Influence of Environmental Contamination on Cytochrome P-450 Mixed-Function Oxygenases in Fish: Implications for Recovery in the Wild Harbor Marsh[1]

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Levels of hepatic cytochrome P-450 and the catalytic functions benzo[*a*]pyrene hydroxylase and aminopyrine demethylase were significantly greater in populations of mummichog, *Fundulus heteroclitus*, sampled from contaminated marshes than in those fish from nearby reference marshes. Aminopyrene demethylase, but not benzo[*a*]pyrene hydroxylase, was also elevated in fish from one of the reference marshes. The levels of cytochrome P-450 and benzo[*a*]pyrene hydroxylase were highest in fish from the Wild Harbor Marsh, site of the 1969 *Florida* spill. A correlation between size and benzo[*a*]pyrene hydroxylase activity was identified in some of the populations studied, although influence by this factor did not mask the population differences. The results are consistent with the hypothesis that elevated levels of mixed-function oxygenase activity in fish from Wild Harbor are the result of oil spilled 8 yr ago, and that recovery of this marsh is not yet complete.

Key words: mixed-function oxygenases, benzo[*a*]pyrene hydroxylase, aminopyrine demethylase, cytochrome P-450, *Fundulus heteroclitus*, environmental contamination, oil spill recovery, petroleum

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Les niveaux de cytochrome P-450 hépatique et les fonctions catalytiques benzo[a]pyrène hydroxylase et aminopyrène déméthylase sont nettement plus élevés dans les populations de le choquemort, *Fundulus heteroclitus*, échantillonnés dans des marais contaminés par comparaison avec ceux de poissons provenant de marais témoins. Dans un des marais témoins, l'aminopyrène déméthylase est élevée, mais la benzo[*a*]pyrène hydroxylase ne l'est pas. Les niveaux maximums de cytochrome P-450 et de benzo[*a*]pyrène hydroxylase se rencontrent dans les poissons du marais de Wild Harbor, site du déversement du *Florida* de 1969. Dans quelques-unes des populations étudiées, nous avons établi une corrélation entre la taille et l'activité du benzo[*a*]pyrène hydroxylase, bien que l'influence de ce facteur ne masque pas les différences entre les populations. Ces résultats sont conformes à l'hypothèse que les niveaux élevés d'activité des oxygénases à fonction mixte chez les poissons de Wild Harbor sont le résultat d'un déversement de pétrole qui se produisit il y a 8 ans, et que ce marais ne s'est pas encore rétabli.

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CYTOCHROME P-450 is a generic name given to a family of hemoproteins which exhibit Soret-absorption maxima at or near 450 nm in the CO-bound reduced state and which are involved in foreign-compound metabolism. Microsomal cytochrome P-450 dependent mixed-function oxygenase systems are perhaps ubiquitous in vertebrate species, including fishes. Fish hepatic microsomal cytochrome P-450 possesses optical and magnetic properties qualitatively similar to the better known cytochromes P-450 from mammals; i.e. reduced, CO-ligated fish cytochrome P-450 exhibits a Soret-absorption maximum near 450 nm, and EPR (electron paramagnetic resonance) spectra of fish cytochrome P-450 exhibit low-spin *g* values near 2.4, 2.24, and 1.9 (Chevion et al. 1977). As with mammalian systems, biotransformation of a great variety of substrates is catalyzed by cytochrome P-450 systems in fish, ranging from endogenous compounds such as steroid hormones to foreign compounds such as aromatic hydrocarbons

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(Inano et al. 1976; Diamond and Clark 1970).

Treatment of fish with selected compounds such as 5,6-benzoflavone or the polynuclear aromatic hydrocarbon 3-methylcholanthrene will, as in mammals, induce increased levels of hepatic benzo[*a*]pyrene hydroxylase activity (Bend et al. 1973; Chevion et al. 1977). Treatment with petroleum can produce a similar induction (Payne and Penrose 1975; Vandermuelen et al. 1978) and it is this feature of cytochrome P-450 systems in fish that lends itself to potential use as an indicator of effects of environmental contamination by petroleum hydrocarbons and other organic pollutants.

