FACTORS INFLUENCING THE INTESTINAL ABSORPTION AND METABOLISM OF POLYNUCLEAR AROMATIC HYDROCARBONS

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by

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ABSTRACT

In this study various factors governing the intestinal absorption and metabolism of polynuclear aromatic hydrocarbons (PAHs) were examined.

In the first part, the effects of gastrointestinal hormones and post-resection intestinal adaptive hypertrophy on xenobiotic-metabolizing enzymes, benzo(a)pyrene hydroxylase (BPH) and UDP-glucuronyl transferase (UDP-GT) were observed. Fasted rats were injected with either saline, 250 μg/kg pentagastrin, 20 μg/kg cholecystokinin-octapeptide (CCK-OP) or 75 units/kg secretin daily for three days and killed on the fourth day. Microsomal preparations were made from the intestinal mucosa and used in the enzyme assays. Pentagastrin produced a 236% increase in BPH activity in colonic mucosa but the rest of the intestinal segments remained unaffected. CCK and secretin did not cause any change in BPH activity in the intestine. UDP-GT activity in all parts of the rat intestine was unaffected by the hormonal treatment. It is concluded that under the present experimental situation only pentagastrin has a significant effect on BPH activity in the colon.

Fasted rats were killed 4 weeks after a 70-cm resection of proximal intestine. Remaining ileal segments were thickened and increased in diameter. The mean villous height in the remnant ileum was 177% and 130% greater than the villous height in the control ileum and jejunum, respectively. The total protein content in the remnant ileum also increased. UDP-GT activity per mg. of protein showed a statistically significant drop in the remnant ileal mucosa but the BPH activity remained unchanged. It is proposed that the presumably less mature hyperplastic

cells have diminished UDP-GT activity. The unchanged BPH activity remains unexplained.

In the second half of this study, factors influencing the bioavailability of PAHs from the intestinal content were observed.

Rats with biliary and duodenal fistulae were administered radiolabelled hydrocarbons - 2,6-dimethylnaphthalene, phenanthrene, 7,12-dimethylbenzanthracene, anthracene and benzo(a)pyrene - dissolved in corn oil only or corn oil with exogenous bile. Subsequent 24-hour biliary and urinary excretion of radiolabel was monitored to assess the efficiency of absorption with and without bile. The following values for absorption without bile (as a % of absorption with bile) were obtained : 2,6-DMN-91.6%, phenanthrene-96.7%, anthracene-70.8%, 7,12-DMBA-43.4% and BP-22.9%. The values for anthracene, 7,12-DMBA and BP were significantly less than 100% but the values for 2,6-DMN and phenanthrene were not. Since the water solubility of the structural isomers phenanthrene and anthracene are 1.29 mg/L and 0.073 mg/L respectively, it is proposed here that for these PAHs, a water solubility of approximately less than 1 mg/L, makes the presence of duodenal bile a prerequisite for efficient absorption.

In continuation of the studies with the PAHs, it was established in the last part of this study that the bioavailability of 2,6-DMN from the intestinal content was not affected by the nature of the dietary vehicle (lipid or non-lipid vehicle) and concomitant fat digestion and absorption. Also, the biliary metabolites of 2,6-DMN undergo an efficient enterohepatic circulation.

[KEY WORDS]

Anthracene

Benzo(a)pyrene(BP)

Benzo(a)pyrene Hydroxylase (BPH)

Cholecystokinin

7,12-Dimethylbenzanthracene (DMBA)

2,6-Dimethylnaphthalene (DMN)

Intestinal resection and PAHs metabolism.

Pentagastrin

Phenanthrene

Polynuclear Aromatic Hydrocarbons (PAHs)

Secretin

UDP-Glucuronyl transferase (UDP-GT)

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ABBREVIATIONS

BP	:	Benzo(a)pyrene
BPH	:	Benzopyrene hydroxylase
CCK-OP	:	Cholecystokinin-octapeptide
cpm	:	Counts per minute
7,12-DMBA	:	7,12-dimethyl benzanthracene
2,6-DMN	:	2,6-dimethyl naphthalene
GIT	:	Gastrointestinal tract
KCl	:	Potassium chloride
3-MC	:	3-methylcholanthrene
$MgCl_2$	:	Magnesium chloride
NaCl	:	Sodium chloride
NADP	:	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	:	Sodium bicarbonate
ONB	:	Over night bile (8-24 hour bile)
PAH	:	Polynuclear aromatic hydrocarbons
r.p.m.	:	Rounds per minute
SEM	:	Standard error of the mean
UDPGA	:	Uridine diphosphoglucuronic acid
UDP-GT	:	UDP-Glucuronyl transferase
UWL	:	Unstirred water layer

Chapter 1

INTRODUCTION

1.1. Background Information

1.1.1. Polynuclear Aromatic Hydrocarbons (PAHs)

An environmental chemical basis for cancer was first proposed in 1775 when Pott attributed the formation of malignant human tumours to prolonged contact with carbon soot. The perception of cancer as a disease primarily related to the environment has been progressively strengthened in the last few decades. This radical change has encouraged increasing attention to the nature of the environmental influences. Of all such environmental agents implicated, chemicals have been receiving profound attention as carcinogens. Chemical carcinogens constitute a large group of naturally occurring and man-made compounds of diverse molecular structure, that are ubiquitous in human environment (Higginson and Muir, 1973). Ever since benzo(a)pyrene (BP) was recognized as a carcinogen at the beginning of this century the presence of it and other PAHs in the environment has received continuous attention. Many PAHs have been shown to be carcinogenic by extensive experiments on animals (IARC, 1973; Miller and Miller, 1974). PAHs can be defined as organic compounds containing two or more benzenic ring structures (Fig. 1.1) which may or may not have substituted groups attached to one or more rings. PAHs are formed whenever organic substances are

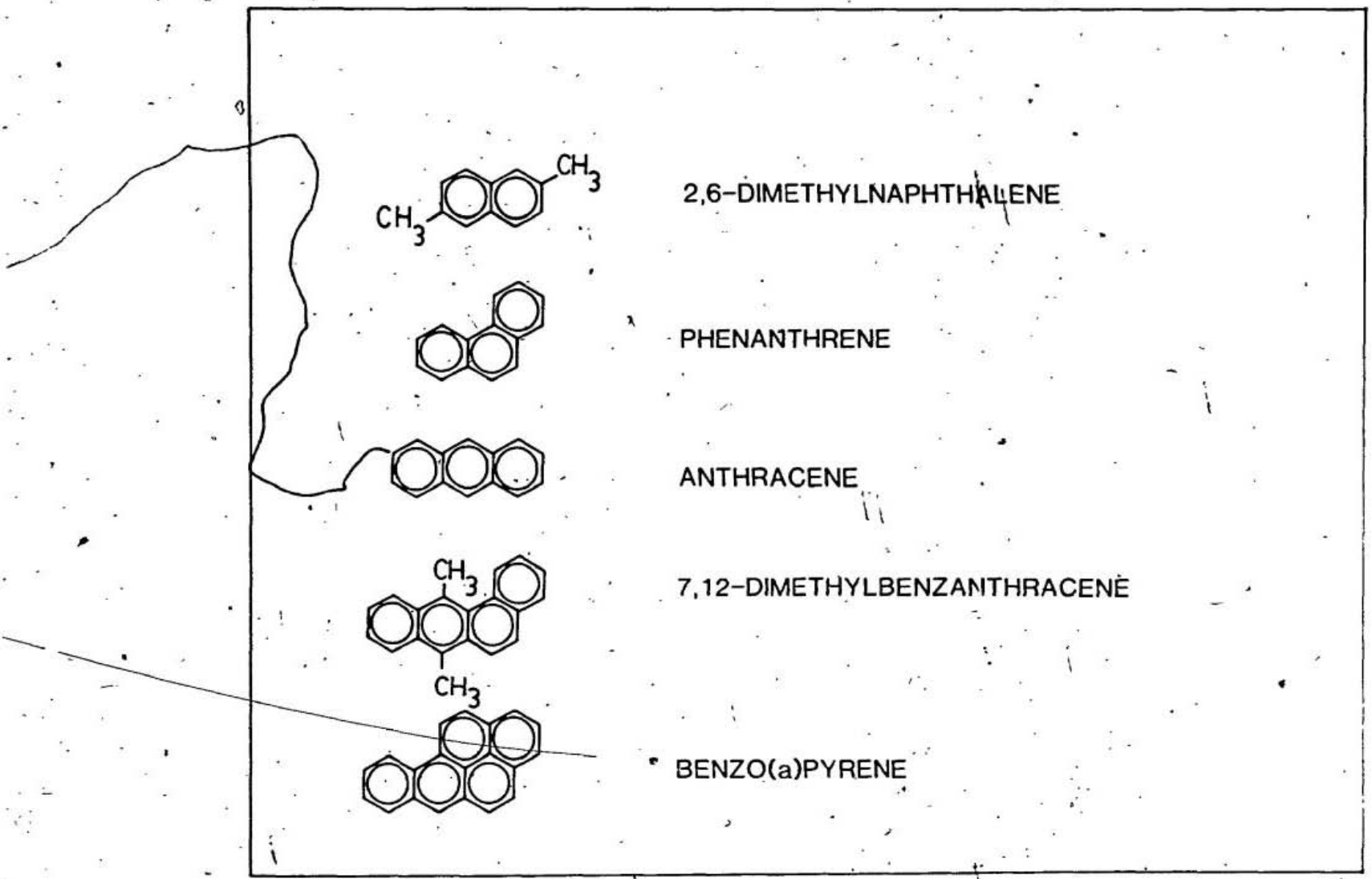
exposed to high temperatures. In this process, called "pyrolysis", the aromatic products that are formed are more stable than their precursors. This stability renders them as persistant environmental pollutants which have accumulated in the food chain throughout the world. To date about one hundred PAHs have been identified in the environment and in human food (Tilgner and Daun, 1969; US EPA, 1975).

Direct evidence for the carcinogenic effect of these compounds in man is mainly confined to the association of malignancy with occupational exposure to them. In this respect, soot, coal, tar, pitch and some mineral oils have all been found to contain high levels of PAHs and people exposed to such products in their occupation often have an increased incidence of cancer (Swallow,1976). Smoking fish or meat increases the PAHs content in them (Gray and Morton, 1981) and some groups of world population who consume such food regularly have a greater incidence of cancer of the gastrointestinal tract (NRC, 1982). Contamination of nature by PAHs is widespread as a result of the huge production volume and varied sources. Among the major sources of PAHs are the incomplete combustion of wood, coal and petroleum and the spillage of raw or refined petroleum. Man-made emission of PAHs (measured as BP, injected into the atmosphere) in the US alone was estimated to be 1320 tons per year (US NAS, 1972).

The possible sources of PAHs contamination of human food are numerous (Howard and Fazio, 1969; Tilgner and Dao, 1969;. Lo and Sandi, 1978). Such contamination can occur from the methods of preparation, such as curing meat and fish by smoke (Gray and Morton, 1981), and from pyrolysis of fat in charcoal-

Figure : 1.1

Structure of some polynuclear aromatic hydrocarbons used in this study.


2,6-DIMETHYLNAPHTHALENE
PHENANTHRENE
ANTHRACENE
7,12-DIMETHYLBENZANTHRACENE
BENZO(a)PYRENE

broiled meat (Lijinsky and Shubik, 1964). PAHs derived to a large extent from vehicle and industrial exhaust are deposited from the atmosphere on leafy vegetables grown in urban regions, thus contributing to the dietary load of this class of substances (Lo and Sandi, 1978; Shabad, 1980). About ten percent of BP detected in lettuce, leeks and tomatoes can be removed by cold water rinsing, an indication that it was originally deposited externally. PAHs derived from tobacco smoke and air pollution can also be trapped in the respiratory tract and gain access to the intestine when respiratory secretions are swallowed. Aside from the sources already mentioned, the most common source of PAHs in food are potential food contaminants of petroleum origin (Haenni, 1968). PAHs are important components of crude oil and petroleum products (Clark and Brown,1977). In this energy-hungry world, off-shore drilling, tanker accidents and various other man-made and natural disasters lead to extensive pollution of the marine environment with petroleum products and crude oil. Investigation of these products have shown that many marine animals concentrate and metabolize PAHs, including naphthalene, methylnaphthalene and dimethylnaphthalene (Anderson, Neff, Cox, Tatem and Highwater, 1974b; Dunn and Stich, 1975; Varanasi and Malins, 1977). Since sea food constitutes a major share of human food around the world, this may prove to be a significant route of entry of PAHs into the human gastrointestinal system.

Xenobiotics undergo several types of metabolic reactions in the organism. These include oxidative, reductive and hydrolytic reactions (summarised as Phase I reactions) as well as synthetic or conjugation reactions (summarised as Phase II reactions). Most phase I reactions involve an enzyme system which catalyzes

oxidative and reductive reactions and often introduces a free hydroxyl group into the xenobiotic molecule. These enzyme systems require NADPH, molecular oxygen and an electron transport system consisting of NADPH cytochrome c reductase, lipid, and a carbon-monoxide binding pigment generally known as cytochrome P-450. This requirement of the system for NADPH and oxygen classifies them in the mixed function oxidase (MFO) category (Mason, 1957).

The products of phase I reaction usually undergo phase II reactions in which they are conjugated with hydrophilic residues such as glucuronic acid or sulphates. Conjugation with glucuronic acid, catalyzed by the microsomal UDP-glucuronyl transferase (UDP-GT; GT EC 2.4.1.17) is quantitatively the most important phase II reaction of drug metabolism (Smith and Williams, 1966).

The most common pathways in PAHs metabolism are the oxidative and synthetic reactions. The oxidative reactions usually result in the formation of a polar oxygenated group on the substrate molecule. This can be the site for a subsequent synthetic reaction producing water soluble entities (Williams and Millburn, 1975). The end products of such reactions are usually non-toxic water-soluble substances which are readily excreted in the urine or bile. However the oxidation may occasionally lead to an activation of the compound to highly reactive ultimate carcinogen, with increased toxicity as a consequence (Miller and Miller, 1974; DiGiovanni and Juchau, 1980; Gelboin, 1980; Sims, 1980; Levin, Wood, Chang, Ittah, Croisy-Delcy, Yagi, Jerina and Conney, 1980).

Farber (1982) in an extensive review of chemical carcinogenesis stated that

the fate of a chemical carcinogen and its ultimate cytotoxicity may depend largely upon the balance between activation and inactivation in the tissues. Since, the gastrointestinal tract is exposed to a variety of PAHs and possesses the enzyme systems to metabolize them, the modifying factors on the activity of these enzymes may be important in the genesis and availability of the ultimate carcinogens.

Since the primary focus of this study is on intestinal absorption and metabolism of PAHs, it is appropriate to summarise first, the current views on PAHs absorption and metabolism.

1.1.2. Absorption of Polynuclear Aromatic Hydrocarbons (PAHs)

The mammalian diet contains, in small amount, a wide spectrum of hydrocarbons, including the PAHs. The possible sources of PAHs contamination of human food have been mentioned before.

It is established that absorption of trace lipids such as sterols and fat-soluble vitamins depends upon concomitant digestion and absorption of fat and bile salts are obligatory in this process (Hollander, 1981). It is thought that the lipophilic PAHs will also be handled in similar fashion. The absorption of PAHs and organochlorine compounds from a lipid vehicle has been repeatedly affirmed (Daniel, Prat and Prichard, 1967; Wilson, Ziprin and Clark, 1982). Dao (1969) showed that the absorption of 3-methylcholanthrene (3-MC) in rats, when given in an aqueous suspension, is only six percent of the extent achieved when fed in sesame oil.

Because lipid absorption is a passive process (Sallee and Dietschy, 1973), a sufficient concentration gradient must exist across the mucosa. However, prior to reaching the mucosa the lipids have to diffuse through three different layers, the largest being the "unstirred water layer". This layer is about 200 to 500 μm in width (Westergaard and Dietschy, 1974) and its barrier function is greatest for hydrophobic molecules (Thomson and Dietschy, 1981).

Micellar solubilization appears necessary for passively absorbed nutrient lipids and trace lipids and might be a prerequisite for PAH absorption. Since the micelles are readily water soluble, they diffuse through the unstirred water layer carrying along the products of lipid digestion as well as the trace lipid and the PAHs. Micelles dissociate near the enterocyte membrane, an action probably favoured by an acid microclimate (Shiau, 1981), and fatty acids, monoglycerides, trace lipids, as well as lipophilic xenobiotics such as PAHs are absorbed into the lipid phase of the brush border. The solubilization of the PAHs in the mixed bile-salt micelles or in the bilayer vesicles, which are formed in the presence of low concentration of bile-salts, provides a "hydrocarbon continuum", as suggested by Patton (1981), which allows non-polar molecules to move from a non-dispersible oil phase to a phase dispersible in an aqueous medium and yet remain constantly in association with a hydrophobic microenvironment. McMahon and Thomson (1970) showed that while a polar lipid, oleic acid, was absorbed nearly as well from an emulsion as from a bile salt micellar solution, uptake of the non-polar lipid α-tocopherol from the emulsion into the intestinal mucosa was lower than that from a micellar solution. This indicates the importance of micellar solubilization for non-polar lipids.

PAHs dissolve readily in mixed bile-salt lipid micelles (Laher and Barrowman, 1983). Non-polar solutes like PAHs are more readily solubilized when polar lipids are present and mixed micelles rather than pure bile salt micelles are formed (Carey and Small, 1970). Savary and Constantin (1967) found the hydrocarbon hexadecane to undergo micellar solubilization, which was greater in mixed than in pure micelles.

Sometimes the micellar solubilization of PAHs may have a negative effect on their absorption. This may occur if a lipophilic substance has, by itself a reasonable water solubility. Its insertion in micelles may then decrease its thermodynamic activity as compared to a monomolecular solution and thus delay its absorption (Amidon, Higuchi and Ho, 1982).

The nature of uptake of hydrocarbons at the enterocyte membrane is an area of uncertainty. The aqueous solubility of monoglycerides and fatty acids is low but it is conceivable that these substances undergo monomolecular uptake from aqueous solution close to the membrane as proposed by Dietschy and his colleagues (Thompson and Dietschy, 1981). Hydrocarbons have a much lower water solubility than the fatty acids and the monoglycerides. If they are taken up from a monomolecular solution, their concentration in this solution must be very low indeed. Is it possible that hydrophobic forces cause intimate contact between particles of the hydrocarbon and the enterocyte membrane ?

The intestine is not only an organ of absorption but also a secretory organ. Hydrophilic organic compounds of differing chemical structure are actively

secreted into the intestinal lumen. Heavy metal ions and highly lipophilic xenobiotics are also delivered in to the gut lumen (Bungay, Dedrick and Mathew, 1981; Richter, Fichtl and Schafer, 1982). Faecal elimination of lipophilic xenobiotics can be augmented by oral administration of paraffins (Richter et al., 1982) or cholestyramine - a styrene-divinyl benzene copolymer containing quaternary ammonium functional groups (Guzelium, 1982), and has been used in attempts to detoxify human beings (Guzelium, 1982).

The intestinal absorption of three carcinogenic PAHs, BP, 3-MC, and 7,12-DMBA, has received partieular attention (Bock, Clausbruch and Winne, 1979; Daniel et al, 1967; Grubbs and Moon, 1973; Laher, Rigler, Vetter, Barrowman and Patton, 1984). When fed in nutrient lipids, they are absorbed and at least in part transported as solutes in chylomicrons in lymph (Daniel et al., 1967). However, recent studies suggest that the portal venous route may be of great quantitative importance in the transport of these compounds from the intestine to the tissues (Bock et al., 1979; Laher and Barrowman, 1983; Laher et al., 1984), mainly in the form of metabolites produced in the enterocytes. A study of BP absorption from the rat jejunum in situ has shown that 40% of the instilled material is recovered in portal venous blood mainly in the form of metabolites, a considerable proportion being glucuronide conjugates (Bock et al., 1979).

In the second half of this study the absorption and excretion of a number of PAHs were observed in rat models. The compounds choosen were 2,6-dimethylnaphthalene (2,6-DMN), anthracene, phenanthrene, 7,12-dimethylbenzanthracene (7,12-DMBA) and benzo(a)pyrene(BP). All these

compounds are quite common in our environment. 2,6-DMN is a major constituent of crude oil and petroleum products. The acute toxicity of whole oil is often directly related to the concentration of naphthalene and its derivatives in the oil (Anderson et al., 1974a). 2,6-DMN is also used in dye and several other important industries. Although the commercial importance of anthracene and phenanthrene is not very high, even then these are found in dye, plastic, pesticide and several other industries. The widespread pollution of the environment by BP has been stated before. The importance of bile in the absorption of some of the PAH from the intestine is well known (Laher and Barrowman, 1983). In our study, we observed the role of bile in the bio-availability of the above compounds in a series of experiments. Since the number of aromatic rings in these compounds increase from two to five and they also have different degree of water solubility, we speculated that the role of bile may be different in each case. With 2,6-DMN, we took our studies a few steps further observing the bioavailability of this compound, when administered in a non-lipid vehicle and when both bile and pancreatic secretions are absent from the duodenum. Due to the importance of enterohepatic circulation of the metabolites which may be more toxic than the parent compound, we also observed the enterohepatic circulation of the metabolites of 2,6-DMN.

1.1.3. Intestinal Xenobiotic Metabolism

The gastrointestinal system is exposed to a great variety of lipophilic xenobiotics, of which PAHs are a significant fraction. Until recently it was thought that the role of intestinal mucosa in xenobiotic disposition was limited to absorption only. The possible role of the intestine in the metabolism of xenobiotics was first reported by Herter and Wakeman (1899) who found that the epithelium of the small intestine is highly effective in removing phenol. Forty years later Marenzi (1939) was able to show that the intestine posseses a remarkable role in the elimination of phenol in the form of conjugates. From that humble beginning, extensive research has been done in this field, and the xenobiotic metabolizing activity of the intestinal mucosa is now well documented. In recent years, development of sensitive analytical methodology has revealed that xenobiotics interact with intestinal mucosal endoplasmic reticulum during the absorptive process (Wattenberg, 1970, 1971, 1972; Chhabra, 1979; Hartiala, 1973; Lake, Hopkins, Chakrabarti, Bridges and Parke, 1973; Chhabra, Pohl and Fout, 1974). Although it has long been established that liver is the main site for metabolic degradation of xenobiotics (Fout, 1962; Conney, 1967; Gillette, 1971; Remmer, 1972; Mannering, 1971), the contribution made by the intestine is not to be ignored. True, the rate of most enzymatic reactions in the intestine are lower than those in the liver by almost 15 to 50 percent (Chhabra and Fout, 1976). However, this low rate of metabolism in the intestine does not rule out the importance of this tissue in xenobiotic metabolism since the surface area of the intestine and the duration of a foreign chemical residence in it may be a determining factor in the contribution of the intestine to the overall metabolism of xenobiotics in animals and humans.

There are two different systems of intestinal metabolism of xenobiotics. The first is located in the intestinal lumen and catalyzed by microorganisms (Scheline, 1973). The other is located in the intestinal mucosa and is catalyzed by the enzyme systems of the enterocytes. It has become increasingly apparent that intestinal commensal microorganisms are capable of a variety of reactions involving the xenobiotics resulting in the alteration of the activity and toxicity of these compounds (Renwick and Drasar, 1976; Batzinger, Bueding, Reddy and Weisburger, 1978).

The liver is the major organ where the phase I and phase II reactions of xenobiotic metabolism take place. But like the hepatocytes, the enterocytes are also capable of performing both phases of biotransformation of xenobiotics. The required enzyme systems are localized in the endoplasmic reticulum, which proves the efficiency of nature because the non-polar substances like the xenobiotics concentrate around the reticulum in the cell.

The intestinal cytochrome P-450 content and the rates of most phase I reactions are about 15 to 50 percent below the corresponding hepatic values (Chhabra and Fout, 1976). Some phase II reactions, such as glucuronidation by UDP-GT have been reported to be higher in the intestine than in the liver.

Studies of the distribution of xenobiotic metabolizing enzymes along the entire length of intestine show that the activity of these enzymes is highest in the proximal part of the intestine and progressively declines towards the distal end (Hoensch, 1976; 1979; Koster, Frankhuijzen-Sierevogel and Noordhoek, 1985).

Chhabra and Fout (1976) found maximum activity in the proximal 76 cm of the rabbit intestine. The rat and the mouse also show similar distribution patterns in the intestinal xenobiotic metabolizing enzymes (Wattenberg, Leong and Strand, 1962). A study on the distribution of these enzymes among mucosal cell populations and along the villous-crypt axis, showed that poorly differentiated, actively dividing crypt cells possess only minute activity, whereas the highly specialized, mature villous tip cells exhibit much greater activity (Hoensch, Woo, Raffin and Schmid, 1976; Hoensch, Hutt and Hartman, 1979).

In experimental animals it has been shown that the xenobiotic metabolizing monooxygenase activity of the small intestine can be affected by several conditions that either elevate or reduce its activity (Hoensch and Hartman, 1981). In a number of excellent reviews, several workers have shown various modifying factors act on intestinal xenobiotic metabolism (Wattenberg, 1972; Vesell, Lang, White, Passananti, Hill Clemens, Liu and Johnson, 1976; Nebert and Felton, 1976; Nebert and Gelboin, 1969; Hoensch, Steinhardt, Weiss, Maier and Malchow, 1984; Williams, 1978; Benford and Bridges, 1983; Campbell and Hayes, 1974; Clayson, 1975; Jori, Salle and Santini, 1971). Age, sex, species, genetics, diet, nutrition, environment and many other factors have been shown to modify intestinal metabolism of xenobiotics.

The monooxygenase activity was found to differ in different age groups of animals. Working with rabbit intestine, Tredger (1976) and his colleagues could detect little or no activity during the first week after birth. They monitored a gradual increase in activity during the next three weeks which by 30 to 40 days

exceeded the normal adult levels. By the 75th. post-partum day the activity settled to normal adult levels. Lucier (1977) and his colleagues found a similar variation in the UDP-GT activity in the small intestine of guinea pigs and rabbits.

