific class of inducing compounds which includes polycyclic aromatic hydrocarbons (PAH), dioxin derivatives and certain PCBs and PBBs.

The objective of this report is to investigate the possibility that these high cytochrome $P\text{-}450_1$ dependent activities found in native flounder could also be the result of exposure to pollutants of the PAH class. To accomplish this, comparisons were made between untreated flounder and flounder which were treated with the PAH-type inducers 1,2,3,4-dibenzanthracene (DBA) and 5,6-benzoflavone (BNF).

METHODS

Animal capture, storage conditions, and tissue preparation (liver) were as described earlier (Bend et al., MDIBL Bull., 19, 11, 1979) as were the assays for 7-ERF activity and AHH activity in the presence and absence of ANF. Glutathione transferase (G-T) activity was assayed according to Booth et al., Biochem. J., 79: 516, 1961, at 30°C with 1 mM of 2,4-dinitrochlorobenzene (DNCB) as substrate and 5 mM glutathione as co-substrate.

That portion of flounder gut from just distal to the pyloric sphincter to proximal to the anal sphincter was removed and washed with at least 20 ml of cold isotonic KCl buffer (1.15% KCl, 1.25 mM HEPES buffer at pH 7 6). The washed intestines were then cut open lengthwise, the mucosa scraped off and homogenized (33% w/v) in the isotonic KCl buffer. Whole homogenate was used as the enzyme source for all assays except the cytosolic G-T where 100,000 g supernatant fractions were used.

Sodium dodecyl sulfate (SDS) gel electrophoresis was done according to Laemmli (Nature, 227: 680, 1970), using 7.5% polyacrylamide gels (PAG) with a 3% stacking gel. Samples were stained in 0.115% Commassie Brilliant Blue for at least 45 minutes prior to destaining in 25% ethanol/8% acetic acid.

Treatment of fish was by IP injection of either a single dose of BNF at 50 mg/kg in corn oil (13 mg/ml) or 3 doses, 24 hours apart, of DBA at 10 mg/kg also in corn oil (8 mg/ml). The animals were sacrificed either 9 or 11 days after the initial injection.

RESULTS

Electrophoresis of SDS treated hepatic microsomes from various flounder was performed. Figure 1 shows an

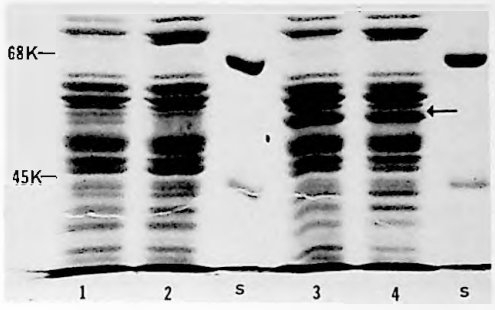

Figure 1.--SDS Polyacrylamide gel electrophoresis of hepatic microsomes from untreated, DBA treated,[1] and BNF treated[2] winter flounder. Standards are 5 μg each of BSA (mol. wt. 68K) and ovalbumin (mol. wt. 45K). Arrow indicates area of 57,000 mol. wt. 225 μg of microsomal protein was applied to each channel. Hepatic AHH and 7-ERF activities and whether the microsomes are from untreated or treated flounder are as follows:

channel	fish status	AHH	7-ERF
		1.6[3]	170[3]
1	untreated		
2	untreated	0.7	106
3	treated (BNF)	13.0	1787
4	treated (DBA)	10.6	1147
s	standards		

[1]-10 mg/kg of 1,2,3,4-dibenzanthracene; see text for details.

[2]-50 mg/kg of 5,6-benzoflavone; see text for details.

