SALMON LIPID STORAGE SITES AND THEIR ROLE IN CONTAMINATION WITH WATER-SOLUBLE PETROLEUM MATERIALS

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ABSTRACT

The Braer tanker wreck demonstrated the danger of petroleum spills to salmon farms. The water soluble fraction (WSF) of crude petroleum oils is quite different from the parent oils. It is rich in methyl- and alkyl-substituted monoaromatic and lower molecular weight polyaromatic hydrocarbons, guaranteeing strong petroleum flavor. Both n-alkanes above C_9 and more complex hydrocarbons above the methylnaphthalenes are virtually absent. The WSF in salmon intercellular fluids is rapidly depurated but a portion dissolves into the adipocyte cells in the salmon muscle and is retained much longer. The distribution of adipocytes along the myosepta of white muscle and in the belly flaps has been defined by histochemistry and their isolation by collagenase, centrifugation, and flotation is described. In dark muscle small fat droplets are instead found among and within the cells of muscle fibers.

INTRODUCTION

Fish tainted by the water-soluble fraction (WSF) of petroleum hydrocarbons are unmarketable and must be held in clean water until the objectional flavor disappears. It is generally accepted that the species-specific differences in bioaccumulation and release of hydrocarbons are related to the amount of tissue lipids and therefore species with higher lipid contents have a higher potential for tainting by hydrocarbons (Hebert and Keenleyside 1995). Muscle is the major lipid storage site in Atlantic salmon but the distribution of lipids is uneven. Lipids present in muscle adipocytes located in the connective tissue (myosepta) have a different composition from cellular membrane lipids. The adipocyte lipids are

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basically triacylglycerol and are functionally different from lipids inside white muscle (WM) fibres (Zhou *et al.* 1995). Unfortunately, there has been only limited work looking at these two different lipid pools and their potential role in tainting of aquatic species. The literature on the accumulation and release of hydrocarbons in salmon (Heras *et al.* 1993) and other species has been based on whole tissue analysis. The objective of the present research was to study the uptake and depuration of the WSF in Atlantic salmon by analysis of blood, white muscle and adipocytes in an attempt to better understand some of the variables which affect the potential for hydrocarbon uptake.

MATERIALS AND METHODS

Exposure of Salmon to WSF

Flotta North Sea crude petroleum was stirred with cold seawater in a ratio of 1:99 (v/v) for 24 h followed by 48 h of settlement to obtain a WSF stock solution of about 4 to 6 ppm total hydrocarbon. Market-sized Atlantic salmon (*Salmo salar*, average weight 2434 g), previously acclimated to the exposure tank, were starved for 24 h before conducting the experiment. Eighteen salmon were placed in a 2 m diameter tank (water depth 30 cm) and exposed to 0.2 ppm WSF for 96 h, followed by a depuration period of 20 days. The WSF exposure concentration was chosen from the results of pilot studies. Water temperature was 4.5 - 6.5C, photoperiod was 12 h light, 12 h dark. Water samples of both head tank and exposure tank were taken twice daily for hydrocarbon analysis. Control fish were kept in clean seawater under the same conditions. During depuration, fish were taken at different time intervals. Adipocytes, blood, and white muscle (WM) were analyzed for their hydrocarbon and lipid contents. All exposure experiments were done at the Aquatron Laboratory of Dalhousie University, Halifax.

Fish Analysis

Fish were transported alive in chilled seawater to the Institute laboratory at the Technical University of Nova Scotia. The fish were anaesthetized and blood was taken from the tail. Fish were then killed by a blow on the head, gutted, and washed with cold water. Several portions of dorsal WM were excised, pooled, and frozen at -35C for hydrocarbon and lipid analyses. To isolate adipocytes, the belly flaps (free of muscle fibers) were cut into small pieces and digested by collagenase in Krebs-Ringer phosphate buffer containing 1% albumin. Digestion was done in a water bath for 1 h at 30C under an O_2 atmosphere. The suspension of adipocytes was strained through a 350 μm metal mesh and centrifuged at 100 × g for 30 s. The adipocyte upper layer was washed once with buffer. To separate free fat from adipocytes, dialysis tubing was filled with the same buffer and the adipocyte suspension was loaded below a clamp at the bottom. On release, fat droplets rose

faster than adipocytes, allowing their separation. Purified adipocytes were frozen in a block and held at -35C until analysis.