Sex hormones appear to be important in the activity of mixed function oxidase in rat liver, higher activity being detected in male livers (Chhabra and Fout, 1976), but no such difference has been found in the intestine (Chhabra and Fout, 1976).

A rhythmic diurnal variation in the activity of intestinal microsomal xenobiotic metabolizing enzymes has been reported by Chhabra and Fout (1976). They found two peak activities in both rat and rabbit, one in the early morning and the second in the late afternoon.

Diet plays a very important role in the regulation of these enzymes. In fact, it has been suggested that the activity may largely or entirely be an effect of exogenous inducers in the diet (Wattenberg, 1971). Starvation decreases intestinal aryl aromatic hydrocarbon hydroxylase (AHH) activity in rats (Wattenberg et al., 1962). Rats maintained on semi-purified diet showed a decrease in AHH activity, which again increased when various vegetables were added to the semi-purified diet (Wattenberg, 1971). Nutrients essential for the structural integrity of microsomal membrane, such as cholesterol, and for the molecular structure of cytochrome P-450, such as iron, have been found to play important roles in the continued activity of these enzymes. Dietary iron (Hoensch et al., 1976) and the quantity and quality of dietary fat (Wattenberg et al., 1962) influences the AHH

activity in rat intestine. Dietary lipids seem to play very little role in UDP-GT activity but a low-protein diet increases its activity (Marselos and Laitinan, 1975).

Xenobiotics themselves are very potent inducers of the xenobiotic metabolizing enzymes. PAHs are particularly noteworthy in this respect. In a number of studies, pretreatment of the animals with PAHs resulted in a massive induction of AHH activity in the intestine (Gelboin and Blackburn, 1964; Nebert and Gelboin, 1969). A similar inducement of UDP-GT activity in the rat small intestine has been reported (Aitio, Vainio and Hanninen, 1972).

Gastrointestinal hormones have a wide range of trophic actions on the digestive system (Johnson, 1976). Recently, it has been shown that pretreatment of rats with the gastrointestinal hormones, pentagastrin, cholecystokinin (CCK) and secretin, causes an increase in the hydroxylation of xenobiotics in the colonic mucosa (Fang and Strobel, 1981). In the first part of this study an attempt has been made to reproduce the work of Fang and Strobel in rat colonic mucosa and at the same time the effect of these hormones on drug metabolism in the rat small intestine has been observed.

It is well established that, following proximal small bowel resection, the residual small intestine undergoes morphological and functional adaptive changes (Dowling and Booth, 1967). An increase in the villous height and in the size of the crypt cell compartment has been observed without changes in the individual mucosal cell size. After proximal resection the remaining ileum also shows an increased capacity to transport glucose and amino acids (Dowling and Booth,

1967) when expressed per unit length of the intestine. Similarly, the activies of some brush-border membrane-associated hydrolytic enzymes have been observed to be increased (McCarthy and Kim, 1973) after resection. With the above background information, an attempt was made to observe the changes in the xenobiotic metabolism capacity of the residual adapted ileum, following massive proximal resection of the small intestine.

To observe the effects of the above mentioned factors on xenobiotic metabolism, both phase I and phase II reactions were studied. Benzopyrene Hydroxylase (BPH), a much studied and well described microsomal enzyme with BP as substrate was taken as a representative of phase I reaction and UDP-Glucuronyl transferase (UDP-GT) with 1-naphthol as substrate represented the phase II reactions.

1.2. Objectives

1. To observe the effect of pentagastrin, cholecystokinin octapeptide (CCK-OP), and secretin on the activity of benzo(a)pyrene hydroxylase (BPH) and UDP-glucuronyl transferase(UDP-GT) in rat small and large intestine.

2. To perform resection anastomosis in the small intestine of the rat and observe the adaptive hypertrophy in the ileal remnant after a period of 30 days; and compare the anatomical hypertrophy with changes, if any, in the activity of the microsomal xenobiotic metabolizing enzyme systems, using BPH and UDP-GT as indicators.

3. To observe the role of bile in the absorption and excretion of the

polynuclear aromatic hydrocarbons, 2,6-dimethylnaphthalene (DMN), phenanthrene, anthracene, 7,12-dimethylbenzanthracene (DMBA) and benzo(a)pyrene (BP). For 2,6-DMN the experiments will include the study of the role of dietary vehicle in the bioavailability of this compound, the effect of absence of both bile and pancreatic secretions from the duodenum on its absorption and finally the enterohepatic circulation of the metabolites of this compound.

4. To correlate the water-solubility of a PAH and its requirement for bile during intestinal absorption and to determine the efficiency of the absorption process of the PAHs in the absence of dudenal bile.

Chapter 2

MATERIALS AND METHODS

2.1. Effects of Gastrointestinal Hormones on Xenobiotic Metabolism in Rat Intestine

2.1.1. Animals

Male Sprague-Dawley rats were purchased from Canadian Hybrid Farms (Nova Scotia, Canada) and were kept in the Animal care facilities until the day of the experiment. They were maintained at 74°F and 55% humidity in Shoe-Box cases (solid plastic rectangular box with a separate rod lid incorporating the feeder) with saw dust bedding. They were allowed free access to food (Purina Rat Chow, Ralston Purina Company) and tap water until the day before the experiment.

2.1.2. Hormone treatment

Three chief gastrointestinal hormones, pentagastrin, cholecystokinin (CCK) and secretin were used in this study. The hormones were obtained from standard suppliers and were of the highest purity available. Pentagastrin was obtained from Ayerst Canada (Peptavlon; Pentagastrin injection, BP, 5 mg/2ml), secretin (secretin, porcine; synthetic pentacitrate salt; 3800 Cl units/mg) and cholecystokinin octapeptide (cholecystokinin fragments, amide sulphated) were obtained from Sigma (MO,USA).

The hormones were administered according to the doses proposed by Fang and Strobel (1984). Pentagastrin was used directly from the Peptavlon vials. Pentagastrin treated rats received a single daily intraperitoneal injection of 250 μg/kg of body weight for three consecutive days. The control animals received 0.9% NaCl solution.

Secretin was dissolved in 0.9% NaCl solution containing cysteine hydrochloride, 1 mg/ml, as a reducing agent. Secretin-treated rats received one daily subcutaneous injection of 75 units/kg of body weight for three consecutive days. Control animals received subcutaneous saline solution.

Cholecystokinin octapeptide was prepared as a concentrated stock solution of 0.5mg/ml in 0.5N $NaHCO_3$ and diluted into 0.9% NaCl solution before use. CCK-treated rats received one daily subcutaneous injection of 20μg/kg of body weight for each of three days. Controls again received saline solution like before.

All the hormones were administered to the animals once daily between 9AM and 10AM, using an 1ml disposable syringe with 9.5 mm needle. The intraperitoneal injections were made in the lower abdomen on the right side, taking care not to puncture any abdominal organs. The subcutaneous injections were made either on the left or on the right flank. Every effort was made to cause a minimum of trauma to the animals.

The animals were sacrificed on the fourth morning for microsomal preparation.

2.1.3. Microsomal Preparation

The isolation of intestinal microsomes with intact cytochrome P-450 and monooxygenase activity is associated with considerable difficulties (Chhabra et al., 1974). The major obstacle in the procedure is protecting the xenobiotic metabolizing enzymes from denaturation by the intestinal proteases.

The methodology followed in this study is according to the technique used by Stohs and his co-workers (Stohs, Grafstrom, Burke and Orrenius, 1976).

The animals, which were fasted overnight, were lightly anaesthetized with diethylether. The abdomen was opened by a midline incision, starting from just above the penis to the xiphoid process. For identification of abdominal organs, the anatomy described by Rene Lambert (1965) was followed. The pyloric end of the stomach was everted to expose the beginning of the duodenum. The duodenum was cut with fine scissors, slightly distal to the pylorus. The pancreatic tissue and fat were removed from the duodenal wall as much as possible using blunt dissection. The duodenum was separated from the rest of the intestine by cutting close to the ligament of Treitz. Again, using blunt dissection, the next 40 cm. of small intestine was freed from the mesentery. The proximal 10 cm. of this segment was collected as the jejunum sample, the next 20 cm was discarded and the last 10 cm was taken as the ileum sample. For large intestine, the complete length of it, starting from the caecum to the anal canal was taken. The rectum was severed close to the anus and using blunt dissection the rectum, the whole of colon and the caecum were separated from the underlying fatty tissue. The caecum was cut free close to the ilio-caecal junction.

Immediately after collection each sample was dropped in a beaker containing ice cold buffer (isotonic KCl in 0.05 M tris HCl buffer with 5 mM $MgCl_2$, pH 7.5). To remove food particle and faecal matter, each segment was flushed with the same ice cold buffer using a 10 cc disposable syringe. Each segment was then cut logitudinally to expose the mucosa. Any remaining food particles were then removed with a cotton swab. The mucosa was scraped with the blunt edge of a scalpel blade and placed in a plastic weigh boat kept in ice. The weight of the mucosa was determined by weighing the boat and mucosa together and then weighing the boat alone after the mucosa had been transferred to the homogenization tube. The 30 cc glass homogenization tube (Wheaton, USA) contained 20 cc of the tris/KCl buffer, 5 ml glycerol and 75 units of heparin. To this, 5 mg trypsin inhibitor per gram of mucosa was added.

Homogenization was carried out in a 4°C cold room, with a teflon pestle attached to an electrc drill (Black and Decker 3/8" drill). The pestle was rotated in the tube by the drill (1200 r.p.m.) while the tube was moved up and down for 12 strokes. Rapid rotation of the pestle with the vertical excursions of the tube causes disentegration of the tissue and disruption of the cells yielding a homogenate consisting of diluted cell cytoplasm, intracellular particles and some unbroken cells. Fractional centrifugation was then used to separate the various components.

The homogenates were transferred to polycarbonate centrifuge tubes and centrifuged at 10,000 g for 10 minutes (RC 2-B Centrifuge, SS-34 Sorval rotor). The supernatant was decanted off to a Beckman polycarbonate centrifuge bottle

and centrifuged in the Beckman L5-65 type / H ultracentrifuge at 105,000 g for 60 minutes (50.2TI rotor; 35,000 rpm; 4°C). After discarding the supernatant the pellet was resuspended in 25 ml of 0.15M KCl by dislodging the pellet with a Pasteur pippete and homogenizing in a 30ml tube with a motor driven teflon pestle. The resuspended pellet was centrifuged in the same ultracentrifuge at 105,000 g for 60 minutes. The final pellet was resuspended in potassium phosphate buffer (pH 7.4, 10 mM, containing 1.1 mM $MgCl_2$). The volume of the buffer varied between 3 to 5 ml depending upon the wet weight of the mucosa (~ 1 ml/100 mg wet weight).

Protein estimation for the microsomal suspension was done prior to freezing.

The samples were then frozen with liquid nitrogen and stored at -70°C in a Revco freezer. Duration of storage never exceeded 7 days. Microsomes frozen under nitrogen have been shown to maintain unchanged levels of AHH activity for at least a week (DePierre, Moron, Johannesen and Ernster, 1975).

2.1.4. Protein Assay

Estimation of protein in the microsomal preparation was done on the principle of protein-dye binding, using the Bio-Rad protein assay kit and is based on the differential colour change of a dye in response to various concentrations of protein. It involves the binding of Coomassie brilliant blue G-250 to protein, which causes a shift in the absorption maximum of the dye from 465 to 595 nm. and this increase is monitored.

2.1.4.1. Dye Reagent Preparation

Dye reagent concentrate was diluted five fold and filtered prior to use. One volume of the concentrate was diluted with four volumes of deionised water, filtered through Whatman no.1 paper and stored in a glass beaker at room temperature. Fresh diluted reagent was prepared before each assay.

2.1.4.2. Protein Standard

The Bio-Rad protein standard I is a lyopholized bovine gamma globulin and was reconstituted by adding deionized water to obtain a final concentration of approximately 1.4 mg/ml. The rehydrated protein was stored at 4°C for up to 60 days.

2.1.4.3. Assay Method

Protein standard solutions containing 40, 80, 159, 477, 795, and 1113 µg per ml in a volume up to 0.1 ml were pipetted into 16x100 test tubes, using an Eppendorf repeater pipette. The volume in the test tubes was adjusted to 0.1 ml with the appropriate amount of buffer. Five millilitres of the dye reagent was added to each test tube and the contents mixed by gentle sucking and expelling with a pasteur pipette. A reagent blank was prepared from 0.1 ml of buffer and 5ml of dye reagent. Each tube was prepared in triplicate.

The unknown protein samples (microsomal preparation) were diluted 1:1 with the buffer and 0.1 ml of the diluted sample was added to each assay tube. After a period of 15 to 30 minutes, the absorbance at 595 nm was measured against the reagent blank in 3 ml cuvettes (Hellma, 10 mm light path, optical glass cuvettes) using a Unicam SP-1800 spectrophotometer. A standard curve was

obtained by plotting the concentration of protein in the standard solution against the corresponding absorbance. The standard curve was prepared each time the assay was performed and was linear in all cases. The assay was reproducible upto a period of one hour after sample-dye mixing. This curve was used to determine the protein concentration in the unknown samples.

2.1.5. Enzyme Assays

A survey of the literature indicates wide variation in the incubation conditions utilized to measure microsomal enzyme activity. The same reaction has been studied using varying concentrations of substrate, enzyme or co-factor and with varying incubation times. Thus meaningful comparison between laboratories for the same reaction is often difficult. For this reason, standard procedures for enzyme assays were adopted, and once standardization was reached, every effort was made to follow the procedure precisely. As mentioned earlier, two enzymes were selected to represent the two phases of xenobiotic metabolism. Aryl hydrocarbon hydroxylase (BP-hydroxylase, Aryl n-hydroxylase, EC 1.14.1.1) activity representing phase I and UDP-GT activity representing phase II were determined using procedures described below.

2.1.5.1. Benzo(a)pyrene Hydroxylase Activity

The method followed was described by Kandaswami and O'Brien (1983) and is a modification of the method of Van Cantfort, De Graeve and Gielen (1977). The principle of this radioactive assay is based on the extraction of the unreacted substrate at the end of the reaction leaving the hydroxylated derivatives in the aqueous phase of the reaction mixture.

The substrate was prepared in the following manner. 5 mCi [G-^{3}H]-BP in toluene was dried down under a gentle stream of nitrogen in a fume hood, 40.96 mg of unlabelled BP were added and the resulting mixture redissolved in 2 ml of toluene and stored at -20°C in 100 µl aliquots. Before use, a 100 µl aliquot was dried under nitrogen and redissolved in 1ml of acetone. 20 µl of this solution was used in each assay tube. Frozen microsomes were thawed at room temperature for about an hour and stored in ice.

The microsomes were incubated at 37°C with a NADPH-regenerating system for 15 minutes. The final incubation volume of 2ml contained 10 mM potassium phosphate buffer (pH 7.4), 1.1 mM $MgCl_2$, 50 µl NADPH-regenerating system (3 mM isocitric acid, 0.3 mM NADP, 7 units isocitric dehydrogenase), 80 µM BP (31.25 mCi/mM) and approximately 0.2 mg of microsomal protein. The background for the assay was determined by incubating boiled enzyme. Assays were routinely done in triplicate.

The incubation mixtures were pre-warmed to 37°C in a water bath for about 10 to 15 minutes and then the reaction was started by the addition of 20 µl of BP solution. Assays were done in 13x100mm screw-capped tubes in a shaking water bath (Forma-Scientific, Thermo-Shake bath model: 2562), oscillating at 30 cycles/minute. Four ml of ice cold ethylacetate-acetone (2:1 v/v) was added to stop the reaction and to extract the unreacted BP. The tubes were then transferred to a rotary agitator (Baltimore Biological Laboratory, tube rotator, or, Scientific Industries,Inc. model: 151), mixed for 5 minutes and centrifuged at 600 g for 5 minutes (Clay Adams, Dynac II centrifuge; or International Equipment Co.

model CL). Centrifuging resulted in the separation of the lower aqueous layer from the organic layer by a compact interface. The organic layer and the interface were suctioned off and the extraction process repeated. After removing the organic layer again, 500 µl of the lower aqueous phase was pipetted out for liquid scintillation counting.

The enzyme activity was expressed as nmol of BP hydroxylated/min./mg. protein.

2.1.5.2. UDP-Glucuronyl Transferase Activity

The procedure followed was according to Hoensch et al. (1984). The substrate was prepared in the following manner. 250 µCi of [^{14}C]-1-naphthol was dissolved in 11.1 ml ethanol, 200 mg unlabelled 1-naphthol added and the mixture stored at room temperature in the dark. 10 µl of this solution was used in each assay.

The final incubation volume of 0.5 ml, contained, 10 mM potassium phosphate buffer (pH 7.4), 0.225 µCi [G-^{14}C]-1-naphthol, 2.5 mM 1-naphthol, 1.1 mM $MgCl_2$, 0.25 mg Brij 58, 3 mM UDPGA and approximately 0.2 mg microsomal protein. The background was determined by omitting the UDPGA from the incubation mixture. The mixtures were not prewarmed and assays were carried out in duplicate. Addition of UDPGA started the reaction, which was carried out in 13x100 mm culture tubes in a shaking waterbath at 37°C. Reaction was stopped after 10 minutes with 1 ml 0.6 M glycine- 0.4 M tri-chloroacetic acid. The tubes were then centrifuged at 2500 g for 5 minutes to precipitate the protein. The supernatant was decanted into 16x100 mm screw-capped tubes.

Following extraction of the supernatant with 8cc of chloroform in a rotating agitator for 5 minutes to remove the unreacted 1-naphthol, the tubes were then spun again in a desk centrifuge at 200 g for 5 minutes. 500 µl of the upper aqueous phase was collected for liquid scintillation counting.

The enzyme activity was expressed as nmol. of 1-naphthol conjugated /min./mg. protein.

2.2. Effect of Post-Resection Hypertrophy of the Small Intestine on Xenobiotic Metabolism

2.2.1. Animals

Male Sprague-Dawley rats purchased from Canadian Hybrid Farms (N.S., Canada) were used in the study. They were maintained in the Animal Care facilities under standard conditions as described previously. At the time of operation the rats weighed between 275 and 325 gms.

2.2.2. Surgery

Diethylether vapour was used to induce and maintain anaesthesia. Induction was carried out by putting the rat in a glass jar containing cotton swabs soaked in ether, the top being closed by a heavy glass plate. Anaesthesia was rapid, in 2 to 5 minutes, and the animal was removed 15 to 20 seconds later after it was clearly anaesthetized. Anaesthesia was maintained by means of a mask, a 150 ml glass beaker lined with cotton wool soaked in ether. Ether was added from time to time as required. During the procedure the animal was observed for signs of respiratory collapse and the mask was withdrawn or replaced as required.

The animal was placed in supine position on a dissection board and the abdomen was shaved with an electric clipper. The abdomen was opened by a midline substernal incision 3 cm long, using a No:22 blade on a No:4 handle. The incision of the muscular and peritoneal layers was made with a fine pair of scissors, after raising the abdominal wall with forceps to avoid injuring the underlying viscera, and it was extended upwards or downwards as required. Incision through the linea alba kept bleeding to a minimum. Haemostasis, when required, was achieved by light pressure.

The caecum was then identified and delivered through the wound. The ileum was traced cranially for 15 cm from the ileo-caecal junction. This point was selected as the lower end of the segment that was to be resected. The upper end was a point 3 cm distal to the attachment of ligamentum Treitz. The mesentery was spread to visualize the blood vessels supplying the segment. Each group of vessels was double ligated with 4-0 silk, keeping a distance of about 0.5 cm between the ligatures. The vessels were divided between the ligatures using a fine pair of scissors. Next, with two Schwartz micro-serre fine clamps, the intestine was occluded just above and below the selected segment. This prevented spilling of the intestinal contents and stopped the bleeding from the cut ends. The selected segment was resected with a scissor and placed in ice cold buffer (Isotonic KCl with $MgCl_2$ in Tris/HCl). Portions of the segment from the upper (jejunal) and lower (ileal) end were removed for histological and enzymatic study.

The two clamps were brought close together so as to approximate the two cut ends. End to end anastomosis was then carried out. The procedure followed

was similar to that previously described (Lambert, 1965). Two stay sutures were used to approximate the cut edges and an anastomosis was carried out. The suture material used was 6-0 silk on an atraumatic curved needle. The first stay suture of the transfixing or through and through type was placed at the antimesenteric border of the intestine. An artery forceps was attached to the stay suture to exert traction on the anastomosis. The second stay suture was inserted in the same way 180° away from the previous one, on the mesenteric border. In this way two suture lines, anterior and posterior, were formed. The anterior suture line, defined clearly by the traction exerted on the stay sutures, was closed by interrupted invaginating (Lembert's suture) stitches. Usually three stitches were required for each suture line. The posterior suture line was brought into view by reversing the anastomosis. This was easily done by pulling the first stay suture below the anastomosis through the gap in the mesentery. This reversing of the stay sutures brought the posterior surface into view. Suturing of the posterior suture line was carried out in the same way as the previous one. A small quantity of crystalline penicillin was smeared on the anastomosis line when suturing was complete.

During the length of the operation, the exposed intestine and the mesentery were soaked with warm normal saline from time to time to prevent dehydration. The anastomosis was then returned to the abdominal cavity and a small quantity of penicillin powder was sprinkled in the peritoneal cavity before closing.

The abdomen was closed in two layers, muscle and peritoneum in one layer and the skin in the second layer. The abdominal organs were carefully replaced

into the peritoneal cavity with particular care to maintain the normal anatomical relations.

The muscular and peritoneal layers were closed with 4-0 silk using a simple continuous suture. The skin was closed by 6 to 8 Michel suture clips (size 7.5mm X 2mm) applied with a Fine Science Tools Michel clip applicator. A little xylocaine (2%) cream was smeared on the abdominal wound to reduce post operative pain and restlessness.

2.2.3. Post-Operative Care

Immediately after surgery the rats were transferred to clean cages with fresh saw-dust bedding. They were allowed water ad libitum but food was withheld for 24 post operative hours. After this period the rats were returned to the Animal Care facility and kept there for 30 days.

2.2.4. Sacrifice

At the end of 30 days, the rats were brought to the laboratory and fasted overnight. Next morning they were anaesthetized with diethyl ether and the abdomen opened by a midline incision. The end to end anastomosis was identified and 10 cm of the "ileum" distal to the anastomosis was removed. A small segment of ileum was sent for histological preparation and from the rest of the sample, microsomes were prepared by the method already described. In some cases the jejunum, from the anastomosis to the ligament of Treitz, was also removed and processed as described above. The rats were then killed with an overdose of diethylether.

2.2.5. Histological Study

Histological sections were prepared from the proximal (jejunal) and distal (ileal) ends of the section of intestine removed, and from the proximal end of the resected ileum at the end of the study. The intestinal pieces were everted by cutting longitudinally and fixing on a card board with insect-pins, mucosa upwards. The samples were then fixed in formalin, embedded in paraffin, cut parallel to the villous/crypt axis and stained with haematoxylin and eosin. The sections were then observed microscopically (Carl Zeiss, x10 objective) and the mucosal thickness measured with the aid of a calibrated eye piece (Maxta, Graticules, England).

2.2.6. Enzyme Assays

Preparations of microsomes and enzyme assays were similar to that described previously.

2.3. Study on the Intestinal Absorption of PAHs

2.3.1. Animals

Male Sprague-Dawley rats, purchased from the Canadian Hybrid Farms (N.S.,Canada) and maintained under standard conditions, as described before, were used in the study. The rats were fasted overnight before the surgery and weighed between 275 to 325 gms at that time.

2.3.2. Surgery

In the same manner as the previous experiment described under section 2.2, anaesthesia was induced and maintained with diethylether.

2.3.2.1. Bile Duct Cannulation

Laparatomy was carried out as described in section 2.2. and the common bile duct was identified by drawing the duodenum to the left through the abdominal incision. Using blunt dissection the duct was cleared from the surrounding tissues for a distance of about 1 cm distal to its bifurcation. With curved fine tissue forceps, two small lengths of 4-0 silk were passed around the duct in the cleared portion, one caudally and one cranially. The duct was then ligated at the lower end of the cleared segment. This made the duct turgid and more visible. With Mini-Vannas spring scissors (Fine Science Tool Inc. cat. no: 15000-00), the bile duct was cut transversely across half of its diameter approximately 10 mm from the liver hilum. A catheter of polyethylene tubing (PE-10, Clay Adams; internal dia. 0.28 mm, external dia. 0.66 mm) 20 to 25 cm long and one end slightly bevelled, was then introduced into the duct through the incision and was gently pushed upwards for a small distance. When the bile flow in the catheter was judged to be satisfactory, the catheter was secured in the duct by the previously placed cranial suture. The catheter was then exteriorized through a stab wound in the right flank using a 16-G hypodermic needle.

2.3.2.2. Duodenum Cannulation

In order to avoid variations in gastric emptying time, it was an obvious choice to introduce the test meal via a duodenal catheter rather than a gastric catheter.

Following the bile duct cannulation, a small stab incision was made with a 16-G needle on the lesser curvature of the stomach, about 1 cm distal to the pylorus. A piece of polyethylene tubing (P.E. 50, Clay Adams; I.D. 0.58 mm, O.D. 0.965 mm) 15 cm long with one end bevelled, was introduced through the incision and passed caudally into the duodenum for 3 to 4 cm, until the end could be palpated in the duodenum. The catheter was secured by a purse-string suture using 4-0 silk on an atraumatic curved needle. Patency was checked by injecting 1ml of saline through the cannula into the duodenum and examining the site for leakage. The cannula was exteriorized through a stab wound in the left flank.

2.3.2.3. Biliary Fistula with Pancratic Obstruction

Obstruction of the external pancreatic ducts prevents entry of the exocrine pancreatic secretions into the duodenum but the endocrine secretions continue.

