INTESTINAL ABSORPTION: STUDIES OF ABSORPTION OF CERTAIN NATURAL LIPIDS AND LIPOPHILIC XENOBIOTICS

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by

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ABSTRACT

The uptake of nutrient lipids by the absorptive cell of the small intestinal mucosa is influenced by a number of factors. Some lipophilic xenobiotic compounds are also absorbed by the enterocyte in conjunction with the nutrient lipid. The interaction that occurs between these compounds and nutrient lipids is of toxicological and physiological interest. The chylomicron appearance time (the interval between introduction of ^{14}C-oleic acid and the first appearance of ^{14}C-labeled chylomicrons in thoracic duct lymph) is an effective tool in monitoring changes in the handling of the first lipid molecules being processed by the enterocyte. The chylomicron appearance time in control animals is highly reproducible and agrees well with previously published work.

Polycyclic aromatic hydrocarbons such as 7,12-dimethylbenz[a]anthracene (DMBA) are known carcinogens and are readily absorbed from the gastrointestinal tract. Due to their lipophilic nature, a substantial proportion of the absorbed xenobiotic can be transported in lymph chylomicrons. Of the PAHs tested, only DMBA was found to exert any effect on the chylomicron appearance time resulting in an appearance time of 23.08 ± 2.10 minutes as compared to a control value of 10.81 ± 1.03 minutes ($p < 0.001$). These results indicate that the inclusion of DMBA in a nutrient lipid test meal significantly retards the normal process of uptake and/or processing of nutrient lipids within the enterocyte.

Exposure to 10μmol DMBA several hours prior to test meal administration, still resulted a delay in chylomicron appearance time. Triglyceride flux analysis for three hours following test meal administration indicates a transient effect of

DMBA since the total triglyceride outputs in both control and DMBA-treated rats were not significantly different.

Subsequent studies used the chylomicron appearance time technique to determine the effects of other lipophilic compounds on lipid absorption. Cholesterol had no effect on the chylomicron appearance time but the hydrophobic surfactant Pluronic L-81 delayed the the appearance of the radiolabel. [16.60 $\pm$ 0.77 minutes, $p \ll 0.01$]. This result supports the report attributing hypolipidemic activity to this compound.

The efficiency of the α-glycerophosphate pathway compared with the monoglyceride pathway for triglyceride resynthesis was examined using the chylomicron appearance time technique. Three groups of rats were used for this study. Group A received a lipid emulsion test meal containing 1-monoolein as its source of monoglyceride. Group B received a test meal containing 2-monoolein and Group C received a test meal without any monoglyceride present. The chylomicron appearance times for these three groups did not differ significantly. This indicates that the α-glycerophosphate pathway is capable of handling the first few molecules of lipid as efficiently as the monoglyceride pathway despite the higher energy cost required to do so.

[KEY WORDS]

Anthracene

1,2-Benzanthracene

Benzo[a]pyrene

Cholesterol

Chylomicron Appearance Time

7,12-Dimethylbenzanthracene

hydrophobic surfactant

Pluronic L-81

Polycyclic Aromatic Hydrocarbons

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ABBREVIATIONS

DMBA	7,12-Dimethylbenz[a]anthracene
BaP	Benzo[a]pyrene
A	Anthracene
1,2-BA	1,2-Benzanthracene
cpm	counts per minute
PAH	polycyclic aromatic hydrocarbon
CAP	chylomicron appearance time
SE	standard error of the mean
FABP	fatty acid binding protein
TGFA	triglyceride fatty acid
VLDL	very low density lipoproteins
SER	smooth endoplasmic reticulum
VI	viscous isotropic

Chapter 1
INTRODUCTION

1.1. Background information

1.1.1. Lipid digestion and absorption

The lipid component of the North American diet contributes approximately 43% of energy derived from the total diet. This lipid component is composed of triglycerides, cholesterol and phospholipids (Guthrie, 1983) with dietary triglyceride accounting for over 90% of total lipid intake. Due to the importance of this dietary component, the mechanism of triglyceride digestion and absorption has been intensively studied.

The pathway from ingestion of dietary triglyceride to absorption can be divided into six general steps: i) intraluminal digestion ii) micellar solubilization iii) permeation from the lumen to the cell iv) intracellular reesterification v) chylomicron formation and vi) transport of the chylomicron from the cell to the circulation (Shiau, 1987).

The ultimate aim of triglyceride digestion is to produce compounds that are more hydrophilic than the parent nonswelling lipid (Shiau, 1987). The digestion process begins in the mouth where body temperature liquifies many fats such as

butter and margarine and mechanical action breaks down large lipid particles: a process which serves to increase the surface area of the lipid increasing its bioavailability for digestive enzymes. Lipolytic enzymes hydrolyse triglyceride resulting in the production of diglycerides, monoglycerides, fatty acids and glycerol. This lipase activity begins with lingual lipase secreted from serous glands (von Ebner's glands) on the dorsal aspect of the tongue. The optimal pH of 4 of this enzyme allows it to continue hydrolysis in the acidic gastric medium. This enzyme lacks positional specificity yet it preferentially attacks the medium chain triglycerides. Once exposed to bile salts, the action of lingual lipase is inhibited (Liao, Hamosh and Hamosh, 1984).