This report is concerned with the hepatic mixed-function oxygenase activities of benzo[*a*]pyrene hydroxylase and aminopyrine demethylase in populations of *Fundulus heteroclitus* (killifish or mummichog) from several sites, including the Wild Harbor Marsh, an area severely contaminated by a highly aromatic No. 2 fuel oil spilled from the *Florida* in 1969. *Fundulus* were selected for this study as they are common in marshes and estuaries where they reside in a fairly restricted area (Lotrich 1975) and thus reliably reflect the environment from which they are sampled. The results are considered generally as they relate to the potential use of mixed-function oxygenases as environmental indicators, and more specifically as they relate to the extent of biological recovery evident in Wild Harbor Marsh 8 yr after the oil spill.

Materials and Methods

Fundulus were sampled by seining in each of four marshes on Cape Cod, Mass.: Monomoscoy, a small marsh on Monomoscoy Island bordering the East River in Waquoit Bay; Little Sippewissett, Great Sippewissett, and Wild Harbor, each bordering Buzzards Bay. Wild Harbor was chosen as a petroleum-contaminated site. Great Sippewissett was also selected as a contaminated site, a portion of this marsh having been experimentally treated for the past 6 yr with sludge fertilizer containing high levels of chlorinated and petroleum-derived hydrocarbons (Krebs et al. 1974). Monomoscoy and Little Sippewissett were chosen as reference marshes, with no known sources of organic contamination.

Fish were collected from approximately the same locations each time in Wild Harbor and Little Sippewissett, and exactly the same locations in Monomoscoy and Great Sippewissett. The animals were returned to the laboratory alive and in most cases maintained in their own water until killing and analysis which took place within 24 hr. At each sampling fish were grouped according to sex and size; i.e. a given group contained fish that were all ±10% of the group mean body weight. In all cases groups were analyzed at least in pairs.

Whole livers from fish in each group were pooled (excluding any individuals parasitized or with punctured gallbladders), homogenized 20% wt/vol in 0.065 M phosphate buffer, pH 7.3, containing 3 mM $MgCl_2$ and 1.15% KCl, and a 9000 × *g* supernatant (postmitochondrial supernatant; PMS) fraction prepared. PMS preparations were diluted to 2.0–3.0 mg protein/mL and cytochrome P-450 was analyzed optically in CO-bound, dithionite-reduced samples vs. CO-bound reference, with a Cary 118C recording spectrophotometer. Cytochrome content was determined using an $OD_{450-490}$ extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$ (Omura and Sato 1964).

Benzo[*a*]pyrene hydroxylase was determined in a 1.0-mL reaction mixture containing 5 μmol NADP (Sigma), 15 μmol G-6-P (Sigma), 2 U G6PD (Sigma), 3×10^{-3} M $MgCl_2$, and 1.5–2.0 mg PMS protein, in 0.1 M phosphate. The pH of the reaction mixture was 7.0. The generating system was allowed to preincubate for 15 min. The reaction was initiated by addition of 15.6 μg of benzo[*a*]pyrene (Aldrich) in 40 μL of methanol and incubated in a shaking water bath at 29°C for 30 min. These conditions were determined to be optimal in this laboratory. The reaction was stopped and the hydroxylated product was extracted and assayed fluorometrically in a procedure similar to that of Nebert and Gelboin (1968), with authentic 3-OH-benzo[*a*]pyrene as a reference standard.

Aminopyrine demethylase was assayed in a 1.5-mL reaction mixture containing an NADPH generating system and PMS protein as above, in 0.166 M HEPES (Sigma), pH 7.6. The reaction was initiated by adding twice recrystallized aminopyrine, incubated at 29°C for 15 min, and stopped by adding 0.5 mL $ZnSO_4$ (25% saturated) and 0.5 mL BaOH (saturated). Formaldehyde was determined according to Nash (1953) as modified by Cochin and Axelrod (1959). Formaldehyde standards were incubated and treated as samples. Controls for benzo[*a*]pyrene hydroxylase and aminopyrine demethylase consisted of reactions carried out without the NADPH generating system.