Bile duct cannulation was carried out as described previously. Traction on the duodenal loop brought the whole length of the bile duct into view. Using curved fine tissue forceps, the common bile duct near its entry into the duodenum, was cleared from the surrounding pancreatic tissue by blunt dissection. The duct was then double ligated as close to the duodenal wall as possible with 4-0 silk.Duodenal cannulation was done as described previously.

[redacted]. Closure of Abdomen

The abdomen was closed in the same manner as described previously in section 2.2.

2.3.3. Post-operative Care and Sacrifice

Immediately following surgery the rats were placed in Bollman-type restraining cages. These provided effective and humane immobilization and prevented access to the cannulae by the rats. Each rat was provided with a water bottle in such a way that it had easy access to the nozzle of the bottle. No food was provided.

Following over-night recovery and prior to test-meal administration, the animals were observed for 30 minutes to ensure adequate flow of bile. The temperature, humidity and the photoperiod were maintained as in the pre-operative period (74°F, 55% humidity).

Following the experiment the rats were anaesthetized by putting them in a glass jar containing gauzes soaked in diethylether. The anaesthetized animals were then sacrificed by cervical dislocation. At necropsy the animals were examined for proper placement of catheters and leakage into the abdominal cavity. Rats with dislodged cannulae were rejected from the study.

2.3.4. Test Meal Preparation

To avoid physiological variations in gastric emptying time in different animals, test meals were administered intraduodenally via the duodenal catheter. Corn oil (Mazola) or ethanol was used as vehicle according to the experimental protocol. Ethanol was chosen to represent a non-lipid vehicle and because it is a good solvent for 2,6-DMN. In the procedure where the presence of bile was required in the duodenum, the hydrocarbon solution was mixed with pooled rat bile before administration.

2.3.4.1. 2,6-Dimethylnaphthalene (DMN) in Lipid

A toluene solution of [G-^{3}H]-2,6-DMN (2.1 mCi/mMol) was dried under a gentle stream of nitrogen and enough unlabelled 2,6-DMN was added to yield a mixture having approximately 1 million cpm/mg. Corn oil was then added and mixed to yield a final concentration of 1 mg 2,6-DMN/0.2ml oil. The mixture was vortexed vigorously while protected from light and observed from time to time to ensure that no undissolved particles were left. Each animal received 0.2 ml of this mixture in the test dose. Prior to test meal administration aliquots were removed for liquid scintillation counting.

2.3.4.2. 2,6-Dimethylnaphthalene (DMN) in Bile-Lipid Vehicle

The above procedure was followed and to the lipid solution of 2,6-DMN, pooled rat bile was added to make a final oil to bile ratio of 1:2.5 v/v (0.2 ml corn oil solution of 2,6-DMN and 0.5 ml bile). Emulsification was achieved by vortexing. Aliquots were removed for liquid scintillation counting.

2.3.4.3. 2,6-Dimethylnaphthalene (DMN) Metabolites

Two donor rats with biliary and duodenal catheters received an intraduodenal bolus dose of labelled 2,6-DMN in 0.2 ml corn oil mixed with bile. The animals received 0.5 ml exogenous bile every hour for 4 hrs. Endogenous bile was collected for 4 hrs. and pooled. Samples of this bile were subjected to liquid scintillation counting. The pooled bile contained radiolabelled metabolites of 2,6-DMN. Recipient rats with biliary and duodenal catheters received 1 ml donor pooled bile intraduodenally as a bolus dose.

2.3.4.4. 2,6-Dimethylnaphthalene (DMN) in Ethanol-Bile Vehicle

A toluene solution of [^{3}H]-2,6-DMN (2.1 mCi/mMol) was dried under nitrogen and unlabelled 2,6-DMN was added to yield a mixture having approximately 1 million cpm/mg. Ethanol was added to this mixture to obtain a concentration of 1 mg/0.2 ml of ethanol. Each animal received 0.2 ml of this solution intraduodenally, mixed with 0.5 ml of rat bile. Aliquots were removed for liquid scintillation counting prior to administration.

2.3.4.5. Phenanthrene in Lipid and Bile-Lipid Vehicle

The procedure followed was exactly like 2.3.4.1 and 2.3.4.2 using [^{14}C] phenanthrene, unlabelled phenanthrene, corn oil and bile.

2.3.4.6. Anthracene in Lipid and Bile-Lipid Vehicle

A benzene solution of [^{14}C]-anthracene (15.1 mCi/mMol) was dried under a gentle stream of nitrogen and sufficient unlabelled anthracene was added to it, to obtain an activity of 1 million cpm/mg. Corn oil was then added to yield a final concentration of 1 mg/0.2 ml oil. Excess benzene was added to the mixture and vortexed vigorously to obtain a homogeneous solution of anthracene in oil and

benzene. The benzene was then evaporated off under nitrogen. Aliquots were removed for liquid scintillation counting prior to administration. For the bile/lipid vehicle, 0.2 ml of the above solution was mixed with 0.5 ml rat bile by vortexing.

2.3.4.7. 7,12-Dimethylbenzanthracene(DMBA) in Lipid and Bile-Lipid Vehicle

The procedure followed was similar to 2.3.4.1 and 2.3.4.2 using [^{3}H]-7,12-dimethylbenzanthracene (40.3 mCi/mmol), unlabelled 7,12-DMBA, corn oil and bile.

2.3.4.8. Benzo(a)pyrene (BP) in Lipid and Bile-Lipid Vehicle

The procedure followed was exactly like 2.3.4.1 and 2.3.4.2 using [^{3}H]-BP-(50.5 Ci/mmol), unlabelled BP, corn oil and bile.

2.3.5. Test Meal Administration

All dosing was carried out in the morning between 9:30 AM and 10:00 AM, using an appropriately sized syringe containing the test meal. A predetermined volume of the test meal was drawn up in a 1 ml disposable syringe, which was then weighed on a Mettler A30 analytical balance. The test meals were administered as a bolus dose intraduodenally through the duodenal catheter and using a separate syringe, the residual test meal in the catheter was flushed into the duodenum with saline or bile, according to the experimental protocol. The empty test meal syringes were then reweighed to determine the exact quantity given to each animal. Using this quantity and the cpm in the test meal aliquots, the exact radioactivity given to each animal was calculated.

TABLE 2-1 : SUMMARY OF EXPERIMENTAL PROCEDURES.

NO.	HYDROCARBON (PAHs)	WEIGHT OF PAH	VOLUME TEST MEAL	VEHICLE	DUODENAL ENVIRONMENT	SAMPLE COLLECTED	ANIMALS
1.	2,6-DMN	1 mg	0.7 ml	corn oil[1]+bile	bile present[2]	urine[3]+bile[4]	6
2.	2,6-DMN	1 mg	0.2 ml	corn oil	bile absent	"	"
3.	phenanthrene	1 mg	0.7 ml	corn oil +bile	bile present	"	"
4.	phenanthrene	1 mg	0.2 ml	corn oil	bile absent	"	"
5.	anthracene	1 mg	0.7 ml	corn oil +bile	bile present	"	"
6.	anthracene	1 mg	0.2 ml	corn oil	bile absent	"	"
7	7,12-DMBA	1 mg	0.7 ml	corn oil +bile	bile present	"	"
8.	7,12-DMBA	1 mg	0.2 ml	corn oil	bile absent	"	"
9.	BP	1 mg	0.7 ml	corn oil +bile	bile present	"	"
10.	BP	1 mg	0.2 ml	corn oil	bile absent	"	"

1. Mazola brand.
2. Exogenous bile supplementation.
3. 24-hour cumulative collection.
4. 8-hour periodic collection and 16-hour (over night bile, ONB) cumulative collection.

2.3.6. Sample Collection

2.3.6.1. Bile

Bile was collected at timed intervals into preweighed disposable glass culture tubes for the first 8 hrs using a fraction collector (LKB 7000A Ultra Rac, LKB-Produkter AB, Sweden). For the next 16 hours (8-16 hr. bile; overnight bile, ONB) bile was collected in preweighed 25 ml Pyrex flasks which were partially stoppered to retard evaporation. The culture tubes and the flasks were reweighed on a Mettler A30 analytical balance to determine the weight of bile excreted. Using a specific gravity value of 1.00, the bile weight (gm) was equivalent to bile volume (ml) excreted. Aliquots were removed for liquid scintillation counting.

2.3.6.2. Urine

Urine was collected continuously for 24 hours into preweighed 150 ml beakers. A glass funnel was used to catch the excreted urine and a wire mesh prevented contamination of the urine with faeces. The beakers were then reweighed on a Sartorius type 2250 precision balance to determine the weight of urine excreted. Using a specific gravity value of 1.00 the urine weight (gm) was equivalent to urine volume (ml) excreted. Aliquots were removed for liquid scintillation counting.

2.3.7. Liquid Scintillation Counting

50 μl of each sample to be analyzed was combined with 10 ml of liquid scintillation cocktail (Aquasol-2) and subjected to direct scintillation counting in a Beckman LS 8100 scintillation counter using library programme #1. Quenching was corrected by means of an external standard (^{137}C

2.3.8. Experimental Procedures

All experiments were performed on the first post-operative morning and involved intraduodenal test meal administration and subsequent collection of excreted bile and urine. Any animal which showed abnormality in overnight bile excretion or urine production was discarded from the experiment and subjected to autopsy.

There were two basic experimental protocols: administration of the hydrocarbon in presence of bile and administration in the absence of bile (Tab. 2.1). In the case of 2,6-DMN, the absorption and excretion of the compound when given in ethanol and when both bile and pancreatic secretion are absent from the duodenum, and the enterohepatic circulation of its metabolites were also observed.

2.3.8.1. Procedure I

Biliary and urinary excretion of radiolabel following labelled hydrocarbon administration; [^{3}H]-2,6-DMN, [^{14}C]-phenanthrene, [^{14}C]-anthracene, [^{3}H]-7,12-DMBA and [^{3}H]-BP in corn oil. Bile present in the duodenum:

In animals with biliary and duodenal catheters 1 mg of labelled hydrocarbon (Table: 2-1) dissolved in 0.2 ml corn oil and mixed with 0.5 ml rat bile were administered intraduodenally as a bolus dose through the duodenal catheter. An intraduodenal supplementation of 0.5 ml rat bile every hour for 8 hours followed the test meal administration. Bile was collected every 30 minutes for 8 hours, then a cumulative 16 hours collection was made. A cumulative 24 hours collection of urine was also made. Collected samples were analyzed for radioactivity.

2.3.8.2. Procedure II

Biliary and urinary excretion of radiolabel following labelled hydrocarbon administration; [^{3}H]-2,6-DMN, [^{14}C]-phenanthrene, [^{14}C]-anthracene, [^{3}H]-7,12-DMBA and [^{3}H]-BP in corn oil. Bile absent from duodenum:

This procedure was similar to procedure I except that bile was omitted from the test meal and there were no post-testmeal bile supplementation.

2.3.8.3. Procedure III

Biliary and urinary excretion of radiolabel following administration of [^{3}H]-2,6-DMN in ethanol. Bile present in duodenum:

In animals with biliary and duodenal catheters, 1 mg of 2,6-DMN dissolved in 0.2 ml ethanol and mixed with 0.5 ml rat bile, was delivered as a bolus dose intraduodenally and an intraduodenal supplementation of bile was made at a rate of 0.5 ml every hour for 8 hours. Sample collection and radiolabel analysis were done similar to previous procedures.

2.3.8.4. Procedure IV

Biliary and urinary excretion of radiolabel following administration of [^{3}H]-2,6-DMN in corn oil. Both bile and pancreatic secretion absent from the duodenum:

In animals with biliary and duodenal catheters, and in which a complete pancreatic obstruction existed, 1 mg of [^{3}H]-2,6-DMN dissolved in 0.2 ml of corn oil were delivered as a bolus dose. Collection of bile and urine and analysis for radiolabel were done similar to previous procedures.

2.3.8.5. Procedure V

Biliary and urinary excretion of radio label following administration of radiolabelled biliary metabolites of [^{3}H]-2,6-DMN. Bile present in the duodenum:

A 1 ml aliquot of a 4 hour collection of pooled bile containing labelled metabolites of 2,6-DMN was administered as a bolus dose intraduodenally. Exogenous bile supplementation was made at a rate of 0.5 ml per hour for 8 hours. Excreted bile and urine were collected and analyzed for radiolabel like the previous experiments.

2.4. Statistical Analysis

The mean and standard error of the mean (SEM) were calculated for each group of results. Graphical presentation of data has included standard error bars whenever possible unless at the expense of clarity. Student's t-test for unpaired values were performed using an Apple IIe computer using a Statistical Analysis Package (Tallarida and Murray, 1982), to test statistical significance between groups of data within an experiment. Significance was established at $p < 0.05$ unless otherwise specified.

Chapter 3

RESULTS

3.1. Effect of Gastrointestinal Hormones on Benzo(a)pyrene Hydroxylase (BPH) and Glucuronyl Transferase (UDP-GT) Activities in Small and Large Intestine of the Rat

This study was undertaken to observe the effects of pharmacological doses of synthetic GI hormones (pentagastrin, cholecystokinin-octapeptide, and Secretin) on xenobiotic metabolizing enzymes in rat intestine.

3.1.1. Pentagastrin Treatment(Table: 3-1, Fig. 3-1)

Our results showed a gradient of activity of BPH from duodenum through jejunum and ileum and colon. UDP-GT activity showed a similar decrease along the length of the small intestine but activity was higher in the colon than in the ileum.

Although the animals received a large dose of pentagastrin (250 μg/kg body wt./day), there was no significant change in either BPH or UDP-GT acvtivity in the duodenum, jejunum or ileum. However in the colonic mucosa pentagastrin treatment significantly increased ($P < 0.05$) BPH activity by almost 236% as compared to control animals. In the case of UDP-GT activity, no such stimulation was seen in the colonic mucosa.

Figure : 3.1

Effect of pentagastrin on benzo(a)pyrene hydroxylase (BPH) and glucuronyl transferase (UDP-GT) activities in the rat intestine.

Hormone treated animals: white

Control: dotted

Each value represents the mean (± SEM)

(*) Significantly different ($P < 0.05$)

nmol. BP Hydroxylated /mg.protein /min.
300
250
200
150
100
50
BPH Activity
Duodenum
Jejunum
Ileum
Colon
UDP-GT Activity
nmol. 1-naphthol conjugated /mg. protein /min
8
7
6
5
4
3
2
1
Duodenum
Jejunum
Ileum
Colon

Figure : 3.2

Effect of cholecystokinin on benzo(a)pyrene hydroxylase (BPH) and glucuronyl transferase (UDP-GT) activities in the rat intestine and pancreas.

Hormone treated animals: white

Control: dotted

Each value represents the mean (± SEM)

nmol. BP Hydroxylated /mg.protein / min.
300
250
200
150
100
50
BPH Activity
Duodenum
Jejunum
Ileum
Colon
Pancreas
UDP-GT Activity
nmol. 1-naphthol conjugated /mg. protein / min
7
6
5
4
3
2
1
Duodenum
Jejunum
Ileum
Colon
Pancreas

Figure : 3.3

Effect of secretin on benzo(a)pyrene hydroxylase (BPH) and glucuronyl transferase (UDP-GT) activities in the rat intestine.

Hormone treated animals: white

Control: dotted

Each value represents the mean (± SEM)

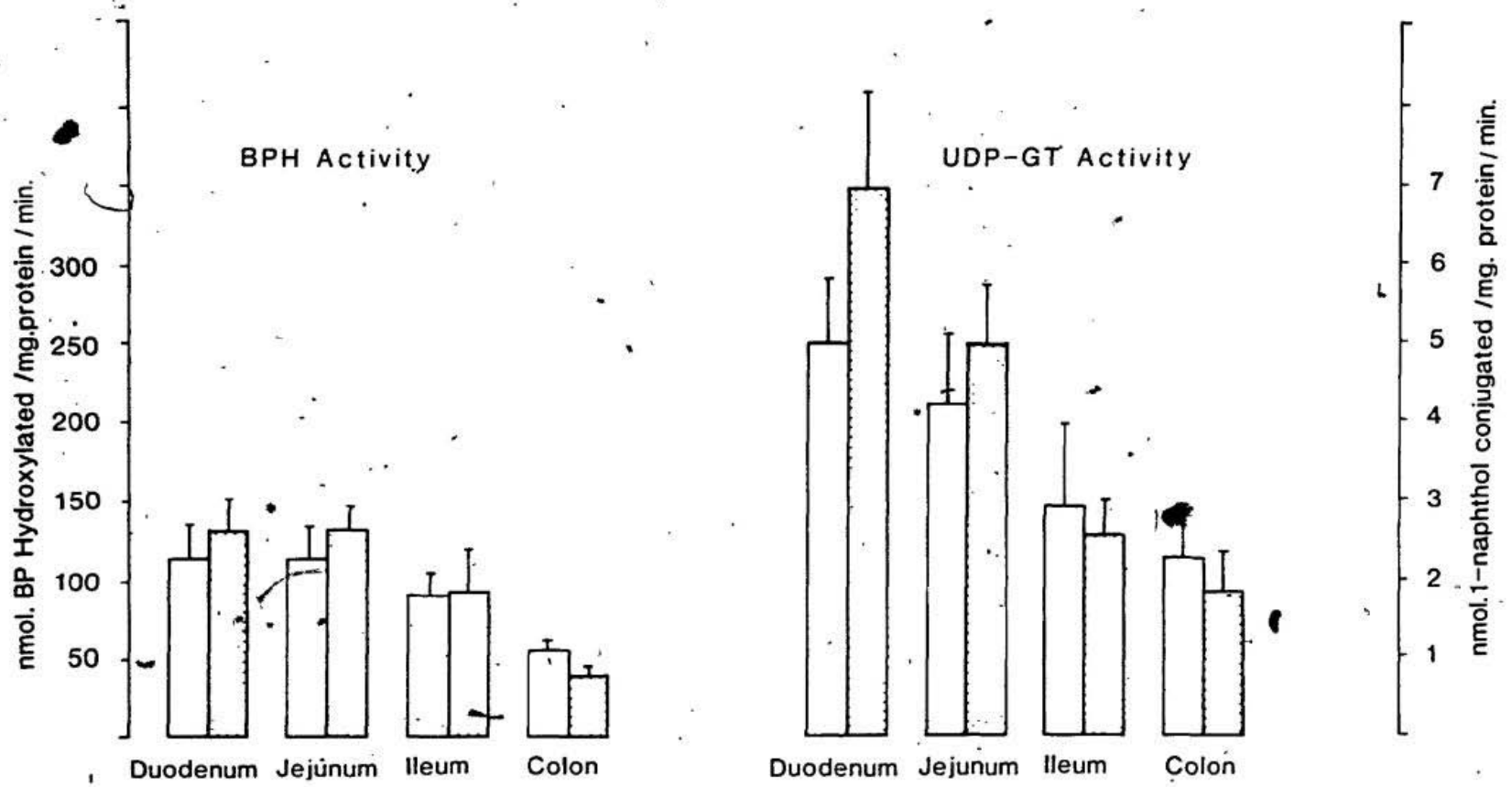

BPH Activity
nmol. BP Hydroxylated /mg.protein / min.
300
250
200
150
100
50
Duodenum
Jejunum
Ileum
Colon
UDP-GT Activity
nmol.1-naphthol conjugated /mg. protein / min.
7
6
5
4
3
2
1
Duodenum
Jejunum
Ileum
Colon

TABLE 3-1 : BENZO(a)PYRENE HYDROXYLASE (BPH) AND GLUCURONYL TRANSFERASE (UDP-GT) ACTIVITIES IN RAT INTESTINE AFTER TREATMENT WITH PENTAGASTRIN.

TISSUE	BPH ACTIVITY		UDP-GT ACTIVITY	
	PENTAGASTRIN	CONTROL	PENTAGASTRIN	CONTROL
Duodenum	201.55 ± 23.5	152.14 ± 28.90	7.57 ± 1.93	8.50 ± 1.62
Jejunum	170.25 ± 22.84	167.88 ± 19.80	5.08 ± 1.36	5.10 ± 0.94
Ileum	147.50 ± 20.43	121.55 ± 22.80	3.05 ± 0.62	1.86 ± 0.42
Colon	192.66 ± 45.02*	81.37 ± 12.86	5.32 ± 0.60	4.22 ± 0.76

1. BPH activity expressed as nmol. BP hydroxylated/mg prot./min.
2. UDP-GT activity expressed as nmol. 1-naphthol conjugated/mg prot./min.
3. Each value represents the mean (± SEM).
4. (*) Significantly different ($P < 0.05$)

TABLE 3-2 : BENZO(a)PYRENE HYDROXYLASE (BPH) AND GLUCURONYL TRANSFERASE (UDP-GT) ACTIVITIES IN RAT INTESTINE AFTER TREATMENT WITH CHOLECYSTOKININ-OCTAPEPTIDE (CCK-OP).

TISSUE	BPH ACTIVITY[1]		UDP-GT ACTIVITY[2]	
	CCK-OP	CONTROL	CCK-OP	CONTROL
Duodenum	109.0 ± 14.4 [3]	77.8 ± 9.13	7.60 ± 1.37	6.42 ± 0.66
Jejunum	69.0 ± 17.9	100.2 ± 26.08	4.49 ± 0.52	4.34 ± 0.92
Ileum	89.2 ± 10.8	89.8 ± 19.93	2.96 ± 0.64	2.82 ± 0.51
Colon	60.5 ± 15.9	65.8 ± 14.33	2.28 ± 0.22	2.52 ± 1.07

1. BPH activity expressed as nmol. BP hydroxylated/mg prot./min.
2. UDP-GT activity expressed as nmol. 1-naphthol conjugated/mg prot./min.
3. Each value represents the mean (± SEM).

TABLE 3-3 : BENZO(a)PYRENE HYDROXYLASE (BPH) AND GLUCURONYL TRANSFERASE (UDP-GT) ACTIVITIES IN RAT INTESTINE AFTER TREATMENT WITH SECRETIN.

TISSUE	BPH ACTIVITY		UDP-GT ACTIVITY	
	SECRETIN	CONTROL	SECRETIN	CONTROL
Duodenum	115.28 ± 13.20	82.71 ± 9.34	4.93 ± 0.93	6.99 ± 1.02
Jejunum	115.28 ± 18.11	131.3[illegible] ± 20.98	4.20 ± 0.89	5.01 ± 0.73
Ileum	91.42 ± 17.22	92.66 ± 26.71	2.93 ± 1.00	2.56 ± 0.44
Colon	54.57 ± 4.30	40.25 ± 4.30	2.22 ± 0.66	1.87 ± 0.50

1. BPH activity expressed as nmol. BP hydroxylated/mg prot./min..
2. UDP-GT activity expressed as nmol. 1-naphthol conjugated/mg prot./min.
3. Each value represents the mean (± SEM).

3.1.2. Cholecystokinin Treatment (Table:3-2, Fig.3-2)

Cholecystokinin (CCK), another major GI hormone, has an identical -COOH terminal sequence to that of gastrin, the terminal pentapeptide being the same in the two hormones. Although Fang and Strobel (1981) observed a marked increase in the drug metabolizing activities of enzymes in colonic mucosa after CCK treatment, under our experimental conditions no such increase was seen in the activity of BPH and UDP-GT in either the colon or the small intestine. The gradient of activity of UDP-GT from duodenum to colon was demonstrated but no such clearly demonstrable gradient for BP was evident in either the control or the treated animals.

Because of the well known trophic action of CCK on rat pancreas (Barrowman and Mayston, 1973), the action of this hormone on BPH and UDP-GT activities in pancreas were also observed. Both the hormone activities were quite low in the pancreas (Fig. 3-2) and there was no statistically significant increase in their activities after hormone treatment.

3.1.3. Secretin Treatment (Table: 3-3, Fig. 3-3)

As in the case of CCK, no stimulation of BPH and UDP-GT activity in rat duodenum, jejunum, ileum or colon was observed, after treatment with secretin. Like the other two experiments, a gradual fall in the activity of these enzymes from foregut to the hind gut was demonstrated.

3.2. Effect of Post-Resection Hypertrophy of the Small Intestine on Xenobiotic Metabolising Enzymes

The animals tolerated anaesthesia and surgery well. Post operative mortality was low, one animal in a group of six. Autopsy revealed that death was due to subacute intestinal obstruction. The other animals recovered quickly from the operation.

After a period of 30 days, at laparatomy, inspection showed dilation and thickening of the remnant. The enlargement was most marked in the residual ileum. The ileal enlargement was tapered being most marked just distal to the anastomosis and almost normal at the ileo-caecal junction.

Microscopically all layers of the intestine showed thickening, although the hyperplasia was most marked in the mucosa. There was an increase in the height of the villi and the tips were rounder and broader. Microscopically the most striking feature was the pronounced crypt hyperplasia, with loss of identity of crypt-villous junction. Many crypts showed cystic dilation. Another feature was a slight increase in the number of goblet cells in both villuos and crypt epithelium.

Because of the loss of crypt villous junction, it was difficult to measure the villous or the crypt height separately. Since the scope of this thesis does not involve a detailed histological examination, it was decided to compare the whole thickness of the mucosa before and after adaptation. Measurements were taken from the muscularis mucosae to the tip of the villous. At least ten readings were taken from each section. Table: 3-4 shows the mean thickness of jejunul and ileal

Figure : 3.4

Effect of adaptive ileal hypertrophy following proximal resection and anastomosis, on benzo(a)pyrene hydroxylase (BPH) and glucuronyl transferase (UDP-GT) activities.

Control: White

After adaptation (hypertrophied): Dotted

Each value represents the mean (± SEM) for 3 to 6 determinations.

(*) Significantly different.