[3]-activity in FU/min/mg protein or pmoles resorufin/min/ mg protein.

electrophoretic pattern obtained with hepatic microsomes from 2 untreated flounder (nos. 1 and 2), a flounder treated (see methods) with BNF (no. 3), and a flounder treated with DBA (no. 4). An intensely staining band, molecular weight approximately 57000, was observed in the microsomes from flounder treated with DBA or BNF (nos 3 and 4). A faint band of the same molecular weight is observed in both of the untreated flounder (nos. 1 and 2). The presence and staining intensity of the band correlates with the AHH activity of the liver homogenates from these fish, 1.6, 0.7, 13.0 and 10.6 FU/min/mg protein for nos. 1-4 respectively. Thus, the electrophoretic band was present, but faint, in the untreated flounder with low hepatic AHH activity (nos. 1 and 2) and was a major band in the flounder treated with BNF (no. 3) or DBA (no. 4) whose AHH activities were quite high. 7-ERF values also parallel this trend. Similar data was obtained when hepatic microsomes from untreated winter flounder with divergent AHH activities were subjected to SDS-PAG electrophoresis as shown in Figure 2. Microsomes were electrophoresed in order of increasing AHH activity from left to right (from 1.09 to 6.08 FU/min/mg homogenate protein). The band of approximate molecular weight 57,000 (see arrow) appeared to become more prominent as the activity of the liver homogenates increased.

This polypeptide band is probably an inducible form of cytochrome P-450 since it was readily apparent in a DBA-treated flounder (channel 2, Figure 3) with elevated AHH and 7-ERF activities but was barely visible in hepatic microsomes from a flounder whose homogenate had very low AHH activity and no detectable 7-ERF activity (channel 1, Figure 3). Carbon monoxide reduced difference absorbance spectra of microsomes from untreated winter flounder livers have demonstrated a correlation between elevated AHH activity and the degree of spectral shift towards a maximum of 448 nm, the wavelength of maximum absorbance (in mammals) of a cytochrome species induced in response to the presence of PAH-type compounds (Bend et al., MDIBL Bull., 19, 111, 1979). It has also been demonstrated that a substantial amount of cytochrome P-448 was present in livers of PAH treated skates

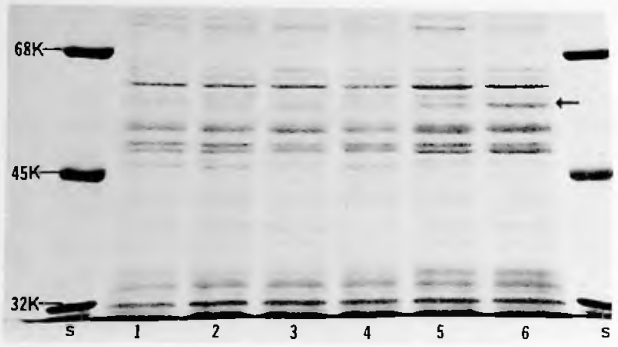

Figure 2.--SDS polyacrylamide gel electrophoresis of hepatic microsomes from untreated winter flounder. Standards are 5μg each BSA (mol. wt. 68K), ovalbumin (mol. wt. 45K) and carbonic anhydrase (mol. wt. 32K). Arrow indicates area of 57K mol. wt. All sample channels contain 50 μg of hepatic microsomal protein. AHH and 7-ERF activity of each flounder is as follows:

channel no.	AHH	7-ERF
	[1]	[1]
1	1.09	150
2	1.66	134
3	2.01	133
4	3.45	261
5	3.89	300
6	6.08	430
s	standards	

[1]-activity in FU/min/mg protein or pmoles resorufin/min/mg protein

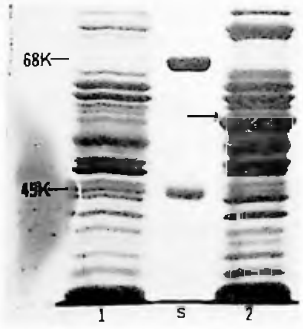

Figure 3.--SDS polyacrylamide gel electrophoresis of hepatic microsomes from an untreated and DBA treated[1] winter flounder. Standards (s) are 5 ug each BSA (mol. wt.= 68K) and ovalbumin (mol. wt.= 45K). Sample channels each contain 225 ug of microsomal protein, AHH and 7-ERF activities and treatment status are below by channel no.:

channel	fish status	AHH	7-ERF
		[2]	[3]
1	untreated	0.31	0
2	DBA treated	4.00	1608
s	standards		

[1]-10 mg/kg of 1,2,3,4-dibenzanthracene; see text for details

[2]-activity in FU/min/mg protein or pmoles resorufin/min/mg protein.