Protein was determined according to Lowry et al. (1951), with bovine serum albumin as a standard. All analyses were performed in duplicate, and data were analyzed by regression analysis and standard *t*-tests performed on pooled variance estimates (Sokal and Rolf 1969).

Results

As indicated in Table 1, fish from each marsh were sampled in 1976 and 1977, and in this report data from the 2 yr are considered together. The numbers of males and females were equivalent and the range of sizes sampled was more than fivefold in each marsh, yet the mean size of animals in all groups did not differ significantly from marsh to marsh. Although animals were grouped by sex as well as size, the data presented are means for all groups within a marsh unless otherwise indicated.

The PMS protein content per gram of liver appeared to be somewhat lower in the fish from Little Sippewissett than in the other marshes (Table 1). However, the variance was rather high in all cases and the differences between marshes were not significant.

BENZO[*a*]PYRENE HYDROXYLASE

With Monomoscoy as the primary reference, benzo[*a*]pyrene hydroxylase activity per milligram of PMS protein (Table 2) exhibited a trend to greater activity in fish from contaminated marshes, yet the differences were not significant, perhaps a feature related to the high variance in PMS protein content. When the data were normalized to liver weight, the

TABLE 1. Sites, dates, and sizes sampled, and hepatic PMS protein in *Fundulus heteroclitus*.

Marsh	*N* fish	*N* groups (♂–♀)	Dates sampled	$\bar{x}$ size (± SD) (g)	Size range (g)	$\frac{\text{mg PMS protein}}{\text{g liver}}$
Monomoscoy (reference)	64	6 (3–3)	July 1976 Aug. 1977	7.61 (±3.14)	2.60–13.23	93.2 (±16.5)
Little Sippewissett (reference)	49	8 (4–4)	July, Oct. 1976 July 1977	8.55 (±6.48)	2.78–25.57	81.6 (±12.7)
Great Sippewissett (contaminated)	53	7 (4–3)	July 1976 July, Aug. 1977	6.26 (±2.79)	1.90–14.19	92.5 (±18.7)
Wild Harbor (contaminated)	149	23 (11–12)	July, Oct. 1976 June, July, Aug. 1977	8.92 (±4.32)	2.94–17.74	97.1 (±19.0)

TABLE 2. Hepatic benzo[*a*]pyrene hydroxylase and aminopyrine demethylase activity in *F. heteroclitus*.

Marsh	$\frac{\text{Units}}{\text{mg PMS protein}}$	$\frac{\text{Units}}{\text{g liver}}$ (×10³)	$\frac{\text{Units}}{\text{g body weight}}$
	Benzo[a]pyrene hydroxylase^{a}		
Monomoscoy	89.2 (±28.4)^{c}	7.58 (±1.72)	143.3 (±36.0)
Little Sippewissett	73.8 (±32.9)	5.72 (±1.43)	178.3 (±63.6)
Great Sippewissett	101.1 (±19.2)	9.00 (±1.00)^{d}	246.7 (±40.7)^{i}
Wild Harbor	108.8 (±31.5)	10.15 (±2.29)^{f}	278.8 (±70.9)^{j}
	Aminopyrine demethylase^{b}		
Monomoscoy	16.4 (±4.8)	1.43 (±0.62)	27.8 (±13.8)
Little Sippewissett	44.6 (±4.2)^{j}	4.16 (±0.24)^{i}	105.7 (±11.0)^{j}
Great Sippewissett	25.7 (±6.9)^{d}	2.12 (±0.15)^{e}	57.0 (±7.5)^{h}
Wild Harbor	36.4 (±11.4)^{g}	3.72 (±0.77)^{i}	95.0 (±41.8)^{g}