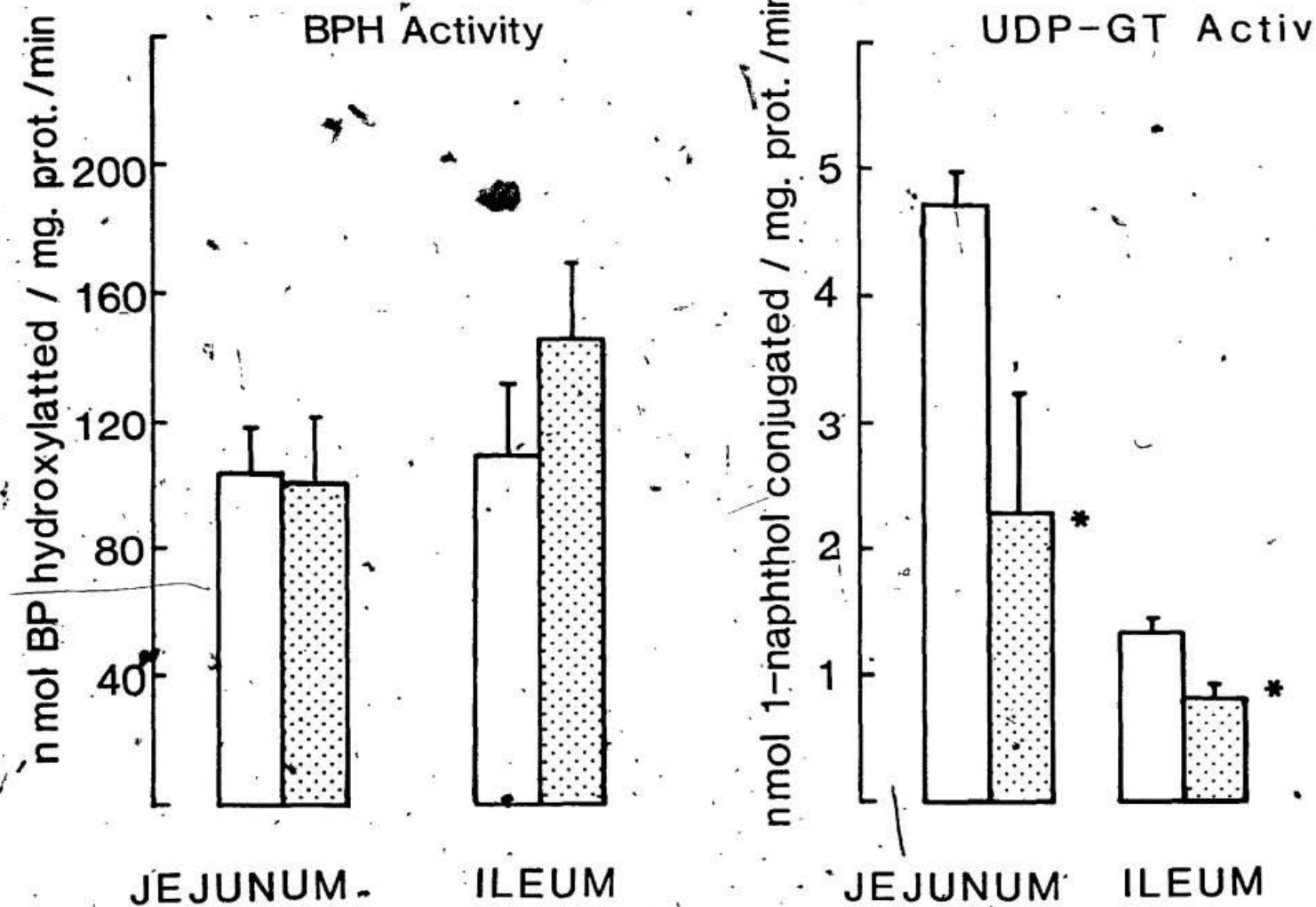

BPH Activity
n mol BP hydroxylatted / mg. prot. /min
200
160
120
80
40
JEJUNUM
ILEUM
UDP-GT Activity
n mol 1-naphthol conjugated / mg. prot. /min.
5
4
3
2
1
*
*
JEJUNUM
ILEUM

TABLE 3-4 : CHANGES IN THE MUCOSAL THICKNESS IN THE REMNANT ILEUM OF THE RAT FOLLOWING PROXIMAL RESECTION AND ANASTOMOSIS.

Animal	Jejunum (± SEM) um. (before resection)	Ileum (± SEM) um. (before resection)	Ileum (± SEM) um (after adaptation)
1.	649 ± 8.61	473 ± 11.0	848 ± 6.80
2.	676 ±25.80	492 ± 13.5	854 ±19.40
3.	683 ±12.20	528 ± 9.8	928 ±18.30
4.	660 ±55.00	448 ± 13.0	830 ±12.50
5.	664 ±12.50	522 ± 10.8	910 ±22.00
MEAN	666.4 ± 13.40	492 ± 33.5	874 ± 42.5*

1. Each entry represents the mean for at least 10 readings from a section.
2. The increase in thickness is almost 178 %.
* Significantly different from the control (Ileum).

TABLE 3-5 : CHANGES IN BPH AND UDP-GT ACTIVITIES IN THE REMNANT ILEUM OF THE RAT FOLLOWING PROXIMAL RESECTION AND ANASTOMOSIS.

	Total microsomal protein (mg/ 10 cm Length)				BPH Activity (nmol /mg prot/min)				UDP-GT Activity (nmol /mg prot/min)			
	Jejunum		Ileum		Jejunum		Ileum		Jejunum		Ileum	
No.	(A)	(B)[1]	(A)	(B)	(A)	(B)[1]	(A)	(B)	(A)	(B)[1]	(A)	(B)
1.	5.42	7.68	5.82	11.52	148	72	167	77	5.55	1.37	1.38	0.98
2.	4.63	9.70	1.92	7.65	88	130	120	202	4.52	4.05	1.57	.0.44
3.	3.00	8.70	1.48	7.60	105	101	105	146	4.82	2.70	1.10	0.88
4.	3.05		4.72	8.65	101		44	161	4.03		1.31	0.64
5.	4.10		2.30	5.74	74		109	143	4.70		1.30	0.09
Mean	4.05	8.69*	3.24	8.23**	103.2	101	109	145.8	4.71	2.28*	1.33	0.76*
SEM	±.48	±.71	±.95	±1.05	±13.9	±20.5	±21.9	±22.5	±.26	±.98	±.08	±.11

(*) Significantly different ($P<0.05$)
1. Readings from three animals only.
(A) Before adaptive hypertrophy.
(B) After adaptive hypertrophy.

mucosa before resection and the ileal mucosa after the adaptation. The ileal mucosa shows an approximately 177% increase in mucosal thickness over the control ileum and approximately 131% over the control jejunum.

The total microsomal protein content in the mucosa of both jejunum and ileum were found to be significantly increased after adaptation. The BPH activity (expressed in nanomoles of product formed per mg. of prot. per min.) was not significantly altered either in jejunum or ileum, after adaptation, as compared to the control. The UDP-GT activity (expressed in nanomoles of product formed per mg. of prot. per min.) in jejunal mucosa and the ileal remnant after the adaptation showed a significant drop in UDP-GT activity (Table: 3-5, Fig. 3-4)(63% of the control, $P < 0.05$).

3.3. Study on the Intestinal Absorption of PAHs

3.3.1. Role of Intraduodenal Bile

The importance of bile in the absorption of polynuclear aromatic hydrocarbons is well established. Although a great deal of study has been done on the absorption of PAHs, most of the workers were concerned with such compounds as BP, 7,12-DMBA or 3-MC. To our knowledge, very little has been done to observe the role of bile in the absorption of lower molecular weight PAHs like 2,6-DMN, anthracene and phenanthrene. In our study we included PAHs with gradually increasing molecular weight and increasing aromatic ring structures having gradually decreasing water solubility.

It is well known that a major portion of a systemically administered dose of

PAH is efficiently excreted in bile and urine. Thus monitoring these two samples provides a reliable index of bioavailability. Since by establishing a biliary cannula, the normal physiological condition in the duodenum is altered, in the control animals this was corrected by infusing 0.5 ml of exogenous bile intraduodenally every hour. This volume of bile supplementation is in agreement with the normal physiological rate of bile secretion in rats (Thompson and Vars, 1953). In the other group of animals, no such supplementation was made and the results obtained were compared with controls and an assessment was made on the role of bile in the bioavailability of these compounds.

3.3.1.1. 2,6-Dimethylnaphthalene (DMN)

Fig. 3-5 shows the 0-8 hour pattern of biliary radio label recovery at half-hour intervals following an intraduodenal administration of [^{3}H]-2,6-DMN, both in the presence and absence of bile. The figure also shows the total cumulative recovery in 24 hours. In the presence of bile, the rate of biliary excretion of radiolabel peaked 90 min. after introduction of the test meal, reaching a value of 5.90 ± 1.93% of the total administered dose. Then the rate of excretion percentage gradually fell to a value of approximately 0.5% of the total dose at 5 hour post infusion period and was constant thereafter. The cumulative recovery from 0-8 hour bile in the above situation was 29.58 ± 4.8% and from 8-24 hour bile it was 1.43 ± 0.25%. In the urine, 24-hour recovery of radiolabel was 20.95 ± 9.97%. The total recovery of radiolabel in bile and urine combined, in presence of duodenal bile, was 51.97 ± 12.12% (Table: 3-6).

In the absence of bile supplementation the corresponding recovery value in 0-8 hour bile, was 25.97 ± 4.32%, in 8-24 hour bile it was 5.65 ± 0.58% and in

Figure : 3.5

Pattern of radiolabel excreted in bile over an 8-hour period following intraduodenal administration of [^{3}H]-2,6-dimethylnaphthalene in corn oil with or without exogenous bile supplementation.

Collections were taken every 30 minutes.

Each point represents the mean (± SEM) for at least 5 animals.

The right side of the figure shows the total recovery of radiolabel in 24-hours divided into 8-hour biliary recovery, 16-hour biliary recovery (ONB, over night bile) and 24-hour urinary recovery.

Biliary excretion in %
1
2
3
4
5
6
30
120
210
300
390
480
Time in minutes
with bile
without bile
Total excretion in %
0
10
20
30
40
50
60
70
8hr. BILE
ONB
URINE
without bile
with bile

24-hour urine it was 16.0 ± 2.78% of the total administered. The total recovery of radiolabel excreted in bile and urine combined, in absence of duodenal bile, was 47.63 ± 5.16%. If the results of the two experimental situations are compared, only the 8-24 hour biliary recovery in absence of bile was significantly higher ($P < 0.05$) than the corresponding recovery in presence of bile but the total recovery was not significantly different from each other (Table: 3-6; Fig. 3-10, 3-11), that is, decreased and prolonged rate of biliary excretion, without altering total availability.

3.3.1.2. Phenanthrene

Fig. 3-6 shows the 0-8 hour pattern of radiolabel excretion at half hour intervals and also the total excretion of radiolabel in 24 hours, after an intraduodenal dose of [^{14}C]-phenanthrene in the presence and absence of exogenous bile supplementation. In both conditions peak recovery was seen at 1 hour after infusion. The peak excretion rate in the presence of bile, 7.83 ± 1.30%, was slightly higher than the peak obtained in the absence of bile, 4.55 ± 0.67%. In the absence of bile, the recovery pattern formed a plateau at approximately 3 hour after infusion and was maintained at a slightly higher level than the control with bile. The cumulative excretion of radiolabel in the 0-8 hour period, in presence of bile was 45.15 ± 3.18%., as compared to 40.84 ± 2.72% in absence of bile. In the 8-24 hour period, the radiolabel recovery in the presence of bile was 10.35 ± 0.52% and in the absence of bile it was 10.54 ± 3.02%. A similar pattern was noticed in the radiolabel excretion in 24-hour urine. The presence of exogenous bile resulted in 17.23 ± 2.28% recovery whereas the absence of bile resulted in 18.93 ± 2.44% recovery. The total combined recovery

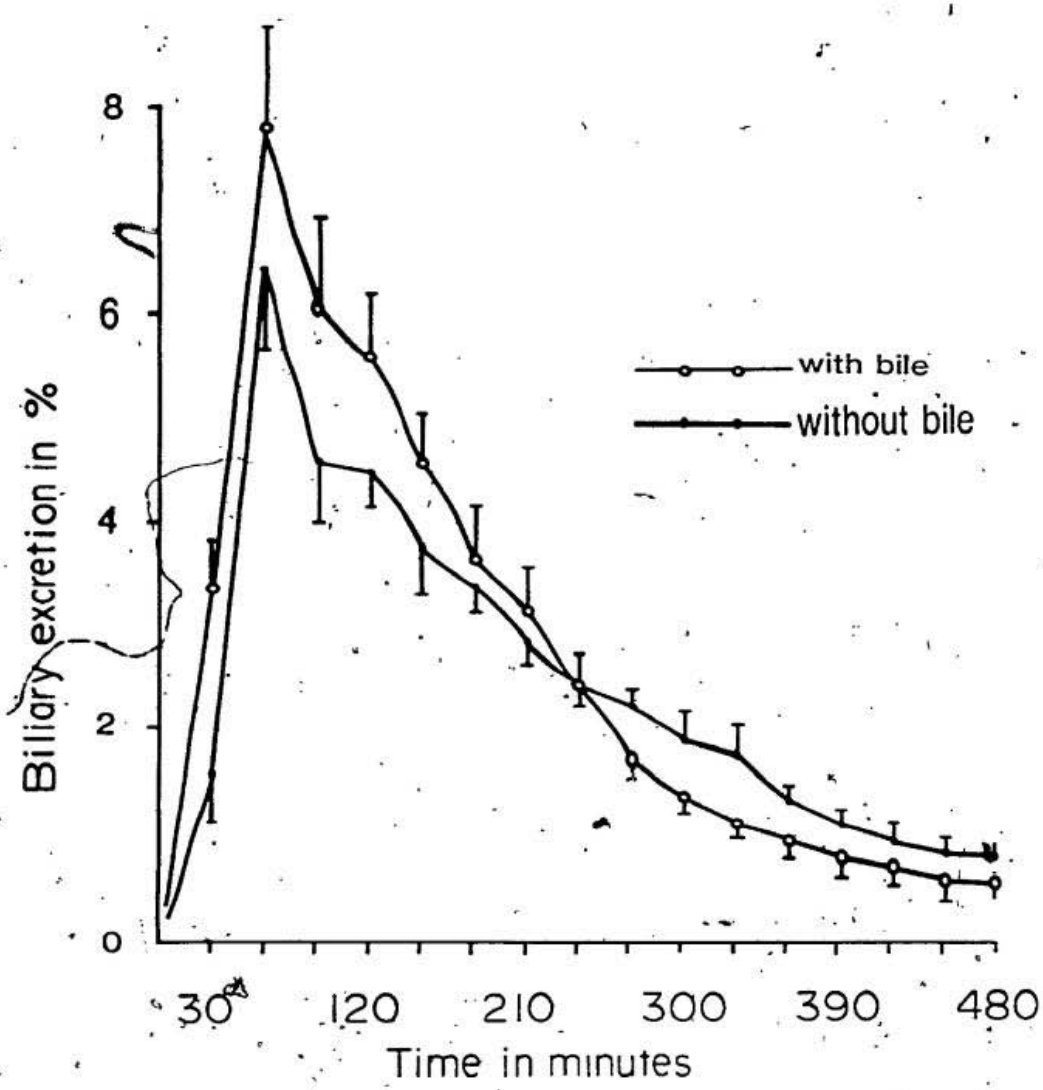
Figure : 3.6

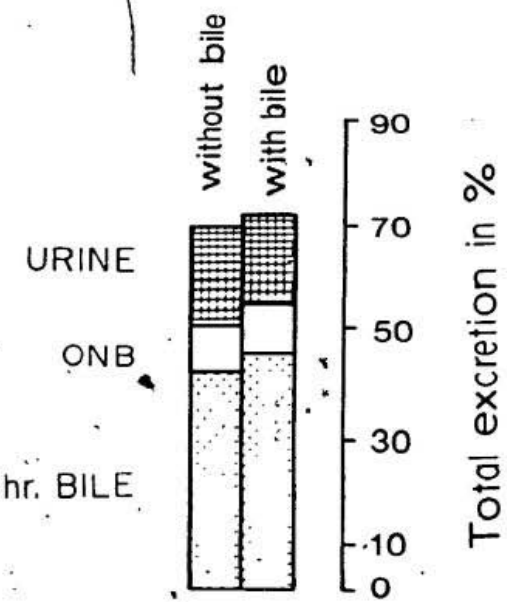
Pattern of radiolabel excreted in bile over an 8-hour period following intraduodenal administration of [^{14}C]-phenanthrene in corn oil with or without exogenous bile supplementation.

Collections were taken every 30 minutes.

Each point represents the mean (± SEM) for at least 5 animals.

The right side of the figure shows the total recovery of radiolabel in 24-hours divided into 8-hour biliary recovery, 16-hour biliary recovery (ONB, over night bile) and 24-hour urinary recovery.


Biliary excretion in %
8
6
4
2
0
with bile
without bile
30
120
210
300
390
480
Time in minutes


without bile
with bile
URINE
ONB
8hr. BILE
90
70
50
30
10
0
Total excretion in %

in bile and urine in 24 hour, in the presence of exogenous bile supplementation was 72.74 ± 4.26% and in absence of bile was 70.32 ± 6.26%. None of these differences was significant. (Tab. 3-6; Fig. 3-10, 3-11).

3.3.1.3. Anthracene

When [^{14}C]-anthracene was administered intraduodenally, the presence of exogenous bile produced a sharp difference in the radiolabel excretory pattern. In the presence of bile the peak recovery rate of 6.14 ± 1.21% was seen at 1.30 hour after infusion as compared to the peak value of 2.42 ± .77% at 2.30 hour in absence of bile (Fig. 3-7). The cumulative biliary recovery from 0-8 hour in the absence of bile was 20.61 ± 1.58% which was significantly ($P < 0.05$) lower than the recovery in the presence of bile, 38.95 ± 5.43%. From 8-24 hours, the cumulative recovery in bile in each group was not significantly different, being 12.37 ± 2.61% in presence of bile and 16.82 ± 3.62% in absence. Likewise the cumulative recovery in urine in both conditions were not significantly different from each other: in presence of bile it was 24.43 ± 5.43% and in absence of bile it was 16.22 ± 1.32% of the administered dose. The total cumulative biliary and urinary radiolabel excretion in presence of bile was 75.77 ± 6.56%, which was significantly ($P < 0.05$) higher (141%) than the recovery obtained in absence of bile, 53.65 ± 4.22% (Table: 3-6; Fig. 3-10, 3-11).

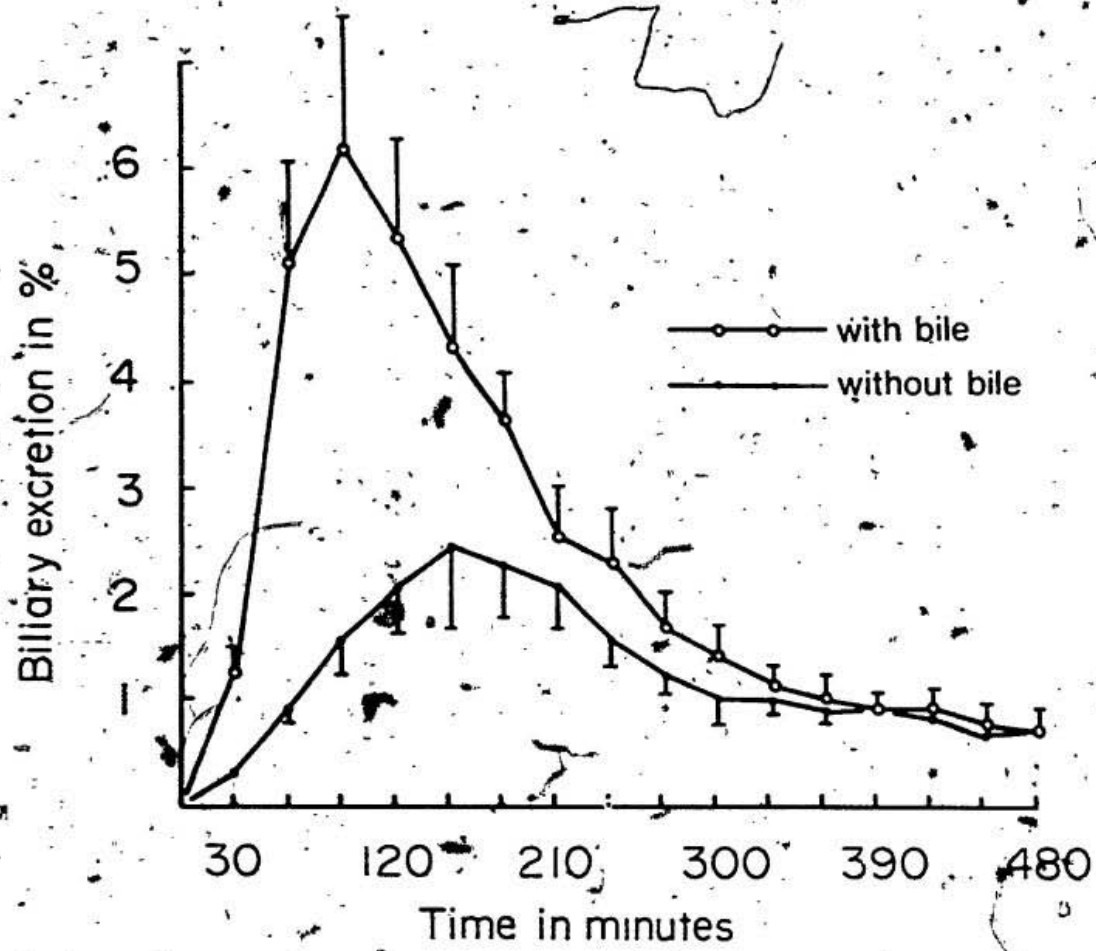
Figure : 3.7

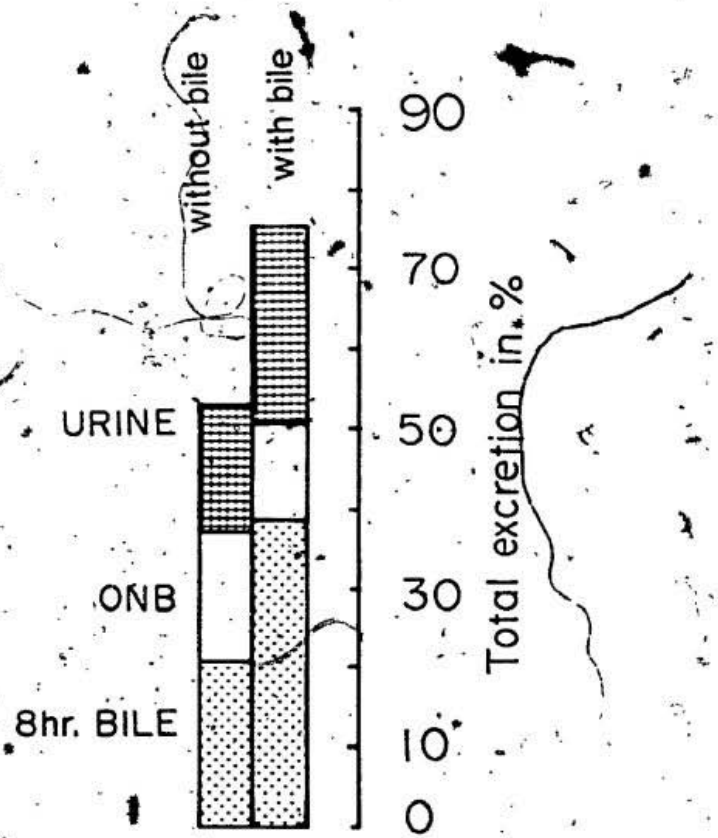
Pattern of radiolabel excreted in bile over an 8-hour period following intraduodenal administration of [^{14}C]-anthracene in corn oil with or without exogenous bile supplementation.

Collections were taken every 30 minutes.

Each point represents the mean (± SEM) for at least 5 animals.

The right side of the figure shows the total recovery of radiolabel in 24-hours divided into 8-hour biliary recovery, 16-hour biliary recovery (ONB, over night bile) and 24-hour urinary recovery.


Biliary excretion in %
1
2
3
4
5
6
with bile
without bile
30
120
210
300
390
480
Time in minutes


without bile
with bile
URINE
ONB
8hr. BILE
90
70
50
30
10
0
Total excretion in %

3.3.1.4. 7,12-Dimethylbenzanthracene(DMBA)

Fig. 3-8 portrays the pattern of biliary radiolabel recovery in the 0-8 hour period following an intraduodenal administration of [^{3}H]-7,12-DMBA, in the presence and absence of bile. Without exogenous bile supplementation, the excretion of radiolabel was relatively uniform throughout this period and no peak excretion rate was observed. Upon inclusion of bile, a peak recovery rate of 2.59 ± 0.19% of the total radiolabel administered was observed at 1.30 hour after infusion. The rate of excretion of radiolabel then fell gradually and after 8 hours the recovery pattern in both control and experimental groups almost coincided. Total recovery in the first 8 hours in the presence of bile was 18.24 ± 2.92% , which is significantly higher ($P < 0.05$) than the recovery in the absence of bile, 6.35 ± 1.06%. Biliary radiolabel recovery in the 8-24 hour period was not affected by the presence of duodenal bile being 3.55 ± 0.45% in the presence of bile and 2.73 ± 0.23 in the absence of bile. Urinary recovery of radiolabel was significantly affected by the absence of bile. Addition of bile resulted in 2.68 ± 0.21% of the total radiolabel being excreted in the urine in 24 hours compared with 1.53 ± 0.18% in its absence. Comparing the total excretion of radiolabel in 24-hrs bile and urine together, a significant difference ($P < 0.05$) was observed between the two groups. The presence of duodenal bile resulted in 24.38 ± 3.39% of the total radiolabel being recovered while in its absence only 10.62 ± 1.33% of the total dose was recovered, a difference of approximately 56% (Table: 3-6; Fig. 3-10, 3-11).

