Coassimilation of dietary fat and benzo(a)pyrene in the small intestine: an absorption model using the killifish

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Abstract Benzo(a)pyrene (BP) was dissolved in dietary fat and fed in a single dose to killifish (*Fundulus heteroclitus*). Fluorescence microscopic examinations of small intestinal content and frozen sections of whole small intestine revealed that during fat digestion BP was codispersed in liquid crystalline product phases produced during lipolysis (1979. Patton, J. S., and M. C. Carey, *Science.* **204:** 145-148) and then coabsorbed with dietary lipid followed by its reappearance in intracellular fat droplets. During the time that the absorbed fat remained in the enterocytes, BP fluorescence was initially concentrated in the intracellular fat droplets and then spread throughout the cytosol of the enterocytes. Tissue analyses showed that BP was rapidly metabolized in the intestine and transported to the gallbladder. These studies show that separation of a dissolved hydrophobic carcinogen from dietary fat occurs primarily after the fat has been digested, dispersed, absorbed, and reassembled in the enterocyte. The inability of the enterocyte to discriminate between dietary fat and dissolved carcinogenic compounds may be a partial explanation of the observed link between high fat diets and the incidence of some cancers. In vertebrates, the intestine and not the liver, appears to be the major site of metabolism of dietary polycyclic aromatic hydrocarbons (PAHs). — **Vetter, R. D., M. C. Carey, and J. S. Patton.** Coassimilation of dietary fat and benzo(a)pyrene in the small intestine: an absorption model using the killifish. *J. Lipid Res.* 1985. **26:** 428-434.

Supplementary key words fluorescence microscopy • intestinal carcinogen metabolism

Fat (triglyceride) comprises over 40% of the calories in the U.S. diet (1). Epidemiologic studies of cancer in humans suggest, but do not prove, that high fat intake is associated with colon and breast cancers (2). A number of mechanisms have been put forth to explain the fat-cancer association, all of which may not be mutually exclusive. *a*) Dietary fat may act to nutritionally promote the development and growth of tumors induced by chemical carcinogens (3, 4). *b*) Fat may be oxidized prior to ingestion, either by becoming rancid or during cooking, and therefore may be contaminated with potentially carcinogenic products of fat oxidation (4, 5). Likewise, intracellular oxidation of fats may produce hydrogen peroxide (4) which may enhance the conversion of procarcinogens to active carcinogens (6). Finally, *c*), since many carcinogens are hydrophobic and tend to concentrate along the food chain in dietary fat, high fat diets may be diets with high carcinogen contents (7). Nitrosamines, aflatoxins, and polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene (BP) have been found in μg/kg concentrations in margarines, vegetable oils, and other sources of dietary fats (8-10).

It has long been known that dietary fat can enhance the intestinal absorption of bulky hydrophobic molecules such as cholesterol and long chain alkanes (11), and the literature is replete with studies in which crystalline hydrophobic drugs or xenobiotics are predissolved in triglyceride oils prior to oral delivery. Dietary fat contains a hydrophobic domain that is maintained during lipolysis and micellar dispersion of the lipolytic products (12). Although the molecular requirements and transport capacity of this hydrophobic domain are just beginning to be understood (13), it is clear that many drugs, natural products, and xenobiotics may be carried. Once dispersed into mixed lipolytic product-bile salt micelles and liquid crystalline vesicles, relatively high concentrations of hydrophobic chemicals can diffuse up to the microvillus membrane where they may be efficiently absorbed (14). However, in the absence of liquid crystalline lipolytic products (i.e., fatty acids and monoglycerides), PAHs, other nonpolar lipids (15), and cholesterol are poorly soluble in bile salt micelles (16-18). Thus the products of dietary fat digestion serve as a delivery vehicle for otherwise insoluble molecules and increase their bioavailability at the absorptive surface.

Abbreviations: BP, benzo(a)pyrene; PAH, polycyclic aromatic hydrocarbon(s); PC, phosphatidylcholine; IMM, intestinal microvillus membrane; TG, triglyceride; VLDL, very low density lipoproteins.