Lipolytic activity in the stomach is largely due to gastric lipase whose activity bears close resemblance to that of the lingual lipase. Because of this similarity the existence of these two enzymes as separate entities has been questioned. Approximately 10-30% of triglyceride is hydrolysed in the stomach to form partial acylglycerols and free fatty acids (Liao, Hamosh and Hamosh, 1983).

Intraluminal digestion

The greatest proportion of triglyceride digestion occurs in the small intestine at the oil-water interface. Here, pancreatic lipase, secreted by the acinar cells of the pancreas, hydrolyses triglyceride at the 1 and 3 positions with a pH optimum of approximately 8 (Borgstrom, 1964). In 1964, Borgstrom demonstrated that the presence of bile salts in the intestine results in the reduction of the pH optimum for this enzyme from 8 to 6 which corresponds with the pH of the small intestine. The mechanism by which this shift occurs is unclear. It has been postulated that the bile salt may induce changes in the conformation of the lipase.

The presence of pancreatic lipase in the small intestine is not sufficient for lipolysis to occur at the prevailing pH and bile salt concentrations. The pancreas secretes a cofactor, colipase, in the form of procolipase. Proteolytic enzymes break down this compound to the active colipase which binds to the lipase in a 1:1 ratio. This binding is facilitated by the presence of bile salts. It has been postulated that the colipase may anchor the lipase at the oil-water interface in the presence of bile salts (Borgstrom and Donner, 1975). The lipase can then act at the interface bringing bile salt micelles close to the site of lipolysis to remove the lipolytic products from the interface. If not removed, the presence of these products accumulating at the oil:water interface results in inhibition of further hydrolysis .

Micellar solubilization

Micellar solubilization of lipid products must occur prior to absorption. Primary bile salts secreted by the liver are converted to secondary bile salts in the intestinal lumen due to bacterial biotransformation. At a critical micellar concentration, bile salt monomers form aggregates called simple micelles. When lipolytic products are incorporated into these micelles, mixed micelles are formed (Carey and Small, 1970). This process increases the water solubility of the lipids by a factor of approximately 1000.

The events of lipid digestion, as described above, can be seen by light microscopy as a four phase series consisting of an oil phase, calcium soap, viscous isotropic (VI) phase and micellar phase (Patton, Vetter, Hamosh, Borgstrom, Lindstrom and Carey, 1985). The oil phase consists of triglyceride and diglyceride existing as a spherical form in water. Lipid digestion by pancreatic lipase can be

observed at the oil-water interface. The first product phase, the calcium soap is seen as a rough brittle shell surrounding the oil droplet. Isolation and analysis of this shell reveals its composition to be over 90% fatty acid and calcium. The viscous isotropic phase follows after calcium soap formation ceases. The composition of the VI phase is unknown since it has not yet been isolated. From the fact that mixtures of protonated fatty acids and monoglycerides exhibit characteristics virtually identical to that of the VI phase it is suggested that these two components make up a large proportion of the this phase. The final phase is the micellar phase where bile salts disperse the VI phase into discrete water soluble disks, or mixed micelles. Small particles of this phase are not visible by light microscopy but a size continuum exists between this phase and the VI phase. An oil droplet remnant is left over after digestion occurs. The composition of this remnant may be concentrated polar constituents of the initial droplet. It is believed that the droplet does not consist of nonpolar lipid as these compounds move freely into the VI phase through a hydrocarbon continuum.

Patton's studies in 1981 resulted in the proposal of a hydrocarbon continuum in triglyceride which remains intact during digestion of triglyceride to monoglyceride and fatty acid. The presence of such a spatial continuum allows trace lipids to flow through into the product phase. Patton observed this method of transport with both β-carotene and the aromatic hydrocarbon perylene.

Permeation from the lumen to the cell

Once digestion occurs, the mixed micelles containing the lipolytic products are presented to the brush border of the enterocyte of the small intestine. Two well defined barriers slow this movement. The first is the unstirred water layer

that lies adjacent to the microvillous membrane of the cell. Lipid transport across this aqueous environment is facilitated by these mixed lipid:bile salt micelles. The second barrier is the lipid membrane. Micelles are thought to release the lipolytic products at the cell membrane. The diffusion of these lipids across the membrane is dependent on the individual permeability coefficient and concentration of each of the products. Long-chain fatty acids which are highly hydrophobic cross at a faster rate than those of a shorter chain length (Sallee, 1979).

Bile salts are reabsorbed from the intestine partly by passive diffusion in the small intestine and partly by active transport in the distal ileum. They return to the liver via the portal vein. About 10% of the intestinal bile salts escape this enterohepatic circulation and is excreted in the feces.