[a] Units = picomoles 3-OH-benzo[*a*]pyrene equivalents per minute.
[b] Units = nanomoles HCHO produced per hour.
[c] Values in parentheses are ± standard deviation.
[d–j] Significantly different from the primary reference (Monomoscoy) at $0.10 > P > 0.05$(d); $P \leq 0.05$(e); $0.02 > P > 0.01$(f); $P \leq 0.01$(g); $P \leq 0.005$(h); $P \leq 0.001$(i); $P \leq 0.0001$(j).

extent of difference between fish from contaminated and uncontaminated marshes remained about the same, although a degree of significance was acquired. However, when normalized to body weight the activity in fish from contaminated marshes was almost twice that in fish from Monomoscoy and the differences were very highly significant. Fish from the two uncontaminated marshes were essentially identical.

The results expressed in Table 2 are means obtained from animals grouped in a wide range of size-classes. Plotting the activity of benzo[*a*]pyrene hydroxylase per gram of body weight in these groups against size discloses a relationship between the two. This relationship is depicted in Fig. 1 for fish from Monomoscoy. Similar results were obtained for fish from a contaminated marsh (Fig. 2), although in this instance the intercept was much greater, while the slope remained essentially the same. Thus the differences in benzo[*a*]pyrene hydroxylase activity between fish from these two marshes become even more pronounced when the data are expressed this way, yet the nature of the relationship between size and activity appears to remain the same in fish from both marshes. The relationship appearing in fish from these two marshes was consistently observed in each pair of groups sampled. The high correlation coefficients and the fact that the points represent fish sampled in different years make this relationship appear even more striking.

In contrast, fish from Wild Harbor Marsh did not exhibit the same relationship between size and activity ($a = 251.8$, $b = 3.16$, $r = 0.189$, $P = 0.200$). There was a high degree of randomness associated with the data here and even in pairs of groups taken from the same seine no such relationship was evident. However, the distinction between Monomoscoy and Wild Harbor fish remains quite obvious and if anything the randomness appearing in Wild Harbor suggests the influence of a disturbing factor, possibly related to the oil, not present in Monomoscoy or even in Great Sippewissett.

Most groups of fish in Little Sippewissett displayed a size–activity relationship like the one seen in Monomoscoy fish, with a similar slope and intercept ($a =$

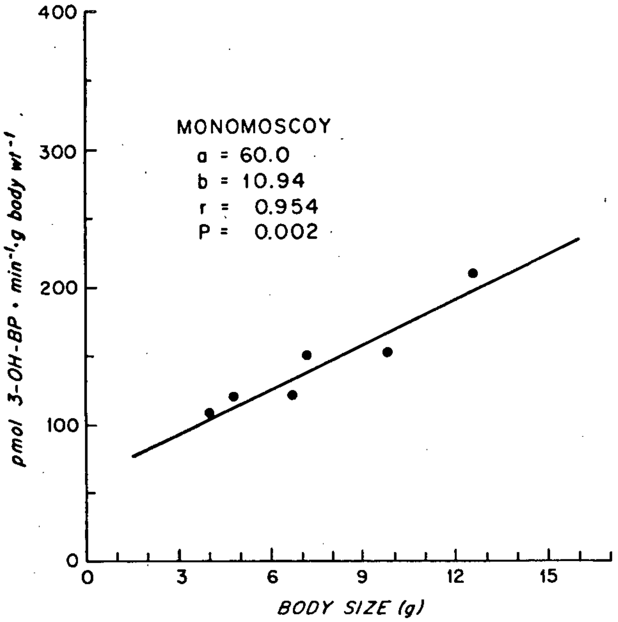

FIG. 1. Relationship between hepatic benzo[*a*]pyrene hydroxylase activity and size in *Fundulus heteroclitus* from Monomoscoy. a = Intercept; b = slope; r = correlation coefficient; P = significance of the correlation. The assumption of linearity to the intercept is not necessarily valid.