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Although intestinal BP absorption can be markedly enhanced by dissolving it in dietary fat (19), at some point during intestinal assimilation dietary lipid and BP separate and travel to different tissues. In rats only 10–20% of absorbed BP that was predissolved in dietary fat is recovered in the lymph (20, 21). Similarly, in the killifish, less than 2% of absorbed BP escapes the enterohepatic circulation (19). In both the rat and the killifish, the majority of BP coabsorbed with dietary fat was recovered in the bile (19, 21). Thus, the objective of this study was to determine the site of uncoupling of dietary lipid and BP during intestinal assimilation. The killifish was used because of its small size, ease of handling, and simple digestive system. Furthermore, the digestion and absorption of fat in the killifish intestine is similar to that in mammals (22).

MATERIALS

BP (gold label ~ 99.0% purity) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Radioactive [^{14}C]BP (16.3 mCi/mmol) was obtained from California Bionuclear (Sun Valley, CA), and its radiopurity was greater than 99+% following thin-layer chromatography on silica gel in a pure benzene solvent system. A-grade sodium taurodeoxycholate (>99%) was obtained from Calbiochem. Gum arabic, porcine pancreatic lipase (Type VI-S ca. 1500 tributyrin units/min per mg at pH 8.0 and 23°C), Sudan Black B (practical grade) and triolein (99%) were purchased from Sigma Chemical Co. (St. Louis, MO). Casein-derived (cas) amino acids were obtained from Difco Laboratories (Detroit, MI). Glutaraldehyde (50% biological grade) was obtained from Polysciences, Inc. (Warrington, PA). Knox brand gelatin was obtained from a local grocer and phosphatidylcholine (PC) was purified from egg yolk on an alumina-silicate column as previously described (23). All other materials were reagent grade or better.

METHODS

Fish

Killifish, *Fundulus heteroclitus,* were collected from tidal creeks on the grounds of the University of Georgia Marine Institute, Sapelo Island, CA. Fish were maintained in flowing sea water tanks and fed a diet of chopped fish and shrimp. All fish were studied within 1 week of capture. Experiments were conducted in the summer months at water temperatures between 25 and 27°C. Both sexes were employed, and all fish weighed between 7 and 9 grams. Killifish possess a simple digestive system consisting of a small intestine without diverticula. Both stomach and large intestine are absent.

BP and food

BP (35 μg) was fed to fish in a single piece of gelatin that weighed 35 ± 2 mg. The gelatin contained 18% triolein (in which the BP was predissolved, 35 μg of BP/6.3 mg of triolein), 2% PC, 10% Knox gelatin, 10% casamino acids, and 60% water. The lipid and half of the water were emulsified by sonication. The emulsion was added to the remaining solution of gelatin and amino acids that had been dissolved by heating. The resulting gel that formed upon cooling could be cut into sections and weighed. Sufficient radioactive BP was added to the food to give ca. 10^6 dpm per 35-mg piece of food. The molar ratio of BP to triolein was ca. 1:72. A standard curve of food weight versus radioactivity was linear.

Feeding

Fish were fed in small schools and each animal voluntarily consumed the entire portion. After 2 min each individual fed fish was transferred to its own 4-l glass aquarium. At timed intervals fish were anesthetized with tricaine methane-sulfonate (250 mg/l, Crescent Chemical, Paradise Valley, AZ), killed by decapitation, and samples were taken for chemical or microscopic analysis.

Time course of BP assimilation

A total of 16 fish were fed the BP-fat food and groups of 4 were killed at 4, 8, 12, and 24 hr. Intestinal content was obtained by flushing the intestine three times with 5 ml of 10 mM taurodeoxycholate in fish Ringers solution and a subsample was counted for radioactivity on a Beckman LS 9000 Liquid Scintillation counter. Intestine, liver, and gallbladder were solubilized in an NCS tissue solubilizer (Amersham, Arlington Heights, IL) and then counted.

Metabolism of BP in the intestine

A total of 48 fish were fed the BP-fat food and groups of 8 were killed at 2, 4, 8, 12, 24, and 48 hr. Intestines were flushed as described above and then homogenized in ethyl acetate–water 3:2 in a Polytron tissue homogenizer. The ethyl acetate phase was then withdrawn and the water phase was extracted twice more with ethyl acetate. The combined ethyl acetate fractions were concentrated under a stream of nitrogen, then chromatographed on silica gel plates in a solvent system of pure benzene (24). In this system, unmetabolized BP runs just below the solvent front, whereas the more polar BP metabolites separate between BP and the baseline. Extraction and separation of BP and its metabolites were carried out under nitrogen and in dim light.