In the cell membrane, the lipolytic products associate preferentially with the lipid matrix. A method of transport is required to move the lipolytic products to the smooth endoplasmic reticulum within the cell. In 1972, Ockner, Manning, Poppenhausen and Ho isolated a protein with a molecular weight of 12,000 from the cytosol of the enterocyte. This protein has a high affinity for fatty acids and is referred to as the fatty acid binding protein (FABP) (Ockner and Manning, 1974). Areas of the small intestine where fat absorption occurs have high concentrations of this protein. The FABP preferentially carries unsaturated long chain fatty acids and it is believed that reesterification of the fatty acids requires FABP transport as a crucial step (Ockner and Manning, 1974).

Intracellular reesterification

Reesterification of lipolytic products to triglyceride occurs in the smooth endoplasmic reticulum. This resynthesis process occurs through a series of biochemical pathways. Generally, fatty acids are activated to acyl CoA derivatives in the presence of CoA, ATP (derived from glucose metabolism) and Mg^{++} by the enzyme acyl CoA synthetase. Triglyceride resynthesis can then follow one of two distinct pathways : i) the monoglyceride pathway or ii) the α-glycerophosphate pathway.

In the monoglyceride pathway, 2-monoglyceride derived from dietary triglyceride digestion is esterified with activated fatty acids to form a diglyceride. Esterification with another fatty acid results in triglyceride formation. Both of these steps require the presence of an acyl CoA acyltransferase. This is the major pathway of triglyceride resynthesis (Shiau, 1987).

When 1-monoglyceride is introduced into the intestinal lumen, its ultimate fate depends largely on the fatty acid moiety involved. 1-monopalmitin is reesterified through the monoglyceride pathway (as described above) to form triglyceride where as 1-monoolein follows a different route, the α-glycerophosphate pathway described below. In addition, the method by which the 1-monoglyceride is presented to the enterocyte is important in determining the mechanism by which the 1- isomer is handled by the absorptive cell.

Luminal hydrolysis can result in the production of 1-monoglyceride. A level of 6% 1-monoolein has been reported from hydrolysis of triolein (Mattson and

Volpenhein, 1964). This monoglyceride is produced as a result of 2-monoglyceride isomerization. The 1-monoolein travels to the intestinal mucosa where it is hydrolysed further to glycerol and fatty acid. These are then used in triglyceride resynthesis. The utilization of 1-monoglyceride as a substrate for the monoglyceride pathway of triglyceride resynthesis is disputed by some researchers (Mattson and Volpenhein, 1964) and supported by others (Brown and Johnston, 1964a; Brown and Johnston, 1964b).

In the absence of monoglyceride, triglyceride is resynthesised by the α-glycerophosphate pathway (Shiau, Popper, Reed, Umstetter, Capuzzi and Levine, 1985). Hexose metabolism results in the formation of α-glycerophosphate which when combined with two molecules of acyl CoA becomes phosphatidic acid. L-α-phosphatidate phosphohydrolase hydrolyses the phosphatidic acid to a diglyceride (Shiau, 1987). A subsequent acylation reaction results in triglyceride formation.

Chylomicron formation

The newly formed triglyceride droplet then acquires phospholipid in the SER. This particle, is transported to the Golgi apparatus where a glycoprotein coat is added. The surface of the fully formed chylomicron is composed of a monolayer of phospholipids, apoproteins, free cholesterol and a small amount of triglyceride. The core contains the majority of triglycerides and cholesterol esters and about one third of the total free cholesterol in the chylomicron. The mechanism by which this packaging occurs and the regulatory mechanisms which govern its assembly are generally unknown. The presence of apoproteins, however, is essential for this process to occur.

Transport of the chylomicra out of the cell

Once formed, the chylomicrons are packaged into secretory vesicles which travel to and fuse with the basolateral portion of the enterocyte cell membrane. The chylomicrons are then exocytosed into the intercellular space. They then pass through gaps in the basement membrane, cross the lamina propria and enter the lacteals, a distance of approximately 50μm (Granger, 1981). Travel of the chylomicrons through the lamina propria is probably by passive diffusion, facilitated by matrix hydration (Tso, Barrowman and Granger, 1986). Thus the fluid movement that occurs concurrently with chylomicron transport may assist the movement of these relatively bulky particles through the interstitium and greatly decrease the chylomicron transit time (Tso and Balint, 1986). Tso, Pitts and Granger (1985) reported that the chylomicron transit time is dependent on the flow rate of lymph when lymph flow is $<40\mu$L/min. At flows above this, there is a minimum transit time of radiolabeled fatty acids between the intestinal lumen and the intestinal lymphatic which is of the order of 10-15 minutes (Tso et al, 1986). Provided the hydration of the interstitium is held relatively constant, changes in the time required to detect these chylomicrons in the intestinal lymphatics can be used to indicate changes in lipid absorption and handling by the enterocyte.