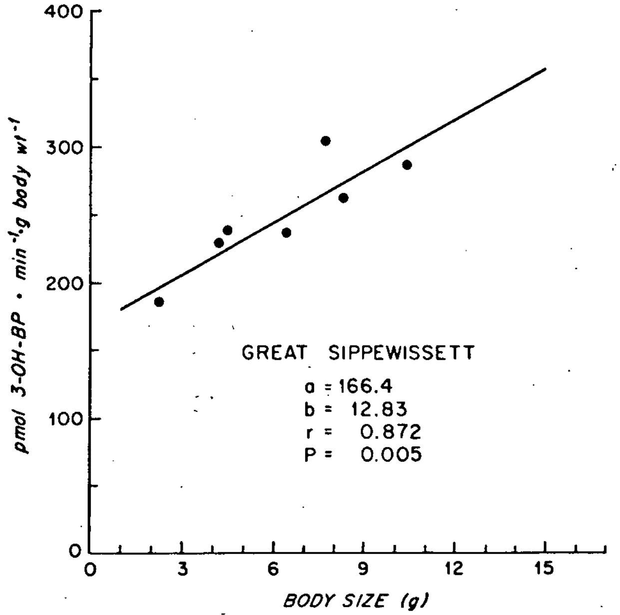

FIG. 2. Relationship between hepatic benzo[*a*]pyrene hydroxylase activity and size in *F. heteroclitus* from Great Sippewissett. a = Intercept; b = slope; r = correlation coefficient; P = significance of the correlation.

74.3, b = 8.54, r = 0.954, P = 0.004). There were, however, other groups in this marsh that did not fit this pattern, for reasons that are not apparent.

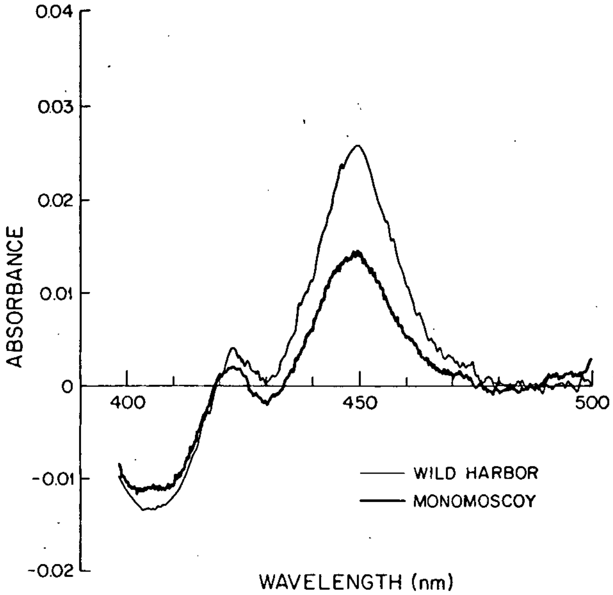

FIG. 3. Carbon monoxide difference spectra of hepatic PMS prepared from *F. heteroclitus*. Fish used in these determinations were adult males sampled after the spawning season. Spectra were determined in 0.1 M phosphate buffer, pH 7.0. Protein concentration in these determinations was 2.75 mg/mL in the Wild Harbor sample and 2.87 mg/mL in the Monomoscoy sample.

There were differences in mixed-function oxygenase activity linked to sex differences and seasonal variation in addition to size, similar to those differences described for other species (Stegeman 1977). These factors did not, however, affect the differences seen between populations.

AMINOPYRINE DEMETHYLASE

The activity of hepatic aminopyrine demethylase was significantly greater in Wild Harbor and Great Sippewissett fish than in those from the primary reference marsh, Monomoscoy. This was true whether the data were normalized to PMS protein, liver weight, or body weight (Table 2). Unlike the situation observed with benzo[*a*]pyrene hydroxylase, however, there was also significantly greater aminopyrine demethylase activity in the fish from Little Sippewissett. In fact, fish from this marsh possessed the greatest demethylase activity. There was no evident relationship between body size and the activity of aminopyrine demethylase in fish from any marsh.