[3]-activity less than 2 pmoles resorufin formed/min/mg protein.

but not in untreated skates (Bend et al., Pesticide and Xenobiotic Metabolism in Aquatic Organisms, 297, 1979). Elcombe et al., (Pesticide and Xenobiotic Metabolism in Aquatic Organisms, 319, 1979), have demonstrated the appearance of a polypeptide at 57,000 daltons in hepatic microsomes from BNF and Arochlor 1242 treated rainbow trout; these compounds also increased the activity of several cytochrome $P\text{-}450_1$ dependent enzyme activities in the livers of these fish.

Table 1 shows typical data from 4 untreated flounder and 3 fish that were administered DBA; included are

Table 1.--Benzo(a)pyrene Hydroxylase (AHH) and 7-Ethoxyresorufin Deethylase Activities in Untreated and DBA Treated[1] Winter Flounder; Effect of *in vitro* 7,8-Benzoflavone (ANF) on Hepatic and Intestinal AHH Activities

Fish No.	Treatment	Hepatic AHH		Hepatic 7-ERF	Gut AHH		Gut 7-ERF
		w/o ANF	w ANF		w/o ANF	w ANF	
90	--	6.02[2]	1.18	748[3]	0[4]	0	0
74	--	4.53	2.71	725	0.170	1.290	0
61	--	0.72	0.31	135	0.010	0.017	0
82	--	0.13	0.34	40	0.110	1.010	0
7	DBA	10.6	1.60	1147	0	0	0
10	DBA	7.70	1.50	630	0.260	1.140	0
1	DBA	4.0	1.30	1608	0	0.026	0

[1] 10 mg/kg of 1,2,3,4-dibenzanthracene (DBA) was injected IP in 8 mg/ml corn oil for 3 successive days, 24 hr. apart. Sacrifice was on the 9th day for nos. 7 and 10 and on day 11 for no. 1.

[2] AHH in FU/min/mg homogenate protein

[3] 7-ERF in pmoles resorufin formed/min/mg homogenate protein

[4] assayed, but not detectable (AHH activity less than 0.009 FU/min/mg protein; 7-ERF activity less than 2 pmoles/min/mg protein).

7-ERF activities and AHH activities in the presence and absence of ANF of liver and gut mucosal homogenates. Fish nos. 90, 74, and 61 were quite similar to the DBA-induced flounder (nos. 7, 10 and 1); they all had elevated hepatic 7-ERF activities and AHH activities which were inhibited by ANF. In contrast, the hepatic AHH activity of fish no. 82 was low and was stimulated nearly 3-fold by ANF. The hepatic 7-ERF for fish no. 82 was also the lowest of any of the fish tabulated. These relationships of hepatic AHH activity, hepatic 7-ERF activity, and alterations of AHH activity in the presence of ANF were confirmed by analysis of large numbers of flounder as reported earlier (Bend et al., MDIBL Bull., 18, 60, 1978).