Light microscopy

At 3, 8, 12, and 24 hr the intestines of fish fed the BP-fat food were frozen in liquid nitrogen, sectioned in a

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cryostat, and viewed with fluorescent illumination or Nomarsky optics. Intestinal contents were not removed and only sections of the proximal intestine were observed. Prior to adding the cover slip, a drop of fixative (2% glutaraldehyde, 5% glycerol, 150 mM NaCl) was placed on top of the sections.

Hydrolysis of BP-fat was observed on a microscope slide using the technique of Patton and Carey (25). Pancreatic lipase (10 μl containing 15 tributyrin units) in 100 mM Tris buffer (pH 8.0), 150 mM NaCl, 1 mM $CaCl_2$ was added to 5 μl of triolein emulsion containing BP (ca. 5 μg BP/mg triolein), and the reaction was observed by fluorescence microscopy. The emulsion was made by hand-shaking a 50:50 (v/v) mixture of triolein and 10% gum arabic in the Tris buffer.

RESULTS

Time course of BP assimilation

Fig. 1 shows the change with time in the relative recovery of BP radioactivity from intestinal contents, intestinal tissue, liver, and gallbladder. By 12 hr almost all luminal BP had been either absorbed or excreted in the feces (Fig. 1). The three tissues, intestine, liver, and gallbladder, together contained >98% of the total body radioactivity at all time points. The remainder of the fish contained <2% of the total body radioactivity. The percentage of total administered BP that was recovered in the fish by 12 hr averaged 50.3 ± 9.0 (n = 3, the fourth fish was anomolous with 2% absorbed). By 12 hr the majority of total body BP (ca. 60%) had moved out of the intestine to the gallbladder (Fig. 1). Renal excretion of BP metabolites, if any, was not monitored in this study. However, the aquaria water was analyzed after 12 hr and >98% of its BP radioactivity was associated with the glass fiber filter pad which contained the fecal material.

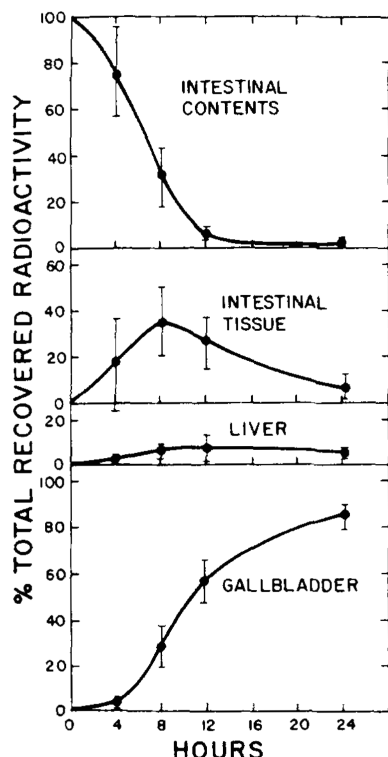

Fig. 1 The effect of time on the recovery of BP radioactivity (BP plus metabolites) from various compartments of the killifish enterohepatic system. Bars = ± 1 standard deviation (n = 4).

Metabolism of BP in the intestine

The effect of time after feeding on the percentage of absorbed intestinal BP that was metabolized is shown in **Table 1.** The percentage of unmetabolized BP dropped from ca. 84% at 2 hr following feeding to 0.3% at 48 hr. The percentage of lipid-soluble metabolites increased from 13% at 2 hr to 77% at 24 hr and then decreased to 22% at 48 hr. The percent of water-soluble metabolites increased from low values of 3-4% between 2 and 12 hr to 78% at 48 hr.