1.1.2. Factors and disease states that influence chylomicron appearance time

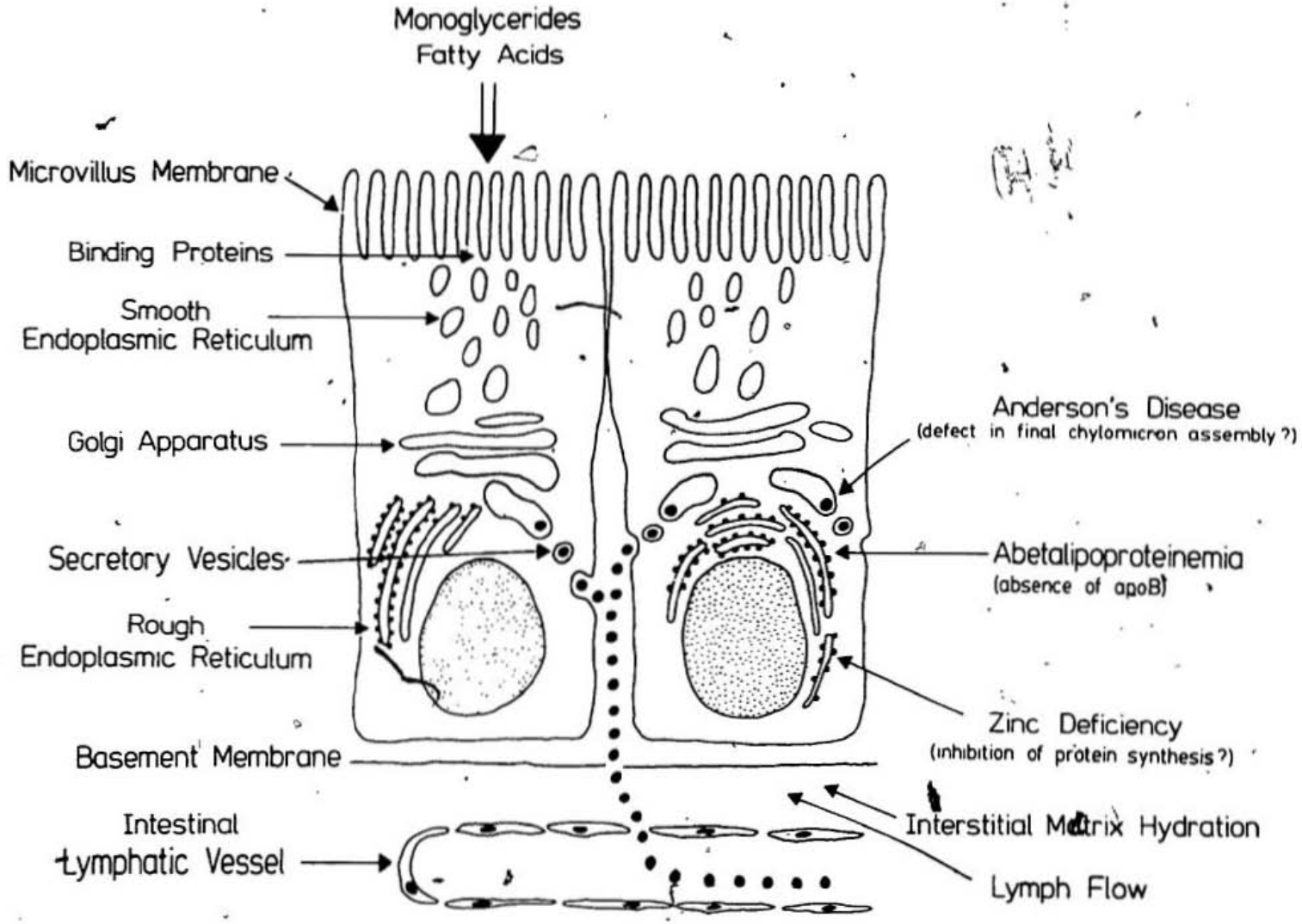
As mentioned previously, the chylomicron appearance time (CAP) ie. the time interval between introduction of a radiolabeled fatty acid in the duodenum and the appearance of the label in lymph chylomicra, can be altered by a number of factors (Figure 1.1).

The rate of fat absorption from the lumen, lymph formation, lymph flow rate and interstitial matrix hydration all appear to have acute effects on the chylomicron appearance time in the lymph (Tso et al, 1986; Tso and Balint, 1986; Tso, Pitts, and Granger, 1985; Tso, Buch, Balint and Rodgers, 1982).

Various clinical conditions have been identified that result in changes in chylomicron appearance time which in turn results in chronic abnormal handling of dietary lipids. Some of these malabsorption syndromes are described below (Figure 1-1.). As mentioned previously, apoproteins are an essential element in chylomicron formation. Inhibition of protein synthesis markedly effects the chylomicron transport of triglycerides into the lymph (Glickman, Kirsch and Isselbacher, 1972). Abetalipoproteinemia, an inborn metabolic abnormality results in a deficiency in apolipoprotein B (apo B) synthesis. This class of apoproteins are required in the early stages of chylomicron formation. Morphological examination of the enterocytes of afflicted patients reveals large unbound lipid droplets within the cell. Chylomicrons are not formed and therefore a malabsorption syndrome is observed (Malloy and Kane, 1982).