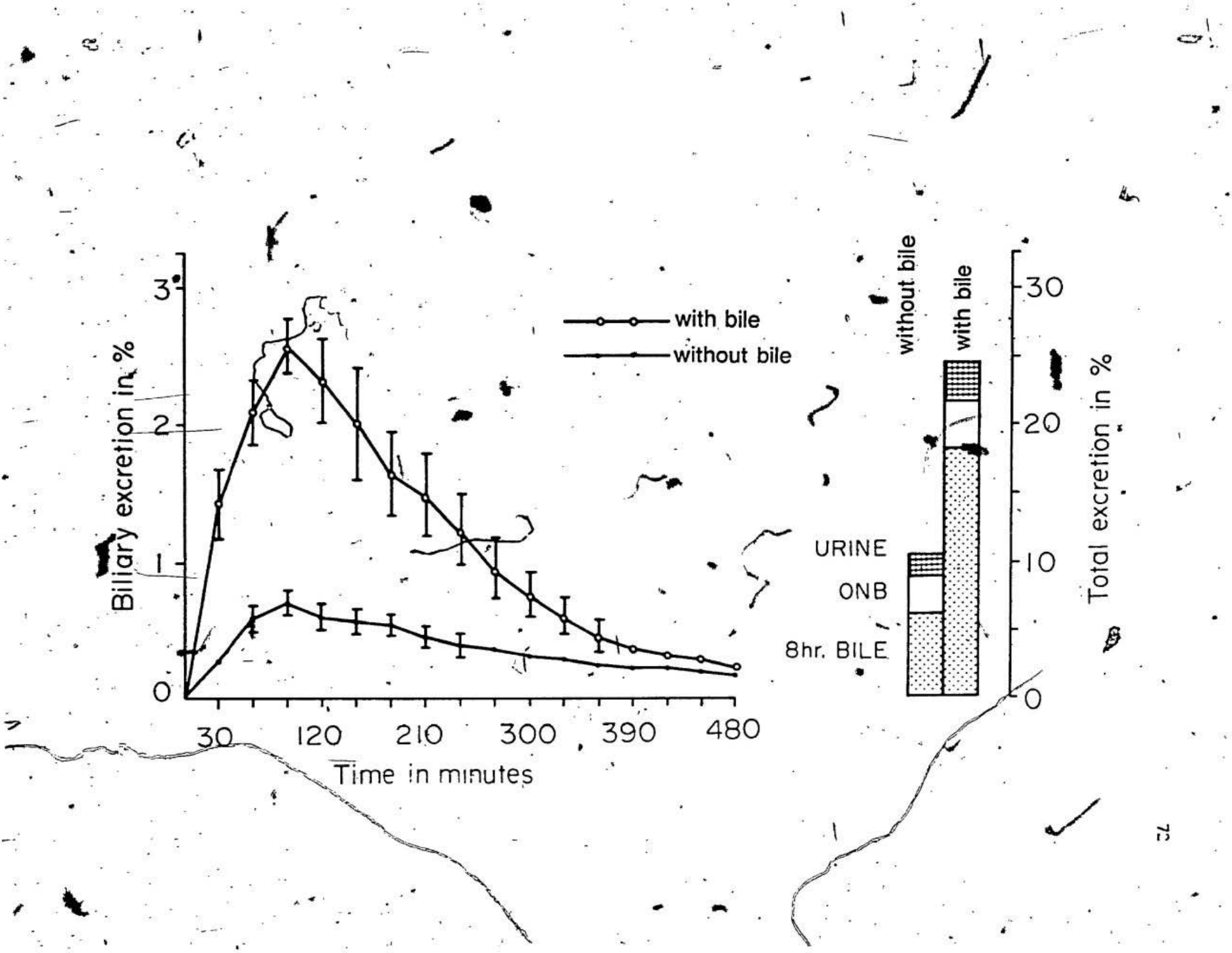
Figure 3.8

Pattern of radiolabel excreted in bile over an 8-hour period following intraduodenal administration of [^{3}H]-7,12-dimethylbenzanthracene in corn oil with or without exogenous bile supplementation.

Collections were taken every 30 minutes.

Each point represents the mean (± SEM) for at least 5 animals.

The right side of the figure shows the total recovery of radiolabel in 24-hours divided into 8-hour biliary recovery, 16-hour biliary recovery (ONB, over night bile) and 24-hour urinary recovery.


Biliary excretion in %
3
2
1
0
with bile
without bile
30
120
210
300
390
480
Time in minutes
without bile
with bile
URINE
ONB
8hr. BILE
30
20
10
0
Total excretion in %

3.3.1.5. Benzo(a)pyrene (BP)

Fig. 3-9 depicits the pattern of biliary radiolabel recovery in 0-8 hour period following an intraduodenal dose of [^{3}H]-BP. In the absence of bile the rate of radiolabel excretion remained relatively constant from 3-6 hours following instillation with no obvious peak occuring. When bile was included, there was a significant ($P < 0.05$) increase in peak biliary excretion of radiolabel to 2.55 ± 0.17% of the administered dose, which occured 3 hours after infusion. Cumulative recovery from 0-8 hours in the presence of bile was 19.92 ± 1.08% which is significantly higher ($P < 0.05$) than the recovery in the absence of bile, 2.95 ± 0.22%. In the 8-24 hour period, the inclusion of exogenous bile did not significantly change the percentage recovery of radiolabel, which was 5.31 ± 0.84% in the presence of bile and 3.11 ± 0.26 in the absence of bile. When the radiolabel excretion in urine was analyzed in the two situations, recovery in the presence of bile (5.23 ± 0.53%) was significantly ($P < 0.05$) higher than the recovery in the absence of bile (0.92 ± 0.17%). Providing an exogenous supplementation of bile produced a combined biliary and urinary excretion of 30.47 ± 1.94 which is significantly ($P < 0.05$) higher than the recovery obtained when bile was excluded (6.99 ± 0.27%) (Table: 3-6, Fig. 3-10, 3-11).

The efficiency of absorption of the representitive PAHs in the absence of bile may be expressed as the percent of absorption in the presence of duodenal bile. It is observed that for 2,6-DMN and phenanthrene, the efficiency of absorption in the absence of duodenal bile is close to 100% (Tab. 3-7). But for anthracene, 7,12-DMBA and BP, the efficiency of absorption gradually decreases in proportion to their water solubility and are significantly lower than the controls.

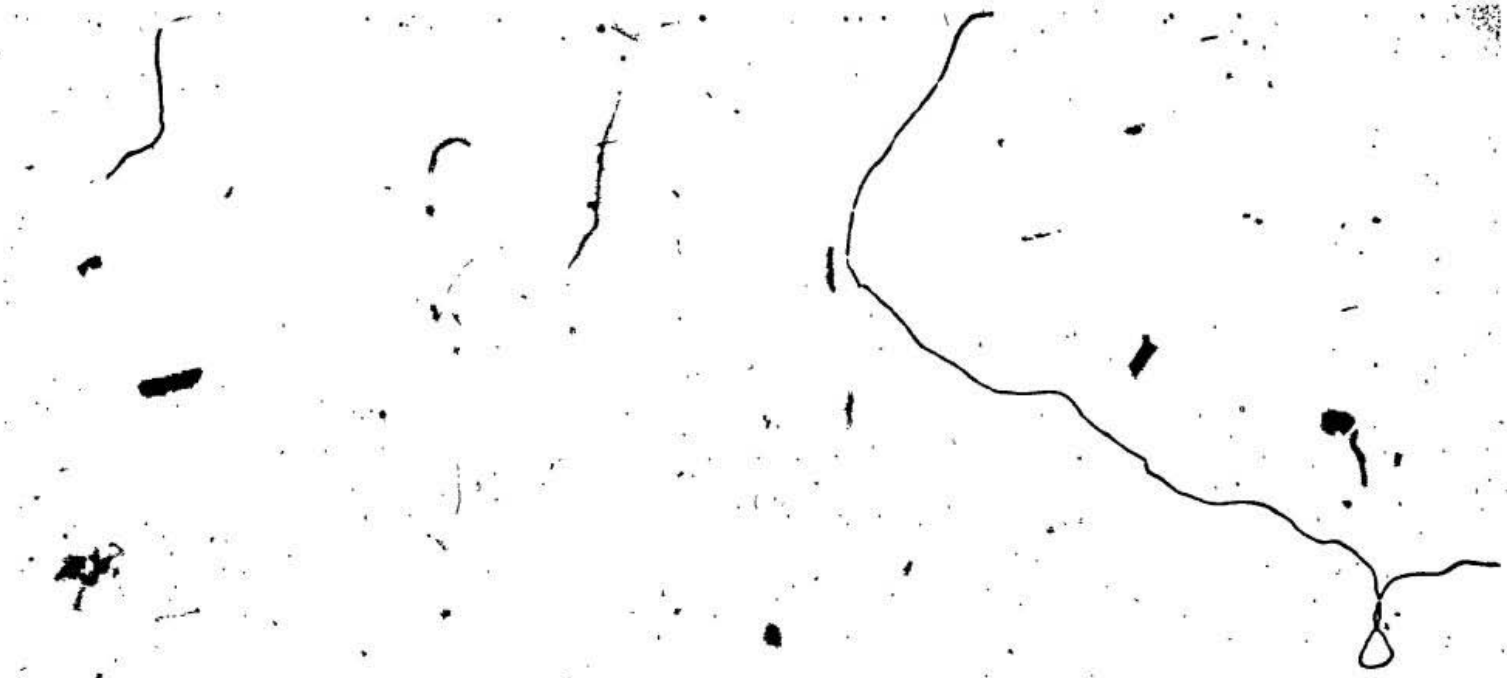
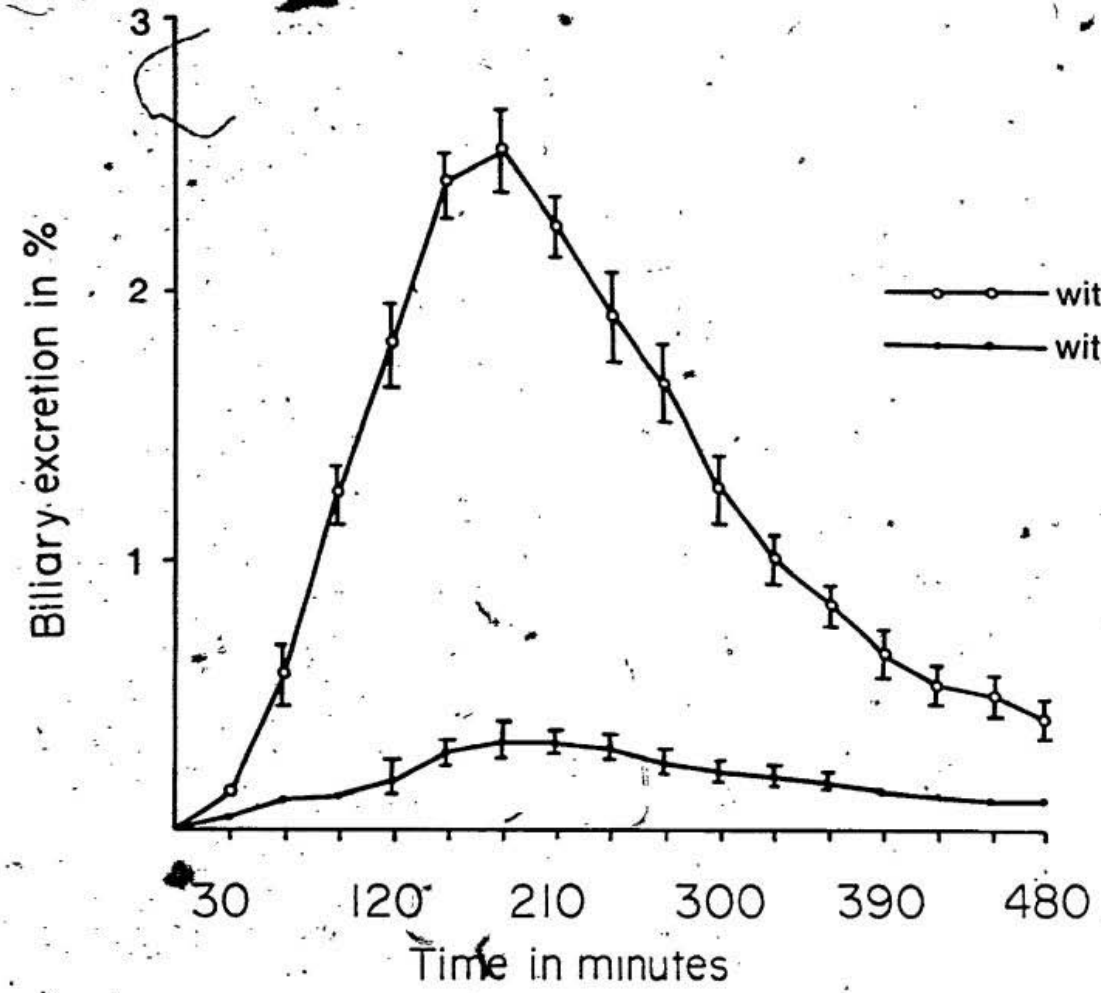


Figure : 3.9

Pattern of radiolabel excreted in bile over an 8-hour period following intraduodenal administration of [^{3}H]-BP in corn oil with or without exogenous bile supplementation.
Collections were taken every 30 minutes.
Each point represents the mean (± SEM) for at least 5 animals.
The right side of the figure shows the total recovery of radiolabel in 24-hours divided into 8-hour biliary recovery, 16-hour biliary recovery (ONB, over night bile) and 24-hour urinary recovery.


Biliary excretion in %
3
2
1
30
120
210
300
390
480
Time in minutes
with bile
without bile

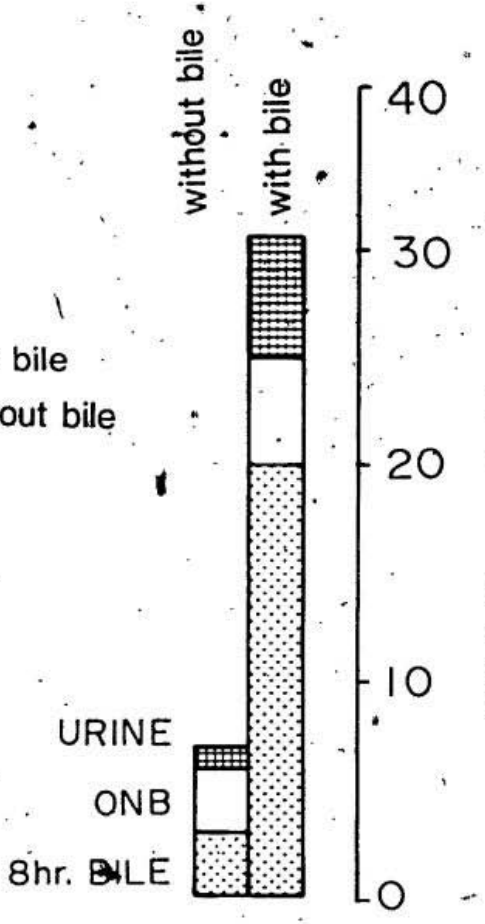

without bile
with bile
URINE
ONB
8hr. BILE
40
30
20
10
0
Total Excretion in %

Figure : 3.10

Urinary excretion of radiolabel in a 24-hour period following intraduodenal administration of labelled PAHs in corn-oil with and without exogenous bile supplementation.
Each value represents the mean (± SEM) for 5 animals.
(*) Significantly different ($P < 0.05$).

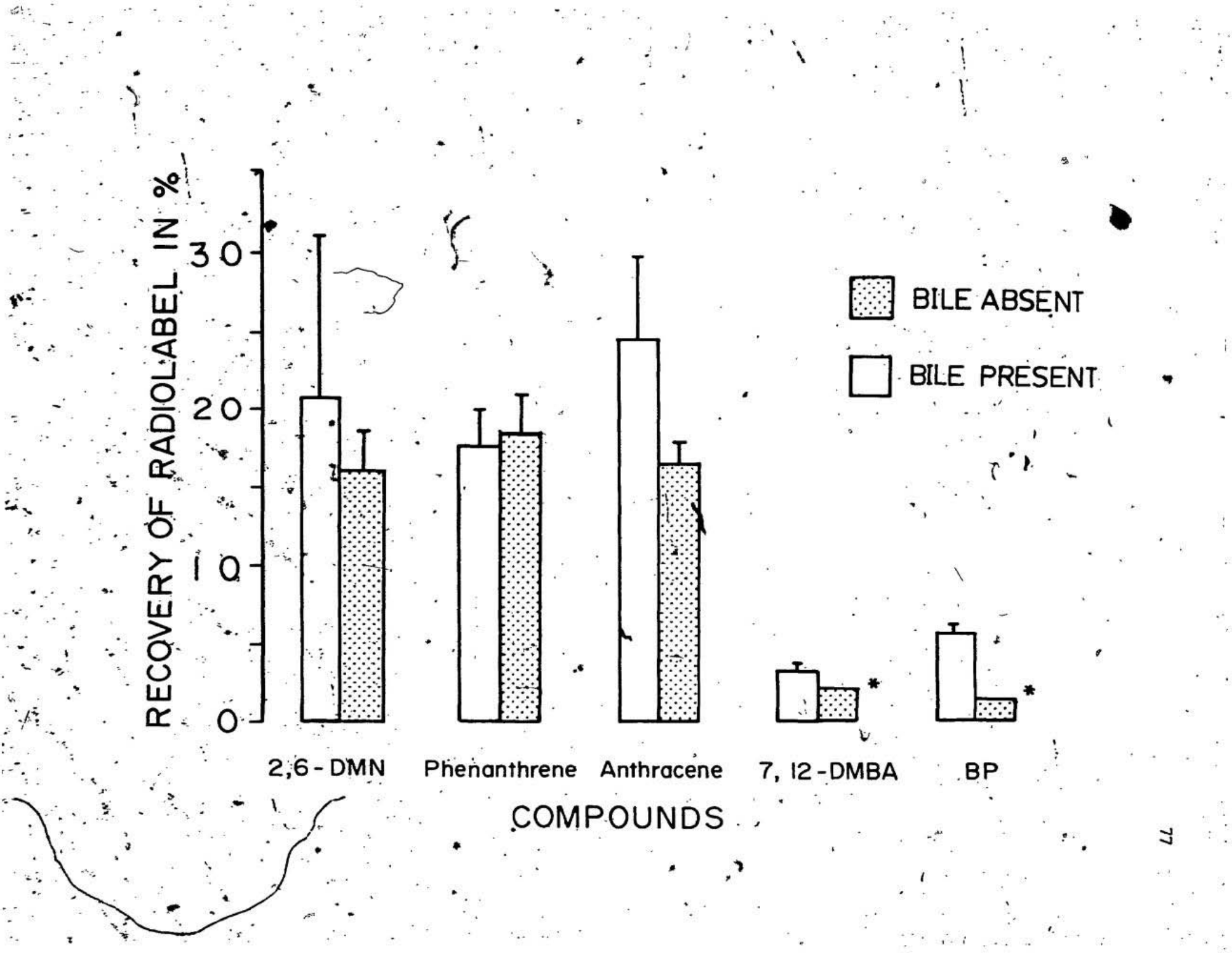

RECOVERY OF RADIOLABEL IN %
30
20
10
0
BILE ABSENT
BILE PRESENT
2,6-DMN
Phenanthrene
Anthracene
7, 12-DMBA
BP
*
*
COMPOUNDS

Figure : 3.11

Total excretion of radiolabel in bile and urine combined in 24-hour period following intraduodenal administration of labelled PAHs in corn-oil with and without exogenous bile supplementation.

Each value represents the mean (± SEM) for 5 animals.

(*) Significantly different ($P < 0.05$)

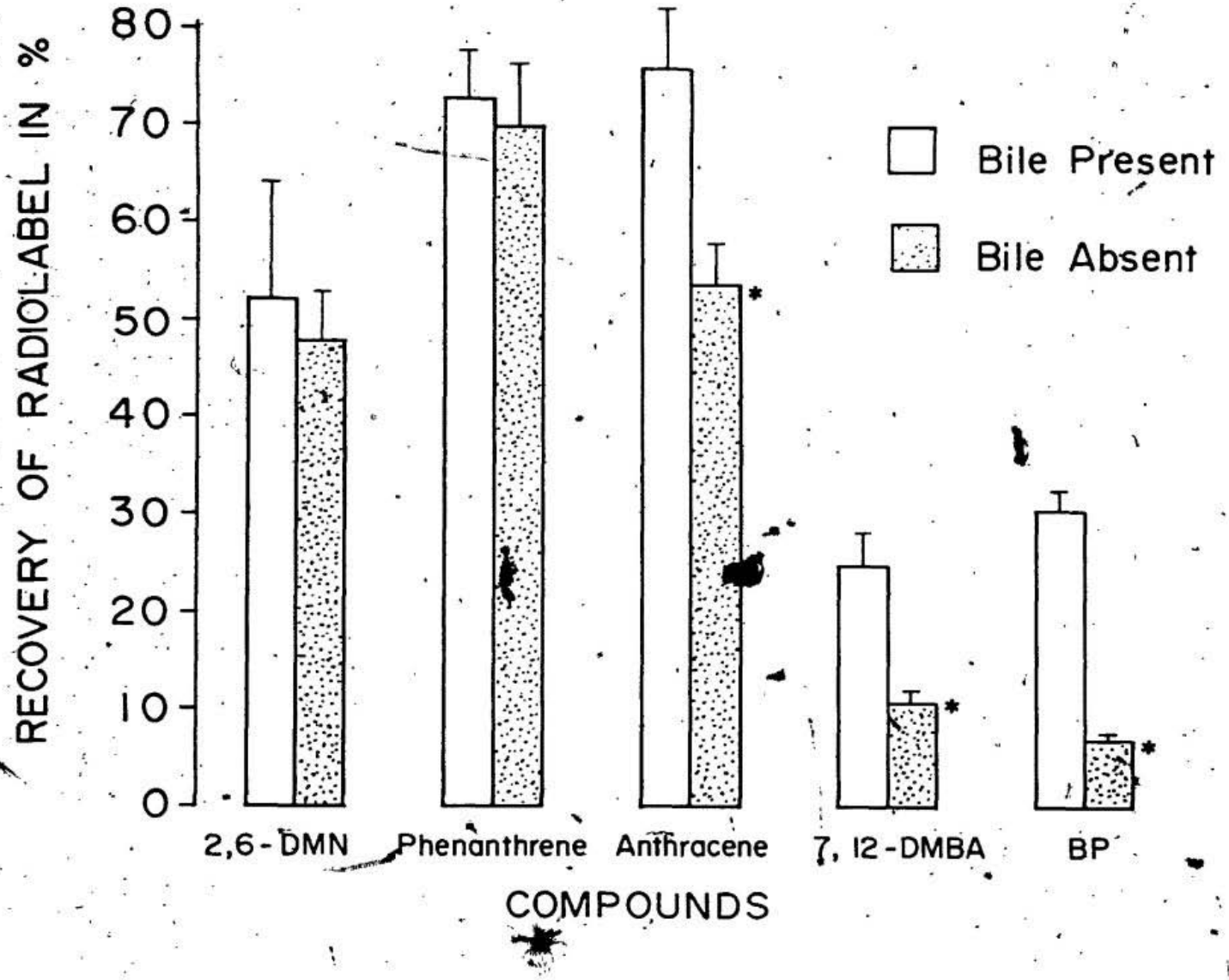

RECOVERY OF RADIOLABEL IN %
80
70
60
50
40
30
20
10
0
Bile Present
Bile Absent
*
*
*
2,6-DMN
Phenanthrene
Anthracene
7, 12-DMBA
BP
COMPOUNDS

Figure : 3.12

Recovery of radiolabel in bile and urine in 24-hours as percentage of the total recovered.

A : With duodenal bile

B : Without duodenal bile

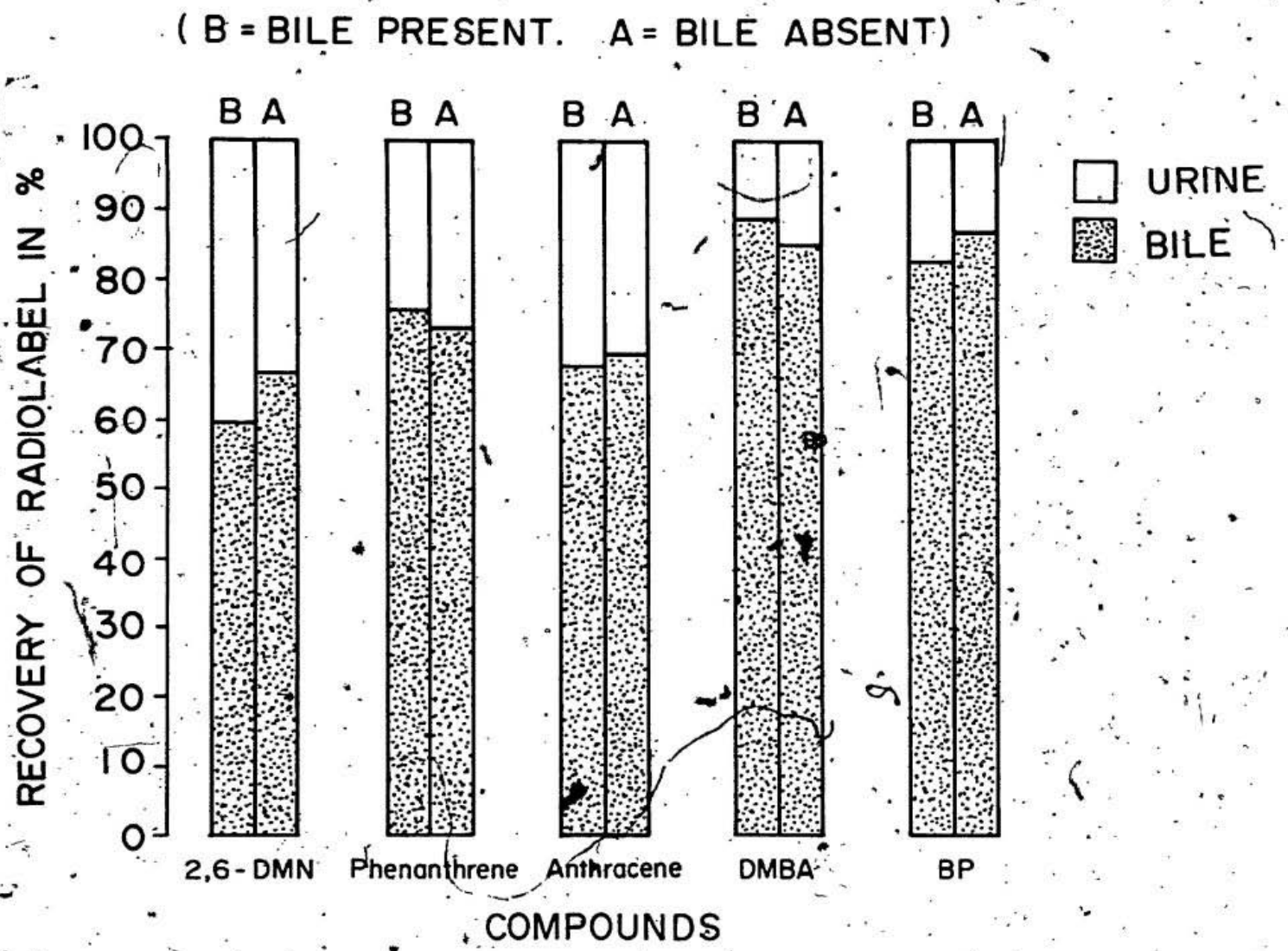

(B = BILE PRESENT. A = BILE ABSENT)
B A
B A
B A
B A
B A
RECOVERY OF RADIOLABEL IN %
100
90
80
70
60
50
40
30
20
10
0
URINE
BILE
2,6 - DMN
Phenanthrene
Anthracene
DMBA
BP
COMPOUNDS

TABLE 3-6 : CUMULATIVE RECOVERY OF RADIOLABEL IN 24-HOUR BILE AND URINE COLLECTIONS FOLLOWING ADMINISTRATION OF RADIOLABELLED PAHs INTRADUODENALLY.