Gut AHH activities of these flounder (Table 1) demonstrated both a lack of correlation with hepatic activities and a difference from liver in terms of response to ANF. For example, in the fish with the highest hepatic AHH activity (no. 90), gut AHH was not detectable (i.e., below minimum detectable activity which was 0.009 FU/min/mg protein). Even though fish nos. 74 and 82 had quite similar gut AHH activities (0.17 and 0.11 FU/min/mg protein) the hepatic AHH activities differed markedly (4.53 vs 0.13 FU/min/mg protein respectively). Analysis of the intestinal mucosa from 48 individual flounder guts for AHH activity showed that, when measurable, activities ranged from 0.01 to 0.49 FU/min/mg protein. There was no correlation between liver and gut AHH activities ($r = 0.05$, $n = 48$). In all instances where gut activity was detected, it was enhanced by ANF. The magnitude of enhancement varied markedly as demonstrated in Table 1. Flounder no. 61 had barely measurable activity in gut which was only slightly elevated by ANF (0.010 to 0.017 FU/min/mg protein). In contrast, gut activity in fish numbers 74 and 82 was enhanced 8- and 9-fold respectively. In several instances, the initial gut activities were enhanced up to 30-fold by ANF. It should also be noted that 7-ERF activity was not present in gut mucosal homogenate from any flounder examined (detection limit was 2 pmoles/min/mg protein).

This lack of relationship between gut and hepatic AHH activities also held for DBA-treated flounder. With the treatment regime used, no induction of intestinal AHH or 7-ERF activity was observed in DBA-treated flounder (Table 1, nos. 1, 7, and 10) which had induced hepatic AHH and 7-ERF activities.

Glutathione transferase activity was measured in livers from 34 individual flounder. The average activity was 1.69 ± 0.77 μmole DNCB conjugated/min/mg cytosolic protein. There was considerable heterogeneity in these fish, specific activities ranged from 0.29 to 3.66. However, there was no correlation between hepatic G-T activity and hepatic AHH activity in these flounder ($r = 0.10$, $N = 34$).

Summary

The presence of an intensely staining polypeptide band (molecular wt. approximately 57,000) upon SDS-PAGE of hepatic microsomes from winter flounder treated with BNF or DBA is demonstrated. The staining intensity of this band in microsomes from untreated and DBA or BNF induced flounder livers appeared to correlate with hepatic AHH and 7-ERF activities. This result strongly suggests that many of the winter flounder captured near Main have induced hepatic monooxygenase systems as a result of exposure to PAH-type inducers present in their natural habitat.

UREA TRANSPORT IN EPITHELIA STUDIED BY AN AUTORADIOGRAPHIC TECHNIQUE

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In man, and in a variety of animal species, the establishment of urea gradients across renal epithelial cells plays an important role in the urinary concentration mechanism. The determination of the exact intracellular and extracellular distribution of urea by conventional techniques is difficult. We have worked for several years to develop autoradiographic techniques to determine the pathway for urea transport across the toad urinary bladder, using the technique of Stirling and Kinter (J. Cell Biol., 35: 585, 1967). A problem with these early studies was the exposure of Epon sections containing the isotopically labeled tissue to water during the sectioning step, with the resulting loss of counts. We have recently employed a modification of the original method developed by Masland and Mills (J. Cell Biol. 83: 159, 1979), in which sections are transferred in a dry state from the microtome to emulsion-coated slides. Toad bladders were mounted on plastic rings, and their luminal surfaces exposed to ^{14}C urea for one minute. The bladders were then snap-frozen in liquid freon at the temperature of liquid nitrogen. They were freeze-dried for 3 days and embedded in Epon. Two micron sections were cut and transferred dry to slides coated with emulsion, and exposed for 5 days at 4°. Grain counts in the luminal bathing medium were compared to those within epithelial cells. The cell-to-medium ratio was 0.57±0.05, a value significantly different from 1.0 ($p<0.01$). Thus, the apical cell membrane is a significant barrier to urea. Following vasopressin, there was a consistent increase in cell-to-medium ratio of ^{14}C urea, with a mean value of 1.02. However, the latter value is regarded as tentative, since back-diffusion from the serosal medium may have contributed to the intracellular counts following vasopressin. The demonstration of a gradient for urea across the luminal membrane in the absence of vasopressin offers support for the usefulness of the autoradiographic technique in the study of solute transport. This work was supported by NIH grant AM-03858.