Microscopy

The partial digestion, in vitro, of a fat droplet containing fluorescent BP (5 μg of BP/mg of triolein) by pancreatic lipase after 20 min in the absence of bile salts is shown in **Fig. 2A.** The fluorescent undigested substrate (TG) is surrounded by pools of fluorescent viscous isotropic product phases (V). BP appears to have been quantitatively transferred into the product phases (V).[2]

The partial digestion, in vivo, of BP-free fat droplets (TG) in killifish intestine after 2 hr is shown in Fig. 2B. Here the frozen section was post-stained with Sudan Black B. Note the viscous isotropic pools of product phase (V) in the lumen (lu) and in close association with the mucosa (mu). Fluorescent BP droplets and product phases were also observed in intestinal content (not shown).

The appearance of absorbed BP in frozen sections of killifish intestine is shown in Fig. 2C and D. In a 3-hr fish, fluorescence is concentrated in the intracellular fat droplets while the lacteals (la) and the rest of the tissue is nonfluorescent. In an 8-hr fish, fluorescence appears not only in the intracellular fat droplets but also throughout the mucosa. In 24- and 48-hr fish (not shown), fluorescence was essentially absent from the intestine although a slight background remained; tiny bright specks of fluorescence were occasionally seen at the base of the epithelial cell layer.

[2]Similar results were seen with perylene, coronene, β-carotene, Sudan III, and Sudan Black B (unpublished observations).

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TABLE 1. Relative concentration of native BP and different classes of metabolites in the intestinal tissue of *Fundulus heteroclitus* after feeding BP in dietary fat

Time	Percent of Total Administered Activity Recovered in Intestinal Tissue	Distribution of ^{14}C Radioactivity between Metabolites		
		Percent Unmetabolized Benzo(a)pyrene	Percent Ethyl Acetate-Soluble Metabolites	Percent Water-Soluble Metabolites
hr				
2	29.7 ± 23.8[a]	84.2 ± 2.9	13.0 ± 2.7	3.4 ± 1.3
4	59.2 ± 20.4	62.3 ± 5.1	34.4 ± 5.8	3.4 ± 0.8
8	18.7 ± 10.3	57.0 ± 12.8	39.7 ± 15.9	3.4 ± 2.9
12	17.8 ± 2.9	48.3 ± 4.2	47.4 ± 3.0	4.2 ± 1.3
24	2.5 ± 3.5	2.3 ± 3.6	77.1 ± 9.4	20.3 ± 11.0
48	0.9 ± 0.5	0.3 ± 0.2	22.3 ± 6.8	77.7 ± 6.6

[a]Mean ± 1 standard deviation (n = eight fish per time period).

DISCUSSION

This study shows that fat and BP are codigested, codispersed, cotransported across the microvillus membrane, cotransported through the cell's cytosol, and coincorporated into newly synthesized fat droplets in enterocytes. Similar observations were made in birds and mammals with the fat-soluble azodye Sudan III (Mr 352) nearly 100 years ago (26, 27). In these early reports, Sudan III predissolved in dietary fat was shown to follow the fat into the enterocytes, lymph, milk, and other sites of fat accumulation in the body (27). However, unlike Sudan III, the majority of BP goes to the gallbladder and does not remain with the chylomicrons. This study extends the observations that were made with the Sudan III to PAH and suggests, in addition, that when uncoupling of hydrophobic solutes from dietary lipid occurs in the intestine, it happens at or near the surface of newly synthesized intracellular fat droplets (Fig. 2C). These observations provide a more detailed view of intestinal fat assimilation. In addition to the well known packaging of dietary fat into lipoproteins, the enterocyte also decontaminates dietary fat as much as possible by metabolizing hydrophobic xenobiotics into more polar metabolites that can then dissociate from the triglyceride.

The direct movement of BP (Fig. 2A) and other hydrophobic solutes from the undigested oil into the product phases of fat digestion supports the idea that there is a hydrophobic space or domain that is essentially continuous during lipolysis (12). Within this domain, dissolved hydrophobic molecules appear to flow freely from oil phase to liquid crystalline product phases. The dispersion of the product phases (i.e., digested fat) into mixed lipid-bile salt micelles and vesicles is a subsequent step in the coassimilation process (28). Although the total carrying capacity of the bile salt-dispersed lipid for hydrophobic molecules like BP is undoubtedly reduced relative to the undigested oil, without the liquid acyl chains of dietary lipid within bile salt micelles, the micellar solubility of BP drops over 50-fold (29).