Hydrocarbons from WM, blood and adipocytes were recovered by steam distillation (Ackman and Noble 1973), with modifications to recover hydrocarbons in dichloromethane, and analyzed by gas chromatography (GC) (Heras *et al.* 1992). The lipid content in each WM sample was determined gravimetrically by $CHCl_3$-MeOH extraction according to Bligh and Dyer (1959). The percentages of hydrocarbons accumulated in adipocytes of WM were calculated assuming that adipocytes from WM and belly flap had similar hydrocarbon accumulations and metabolism, and that the membrane cellular lipids from WM were about 0.7% w/w, the minimum for this species (Polvi and Ackman 1992).

RESULTS

The dissection of fish showed major sections of WM separated by white bands called myosepta. These were cut out, fixed, sectioned and stained with Oil-Red-O. Figure 1 shows that the white muscle cells were essentially free of adipocytes, which were clustered along the collagen fibres of the myosepta. Other tissues such as the belly flaps and the mesentery tissue were found to be the main tissues for adipocyte accumulation (Fig. 2).

FIG. 1. SECTION OF SALMON WHITE MUSCLE SHOWING MYOSEPTA SEPARATING TWO SETS OF DIFFERENT MUSCLE FIBRES

Staining with Oil-Red-O reveals adipocytes loosely attached along the connective tissue and the general absence of such adipocytes elsewhere.

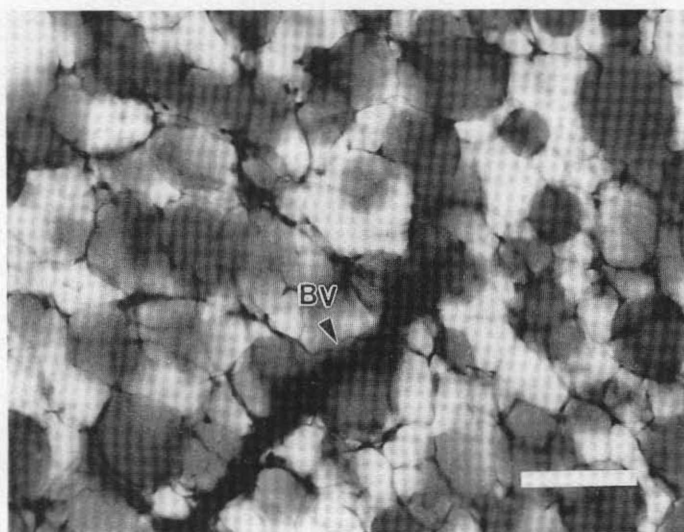

FIG. 2. LIGHT MICROGRAPH OF ADIPOCYTE DISTRIBUTION IN BELLY FLAPS OF ATLANTIC SALMON (FROZEN SECTION, OIL-RED-O STAINING) SHOWING HIGH DENSITY OF ADIPOCYTES AND A TYPICAL BLOOD VESSEL (BV)
Bar = 100 μm.

Uptake

The WSF was mainly composed of single ring aromatic hydrocarbons and only traces of higher *n*-alkanes were detected. Moderate concentrations of naphthalene and methylnaphthalenes in the stock solution were also observed, but virtually no hydrocarbons of higher molecular weight. There was no mortality during the 96 h exposure, but fish exhibited a decreased food intake after the exposure compared to the control fish, probably due to stress from handling. However, lipid contents of WM sampled at different depuration stages did not show any significant difference (Table 1). About 40% of the WM lipids were found in myosepta, mostly inside adipocytes. The technique of digestion-centrifugation-flotation applied in our laboratory allowed us to purify large quantities of fish adipocytes. Both WM and purified adipocytes accumulated hydrocarbons, but adipocytes accumulated 59.4 ppm with a bioaccumulation factor (BAF) of 297.2, while WM cells proper accumulated only 4.15 ppm hydrocarbons with a BAF of 10.1 (Fig. 3A). The hydrocarbon content of adipocytes accounted for 54% of the WM total at time zero. Blood hydrocarbon levels at this time totalled 14.3 ppm (8.2 ppm in plasma and 6.1 ppm in blood cells).