CYTOCHROME P-450

The optical properties of the cytochrome P-450 complement in the PMS fractions displayed qualitative similarity in all groups of fish, with a peak absorption in the Soret region at 450 nm (e.g., Fig. 3). As suggested in Table 3, however, there were quantitative dif-

TABLE 3. Hepatic cytochrome P-450 content in *F. heteroclitus.*

Marsh	P-450[a] / mg PMS protein	P-450 / g liver	P-450 / g body weight
Monomoscoy	0.055 (±0.006)[b]	5.11 (±1.19)	0.099 (±0.043)
Little Sippewissett	0.064 (±0.016)	5.06 (±0.63)	0.156 (±0.032)[d]
Great Sippewissett	0.066 (±0.016)	5.94 (±1.46)	0.164 (±0.054)[c]
Wild Harbor	0.072 (±0.016)[d]	6.70 (±1.25)[e]	0.189 (±0.067)[f]

[a] Nanomoles of cytochrome P-450.

[b] ± Standard deviation.

[c–f] Significantly different from the primary reference (Monomoscoy) at $P \leq 0.04$(c); $P \leq 0.02$(d); $P \leq 0.01$(e); $P \leq 0.005$(f).

ferences in cytochrome P-450 among fish from the various marshes. Only in fish from Wild Harbor was the content of hepatic cytochrome P-450 per milligram of PMS protein or normalized to liver weight significantly greater than in fish from Monomoscoy. However, when normalized to body weight, the content of hepatic cytochrome P-450 was significantly greater in fish from Great Sippewissett and Little Sippewissett, in addition to Wild Harbor.

Based on a rough estimate of turnover number (i.e. activity per unit P-450), it appears that the cytochrome P-450 complement in Wild Harbor fish, for example, is no more efficient at catalyzing benzo[*a*]pyrene hydroxylation than is the cytochrome complement in Monomoscoy fish. Thus while there is a greater benzo[*a*]pyrene hydroxylase activity in fish from the contaminated marshes, it may be a feature of the higher levels of cytochrome P-450. On the other hand, the higher aminopyrine demethylase activities in Wild Harbor and Little Sippewissett fish appear to be associated not just with increased cytochrome P-450 content but also with a cytochrome complement that is more efficient at catalyzing this reaction.

Discussion

The results presented here describe elevated levels of benzo[*a*]pyrene hydroxylase, aminopyrine demethylase, and cytochrome P-450 in *Fundulus heteroclitus* sampled from areas with known sources of organic contaminants, in comparison with fish from ostensibly clean areas. Previous reports dealing with putative induction of fish mixed-function oxygenases in the environment have usually involved a single sampling of animals at a given site, with analysis individually or pooled (Burns 1976; Payne 1976; Pederson et al. 1976; Stegeman and Sabo 1976). In this study there was repeated sampling, extending over a period of years, and the differences observed were highly significant.

The appearance of higher benzo[*a*]pyrene hydroxylase activity is consistent with other recent reports suggesting that levels of this mixed-function oxygenase activity can be linked to environmental contamination (Payne 1976; Pederson et al. 1976; Stegeman and Sabo 1976). This may result from induction of benzo[*a*]pyrene hydroxylase by environmental contaminants similar to that observed to occur upon treatment of some fish species with known "Type II" inducers (Bend et al. 1973; Chevion et al. 1977; Pederson et al. 1976) or with the complex mixtures of compounds that make up petroleum (Payne and Penrose 1975). The areas sampled in Wild Harbor and Great Sippewissett are characterized by high levels of aromatic and, in the case of Great Sippewissett, chlorinated hydrocarbons which could well be acting as such inducers. However, the observation that differences in benzo[*a*]pyrene hydroxylase were most significant when activity was normalized to body weight implies that increased liver size may also be important in conferring an increased capacity for this, and possibly other hydrocarbon biotransformation activities, on these animals.