How dietary lipids and molecules like BP move across the intestinal microvillus membrane (IMM) is poorly understood. According to prevailing theory (14) even the most insoluble hydrophobic molecules, when absorbed from the aqueous phase of intestinal content, are thought to do so as individual monomers. Presumably micellar vesicle dispersion of lipids permits high concentrations to come in close association with the absorptive membrane where rapid monomer flow and then membrane transport occurs. There is no good evidence yet for bulk membrane uptake of lipids (i.e., intact micelles). Thus, according to the monomer uptake hypothesis (14), the hydrophobic domain is destroyed prior to membrane transport. Although it was generally thought that lipid transport occurred passively through the lipid bilayer of the IMM (14), the IMM bilayer is one of the most viscous, high-melting membranes in the vertebrate body (30), and recent findings suggest that there is a specific fatty acid transport protein in the IMM (31). Whether or not this protein is involved with hydrophobic xenobiotic transport is unknown.

In the enterocyte the cytosolic transport of absorbed fatty acids and hydrophobic molecules like BP and its metabolites may be assisted by two proteins, fatty acid binding protein (FABP) and ligandin (32, 33), both of which appear to bind one molecule of BP (or fatty acid) with similar affinities (34). Both FABP and ligandin occur in killifish intestine (P. Van Veld, R. D. Vetter, and J. S. Patton, unpublished observations). The cellular concentrations of these two binding proteins have been estimated to be ca. 10^{-4} M (35) which is at least two orders of magnitude lower than the concentration of fatty acid that occurs in intestinal content during digestion of a fatty meal (36). Thus it is unclear whether or not fatty acids are transported through the cytosol as individual monomers or as small aggregates or micelles (with a hydrophobic domain for carrying molecules like BP).

The continuous diffusion of lipolytic products into the enterocyte is driven by the resynthesis of triglyceride which occurs in the endoplasmic reticulum (37). In fish,

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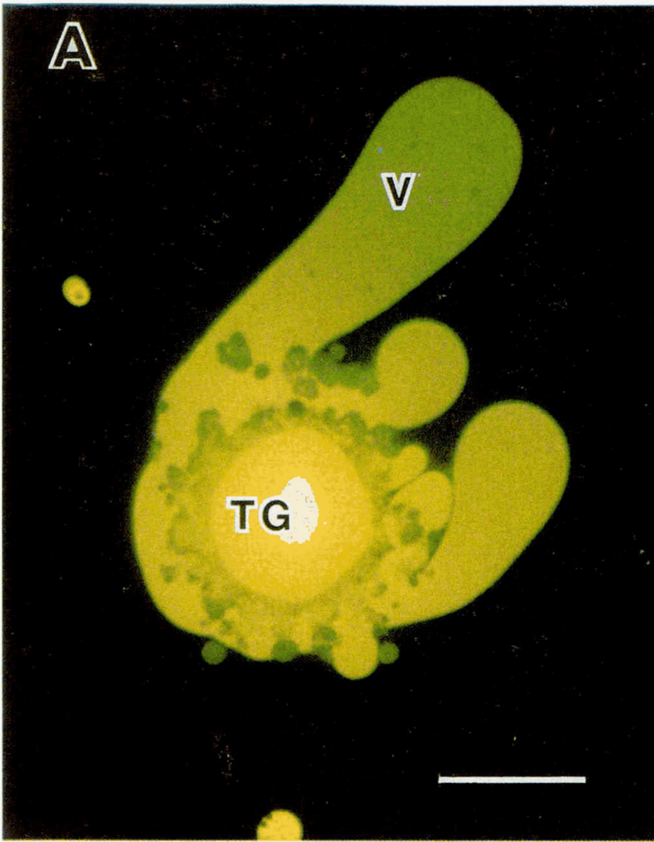