COMPOUNDS	8-HOUR BILE		16-HOUR BILE		URINE		TOTAL	
	BILE(P)	BILE(A)	BILE(P)	BILE(A)	BILE(P)	BILE(A)	BILE(P)	BILE(A)
2,6-DMN	29.58	25.97	1.43	5.65*	20.95	16.00	51.97	47.63
	±4.80	±4.32	±0.25	±0.58	±9.97	±2.78	±12.12	±5.16
Phenanthrene	45.15	40.84	10.35	10.54	17.23	18.93	72.74	70.32
	±3.18	±2.72	±0.52	±3.02	±2.28	±2.44	±4.26	±6.26
Anthracene	38.95	20.61*	12.37	16.82	24.43	16.22	75.77	53.65*
	±5.43	±1.58	±2.61	±3.62	±5.43	±1.32	±6.56	±4.22
7,12-DMBA	18.24	6.35*	3.55	2.73	2.68	1.53*	24.48	10.62*
	±2.92	±1.06	±0.45	±0.23	±0.21	±0.18	±3.39	±1.33
BP	19.92	2.95*	5.31	3.11	5.23	0.92*	30.47	6.99*
	±1.08	±0.22	±0.84	±0.26	±0.53	±0.17	±1.94	±0.27

* : Significantly different ($P < 0.05$)
BILE(P) : Duodenal bile present
BILE(A) : Duodenal bile absent.

TABLE 3-7 : EFFICIENCY OF ABSORPTION OF PAHs IN ABSENCE OF DUODENAL BILE IN RELATION TO THEIR WATER SOLUBILITY.

COMPOUND	MOL. WT.	AQUEOUS SOLUBILITY** (mg /L)	RECOVERY OF RADIOLABEL IN PERCENTAGE		EFFICIENCY OF ABSORPTION IN PERCENTAGE IN ABSENCE OF BILE***
			BILE PRESENT	BILE ABSENT	
2,6-DMN	156	2.0 ±0.02	51.97	47.63	91.64
Phenanthrene	178	1.29 ±0.07	72.74	70.32	96.67
Anthracene	178	0.073 ±0.005	75.77	53.65	70.84*
7,12-DMBA	256	0.061 ±0.0006	24.48	10.62	43.38*
BP	252	0.0038 ±0.00031	30.47	6.99	22.94*

* Significantly lower ($P < 0.05$)
** Mackay and Shiau (1977)
*** % of absorption with bile.

If the total radioactivity recovered in 24-hour is taken as 100% and the percentage of excretion in bile and urine are calculated (Fig. 3-12), it is observed that as the molecular weight of the compounds increases the proportion excreted in the urine gradualy decreases.

3.3.2. Role of Lipid Vehicle in 2,6-Dimethylnaphthalene (DMN) Absorption

This study assesed the role of concomitant lipid absorption on the bioavailability of [^{3}H]-2,6-DMN. A test meal of radiolabelled 2,6-DMN was administered dissolved in ethanol and biliary and urinary radiolabel recovery pattern was compared with that of a test meal given in corn oil.

Fig. 3-13 shows the half-hourly pattern of biliary radiolabel excretion in the first 8 hours after instillation of [^{3}H]-2,6-DMN in ethanol. The peak recovery was obtained within 30 minutes after infusion as compared with that after corn-oil (90 min.)(Fig. 3-5). Although the peak biliary recovery rate did not differ significantly between the two, the biliary recovery pattern of radiolabel in 0-8 hour period when 2,6-DMN was administered in ethanol, fell to approximately 1% of the total dose at 3 hour after infusion as compared to a relatively slower fall in recovery percentage when 2,6-DMN was given in corn oil. The cumulative biliary recovery of radiolabel from 0-8 hour was 27.27 ± 7.79% and in the 8-24 hour peroiod was 6.42 ± 4.90%. The total recovery of radiolabel in 24-hour urine was 14.14 ± 6.02% and thus the combined biliary and urinary recovery of radiolabel in 24-hours, when the test-meal was administered in ethanol was 47.84 ± 15.24%. This is not significantly different from the total recovery when the compound was given in corn oil (51.97 ± 12.10%) (Table: 3-8; Fig. 3-16, 3-17, 3-18).

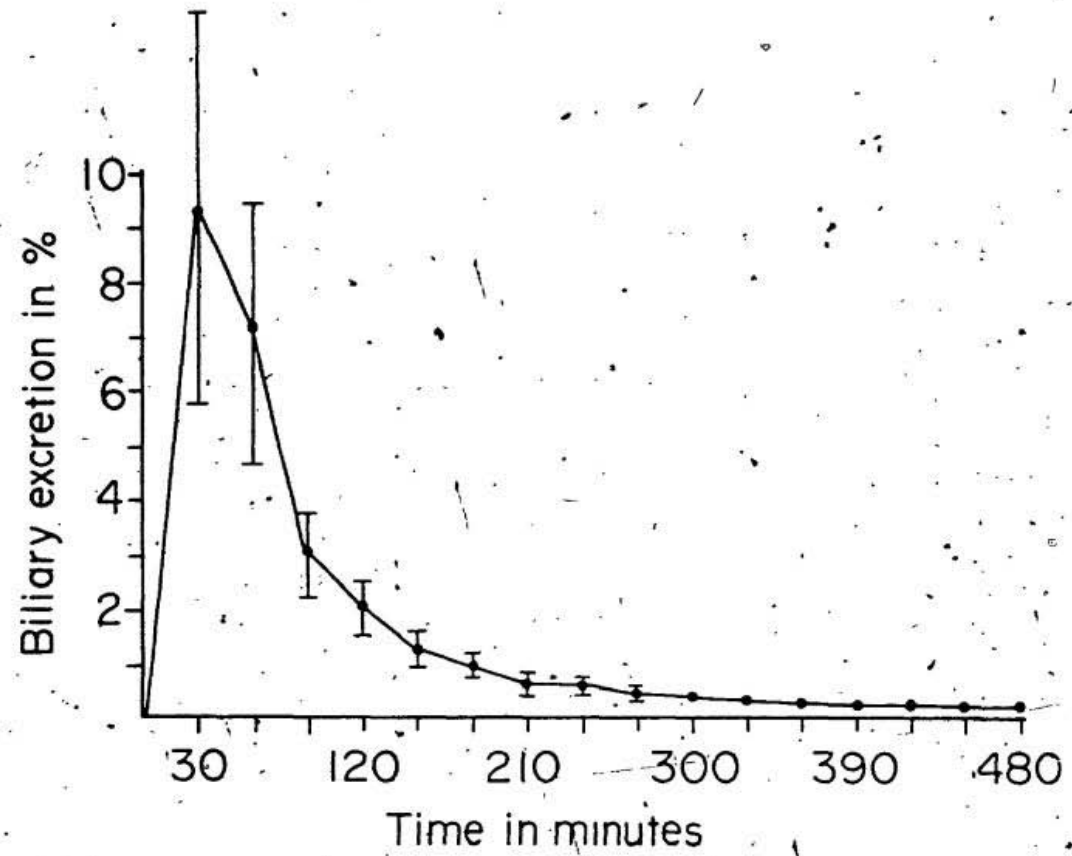
Figure : 3.13

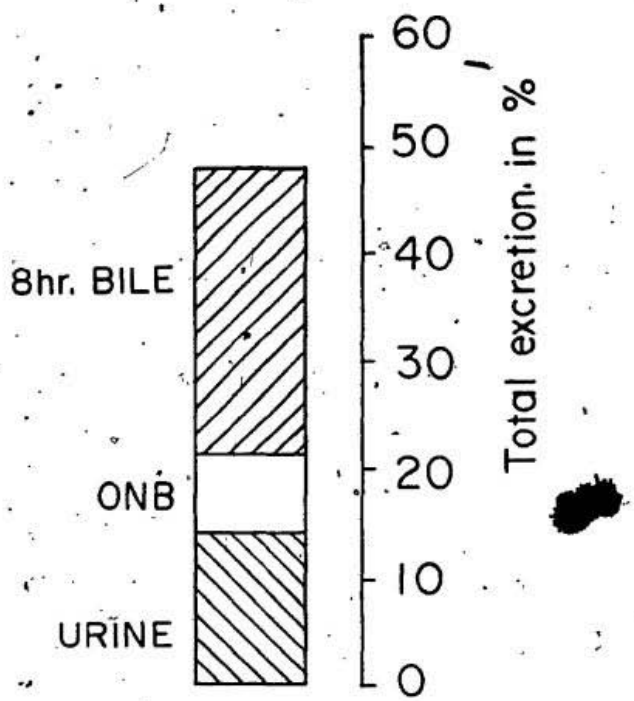
Pattern of radiolabel excreted in bile over an 8-hour period following intraduodenal administration of [^{3}H]-2,6-dimethylnaphthalene in ethanol with exogenous bile supplementation.

Collections were taken every 30 minutes.

Each point represents the mean (± SEM) for at least 5 animals.

The right side of the figure shows the total recovery of radiolabel in 24-hours divided into 8-hour biliary recovery, 16-hour biliary recovery (ONB, over night bile) and 24-hour urinary recovery.


Biliary excretion in %
10
8
6
4
2
30
120
210
300
390
480
Time in minutes


8hr. BILE
ONB
URINE
60
50
40
30
20
10
0
Total excretion in %

3.3.3. Enterohepatic Circulation of 2,6-Dimethylnaphthalene (DMN) Metabolites

The enterohepatic circulation of the metabolites of a number of PAHs has been well established. This experiment was carried out to determine whether the 2,6-DMN metabolites also undergo enterohepatic circulation. Rat biliary metabolites of [^{3}H]-2,6,-DMN were collected and pooled. These radiolabelled derivatives were then infused intraduodenally to recipient rats and the subsequent biliary and urinary excretion of radiolabel was monitored.

Fig. 3-14 shows the pattern of radiolabel recovery at half-hour intervals during the first 8 hour period. The peak excretion of radiolabel (3.11 ± 1.5%) was seen at 1.50 hour post infusion, after which it gradually declined. But at 5.0 hour after infusion there was a sudden rise of radiolabel excretion. The second peak was in sharp contrast to the pattern of radiolabel excretion when the parent compound was given intraduodenally, where there was no such peak (Fig. 3-5). The cumulative excretion of radiolabel in bile in the 0-8 hour period was 28.34 ± 4.80% and in the 8-16 hour period was 7.71 ± 1.60%. In urine, the cumulative 24 hour recovery of radiolabel was 46.18 ± 6.90%, and the total combined radiolabel excretion in bile and urine in 24 hour was 82.25 ± 5.25% (Table: 3-8, Fig. 3-16, 3-17, 3-18).

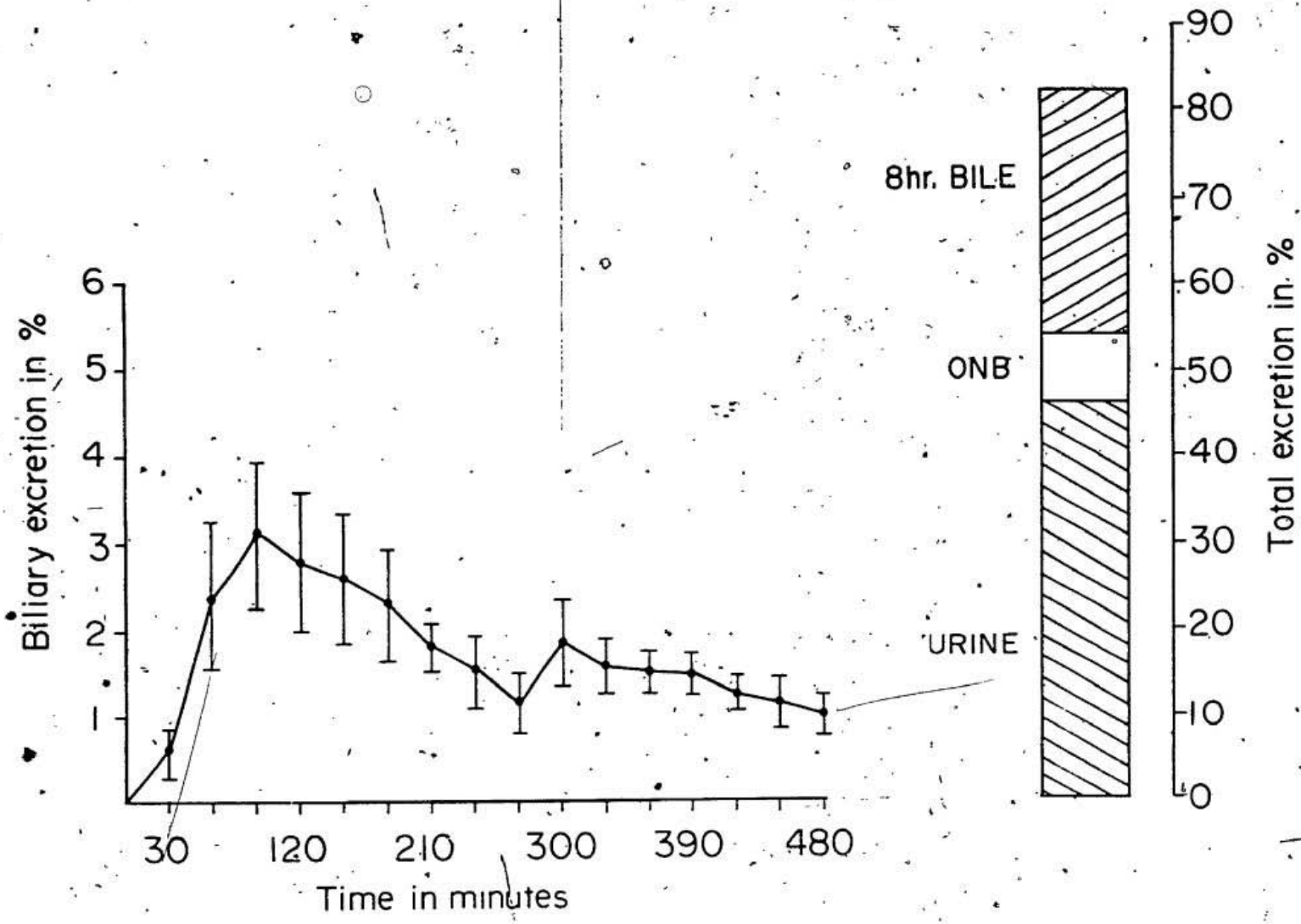
Figure : 3.14

Pattern of radiolabel excreted in bile over an 8-hour period following intraduodenal administration labelled biliary metabolites of [^{3}H]-2,6-dimethylnaphthalene.

Collections were taken every 30 minutes.

Each point represents the mean (± SEM) for at least 5 animals.

The right side of the figure shows the total recovery of radiolabel in 24-hours divided into 8-hour biliary recovery, 16-hour biliary recovery (ONB, over night bile) and 24-hour urinary recovery.


Biliary excretion in %
6
5
4
3
2
1
30
120
210
300
390
480
Time in minutes
8hr. BILE
ONB
URINE
90
80
70
60
50
40
30
20
10
0
Total excretion in. %

3.3.4. Role of Lipid Hydrolysis in 2,6-Dimethylnaphthalene Absorption

Evidence indicates that concomitant fat feeding greatly enhances PAH absorption. Recent studies suggest that during digestion lipids form a series of co-existing phases as a result of the action of pancreatic lipase. These phases form a "hydrocarbon continuum" (Patton, 1981) which greatly facilitates the absorption of non-polar molecules such as the PAHs. In this study both bile and pancreatic secretions were diverted from the duodenum to prevent the action of pancreatic lipase on the corn oil and the affect on 2,6-DMN absorption was observed.

Fig. 3-15 depicits the half-hourly pattern of biliary recovery of radiolabel in the 0-8 hour period in the absence of bile and pancretic secretions, when a test meal of [^{3}H]-2,6-DMN in oil was given intraduodenally. The peak recovery rate of radiolabel (1.49 ± 0.52% of the total dose) was seen at 1.50 hour after infusion, as compared to the peak recovery of 5.90 ± 1.93% in the controls. A gradual fall in the rate of excretion followed but was not as steep as in the controls. A steady rate was achieved at about 4.50 hour after infusion. The cumulative biliary recovery of radiolabel in the 0-8 hour period was 11.25 ± 1.71% which is significantly ($P < 0.05$) lower than in the control animals. The recovery of radiolabel in the 8-24 hour bile on the other hand was significantly higher than in the control animals, at 6.27 ± 1.43%. The 24-hour recovery of radiolabel in urine was 9.97 ± 1.78% which did not significantly differ from the control animals. The total 24-hour recovery of radiolabel in bile and urine combined was 27.50 ± 2.66% which again did not differ significantly from the control group (Tab. 3-6, Fig. 3-19, 3-20, 3-21).

Figure : 3.15

Pattern of radiolabel excreted in bile over an 8-hour period following intraduodenal administration of [^{3}H]-2,6-dimethylnaphthalene in corn oil with both bile and pancreatic secretions absent from the duodenum.

Collections were taken every 30 minutes.

Each point represents the mean (± SEM) for at least 5 animals.

The right side of the figure shows the total recovery of radiolabel in 24-hours divided into 8-hour biliary recovery, 16-hour biliary recovery (ONB, over night bile) and 24-hour urinary recovery.

Biliary Excretion in %
3
2
1
30
120
210
300
390
400
TIME (minutes)
8hr.
BILE
ONB
URINE
Total Excretion in %
35
25
15
5

Figure : 3.16

Cumulative recovery of radiolabel in urine in a 24-hour period following intraduodenal administration of [^{3}H]-2,6-dimethylnaphthalene.

Each bar represents the mean (± SEM) for at least 5 animals.

NORMAL
ABSENCE OF BILE
ENTEROHEPATIC CIRCULATION
VEHICLE ETHANOL
ABSENCE OF BILE AND PANCREATIC SECRETION
EXCRETION IN PERCENTAGE
50
40
30
20*
10
0

Figure : 3.17

Total recovery of radiolabel in bile and urine combined in a 24-hour period following intraduodenal administration of [^{3}H]-2,6-dimethylnaphthalene.

Each bar represents the mean (± SEM) for at least 5 animals.

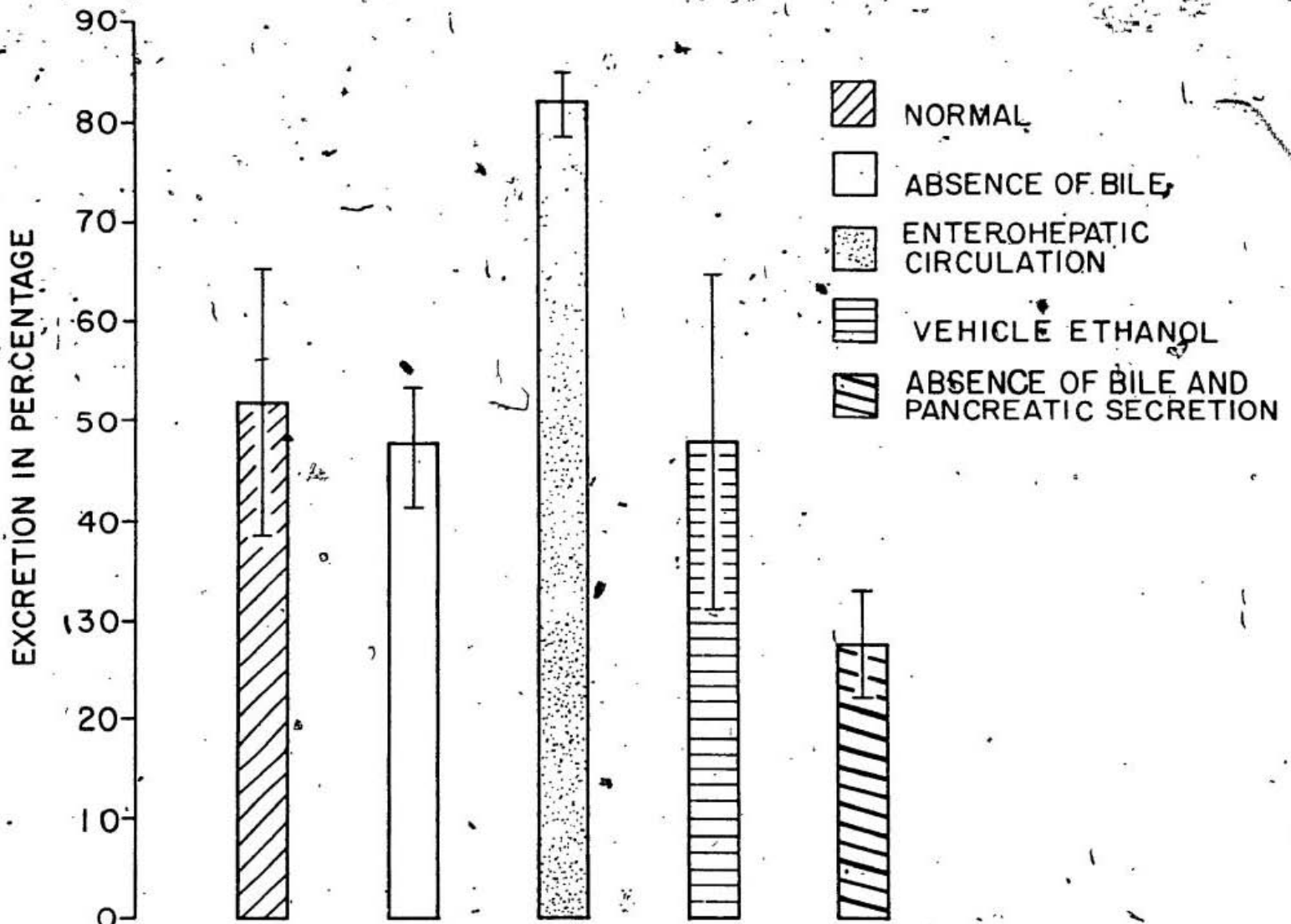

EXCRETION IN PERCENTAGE
90
80
70
60
50
40
30
20
10
0
NORMAL
ABSENCE OF BILE
ENTEROHEPATIC CIRCULATION
VEHICLE ETHANOL
ABSENCE OF BILE AND PANCREATIC SECRETION

Figure : 3.18

Recovery of radiolabel following intraduodenal administration of [^{3}H]-2,6-dimethylnaphthalene in a 24-hour period shown separately as 8-hour bile, 16-hour bile (ONB, overnight bile) and 24-hour urine.

Each bar represents the mean (± SEM) for at least 5 animals.