Zinc deficiency has been linked to impairment of intestinal triglyceride

Figure 1-1: Factors affecting lipid absorption in the enterocyte.
The left side of the figure shows the structures involved in dietary lipid absorption.


Monoglycerides
Fatty Acids
Microvillus Membrane
Binding Proteins
Smooth
Endoplasmic Reticulum
Golgi Apparatus
Secretory Vesicles
Rough
Endoplasmic Reticulum
Basement Membrane
Intestinal
Lymphatic Vessel
Anderson's Disease
(defect in final chylomicron assembly ?)
Abetalipoproteinemia
(absence of apoB)
Zinc Deficiency
(inhibition of protein synthesis ?)
Interstitial Matrix Hydration
Lymph Flow

transport possibly through inhibition of intestinal lipoprotein synthesis. A decrease in net triglyceride transport has been reported by Koo and Turk (1977), in rats fed a zinc deficient diet. Electron micrographs of the intestinal mucosa of these rats show accumulation of large lipid droplets. These droplets are not membrane bound and tend to coalesce. Marked reduction in the granular endoplasmic reticulum and an inactive appearing Golgi apparatus is observed. The droplets are unable to enter the intercellular space and therefore do not appear in the lymph. In studies where a subclinical level of zinc deficiency is induced in rats, marked morphological alterations in intestinal nascent chylomicrons and decreases in apoprotein C and E are observed (Koo, Henderson, Algilani and Norvell, 1985).

Anderson's disease or chylomicron retention disease is a recessively inherited condition of lipid malabsorption. Patients with this disease exhibit no increase in plasma triglyceride levels after being given an oral fat load. Ultrastructural examination of the enterocytes of these patients after fat loading reveal a large number of fat particles vesiculating the endoplasmic reticulum. In addition, these chylomicron sized particles are clustered within the dilated vesicles of the Golgi zone. Fat transport out of the enterocyte appears to be impaired (Roy, Levy, Green, Sniderman, Letarte, Buts, Orquin, Brochu, Weber, Morin, Marcel and Deckelbaum, 1987). The defect in fat transport in this disease is not yet understood but it is postulated that either the final chylomicron assembly or the exocytosis pathway of the completed chylomicron is affected.

In addition to clinical conditions and physical parameters, various xenobiotics have been found to affect lipid absorption. Included are the Pluronic

polyols which are a group of hydrophobic surfactants and colchicine which is a drug used in treatment of acute gout.

1.1.3. Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are an important class of chemical compounds many of which have been found to be potent carcinogens. These compounds are composed of three or more fused benzene rings and are generally formed as a result of incomplete combustion of carbonaceous materials (Blumer, 1976). Research into the possible adverse effects of these compounds on human health has been carried out since dibenz(a,h)anthracene was recognized as a carcinogen in the early part of this century. As of 1978, over 100 PAHs have been identified (Lo and Sandi, 1978). Early studies demonstrated the carcinogenic effect of topical application of coal tars on mouse skin and later the compounds responsible for this carcinogenic effect were shown to belong to the class of PAHs.

Initially, research concentrated on occupational exposure to this class of compounds largely as a result of the proposal for an environmental basis for cancer by Pott in 1775. Pott concluded that the high incidence of scrotal cancer in chimney sweeps was a result of long term exposure to carbon soot. Later it became apparent that human exposure can occur from a number of other sources. One such route of exposure is via PAH contaminated foodstuffs. PAHs appears in a wide variety of food types from a variety of sources. Commonly, food contamination by these compounds is a result of environmental pollution deposited on leafy vegetables or from smoke-cured or charbroiled meats. Heating of oils and other cooking procedures also result in PAH formation (Gray and Morton, 1981).

PAHs such as benzo(a)pyrene formed as a result of industrial emissions have been detected in lettuce, kale, spinach, leeks and tomatoes in significant quantities (Lo and Sandi, 1978). Fortunately, rinsing of these foods with water removes much of the contamination. PAH contamination has also been reported in margarines, butter and vegetable oils produced from contaminated plant raw materials (Hopia, Pyysalo and Wickstrom, 1986).

The bulk of PAH contamination of food results from curing and certain forms of cooking such as grilling over charcoal (Lo and Sandi, 1978). Since the PAHs are generally lipophilic, association with the fat components of meat and fish is common.

The amount of PAH deposition on smoked foods depends largely on the wood deposition temperature (Gilbert and Knowles, 1975). Because the penetrating ability of these compounds is relatively low, surface cuts of meats contain higher concentrations of these compounds than the inner cuts (Gilbert and Knowles, 1975).

Charbroiled meat and fish also contain significant levels of PAHs. Here, the fat content of the food and the distance from the heat source are key factors. PAH contamination in this case is due to pyrolysis of fat. During the charbroiling process quantities of fat drip on the coals. Pyrolysis of this fat occurs and forms polycyclic aromatic hydrocarbons. These are then volatized and deposited on the meat (Lijinsky and Shubik, 1965).