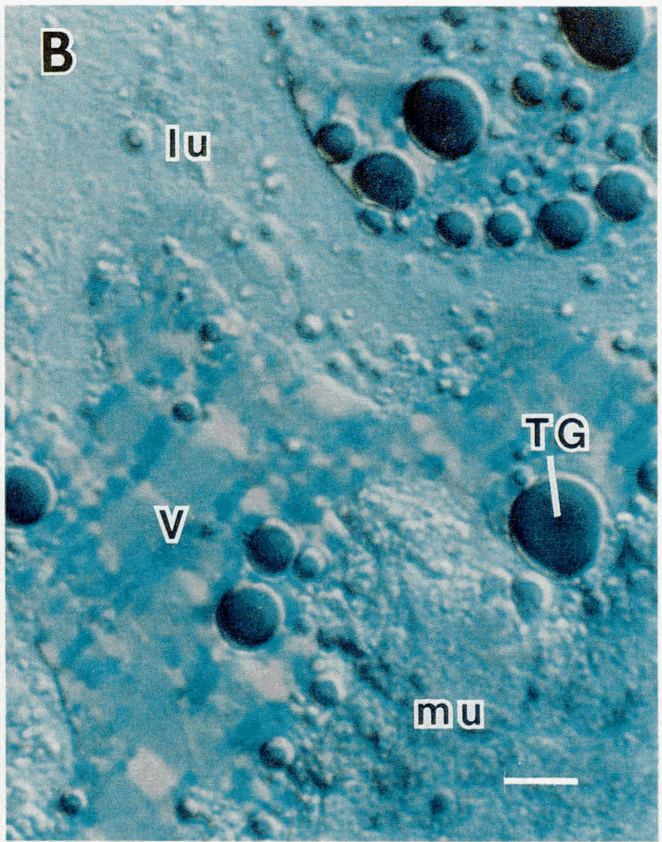

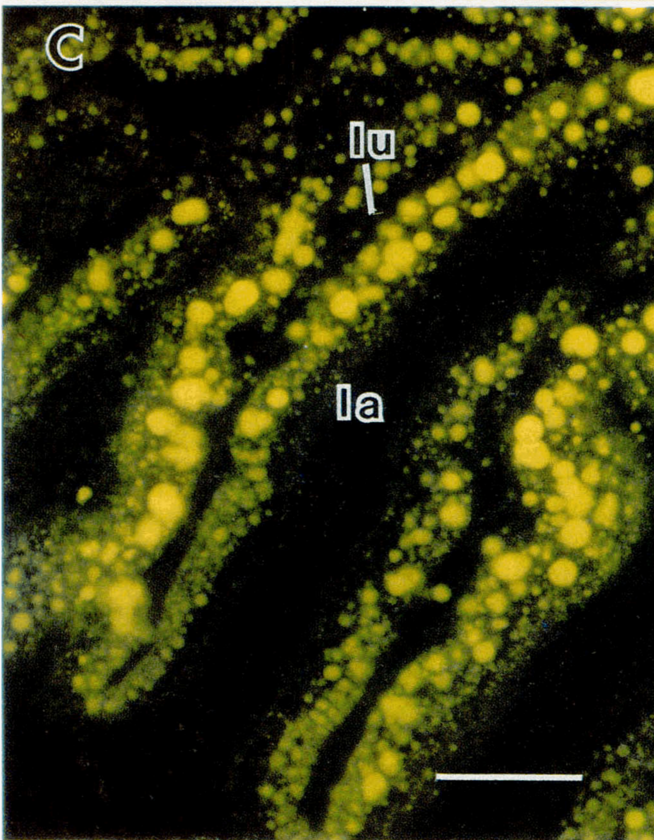

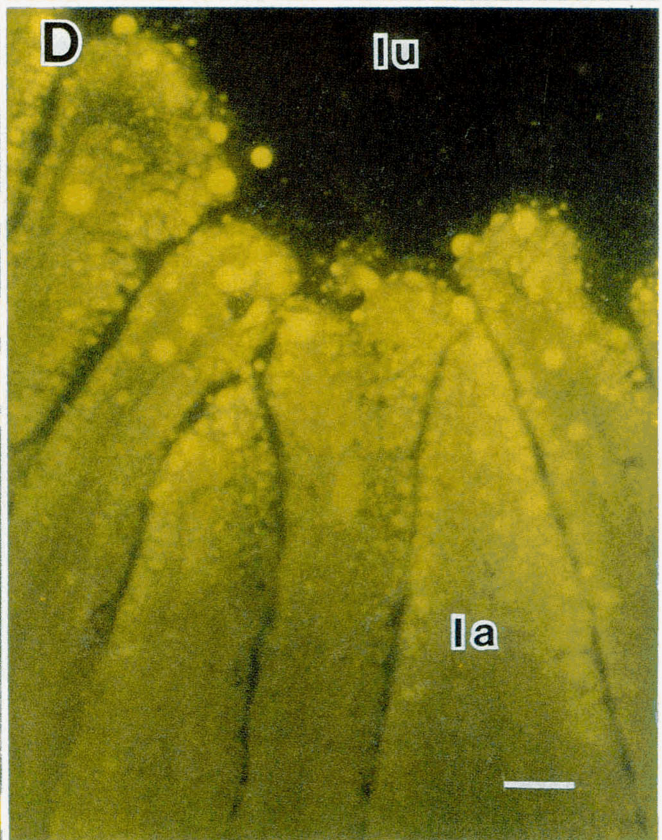

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the large fat droplets (diameters 1–10 μm) that form quickly during lipid absorption (Fig. 2C) are slowly processed and packaged into VLDL-sized lipoproteins (diameters 50–100 nm) in a manner analogous to that in mammals (38). As the newly absorbed fat is processed, it is in contact with the BP-metabolizing enzymes that occur in the endoplasmic reticulum of enterocytes (39, 40). It is possible that some of the BP metabolites that occurred in the intestine in this study originated in the liver and were reabsorbed from the bile (41). However, Bock, Clausbruch, and Winne (42), using closed loop sections of rat intestine, found that >90% of dietary BP was recovered in portal blood as metabolites. Although intestinal metabolism of drugs and xenobiotics is well recognized (43), the liver is generally considered to be quantitatively the most important detoxifying organ in the vertebrate body (44). This study and that of Bock et al. (42) suggest that the enterocytes of the intestine may be much more important than the liver in the metabolism of dietary hydrophobic molecules like BP. Factors that inhibit this metabolism may increase the delivery of PAH carcinogens (or beneficial drugs) to the general circulation and peripheral tissues such as the mammary gland. Since fasting or semisynthetic diets can cause complete disappearance of intestinal monoxygenase activities (43, 45), dietary habits may significantly affect intestinal PAH disposition.