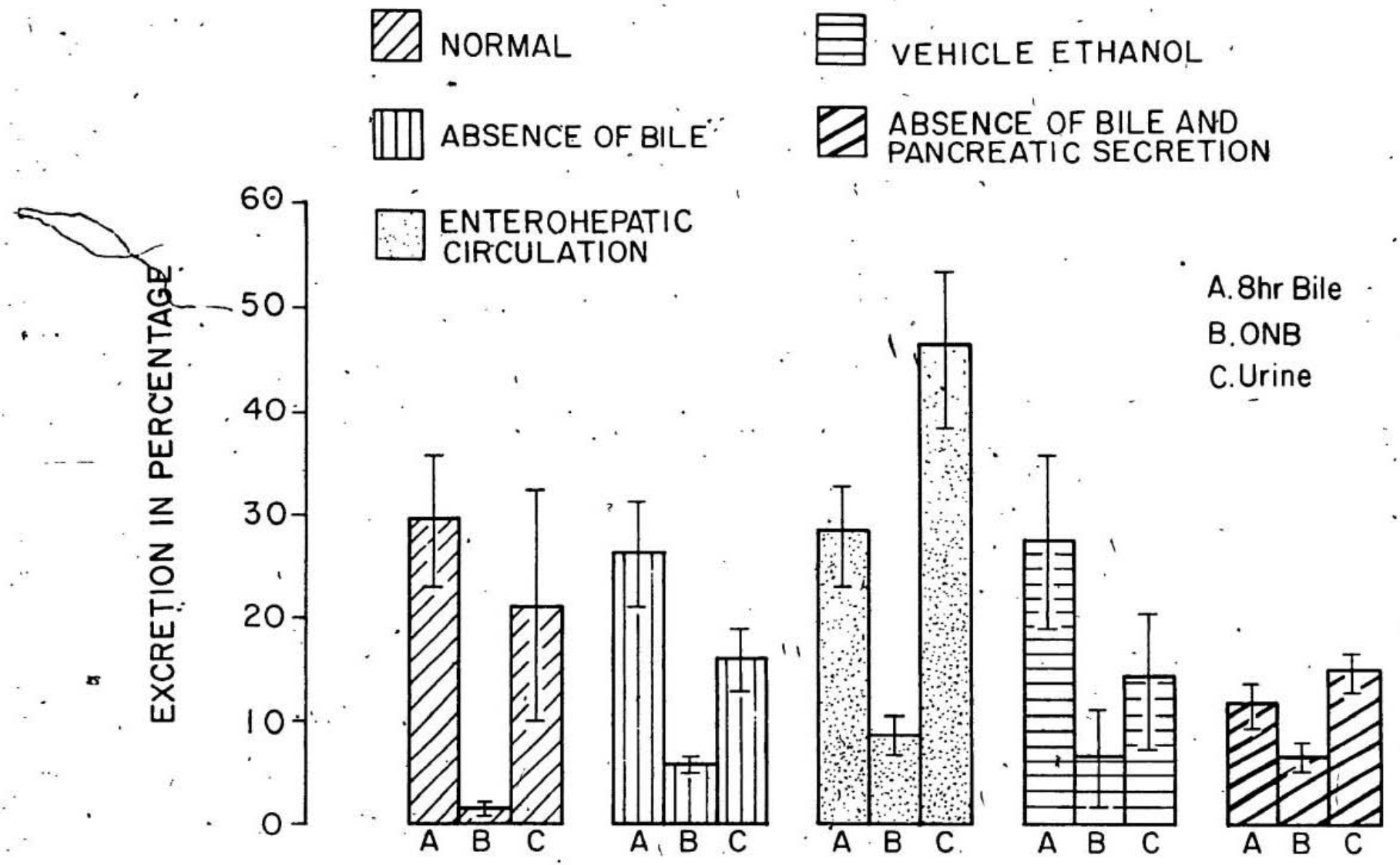

NORMAL
VEHICLE ETHANOL
ABSENCE OF BILE
ABSENCE OF BILE AND PANCREATIC SECRETION
ENTEROHEPATIC CIRCULATION
A. 8hr Bile
B. ONB
C. Urine
EXCRETION IN PERCENTAGE
60
50
40
30
20
10
0
A B C
A B C
A B C
A B C
A B C

TABLE 3-8 : CUMULATIVE RECOVERY OF RADIOLABEL IN 24-HOUR BILE AND URINE COLLECTION, FOLLOWING ADMINISTRATION OF LABELLED 2,6-DMN IN DIFFERENT DUODENAL ENVIRONMENTS. **

DUODENAL ENVIRONMENT	8-HOUR BILE	16-HOUR BILE[4]	24-HOUR URINE	TOTAL
Bile present	29.58 ± 4.80	1.43 ± 0.25	20.95 ± 9.97	51.97 ± 12.10
Bile absent	25.97 ± 4.32	5.65 ± 0.58*	16.00 ± 2.78	47.63 ± 5.16
Vehicle ethanol[1]	27.27 ± 7.79	6.42 ± 4.90*	14.14 ± 6.02	47.84 ± 15.24
Absence of bile[2] and pancreatic secretion	11.25 ± 1.71*	6.27 ± 1.43*	9.97 ± 1.78	27.50 ± 2.66
Biliary metabolites[3] of 2,6-DMN.	28.34 ± 4.80	7.71 ± 1.60*	46.18 ± 6.90	82.25 ± 5.25*

(*) Significantly different ($P<0.05$).
1. Non-lipid vehicle.
2. No lipid hydrolysis by pancreatic lipase.
3. Enterohepatic circulation.
4. Overnight bile, ONB.
(**) Each value represents the mean (± SEM).

Chapter 4

DISCUSSION OF RESULTS

4.1. Effect of Gastrointestinal Hormones on Xenobiotic Metabolizing Enzymes in Small and Large Intestine

4.1.1. Introduction

The structure, physiological properties and actions of the major gastrointestinal (GI) hormones, gastrin, secretin and cholecystokinin (CCK), have been reviewed by Grossman (1976). Johnson (1981) has shown that pentagastrin stimulates colonic mucosal DNA synthesis leading to a rapid increase in mucosal DNA content and cell number. Previous studies have indicated that a short course of treatment with pharmacological doses of pentagastrin, secretin and CCK leads to enhanced xenobiotic-metabolizing enzyme levels in the colonic mucosa of rats (Fang and Strobel, 1981). With this background information, we initiated our study on the effects of GI hormones on xenobiotic metabolism in the small and large intestine. Since a major part of this study involved the activity of microsomal enzymes, a brief discussion of the assay systems is warranted in the beginning.

4.1.2. Microsomal Preparation

One aim of enzymatic analysis is to obtain information on the concentration and localization of metabolites in living cells. It is therefore desirable to prepare the living material in a form suitable for measurements, but without altering the structure or the relative amounts of the substances to be analyzed. Since it is not practical to adhere to this, results are influenced by the methods used.

The difficulties in isolating intestinal microsomes are well documented. The major problem is destruction of the microsomal enzymes by intestinal proteases during extraction. The procedure stated by Stohs et al. (1976) seems to have solved most of these problems and we followed their methodology in obtaining intestinal microsomal preparations with stable enzyme systems. For the sake of uniformity the animals were fasted overnight and were sacrificed between 9 AM and 10 AM to eliminate any diurnal variation (Jori et al.,1971). Fasting reduced food residues and faecal matter in the intestine, facilitating mucosal scraping. Since it is known that the monooxygenase activity is destroyed by proteolytic enzymes (Orrenius, Berg and Ernester, 1969) which are present in the small intestine homogenate, soybean trypsin inhibitor was added to the initial homogenate. The inhibitory effect of trypsin may be due to a solubilization of the reductase from the microsomal membrane or to a decomposition of cytochrome P450 (Lehrmann, Ullrich and Rummel, 1973). Addition of trypsin inhibitor causes six fold increase in microsomal BP-monooxygenase activity (Stohs et al., 1976). Glycerol in the buffer system prevents the conversion of cytochrome P450 into cytochrome P420 (Capdevila, Jakobsson, Jernstrom, Helia and Orrenius, 1975), and Stohs et al. (1976) have reported a drop in BP-monooxygenase activity when

glycerol was eliminated from the homogenate. To improve the yield of intestinal microsomes heparin was added to the initial homogenate. Heparin decreases agglutination of the microsomes in the homogenate (Goodman and Kadis, 1965).

Washing of the microsomal pellet obtained from the first spin, reduced contamination with soluble haemoproteins of the intestine. Stohs et al. (1976) have shown minimum mitochondrial contamination of similar microsomal preparations. Using the stated method, stable intestinal microsomal preparations are obtained since less than 20% of cytochrome P450 is lost upon 8 hour storage of the microsomal preparations on ice (Grafstrom and Greene, 1980). For logistical reasons, we could not use the microsomes on the same day as they were prepared and had to freeze them in liquid nitrogen and store them at -70°C. This procedure caused little or no change in the enzyme activity, since we used the microsome preparations from some animals in BPH and UDP-GT assays before and after freezing and did not find a significant difference in the assays (data not shown).

4.1.3. Enzyme Assay : Benzo(a)pyrene hydroxylase (BPH)

The first step in benzo(a)pyrene (BP) metabolism is catalyzed by BPH, a microsomal bound monooxygenase. This leads to the formation of various reactive arene oxides or epoxides which may be converted to more polar water-soluble metabolites. Various investigators have termed these epoxides as ultimate carcinogens or proximate carcinogens (Heidelberger, 1976) because of their mutagenic activity. Hence, it is important to accurately measure the total activity of the BPH and the factors modifying it.

In most of the published papers, the BPH activity is measured by a fluorimetric method whose main advantage lies with its very high sensitivity (Nebert and Gielen, 1972). The radioactive assay, when compared with the fluorimetric method, shows several advantages over the latter. The radioactive assay allows measurement of virtually all of the products of BP metabolism, whereas the fluorescent method measures only a fraction of the metabolites (3-hydroxy benzpyrene). The radioactive assay for BPH is more convenient than the fluorescent procedure and gives very satisfactory duplicate values (DePierre et al.,1975). Many PAHs are photosensitive and easily transformed by non-enzymatic reactions. This is particularly critical for the fluorimetric method which only measures one or a small number of metabolites, and which, as opposed to the isotopic method shows a critical sensitivity to the room lighting during the manipulation (Van Cantfort et al.,1977). Considering all the above factors we decided to adopt the radioactive assay for our study. While standardizing the assay in our laboratory, we found the assay to be linear with concentration of microsomal proteins at least in the range of 0.2 mg to 0.5 mg per assay mixture. At lower or higher concentration of protein very irregular results were obtained. This may be because the apparent rate of metabolite formation was dependent not only on the substrate concentration but also on microsomal protein concentration (Hansen and Fout, 1972). One possible explanation for such unusual kinetics might involve depletion of free substrate via binding to non-enzymatic sites (Nebert and Gelboin, 1968). For the sake of uniformity, we used 0.2 mg of microsomal protein in all the assay mixtures.

The BPH enzyme system has an absolute requirement for NADPH and

molecular oxygen. NADPH was supplied by a regenerating system consisting of NADP and either glucose-6-phosphate and glucose-6-phosphate dehydrogenase or citric acid and isocitric dehydrogenase. The role of magnesium in the enzyme system may be a stimulatory one (Nebert and Gielen, 1972).

4.1.4. Enzyme Assay : Glucuronyl transferase (UDP-GT)

The intestinal glucuronide biosynthesis may be of paramount importance in the detoxification of xenobiotics entering the body through the GIT. Many substrates of UDP-GT are metabolites of Phase I reactions, some of which are highly cytotoxic. Thus glucuronidation may effectively prevent recycling and thus prevent the formation of ultimate carcinogens.

Assay of UDP-GT activity was done according to Hoensch et al.(1984). Assay of this enzyme is complicated by its often high latency, associated with constraint at or within the microsomal membrane. The enzyme is activated non-specifically by membrane perturbation. Addition of Brij "80" in the assay system further activates the enzyme. The donor substrate UDPGA, must be added to the assay system, and in considerable molar excess because of breakdown there. During standardization we found this assay simple to perform and the enzyme activity showed linearity with microsomal protein concentration. In our assay systems, we used 0.2 mg of microsomal protein per assay mixture.

4.1.5. Effect of Gastrointestinal Hormones

The aggregate mass of endocrine cells in the gut exceed that of all other endocrine glands combined (Grossman, 1976). There are three established GI hormones- gastrin, secretin and cholecystokinin(CCK). They are all straight-chained peptides with no rings and no non-peptide constituents (Gregory, 1974). All three of these hormones come from a group of cells that belong to the APUD (amine precursor uptake and decarboxylation) series and are apparently derived embryologically from the neural crest (Pearse, 1976).

Recently there has been increasing interest in the fact that GI hormones may exert long-term growth influences on their target organs as well as acute secretory and motor actions on the tissues of the alimentary tract and its associated glands. But it must be appreciated that the functional complexity of the GI tract and its associated glands (liver and pancreas), involving multiple feed-back pathways which are largely unknown in detail, render a selective approach to a single organ virtually impossible. Chronic administration of one or more GI hormones to the living organism will therefore always be 'superimposed' on the actual physiological condition in the GI tract and the selective analysis of a single organ will offer only limited insight into a highly complex regulatory situation within the whole system.

It has been established that several GI polypeptide hormones, notably gastrin, secretin, and related structural analogues such as pentagastrin and caerulein, exert significant trophic actions on the gut and pancreas (Barrowman, 1975; Johnson, 1976; Johnson, 1977). In general this has been observed at

pharmacological dose levels and the physiological significance of this phenomenon remains unclear.

There are a number of pathological conditions where endocrine tumours may secrete an abnormal quantity of the GI hormones into the circulation. During these circumstances the concentration of the hormones may be much higher than normal at the target organs. In our study, we tried to elucidate the role of such abnormally high levels of GI hormones in the intestinal xenobiotic metabolism in rat.

Trophic hormones have a number of cellular activities which taken together are known as the pleiotypic response. These consist of stimulation of a) RNA synthesis, b) protein synthesis, c) DNA synthesis, d) glucose transport and e) inhibition of protein catabolism. It is well established that pentagastrin (PG) has a strong pleiotypic action, on the gastric and duodenal mucosa (Johnson, Aures and Yuen, 1969), on the ileal mucosa (Johnson and Guthrie, 1974), on the colonic mucosa (Johnson, 1977) and on the pancreas (Mayston and Barrowman, 1973). Using tissue (Lichtenberger, Miller and Erwin, 1973) or organ culture (Sutton and Donaldson, 1975) systems, investigators have found that gastrin maintains the epithelial cell lines, decreases the doubling time, increases the proliferative population and stimulates mitosis, DNA and protein synthesis when administered in vitro. Other investigators have also found that PG not only stimulates colonic mucosal growth, but at the same time also causes an increase in the level of activity of some of the xenobiotic metabolizing enzymes (Fang and Strobel, 1981).

In our study we observed an increase in BPH activity in colonic mucosa after PG treatment but not in UDP-GT activity. The increase in BPH activity may be explained in the light of previous observations. But the non-response of the UDP-GT is difficult to explain. Fang and Strobel (1981) found a stimulatory affect of PG on xenobiotic metabolizing enzymes in colonic mucosa, including BPH activity, which are all part of phase I reactions. Since some investigators found it difficult to draw conclusions concerning the nature of the type of protein being synthesized after PG stimulation (Johnson et al., 1969), it just may be that UDP-GT, which catalyzes a phase II reaction, is not stimulated by PG. In our study we did not observe any stimulation of BPH and UDP-GT activity in the small intestine after PG treatment. Although previous investigators have found an increase in protein synthesis in the small intestine after PG treatment (Johnson and Guthrie, 1974), they did not specify the nature or type of the protein produced. Maybe the particular type of protein synthesis involved in xenobiotic metabolism in the small intestine is not stimulated by PG. Such asynchrony has also been observed in the rate of synthesis of individual pancreatic enzymes after stimulation by GI hormones (De Caro, Ronconi and Sopranci, 1969; Solomon, Peterson, Elashoff and Grossman, 1978).

Since colon is more sensitive to PG than the duodenum (Johnson, 1977), another way of explaining this non-response of small intestine in the present study is that the dose of PG employed by us may be less than the critical concentration required for stimulation of xenobiotic metabolism in the small intestine. An additional factor that must be considered here is the presence of a relatively high concentration of somatostatin in the small intestine mucosa as compared to the

colonic mucosa and somatostatin is known to antagonize several functions of gastrin (Creutzfeldt and Arnold, 1978).

Of the major GI hormones, CCK and gastrin have an almost identical -COOH terminal sequence. The terminal pentapeptide is the same in the two hormones. Thus one might expect that CCK would have the same trophic action on the GI tract as pentagastrin. Many reports indicate that CCK or its analogue have a trophic action on the rat pancreas (Barrowman and Mayston, 1973; Brants and Morisset, 1976). Although gastrin does stimulate growth of the pancreas, CCK does not stimulate DNA synthesis in the oxyntic gland mucosa (Johnson and Guthrie, 1976). However, the same authors have found a slight but statistically significant increase of DNA synthesis and of RNA and DNA content of duodenal mucosa. Fang and Strobel (1981) found an increase in the activity of xenobiotic metabolizing enzyme activity in rat colonic mucosa after chronic CCK administration. In our study, we did not find any such increase in either colonic or the small intestinal mucosa after pretreatment with CCK.

Since previous investigators found a stimulatory effect of CCK on these enzymes (Fang and Strobel, 1981), it is difficult to interpret the results in our study. In addition to the arguments already presented in the case of PG, several other factors may be at play. Trophic hormones in general do not stimulate the growth of their tissue or organs of origin. The lack of stimulatory effect of gastrin on antral DNA synthesis observed by Johnson (1977) correlates with studies of other growth regulators. The concentration of gastrin in intestinal mucosa decreases dramatically from the antrum to the duodenum bulb, after which a

further gradual decrease is observed (Rehfeld, Stadil, Malmstrom and Miyata, 1975). In addition to antrum and duodenum, a small amount of gastrin is present in the fundic, jejunal and ileal mucosa (Larson and Rehfeld, 1979). This may explain the stimulatory affect of PG on BPH activity in the colonic mucosa observed in our experiment. CCK is produced throughout the intestine and the colon contains abundant CCK nerves (Larson and Rehfeld, 1979). This relatively high concentration of CCK in the colon may be responsible for its failure to increase BPH and UDP-GT activity. Again it has been observed that the stimulatory action of CCK on the pancreas is time and dose dependent; supramaximal stimulation results in inhibition of protein synthesis (Lampel and Kern, 1977; de Caro et al., 1969). From the limited experience available with maximal hormonal stimulation of the pancreas lasting up to 72 hours, it may be concluded that, concomitant with the adaptive increase in all steps in the secretory process, a 'desensitization' of the whole organ versus the hormonal stimulus can be observed from 48 hours of treatment onward (Bieger, Martin-Achand, Bassler and Kern, 1978). Since in our study the rats received a high dose of CCK for 3 days, the above mentioned factor cannot be overruled completely. In conclusion the role of somatostatin and other recently recognized gut hormones on the trophic action of CCK still remain somewhat unexplained.

In a number of studies secretin has been found to have no trophic activity of its own and to inhibit the trophic response to pentagastrin in the mucosa of oxyntic gland, duodenum and ileum (Johnson and Guthrie, 1974). Chronic administration of secretin prevents parietal cell hyperplasia and the increased secretory capacity induced by multiple injections of PG (Stanley, Coalson,

Grossman and Johnson, 1972). Johnson and Guthrie (1978) found identical inhibition of the trophic action of PG on colonic mucosa produced by secretin. The same authors could not find any trophic action of secretin on colonic mucosa when administered by itself. A similar inhibitory effect is also mediated by VIP (vasoactive intestinal polypeptide), a structural analog of secretin (Johnson, 1977).

In the light of the preceeding discussion we assumed that pretreatment with secretin would produce no stimulation of the xenobiotic-metabolizing enzymes in the small and large intestinal mucosa. Our results show that, as expected, there was no change in the activity of BPH or UDP-GT in the small intestinal and colonic mucosa. However, at least one group of investigators has published data which are contrary to our observations (Fang and Strobel, 1981).

In conclusion it must be observed that this study is by no means complete. More detailed and scrupulous studies are required to explain the various points raised.

4.2. Effect of Post-Resection Hypertrophy of Small Intestine on Xenobiotic Metabolism

The results from the resected animals confirm previous observations that there is lengthening, thickening and dilation of the remaining gut, this being particularly noticeable immediately distal to the anastomosis. Thickening of all layers of the bowel together with crypt hyperplasia is also confirmed. Compensatory hypertrophy of the ileal remnant of rat intestine following proximal resection is a well established phenomenon (Dowling and Booth, 1967; Dowling and Gleeson, 1973). These morphological alterations are associated with

an increase in glucose absorbtion in vivo, when expressed in terms of unit length of intestine, but when expressed per unit weight of intestine, sugar absorption is decreased (Menge and Robinson, 1978). This observation indicates that the change in absorption is mainly due to an increase in the number of absorptive cells per unit length of intestine, as a result of the longer villi. This theory is further confirmed by the observation that absorptive capacity of the intestinal remnant is markedly reduced when assesed in vitro (Menge and Robinson, 1978; Weser and Hernandez, 1971). Activities of some brush-border-membrane associated hydrolytic enzymes have been observed to be increased when expressed per unit length of the intestine (Weser and Hernandez, 1971). However, when enzyme specific activity is measured (units of enzyme per mg of protein) there is either no change or there is actually a reduced level of enzyme activity in the hyperplastic intestine (Gleeson and Dowling, 1972; Morita, Pellegrini and Kim, 1981; Weser and Hernandez, 1971). These changes have been attributed to the presence of a relatively immature population of enterocytes in the mucosa of the ileal remnant (Loran and Krocker, 1963). The increase in cell turnover leads to a stimulation of cell migration rate along the villous which leads to reduced life span of the individual cells, consequently the maturation of the enterocytes seldom reaches completion. Proximal resection of the small bowel exposes the remaining gut to abnormally high concentrations of certain nutrients which may act topically to stimulate hyperplasia (Dowling and Booth, 1967; Gleeson, Cullen and Dowling, 1972). Chalone-like substances i.e. substances with inhibitory properties have been extracted from both the large bowel (Houck, Kanagalingham, Kaufman and Sunshine, 1976) and the small bowel (Bergeron and Sassier, 1980) and these may play a part in intestinal adaptation.

In the present study, a significant increase in the microsomal protein content was observed in the remnant ileum after resection. This protein content of the residual ileum reflects the number of heterogenous mucosal cells, ie. villous cells in various stages of differentiation and immature crypt cells. Various investigators have shown the distribution of the xenobiotic-metabolizing enzymes in the crypt-villous axis. Hoensch et al. (1979) found the BPH activity to be highest in the highly specialized, mature villous-tip cells and lowest in the poorly differentiated, actively dividing crypt cells. The distribution of glucuronidation capacity along the crypt-villous axis is not known in detail. Results by Schiller and Lucier (1978) indicate that the activity of UDP-GT is highest in villous cells and lower in crypt cells. Thus the BPH and UDP-GT activity in the microsomal preparation from the mucosa of the remnant ileum, consisting mostly of immature dividing cells, is expected to be lower than in the microsomal preparation from mucosa prior to adaptation.

In the present study the BPH activity in the mucosa of the ileal remnant was not significantly different from the activity before adaptation. Since it has been shown by several investigators that the cell population in a post-resected hypertrophied ileal mucosa consists chiefly of immature cells, this finding is in contrast to the observations of Hoensch et al. (1979) who found lowest activity of BPH in the immature crypt cells. But others in this field have found highest activity of this enzyme in the lower villous region (Porter, Dworacyzk and Gurtoo, 1982). Also, there are reports that some enzyme activities may remain unchanged in the ileal remnant. Weser and Hernandez (1971) did not find any change in the sucrase activity after resection and hypertrophy, although other

disaccharidase activities were lowered. Histochemical studies of several other enzymes in the epithelium after intestinal resection have not shown any changes (Dowling, 1967). Since no efforts were made to identify the maturity of the mucosal cell population in the present study, it is difficult to come to a definite conclusion regarding the BPH activity.

On the other hand, the UDP-GT activity was significantly lowered in the remnant ileum. This compares with the observations of other investigators who found a lower activity of some enzymes (Weser et al., 1971) in the post-resected hypertrophied remnant. It has been shown that after resection the intestinal mucosa may acquire characteristics of metabolic immaturity. Whereas normal rats depend on energy supplied by oxidative phosphorylation in order to transport vitamin A, resected animals utilize energy supplied by anaerobic glycolytic phosphorylation (Loran and Althausen, 1959). This alteration in metabolism is a characteristic of foetal tissue and rapidly dividing immature cells (Ville and Hagerman, 1958). The findings in this study of a reduction in UDP-GT activity in the mucosa of the resected animals are consistent with a younger cell population.

The lack of change in BPH activity is at present unexplained. Further experimentation is required to shed more light in this respect.

4.3. Study on the Intestinal Absorption of PAHs

4.3.1. Role of Intraduodenal Bile in PAHs Absorption

The present study was undertaken to identify certain dietary and physiological factors in the mammalian gastrointestinal lumen which might influence the uptake of several PAHs following intraduodenal administration.

Many toxic compounds in the environment, including certain well-recognized carcinogens, are lipophilic and exist in substantial amounts in the food chain. Most of these toxic compounds, especially the PAHs are highly lipophilic and are found as solutes in dietary fat. Thus the gastrointestinal mucosa constitutes an important interface between man and his toxic environment.

It is established that the absorption of trace lipid nutrients such as sterols and the fat-soluble vitamins is intimately dependent on the normal process of fat digestion and absorption and that bile salts, which play a facilitatory role in triglyceride absorption, are obligatory for absorption of these trace lipids (Hollander, 1981). It seems likely that PAHs will also be handled in a similar fashion to trace nutrient lipids. It has been proposed that lipophilic xenobiotics such as the PAHs, which are largely presented to the GIT as solutes in dietary lipids, remain in solution in the intestinal lumen as lipolysis proceeds by passing through a "hydrocarbon continuum" formed successively by the liquid triglyceride oil, a viscous isotrophic phase consisting of the split products of triglyceride lipolysis, monoglycerides, fatty acids and a bile-salt stabilised liposomal and mixed micellar phase (Patton, 1981).

As explained earlier in this thesis, the mixed micelle formation facilitates the transport of the hydrophobic xenobiotics through the "unstirred water layer", a major barrier to the passage of these compounds as they approach the enterocyte membrane. The final uptake step at the apical membrane of the enterocyte is not clearly understood but it is possible that these substances must pass through an aqueous monomeric phase in which the compound briefly exists as a solute at low concentration in the water adjacent to the cell membrane (Thompson and Dietschy, 1981). From the above discussion it is evident that as a molecule moves from the bulk phase of the intestinal contents into the cell interior it must penetrate at least two barriers, an unstirred water layer (UWL) and the cell membrane. Since the lipophilic xenobiotics are easily absorbed into the lipid cell membrane, the limiting factor in their absorption is the resistance offered by the UWL (Wilson and Dietschy, 1974).