Aminopyrine demethylase activity, like benzo[*a*]pyrene hydroxylase, was found to be higher in fish from the contaminated marshes. Interestingly, the demethylase activity was also very high in animals from Little Sippewissett, one of the marshes selected as being uncontaminated and where there was low benzo[*a*]pyrene hydroxylase activity. The reason for this discrepancy is unknown, although the possibility clearly exists that some unsuspected contaminant(s) may be inducing this mixed-function oxygenase activity in Little Sippewissett fish. PCB's, as an example, have been successfully used to induce aminopyrine demethylase in at least one species of fish (Hill et al. 1976), and the possibility of chlorinated hydrocarbons, or other compounds, entering the marsh from nearby homesites cannot be excluded. Similar circumstances may be working to produce the apparent scatter in benzo[*a*]pyrene hydroxylase in these fish.

It is well known that benzo[*a*]pyrene hydroxylase and *N*-demethylase activities can be differentially induced by selected compounds in mammals, and that this involves different cytochromes P-450 (Lu et al. 1972). Furthermore, it has been found in at least one species of fish that the "Type II" inducer 3-methylcholanthrene will induce benzo[*a*]pyrene hydroxylase activity but not benzphetamine demethylase (Bend et al. 1977). Thus, a lack of correlation between benzo[*a*]pyrene hydroxylase and aminopyrine demethylase activities in *Fundulus* from Little Sippewissett need not be surprising, and suggests the possible involvement of multiple cytochromes P-450 in this species.

A higher cytochrome P-450 content was observed in conjunction with either higher hydroxylase or demethylase activities. The differences seen here were on the same order of magnitude as the differences in cytochrome P-450 content found by Burns (1976) in *Fundulus* sampled from Wild Harbor, Great Sippewissett, and selected uncontaminated marshes, although in that report significance was not determined. If the higher activities are due to induction, then this result is unusual for it stands in sharp contrast to reports describing induction of mixed-function oxygenases in fish, particularly of benzo[*a*]pyrene hydroxylase, without any apparent increase in cytochrome P-450 content (Bend et al. 1977; Chevion et al. 1977).

Aside from the increased levels, there were no differences seen in the optical properties of cytochrome P-450, a situation similar to that described by Burns (1976) for these fish. Induction of benzo[*a*]pyrene hydroxylase by "Type II" inducers in many mammalian species is accompanied by a shift in the absorption maximum of the cytochrome P-450 complement, due to an increase of a cytochrome with different spectral properties (Alvares et al. 1967). While this was initially thought not to be the case in fish (Chevion et al. 1977), the appearance of different spectral forms of cytochrome P-450 upon induction has been determined for little skate, *Raja erinacea*, although this was only after resolution of the cytochromes (Elmamlouk et al. 1977). A similar situation may occur in other cases, including the present one.

The results presented here support the contention that fish mixed-function oxygenases may in some circumstances be used as indicators of environmental contamination by foreign compounds including, but in no way restricted to, some fraction of petroleum. The results also suggest that it may not be sufficient to use a single catalytic function as an indicator. Unknown factors, such as are possibly at work in the Little Sippewissett Marsh or in trout sampled by Pederson et al. (1976), may influence interpretation of results. This includes the possibility that contamination may eventually produce lesions resulting in decreased mixed-function oxygenase activities (Ahokas et al. 1976). Clearly work is required to further clarify those circumstances, probably limited, under which use of mixed-function oxygenases as indicators may be validly interpreted.

Nevertheless, in the case of Wild Harbor, the comparatively greater levels of benzo[*a*]pyrene hydroxylase, aminopyrine demethylase, aldrin epoxidase (Burns 1976), and cytochrome P-450 in *Fundulus* from this marsh strongly suggest that a relationship exists between these higher mixed-function oxygenase activities and the fuel-oil hydrocarbons and that these fish still show effects 8 yr after the spill. This is in agreement with the demonstration that substantial amounts of aromatic hydrocarbons remain in Wild Harbor (Teal et al. 1978) and that other members of the biota there are still being affected (Krebs and Burns 1977). It is not possible, however, to distinguish between whether the high mixed-function oxygenase activities observed currently result from a contemporary event, possibly induction, or from a selection for this condition. Similarly, we do not know what fractions of petroleum or petroleum products may be involved, nor whether apparent induction identified in vitro indicates enhanced biotransformation and elimination of hydrocarbons in vivo.

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