TABLE 1.
LIPID CONTENTS OF WHITE MUSCLE (WM) AND CORRESPONDING ISOLATES OF ADIPOCYTES OF MYOSEPTA IN WM DURING DEPURATION (w/w%)

	Depuration time (days)				
	0	1	4	10	20
WM (%)	4.48	3.11	4.81	4.55	3.25
Myosepta[a]	42.0	35.3	41.8	38.1	37.1

[a] Fraction of lipids stored in myosepta from the total lipids of WM (% w/w).

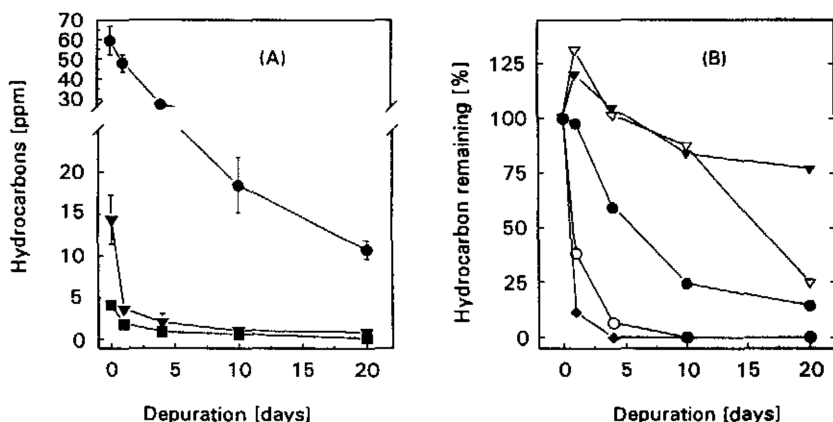

FIG. 3. (A) RELEASE OF HYDROCARBONS FROM ADIPOCYTES, WHITE MUSCLE (WM) AND BLOOD DURING 20 DAYS OF DEPURATION; ● = ADIPOCYTE, ▼ = BLOOD, ■ = WM (B) DEPURATION OF DIFFERENT HYDROCARBONS FROM ADIPOCYTES; ♦ = BENZENE, O = TOLUENE, ● = ETHYLBENZENE + XYLENES, ∇ = PROPYLBENZENES + TRIMETHYLBENZENES + ETHYLMETHYLBENZENES, ▼ = METHYLNAPHTHALENES

Depuration

The depuration rates of hydrocarbons from adipocytes and WM showed important differences (Fig. 3A). Hydrocarbons from WM were released faster than those from inside adipocytes. The WM rapid-release phase lasted one day with a hydrocarbon loss of 55% of the total originally accumulated. Adipocytes released only 19% of their hydrocarbon during the same period, with a depuration rate at least three times slower. The second phase of depuration (between days 1 and 4) was slower and showed a similar rate in both WM and adipocytes (21%/day and 18%/day, respectively). The role of adipocytes in WM became evident here and virtually 100% of retained hydrocarbons were stored inside adipocytes. During the remaining depuration period rates were around 5% per day for the two tissue types. Blood hydrocarbon levels showed a two-phase depuration profile similar to the

TABLE 2.
BIOACCUMULATION FACTOR[a], DISCRIMINATION FACTOR[b], AND HALF-LIVES OF HYDROCARBONS IN WHITE MUSCLE (WM) AND ADIPOCYTES ISOLATED FROM MUSCLE TISSUE OF ATLANTIC SALMON AFTER 96 H EXPOSURE TO 0.2 PPM WSF