1.1.4. Metabolism of polycyclic aromatic hydrocarbons

As outlined in the previous section, the gastrointestinal tract is a key route for human exposure to polycyclic aromatic hydrocarbons. It is believed that trace amounts of lipid-soluable compounds can readily dissolve in dietary fat and then can be transferred to mixed lipid-bile salt micelles (Laher and Barrowman, 1983). Such being the case, lipophilic PAHs may be passively carried into the enterocyte through the hydrocarbon continuum postulated to exist in the small intestinal lumen during fat digestion and absorption (Patton, 1981).

Once absorbed into the enterocyte, PAHs may travel one of two routes into the general circulation. Lindstrom, Barrowman and Borgstrum (1987) showed that a certain portion of 7,12-dimethylbenz[a]anthracene (DMBA) absorbed from the small intestine appears unmetabolized in the chylomicron fraction of the lymph. The relative importance of this route was determined by Laher and Barrowman (1987). From these studies it was found that the lymphatic route contributes 20% of the total recoverable DMBA from both lymph and bile combined. Once in the systemic circulation, it is not known whether DMBA remains with the chylomicron remnant after exposure to lipoprotein lipase activity in peripheral tissues. Irrespective of this, DMBA is largely available for metabolism by the liver followed by biliary excretion (Lindstrom et al., 1987).

The portal venous blood route for DMBA transport from the enterocyte appears to be a more important pathway than the lymphatic route. In the early 1960's PAH metabolism in the enterocyte was described (Wattenberg, Leong and Strand, 1962). The enterocyte possesses enzyme activity that oxidizes PAHs such

as benzo(a)pyrene (BaP) to hydroxyl derivatives and subsequently quinones. Induction of this enzyme activity occurs from prior exposure of the cells to PAHs or other inducers (Wattenberg, 1971). In addition, the glutathione-S-transferase enzymes are induced in the small intestine after benzo(a)pyrene administration and the distribution of this enzyme parallels with that of the benzo(a)pyrene hydroxylase system (Clifton and Kaplowitz, 1977). Once metabolized, the PAH metabolites, probably in a conjugated form, are transported to the liver via the portal vein (Bock, Clausbruch and Winne, 1979).

In the liver, PAHs undergo Phase I (usually oxidations) and Phase II (conjugations) metabolism, the end result being the conversion of the lipophilic parent compounds to water soluble species which and can be excreted in bile (Dipple, 1983; Digiovanni and Juchau, 1980). While PAHs are biologically rather inert compounds, some of their metabolites are highly reactive electrophilic substances which are the ultimate carcinogenic products of this class of compounds (Grover, 1986).

1.1.5. Pluronic L-81

Another group of compounds which appear associated with the lipid portion of the diet are the hydrophobic surfactants of which the Pluronic polyol detergents are members. Compounds in this group have been used as emulsifiers in foods such as milkshakes, and, in small doses, they are well tolerated and free from toxic effects.

The hydrophobic component of these substances varies between these surfactants as does the manner by which they interact with nutrient lipids during

absorption. In 1977, Bochenek and Rodgers showed that rats given a lipid test meal containing a small quantity of the Pluronic compounds showed an decrease in lipid absorption proportional to the hydrophobicity of the Pluronic administered. In animals given these detergents with a hydrophobic component of at least 80%, inhibition of lipid absorption was observed. Detergents containing less than 60% hydrophobicity did not interfere with lipid absorption and, in some cases, even enhanced absorption.

One of the pluronic compounds, Pluronic L-81, which is composed of polyoxyethylene and polyoxypropylene copolymers and has a molecular weight of 2750, and has a hydrophobic component of 90%. Tso, Balint and Rodgers (1980), has shown that this compound depresses lymphatic transport of triglyceride fatty acid (TGFA) and cholesterol in rats given a chronic dose (3-4 wks) of L-81. In addition, the same inhibition of lipid transport can be observed in acute administration studies (Tso, Bishop and Rodgers, 1981).

The mechanism involved in this effect on lipid absorption has been recently investigated. Initially, impairment of digestion and absorption of the lipid in the intestinal lumen was suggested as the mechanism by which Pluronic L-81 exerts its effect (Bochenek and Rodgers, 1977). This theory was based on *in vitro* studies by Green, Heald, Baggaley, Hindley and Morgan (1976). It was later shown that in cases where a large dose of pluronic L-81 was given, the effect was as postulated, but, when small doses were administered, this did not hold true. Instead, lipid travels normally from the lumen into the enterocyte but accumulates within the small intestinal mucosa (Brunelle, Bochenek, Abraham,

Kim and Rodgers, 1979). It is therefore apparent that Pluronic L-81 does not interfere with processes in the intestinal lumen, which in turn, indicates a intracellular event.

In order to investigate this, electron microscopic studies were employed . These studies revealed accumulation of lipid in the enterocyte. Only very low-density lipoprotein-sized particles were transported into the lymph by the Pluronic L-81 exposed cells. These studies show that there is a significant inhibition of intracellular transport of chylomicron-sized particles and a resulting blockage of chylomicron secretion by the enterocyte (Tso et al., 1981).