Uptake of fatty acids, monoglyceride and trace nutrient lipids by the enterocyte membrane is a passive process. It is assumed that the lipophilic xenobiotics will also follow a similar pattern. Diffusion is a movement due solely to the kinetic energy and electrical charge of the molecules and movement takes place only in the direction of the prevailing electro-chemical gradient. Since the xenobiotics are electrically neutral particles, their driving force across the GIT mucosa is their concentration in the bulk intestinal aqueous phase. It is known that the products of fat digestion exist in several physical states in the intestine and that xenobiotics such as PAHs can exist as solutes in the bile-salt micelles in the aqueous phase. Several investigators have observed that absorption of lipids depend ultimately on their concentration in an aqueous dispersion in the lumen

(Borgstrom, 1960; Hofmann and Borgstrom, 1964) and that micellar solubilization enhances uptake by increasing the aqueous concentration. Hence, solubilization of the xenobiotics in the micelle will also enhance their aqueous concentration. Movement across the UWL is a simple diffusion process where the rate of movement depends upon the thickness, aqueous diffusion constant of the particle and the concentration gradient between the bulk water phase and the cell membrane (Thompson and Dietschy, 1981). For the sake of simplicity, if we consider the thickness of the UWL as constant, the factors determining the diffusion through the UWL are the concentration gradient and the aqueous diffusion constant. For the lipophilic substances including the PAHs, the concentration gradient depends on the micellar solubilization which enhances aqueous solubility. Micellar solubilization may slightly increase the diffusional resistance due to increased particle size of the micelle as compared to the monomers, but this increase is modest compared with the possible increase in the driving force (Simmonds, 1974).

In our study we examined the role of duodenal bile on the absorption and subsequent excretion of a number of PAHs in rat. Our selection of the PAHs was governed by several factors. In one extreme of the list we had 7,12-dimethylbenzanthracene (7,12-DMBA) and benzo(a)pyrene (BP), compounds with high molecular weight, very low water solubility and four or five aromatic rings in their structure (Fig.1-1). At the other extreme we had 2,6-dimethylnaphthalene (2,6-DMN), a smaller compound with lower molecular weight and relatively high water solubility. In the middle of the list we had the structural isomers phenanthrene and anthracene. Both these compounds consist of three aromatic

rings: in anthracene the rings are connected linearly while in phenanthrene they are connected angularly (Fig.1-1). This structural difference has a profound effect on their water solubility (Tab.3-7).

We assumed that, since the aqueous solubility of the lipophilic substances determines the driving force across the UWL and micellar solubilization enhances such solubility, then a PAH with relatively high water solubility should be able to attain by itself enough driving force to cross the UWL without the help of the bile salts. Our results are in keeping with this hypothesis in the case of 2,6-DMN. Fig. 3-5 shows the bioavailability of this compound when administered intraduodenally in corn oil in the presence and absence of duodenal bile. The compound is well absorbed in both situations and total excretion of radiolabel in 24 hours is not significantly different from each other. If the biliary recoveries of radiolabel in presence and absence of bile are compared, there is no significant difference in 0-8 hour period but in 8-24 hour period the biliary recovery is significantly higher in absence of bile (Tab. 3-6). This may be due to a faster initial rate of absorption in presence of bile which reduces the concentration gradient on the luminal side resulting in a slower rate of absorption in the later period. The faster rate of absorption in the presence of bile may be explained in the following way: due to the relatively high water solubility, 2,6-DMN is dispersed in the intestinal aqueous phase quite adequately for diffusion through the UWL. At the same time bile-salt micelles solubilize some of the 2,6-DMN increasing the proportion in aqueous dispersion. These two additive factors increase the rate of absorption initially. In the absence of bile the compound is absorbed fairly well due to sufficient aqueous dispersion but the lack of mixed

micelle may reduce the rate of absorption initially so that in the 8-24 hour period the absorption is greater compared to the control group. When overall biliary and urinary excretion of both groups are compared, no significant difference is observed, indicating that the absorption and subsequent excretion of 2,6-DMN in the absence of bile is very efficient.

On HPLC analysis of the collected bile it was found that 10% of the radiolabel was in the parent compound and 90% was present in the biliary metabolites of 2,6-DMN. This shows that like most other PAHs, the biliary route is the principal excretory pathway for this compound and metabolic transformation is a prerequisite for excretion though perhaps not an absolute prerequisite. In an intact animal these biliary metabolites would be available for enterohepatic circulation and since we recovered almost 60% to 65% of the total excreted radiolabel in the bile (Fig.3-12), this recycling must be quite significant.

In our next experimental procedure we used phenanthrene in the test meal, which has a water solubility of 1.29 mg/L as compared to 2.0 mg/L for 2,6-DMN (Mackay and Shiu, 1977). The results obtained are comparable to the 2,6-DMN data. The absence of bile brought no significant difference in the recovery of radiolabel in 0-8 hour bile, 8-24 hour bile and 0-24 hour urine. So it is evident that a water solubility of 1.29 mg/L is sufficient to cause enough aqueous dispersion of the compound to maintain a positive chemical gradient across the UWL even in the absence of bile. Both in the presence and absence of bile, between 73% to 76% of the excreted radiolabel was recovered in bile (Fig. 3-12). This is 10 to 12 percent more than that observed with 2,6-DMN. This may

indicate that the higher the molecular weight of a compound, the greater is the proportion of metabolites being excreted in the bile which are subsequently available for enterohepatic circulation.

If the percentage of recovery of radiolabel from 2,6-DMN and phenanthrene are compared it may be observed that a higher proportion of radiolabel is excreted in the case of phenanthrene as compared to 2,6-DMN (Fig. 3-11). This may be due to a greater tendency of 2,6-DMN metabolites to bind to cellular structures and to accumulate in the body. Although this is only a speculation, its implication from the toxicological point of view cannot be ignored.

For both 2,6-DMN and phenanthrene, the lack of an absolute biliary requirement for bioavailability is attributed to their relatively high water solubility as compared to other PAHs. Other investigators have shown that the nutrient trace lipid, cholesterol, which has similar water solubility (2.0 mg/L) (Merck's Index) as 2,6-DMN, has an absolute requirement for bile salts for absorption (Siperstein, Chaikoff and Reinhardt, 1952). This may be because of the special nature of the bile-salt micelle which facilitates the uptake of cholesterol by the enterocyte membrane. McIntyre (1975) has proposed that cholesterol is absorbed by the micellar collision with the intestinal membrane and monomer concentration is not important and this collision somehow changes the membrane so that cholesterol is absorbed.

Anthracene is a structural isomer of phenanthrene but has a much lower water solubility of 0.073 mg/L (Mackay and Shiau, 1977). When [^{14}C]-anthracene

is given in the test meal, a different pattern of biliary radiolabel recovery was observed compared to phenanthrene. In the absence of bile the peak biliary recovery in the 0-8 hour period was not only lower than in the presence of bile but the time of peak excretion was also delayed and the cumulative recovery during this period was significantly lower. As a consequence, the total excretion of radiolabel in the absence of bile was also significantly lowered, even though there were no significant differences in the 8-24 hour biliary and 0-24 hour urinary excretion. Compared to phenanthrene, anthracene has a slightly different structural configuration and a lower water solubility. Since there was such a difference in the bioavailability of these compounds in the absence of duodenal bile, it is logical to come to the conclusion that water solubility played the critical role. It is evident that a water solubility of 0.073 mg./L for anthracene is not enough to maintain an adequate aqueous dispersion in the intestinal lumen to create the driving force to overcome the resistance of the UWL and that it must rely on micellar solubilization to attain this force.

In the continuation of the study on the role of bile in the absorption of PAHs we next used benzo(a)pyrene (BP) and 7,12-dimethylbenzanthracene (DMBA) in the test meal. BP is a five ring compound with a water solubility of 0.0038 mg/L and DMBA is a four ring structure with a water solubility of 0.061 mg/L (Mackay and Shiau, 1977).

When [^{3}H]-7,12-DMBA was administered intraduodenally, a pattern similar to that with anthracene was observed. Absence of duodenal bile caused significant drop in radiolabel recovery in the 0-8 hour bile (Fig. 3-8), but not in

the 8-24 hour bile or 0-24 hour urine (Tab. 3-6). The total recovery of radiolabel is reduced by almost 56% in absence of bile (Fig. 3-11). It may be noted that although the aqueous solubility of DMBA and anthracene are rather similar (Tab. 3-7), there is a considerable difference in their bioavailability both in the presence and absence of bile. Perhaps the size of the DMBA molecule with an extra benzene ring (Fig. 1-1) and other unidentified factors may play a role in determining the uptake of the PAHs.

As shown in Fig. 3-9 , absence of intraduodenal bile caused a dramatic decrease in the radiolabel recovered in the 0-8 hour bile after intraduodenal administration of [^{3}H]-BP. There was a fall of almost 85% in the biliary recovery during this period when bile was absent. But in the 8-24 hour period the absence of bile did not produce such a drastic change in cumulative biliary recovery. This indicates that in the presence of duodenal bile a major part of an intraduodenal dose of BP is excreted in the bile within the first few hours of administration, confirming previous reports that the principal route of excretion of BP in rats is via bile (Chipman, Frost, Hirom and Millburn, 1981a; 1981b). There was also a significant drop in the excretion of radiolabel in urine in the absence of bile (Fig. 3-10). This reduction in urinary excretion of radiolabel in the absence of bile was not observed in the case of anthracene. It may be that, because of its very low water solubility as compared to anthracene, only a fraction of the total test meal of BP is absorbed (6.99%) in the first place and a negligible percentage finds its way to the systemic circulation.

Our results with BP and DMBA further validate the observations with

anthracene. It seems that at and below a water solubility of 0.079 mg/L, the PAHs have a requirement for bile salts for efficient aqueous dispersion in the intestinal lumen to maintain the kinetic energy required to overcome the resistance of the UWL.

Fig. 3-12 shows that after an intraduodenal dose of a PAH, a variable fraction of the metabolites appear in the urine. This varies from 40.29% for 2,6-DMN to 17.25% for BP. Thus, lower the molecular weight of the parent compound, the higher is the proportion of the metabolites found in the urine. A molecular weight threshold determines whether a compound will be excreted in urine or in bile, compounds with molecular weight below the threshold being excreted primarily in urine. Our results are in agreement with this theory.

From the above discussion a number of conclusions can be derived. All the PAHs included in this study are well absorbed from the intestine in the presence of bile. In the case of anthracene, DMBA and BP, absence of duodenal bile reduces their bioavailability but by no means is it completely abolished. This is an anomaly in nature, since in the absence of bile, absorption of trace nutrient lipids like cholestrol and vitamin A is almost negligible. Water solubility of the individual PAHs, however, seems to play the deciding factor in the bioavailability of these compounds in absence of bile.

The efficiency of absorption of the PAHs in the absence of duodenal bile may be expressed as a percentage of the absorption in the presence of bile. Tab. 3-7 shows the efficiency of absorption of the representative PAHs. In the case of

2,6-DMN and phenanthrene the efficiency is close to 100% in the absence of bile. But the efficiency of absorption of anthracene, a structural isomer of phenanthrene, is 70.84%. From there with the decrease in water solubility, the efficiency gradually decreases : 43.38% for DMBA and 22.94% for BP. From these observations it may be concluded that, up to a water solubility of approximately 1.0 mg/L bile plays little role in the bioavailability of the PAHs but for PAHs with water solubility of less than 1 mg/L, intraduodenal bile becomes a critical factor for the efficient absorption by the enterocytes.

A major proportion of the recovered radiolabel was in bile which is in agreement with previous observations indicating that this is the chief route of PAH excretion in rats (Chipman et al., 1981a; 1981b). The dependence of the biliary excretion in the handling of PAHs is well established. It has been observed that pretreatment with inducers of hepatic microsomal enzymes enhances biliary metabolite excretion (Levine, 1974; 1972; 1970). Extensive studies on the metabolic fate of the PAHs reveals that a number of polar metabolites can be formed in vitro in the presence of rat liver preparations. These include epoxides, dihydrodiols, phenols, ketones and quinones and the conjugated derivatives of these metabolites have molecular weights of over 350, the approximate minimum figure determined by William and co-workers (Milburn, Smith and William, 1967) for substances that have significant biliary excretion in rats. Although, the liver is the main site for such metabolism, a considerable portion of the PAHs may be metabolized by the enterocytes during absorption (Bock et al., 1979). These metabolites may thus be excreted into the lumen of the intestine and are available for enterohepatic circulation.

Although bile is the main route of excretion for the metabolites of these compounds, a significant percentage of radiolabel was excreted in the urine in this study implying their accesibility to the systemic circulation. This may be due to direct transport from the enterocytes to the lymphatics and thence to the systemic blood (Rees, Mandelstam, Lowry and Lipscomb, 1971) and from the hepatic venous blood (Chipman et al., 1981b).

One technical problem related to the use of tritiated hydrocarbons in this study is the loss of a tritium atom during hydroxylation steps. Hydrogen is known to migrate around the aromatic ring systems during such hydroxylation (the NIH shift). The hydroxylation results in the formation of ^3H_2O which enters the body water. This will create a small error when tritiated derivatives in bile and urine are assayed by scintillation counting.

4.3.2. Role of the Dietary Vehicle in 2,6-Dimethylnaphthalene (DMN) Absorption

The experimental model used in this study provides a means for assessing the contribution of concomitant fat absorption to the systemic delivery of intraduodenally administered 2,6-DMN.

Fragments of evidence indicate that concomitant fat feeding greatly enhances PAH absorption. Highly lipophilic PAHs are likely to be found in the lipid portion of the diet. In addition to the possible cancer promoting effects of fat, dietary lipids can markedly increase the bioavailability of hydrophobic molecules such as the PAHs (Falk and Kotin, 1963). Digestible fats when used as vehicles for PAHs, provide profoundly greater systemic availability of

hydrocarbons than do aqueous suspensions (Dao, 1969). The absorption from lipid vehicles of PAH and organochlorine compounds has been repeatedly demonstrated (Daniel et al., 1967; Wilson et al., 1982).

The apparent dependence of hydrocarbon absorption on concomitant triglyceride digestion and absorption may be explained in terms of the "hydrocarbon continuum" conception of Patton (1981). Because of the exceedingly low water solubility of these compounds they pass through a series of solubilizing phases in the intestinal lumen, created by triglyceride and their digestion products, in order to reach the enterocyte membrane. Another possible explanation for the apparent dependence of hydrocarbon absorption on concomitant fat digestion and absorption may be during active triglyceride (TG) absorption, chylomicrons are being manufactured in large quantities and thus the absorbed hydrocarbons can be carried away rapidly by these lipoprotein particles. In contrast, in the absence of active chylomicron formation, the absorbed hydrocarbon will be carried mainly by the very low density lipoproteins. Thus active TG transport will not only facilitate the rapid transport of absorbed hydrocarbon, it will also maintain a low concentration of hydrocarbon intracellularly to facilitate the uptake of hydrocarbon by the enterocytes.

Since 2,6-DMN has a relatively high water solubility compared to BP or DMBA, we assumed that if 2,6-DMN is administered intraduodenally in a non-lipid vehicle like ethanol, instead of oil vehicle, the bioavailability of this compound will not differ significantly. Fig. 3-17 shows that the total radiolabel recovery was not significantly different from the animals where the compound was

administered in oil. This seems to indicate that concomitant fat absorption plays little role in the systemic availability of this compound. If the pattern of radiolabel recovery in the 0-8 hour period is compared between the control (corn oil vehicle) and the experimental animals it is observed that peak recovery occurs earlier when 2,6-DMN is administered in ethanol. This further implies the non-essential role of fat absorption in 2,6-DMN uptake by the enterocytes. When the compound is administered in corn oil, some time is required for the pancreatic lipase to act on the fat droplets and release the 2,6-DMN molecules to the aqueous environment as micellar solutes. But when administered in ethanol, due to high water miscibility of this solvent, the 2,6-DMN molecules are immediately dispersed into the aqueous phase of the intestinal lumen and are available for uptake by the enterocytes.

4.3.3. Enterohepatic Circulation of 2,6-Dimethylnaphthalene (DMN) Metabolites

The role of metabolic activation in converting relatively inert parent PAHs to carcinogenic intermediates is well known (Levin, Wood, Chang, Ryan and Thomas, 1982). Following the production of polar metabolites by the liver the PAHs are readily excreted in bile, the principal route of excretion for PAHs (Levin, 1970). The biliary metabolites of PAHs are known to undergo enterohepatic circulation. Conjugates of BP metabolites in rat bile are hydrolysed by rat and human intestinal microflora (Renwick and Draser, 1976) and a significant portion of biliary metabolites undergo enterohepatic circulation in the rat (Chipman et al., 1981a). Similar recycling of DMBA biliary metabolites has also been shown (Laher and Barrowman, 1984). Enterohepatic circulation has

also been reported in rats for glutathione conjugates derived from propachlor (Bakke, Gustafsson and Gustafsson, 1980) and naphthalene (Pue, Frost and Hirom, 1982). Thus potentially cytotoxic, mutagenic and carcinogenic products excreted in the bile may well be reabsorbed from the intestine and become available to a number of tissues. This systemic availability of hepatic metabolites of PAHs may have a toxicological significance as regards other tissues of the body, in particular, the lungs which present the first capillary bed passed through by hepatic venous blood.

From the above discussion it becomes apparent that many PAH metabolites undergo enterohepatic circulation and it was highly probable that 2,6-DMN metabolites would also do so. Fig. 3-17 shows that 82.29% of an intraduodenal dose of 2,6-DMN biliary metabolites was excreted in bile and urine in 24 hours, establishing the existence of an enterohepatic circulation for metabolites of this compound. A significant proportion of the recycled metabolites (46.18%) was excreted in the urine indicating the accessibility of the metabolites to the systemic circulation. No major difference was observed in the excretory pattern of biliary radiolabel between the parent compound and biliary metabolites: 29.58% of an intraduodenal dose of 2,6-DMN was excreted in bile in the 0-8 hour period compared to 28.34% for the metabolites. This may again be attributed to the relatively high water solubility of 2,6-DMN. An interesting phenomenon is the secondary peak that was observed in the recovery pattern of the metabolites in the 0-8 hour period (Fig. 3-14). This may be due to the fact that some of the metabolites are excreted back into the intestinal lumen by the enterocytes and are reabsorbed. Thus in an intact animal the recycling of the metabolites could be

active not only for the biliary metabolites but also for the metabolites excreted by the enterocytes. The origin of the latter metabolites is not clear. They may reach the enterocytes from the circulation (indirectly from the hepatic venous blood) or they may be produced by the enterocyte microsomal enzymes during the absorption or they may be available from both sources.

So, this study establishes the existence of an enterohepatic circulation for the biliary metabolites of 2,6-DMN. The next step would be to assess the cytotoxicity and mutagenicity of these metabolites when evaluating the toxicological significance of 2,6-DMN.

4.3.4. Role of Lipid Hydrolysis in 2,6-Dimethylnaphthalene (DMN) Absorption

The major dietary lipid species, triglyceride, is efficiently hydrolysed in the small-intestinal lumen by pancreatic lipase together with colipase. This hydrolysis is a prerequisite for absorption. It has been established that absorption of trace nutrients such as sterols and fat-soluble vitamins is intimately dependent on the normal processes of fat digestion and absorption and that bile salts, which play a facilitatory role in triglyceride absorption, are obligatory for absorption of these trace lipids (Hollander, 1981). By the action of the pancreatic lipase in the intestinal lumen, long-chain triglyceride is hydrolysed to monoglyceride and two fatty acid molecules, which then form the bulk of a subsequently formed viscous isotropic phase of low surface tension and containing water (Patton, 1979). There are continuous lipid and aqueous media in this phase and non-polar tracers such as β-carotene and dibenzanthracene can be seen to flow from the triglyceride

phase into the viscous isotropic phase as the former is digested and the latter phase created. PAHs, which are presented to the intestinal lumen as solutes of triglycerides, are likely to behave in a similar fashion.

Laher and Barrowman (1983) have shown the influence of the degree of simulated triglyceride hydrolysis on the aqueous partition of the hydrocarbon DMBA. They observed maximum aqueous partition (micellar solubilization) of DMBA at 40% "hydrolysis" and also noted an inverse relationship between the percentage of hydrocarbon present in the micellar phase and the quantity of triglyceride. Triglyceride hydrolysis is, therefore, necessary for the appreciable partition of hydrocarbon from an oil phase into an aqueous dispersible bile-salt solution, thus forming the chemical gradient necessary for the uptake by the enterocytes.

In our experiment there was no significant reduction in the recovery of total radiolabel in bile and urine after an intraduodenal dose of [^{3}H]-2,6-DMN in corn oil, when both pancreatic secretions and bile were absent from the duodenum. This shows that bioavailability of this compound does not depend on concomitant triglyceride hydrolysis. The cumulative recovery of radiolabel in the 0-8 hour period was significantly lower than the control animals (Tab : 3-8), indicating that in absence of pancreatic hydrolysis, the 2,6-DMN molecules tend to cling to the corn oil droplets effectively reducing their rate of absorption. But, in contrast in the 8-24 hour period the cumulative recovery was significantly higher than the control animals indicating that the 2,6-DMN molecules gradually escape from the fat droplets and are absorbed efficiently, so that in the 24 hours cumulative recovery there was no significant difference.

Thus, triglyceride hydrolysis by pancreatic lipases, which is an essential factor for bioavailability of trace nutrients and many PAHs, does not play an essential role in the intestinal absorption of 2,6-DMN.

SUMMARY

This thesis examines several factors governing the intestinal absorption and metabolism of the PAHs. The study was divided into two main parts.

A wide variety of factors are known to influence the activities of intestinal xenobiotic metabolizing enzymes, benzo(a)pyrene hydroxylase (BPH) and UDP-glucuronyl transferase (UDP-GT). In the initial part of this thesis, the effects of two different factors on these enzyme activities were observed. The trophic action of the gastrointestinal hormones (pentagastrin, cholecystokinin and secretin) on rat intestine is well established. In the beginning of this study, the effects of these hormones on BPH and UDP-GT activities in rat intestine were examined. A number of investigators have observed that, after proximal resection and anastomosis of small-intestine in rat, the remnant ileum undergoes morphological and functional changes. In this study, as a continuation of the factors influencing xenobiotic metabolism in rat intestine, the effect of such post-resection adaptive hypertrophy on BPH and UDP-GT activities was examined.

Our results indicate that pentagastrin treatment produces a significant increase in the BPH activity only in colonic mucosa. No effect of pentagastrin was observed on this enzyme activity in duodenum, jejunum and ileum. The hormone also did not have any effect on UDP-GT activity in any region of the intestine. CCK-OP or secretin did not cause any change in the BPH or UDP-GT activities in either the small or the large intestine. It appears that under the

present experimental conditions, only pentagastrin has a stimulatory effect on the BPH activity in colonic mucosa.

After proximal resection and end-to-end anastomosis in rat small intestine, followed by 4 weeks of recovery, the remnant ileum showed significant increase in the mucosal thickness. The total protein content of the mucosa was also increased indicating a stimulation of protein synthesis. There was no change in the BPH activity (expressed as nmol. BP hydroxylated /mg prot./min) in the hypertrophied ileum as compared to the pre-resection [redacted]ate, but the UDP-GT activity (expressed as nmol. 1-naphthol conjugated/mg prot./min) was significantly lowered. Since the hypertrophied mucosa consists of rapidly dividing relatively immature cells, this may account for the fall in UDP-GT activity when expressed per weight of mucosa. However, the lack of change in BPH activity is difficult to explain.

The second portion of the study examined the absorption of several PAHs in the rat. When radiolabelled PAHs- [^{3}H]-2,6-dimethylnaphthalene(DMN), [^{14}C]-phenanthrene, [^{14}C]-anthracene, [^{3}H]-7,12-dimethylbenzanthracene (DMBA) and [^{3}H]-benzo(a)pyrene(BP) -were administered intraduodenally to rats, radiolabel was excreted in bile and urine and was used as an index of relative absorption. All the compounds studied were well absorbed from the intestine, although with the larger molecules DMBA and BP, the percentage of absorption was relatively low. The representative PAHs were absorbed from the small intestine even in total absence of luminal bile but the efficiency of absorption in absence of bile was related to the aqueous solubility of the PAHs. Although these

PAHs are probably transported from the enterocytes by both portal venous and lymphatic routes, this study didnot attempt to determine the relative importance of these two routes.

The efficiency of absorption of DMN (water solubility 2.0 mg/L) and phenanthrene (water solubility 1.29 mg/L) in absence of bile was not significantly different from the absorption in presence of duodenal bile, 91.64% and 96.67% respectively. It is known that the "unstirred water layer" (UWL) lining the absorptive surface of the intestine constitutes the major obstruction to the passage of lipophilic compounds to the enterocytes. Therefore relatively high water solubility of DMN and Phenanthrene as compared to the other three PAHs studied, might account for these results. This observation is further validated by the dependence of anthracene (water solubility 0.073 mg/L), a structural isomer of phenanthrene, on luminal bile for efficient absorption. In the absence of duodenal bile, the efficiency of absorption (% of absorption in presence of bile) of anthracene was significantly reduced to 70.84%. In the case of DMBA (water solubility 0.061 mg/L) and BP (water solubility 0.0038 mg/L), the efficiency of absorption was further reduced to 43.38% and 22.94% respectively. The significant difference in the bioavailability of structural isomers phenanthrene and anthracene in absence of bile indicate the importance of water solubility in determining the biliary requirement for absorption of the PAHs. The observations made in this study seem to indicate that for the efficient absorption of PAHs whose water solubility is less than approximately 1 mg/L, bile plays an increasingly important role as water solubility of the compounds progressively decrease.

When the factors governing the bioavailability of 2,6-dimethylnaphthalene was further studied several other observations were made. There was no significant difference in the bioavailability of this compound when it was administered in ethanol instead of corn oil. This indicates that DMN does not require concomitant fat digestion and a continuous lipophilic microenvironment (hydrocarbon continuum) for efficient absorptive process. This theory is further strengthened by the observation that the compound was efficiently absorbed even when the intestinal lipolysis was inhibited by diverting both the bile and the pancreatic secretion from the duodenum. It was also observed that, like most other PAHs, the biliary metabolites of DMN undergo extensive enterohepatic circulation. The results obtained indicate that 2,6-DMN is well absorbed from the intestine and the bioavailability of this environmental pollutant seems to be independent of most factors which regulate the bioavailability of other PAHs. The observed enterohepatic circulation of the biliary metaboltes of 2,6-DMN indicates that the metabolites may be available for exerting various toxic effects which may prove to be more important than the parent compound.

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