	Factors at Day Zero					
	Bioaccumulation		Discrimination		Half-life (days)	
Hydrocarbon	WM	Adipocytes	WM	Adipocytes	WM	Adipocytes
Benzene	4.4	97.0	0.21	0.32	0.50	0.57
Methylcyclohexane	30.6	481.0	1.43	1.57	2.50	12.0
Toluene	10.8	232.4	0.50	0.76	0.61	0.81
Ethylbenzene	26.0	428.6	1.22	1.40	0.81	3.9
Xylenes	47.0	570.5	2.19	1.86	0.87	6.8
Isopropylbenzene	20.2	267.2	0.94	0.87	3.30	17.0
Propylbenzene	36.0	427.0	1.68	1.39	3.80	18.0
Ethyl-methylbenzenes	50.7	523.3	2.37	1.70	2.50	15.0
Trimethylbenzenes	73.7	737.7	3.44	2.40	2.90	16.0
Methylnaphthalenes	228.2	2973.5	10.65	9.69	3.50	>20

[a] Ratio between hydrocarbon concentration in WM or adipocytes and exposure water.
[b] Calculated as the concentration ratio of each hydrocarbon to the total hydrocarbon in WM or adipocytes divided by the equivalent ratio in the exposure water.

WM. It is interesting to note that after 20 days of depuration, WM and blood were almost clean and had less than 0.5 ppm of hydrocarbon each, while as much as 10.7 ppm of hydrocarbons was still present in adipocytes.

Depuration of Individual Hydrocarbons

Accumulation of hydrocarbons in adipocytes was much higher than in WM (Table 2), and increased with both the ring substitution by alkyl groups and number of aromatic rings. Thus, BAF of methylnaphthalenes was 23 times higher than their one ring counterpart (toluene) in WM and about 13 times higher in adipocytes. The BAF of methylcyclohexane was unusual, higher in both WM and adipocytes than was found for aromatic hydrocarbons with similar molecular weights. The WM showed more selective properties for the accumulation of low molecular weight hydrocarbons (Table 2, discrimination factor) than did the adipocytes. The depuration of individual hydrocarbons from adipocytes was related to their alkyl substitution and number of aromatic rings (Fig. 3B). All hydrocarbons in WM were released faster than in adipocytes. The general pattern of depuration was a fast release from WM at the beginning of depuration, while for adipocytes only benzene and toluene showed this characteristic (Fig. 3B). As a result, half lives of all hydrocarbons in WM were below 4 days, much shorter than those in the adipocytes (Table 2). Blood hydrocarbon depuration showed a pattern similar to that of WM.

DISCUSSION

Petroleum hydrocarbons in fish have no biochemical functions, hence their deposition levels in different compartments are primarily determined by their lipid-water partition coefficients and diffusion processes (Neely *et al.* 1974), not by biological selectivity. Therefore, the kinetics of bioaccumulation and release from tissues are mainly controlled by the physicochemical properties of the cells and the circulation of fluids around them. Adipocytes of myosepta can be described as a lipid globule compartment (mostly triacylglycerol) surrounded by a thin layer of an external hydrophobic envelope (cell membrane) and of water (cytoplasm); these restrict the exchange of substances. On the other hand, the non-adipocyte compartment of WM and non-adipocyte compartment of blood are similar to each other; both are mainly composed of water, lack lipids stored inside cells, and posses hydrophobic environments created by membranes and some areas of protein. Membranes are a key factor in the partitioning of hydrocarbons, and partitioning is not affected by changes in their composition (Boryslawskyj *et al.* 1988). Diffusion is probably the limiting factor controlling the accumulation rate.

When fish were returned to hydrocarbon-free seawater, the lipid/water partition favoured a gradual release of hydrocarbons from tissue to water. The non-