The lack of inhibition of VLDL production suggests that separate pathways exist for chylomicron and VLDL assembly and transport in the small intestinal mucosa of the rat. Because of this preferential inhibition by Pluronic L-81, this compound is being used as a tool to investigate chylomicron and VLDL assembly and secretion.

Evidence from other sources supports the suggestion of a two pathway system. It was shown that a palmitate infusion causes a marked increase in VLDL transport whereas oleate and linoleate did not. Oleate and linoleate causes an increased chylomicron output (Ockner, Hughes and Isselbacher, 1969). More supporting evidence came when it was shown that the fatty acid composition of the VLDL and chylomicrons differ. Further, Mahley, Bennett, Morre, Gray, Thistlethwaite and LeQuire (1971) showed micrographs of separate populations of VLDL and chylomicrons in Golgi vesicles.

Based on this evidence, Tso, Drake, Black and Sebesin (1984) hypothesised that the intestinal lipid transport would not be altered if a lipid was infused that stimulated VLDL production in conjunction with Pluronic L-81. It has been shown that phosphatidylcholine stimulates VLDL production in humans and has little effect on chylomicron production (Beil and Grundy, 1980). Such being the case Tso and coworkers (1984) showed that the concomitant infusion of egg phosphatidylcholine and Pluronic L-81 resulted in no change in transport of lipid to the lymph as compared to phosphatidylcholine infusion alone.

It is therefore apparent that if chylomicron assembly and secretion are altered by this compound, the chylomicron appearance time should be lengthened with the administration of Pluronic L-81 in a lipid emulsion test meal.

1.2. Objectives

1. To determine the effect, if any, of 7,12-dimethylbenz[a]anthracene, benzo(a)pyrene, anthracene, benzanthracene, and cholesterol on chylomicron appearance time. If an effect is observed, to determine the mechanism by which the compounds exert the effect.

2. To assess the efficiency of the α-glycerophosphate pathway in triglyceride resynthesis in the absence of monoglyceride in the diet.

3. To determine the effect, if any, of a Pluronic polyol (L-81) on chylomicron appearance time.

Chapter 2

MATERIALS AND METHODS

2.1. Animals

Male Sprague-Dawley rats weighing between 275-375 g were purchased from Canadian Hybrid Farms (Nova Scotia, Canada) and were maintained under standard light and temperature conditions (12 hour photoperiod, 74 ° F and 40% humidity). The rats were housed in Shoe-Box cages; plastic rectangular cages with wire bar detachable covers (3 rats/cage) with sawdust bedding. Free access to food (Purina Rat Chow, Ralston Purina Company) and tap water was permitted.

2.2. Surgery

2.2.1. Anaesthesia

Diethylether vapour was used to induce and maintain anaesthesia. Anaesthesia was induced by placing the rat in a glass jar containing ether-soaked cotton swabs. The jar was covered with a heavy plexiglas lid. The rat was removed once anesthesia was achieved (1-2 minutes). Anaesthesia was maintained during surgery by means of a mask consisting of a 150 mL beaker containing ether soaked gauze. The mask was removed or replaced as required with care being taken to monitor the rat for signs of respiratory distress.

2.2.2. Surgery

The rat was placed in a dorsal recumbent position with each limb secured. The abdomen was shaved with an electric shaver and a left sub-costal incision was made in the abdomen. Bleeding from the incision line was usually minimal but if necessary, hemostasis was achieved by applying gentle pressure with gauze. The incision was held open by a Weitlaner retractor.