In summary, BP remained largely associated with dietary lipid during the intestinal events of digestion, dispersion, membrane and cytosolic transport, and cellular resynthesis but was then metabolized to polar derivatives and separated from the newly synthesized fat. Like a sponge, the intestine appears to absorb dietary lipids and many associated hydrophobic solutes rapidly, efficiently, and nonspecifically. It then, as much as possible, sorts out more slowly the useful (fat-soluble vitamins, triglycerides, etc.) from the harmful (pesticides, carcinogens, etc.) before packaging the lipid for delivery to the general circulation. Presumably this sorting process operates by maintaining or increasing the fat solubility of useful molecules (i.e., by esterification reactions) and by decreasing the fat solubility of unwanted molecules (i.e., hydroxylation and conjugation reactions).

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Fig. 2 Scale bar = 50 μm. A, The partial hydrolysis after 20 min of a benzo(a)pyrene-saturated (1.98%) triolein droplet (TG) by porcine pancreatic lipase (~ 1500 tributyrin units of lipase/ml) at pH 8.3 in the absence of bile salts. BP has quantitatively moved into a viscous isotropic product phase (V). BP had no effect on lipase activity at the concentration used. Identical results to those in Fig. 2A (not shown) were found with perylene, coronene, β-carotene, Oil Red O, and Sudan Black B. B, The partial digestion of fat droplets in fish intestine 1 hr after feeding (stained with Sudan Black B). Contents of intestinal lumen: TG, undigested triglyceride; V, viscous isotropic product phase; mu, intestinal mucosa (Nomarsky optics). C, Frozen section of intestinal villi 3 hr after feeding a fatty meal that contained benzo(a)pyrene dissolved in fat (5 μg BP/mg fat). The meal consisted of a single piece (35 mg) of a collagen gel containing 20% triolein; lu, intestinal lumen; la, lamina propria. Note fluorescence only in intracellular fat droplets. D, Same as C except fish was killed after 8 hr. Note fluorescence throughout the intestinal villi.

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