adipocyte pool of hydrocarbons, mainly composed of low molecular weight hydrocarbons, was responsible for the rapid decrease of hydrocarbons observed on the first day of depuration as they were transported by blood to gills for excretion, or to liver for metabolism by the cytochrome P450 system (Fig. 3A). The muscle of cod and scallops is lean and provides a compartment very similar to the non-adipocyte compartment of salmon. The rapid uptake (minutes-hours) and fast depuration (a few days) of the hydrocarbons of this compartment in those two species (Ernst *et al.* 1987; Ernst *et al.* 1989) confirms its characteristics as similar to WM. Adipocytes on the other hand, played the most important role in the uptake and long term release of hydrocarbons in salmon. Although they showed a fast depuration during the first day, it was not as fast as WM and was mostly attributed to the release of the more water-soluble hydrocarbons such as benzene and toluene (Fig. 3B) while the less soluble hydrocarbons showed a slower release during depuration. Possibly the smaller hydrocarbon molecules can also pass more freely than bulkier molecules through the phospholipids of membranes. Adipocytes became the only storage site of remaining hydrocarbons in less than four days of depuration, and after that the slow depuration from the whole white muscle of the fish was a reflection of the depuration of these adipocytes (Fig. 3). Movement of hydrocarbon from one compartment to another depends on the concentration ratio of tissue to water, on diffusion, and also depends on the flow rate of blood through the tissue. Blood hydrocarbon levels were higher than WM because blood collected hydrocarbons from other tissues as well, but showed a similar patten of depuration. The large differences in the bioaccumulation and release of hydrocarbons between the adipocyte and non-adipocyte compartments confirm that the species-specific characteristics of the accumulation, and the retention of hydrocarbons, are actually dependent on the lipid content, storage site and location in the tissue.

Theoretically, depuration would be the reverse process of accumulation, but this does not mean that the rates of accumulation and depuration are the same because the hydrocarbons in the single lipid droplet of the adipocyte must diffuse first to the surface and then pass through the surrounding membrane to the cytoplasmic environment. Hydrocarbons from the surface of some adipocytes could diffuse first into other adipocytes before moving into capillaries because in most myosepta the volume of adipocytes is larger than that of blood capillaries. The latter may in fact be somewhat remote from the adipocytes (Zhou *et al.* 1996). This would create a concentration gradient expected to be smaller for the reverse diffusion process. In addition, lipid to water partition coefficients of hydrocarbons favour the retention in the lipid environment, i.e., lipoprotein membranes and adipocytes. This might be an important factor controlling depuration. Increasing the number of aromatic rings and substitutions on the rings favored the bioaccumulation of individual hydrocarbons in both adipocytes and WM tissue, and decreased their depuration rates. Once high molecular weight hydrocarbons become associated with lipids inside adipocytes, they may take months to depurate. The selective

accumulation of hydrocarbons is the result of the combined effects of a detoxification by catabolism and diffusion of individual hydrocarbons according to their solubility in water. The cytochrome P450 system oxidizes hydrocarbons and produces more polar metabolites easily excreted by gills or bile. Detoxification starts as soon as the P450 is induced by hydrocarbons (George *et al.* 1995) and continues for as long as hydrocarbons are present. This process would act first on the low molecular weight hydrocarbons, and leads to an apparent increase in the accumulation of higher molecular weight molecules. The exposure time was long enough to reach an equilibrium on the uptake-excretion process for the low molecular weight hydrocarbons.

The histology revealed numerous very small lipid droplets in and around the muscle cells of dark muscle. This observation corresponds to the droplets reported by Ingemansson *et al.* (1991) in dark muscle of rainbow trout (*Oncorhynchus mykiss*) and, in general, accounts for the dark muscle of fish reputedly having more lipid than white muscle (Ackman and Eaton 1971). Presumably some of the above arguments apply to this type of depot fat but salmon dark muscle remains to be investigated.

There is a concern that with a longer exposure there would be an increase in the accumulation of larger aromatic molecules above their "tainting" thresholds. Polycyclic aromatic hydrocarbons, such as methylnaphthalenes, were still present in adipocytes when the Institute sensory taste panel did not show significant differences between tainted and control fish after 10 days of depuration. Fatter salmon may take months to depurate (Ritchie and O'Sullivan 1994). This must be brought to the full attention of regulatory bodies because the carcinogenic properties and the persistent retention of high molecular weight hydrocarbons in adipocytes may pose potential health hazards to consumers of marine foods previously exposed to the WSF of petroleum and petroleum products no longer obvious on the surface of the ocean.

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