2.2.3. Duodenum cannulation

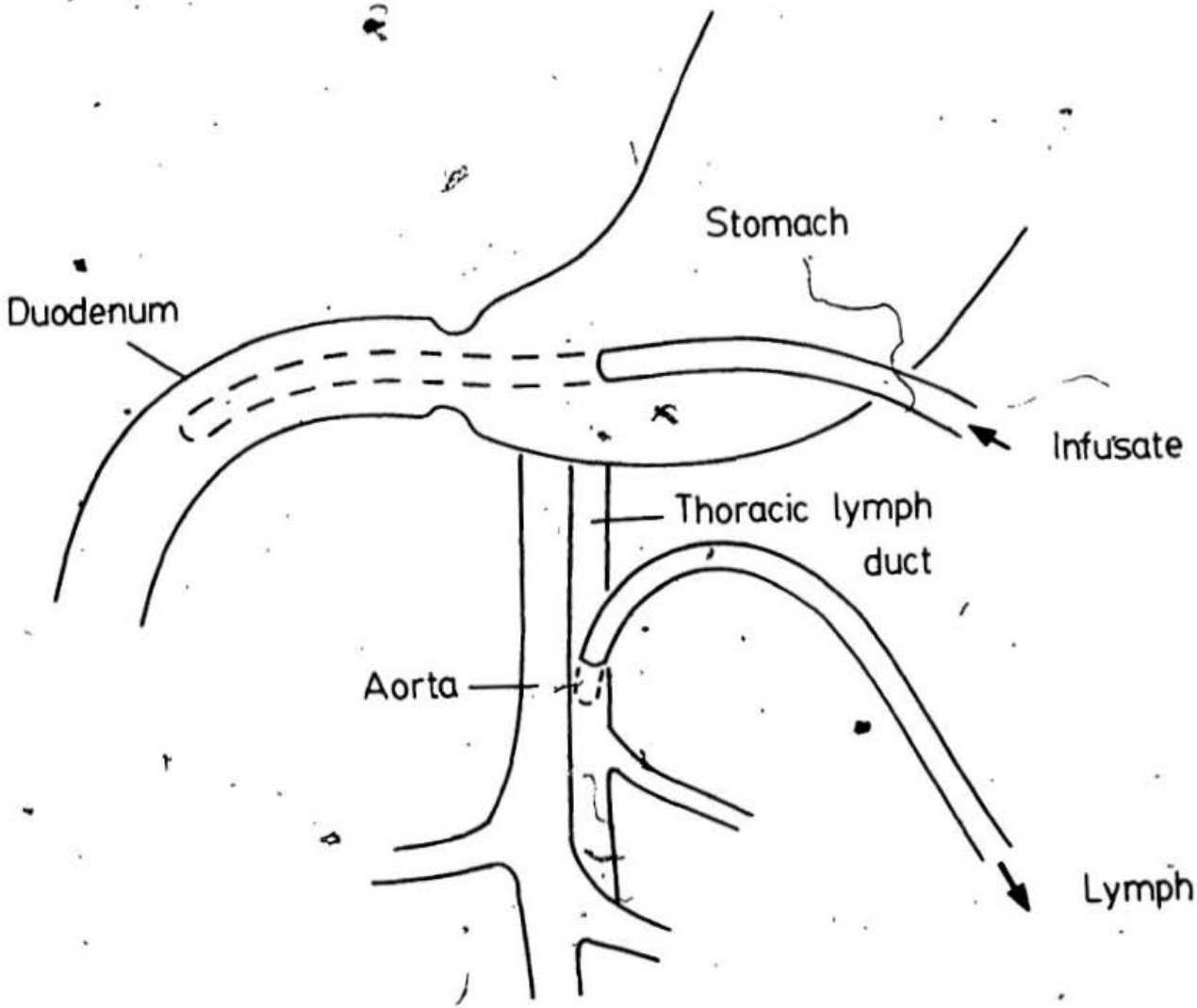
Polyethylene tubing (PE90, Clay Adams, OD=1.27 mm; ID=0.86 mm) measuring 45 cm in length was introduced into the duodenum through a small puncture on the ventral surface of the stomach approximately 1 cm from the pylorus (Figure 2.1).

The tube was passed into the duodenum for approximately 2 cm. and was secured by a purse-string suture of 4-0 silk. The tubing was exteriorized through a stab wound in the right flank of the abdomen. Approximately one mL of 0.9% NaCl saline was infused through the cannula into the duodenum to ensure free passage of material into the duodenum and to check for leakage around the purse-string suture.

2.2.4. Thoracic lymph duct cannulation

Thoracic lymph duct cannulation was performed based on the method of Bollman, Cain and Grindley (1948). The intestinal organs were reflected to the right and covered with a warm saline-soaked gauze. Approximately 2 cm of the thoracic lymph duct was dissected from the aorta and the surrounding tissue by blunt dissection. A strand of 4-0 silk was passed around the vessel as far cranial

Figure 2-1: Placement of the duodenum and thoracic lymph duct cannulae.


Stomach
Duodenum
Infusate
Thoracic lymph duct
Aorta
Lymph

as possible and tied to occlude the vessel. A nick was made in the lymph duct caudal to the ligature and heparinized polyethylene tubing measuring approximately 45 cm in length (PE 90, Clay Adams, OD=1.27 mm; ID=0.86 mm) was inserted and held in place by a drop of cyanoacrylate glue (Krazy Glue) (Figure 2.1). The tubing was exteriorized through a stab wound in the abdominal wall. Gentle suction on the cannula often helped to establish a constant lymph flow.

2.2.5. Closure

The abdominal organs were returned to their proper positions. The abdominal muscle layers were closed using 4-0 silk in a simple interrupted suture pattern and the skin was closed with 7.5 mm Michael surgical clips.

2.2.6. Post-operative housing

The rat was placed in a Bollman-type restraint cage immediately after surgery, prior to recovery from anaesthesia. This provided sufficient and humane immobilization to prevent access to the cannulae. A physiological saline solution (0.9% NaCl) containing 5% glucose was infused into the duodenum cannula at a rate of 2.4 mL/hr for the 24 hr recovery period. Food and water were withheld. The restrained rats were kept at a constant temperature of 74 ° F with a 12 hour photoperiod.

2.2.7. Sacrifice

Rats that did not exhibit a constant lymph flow on the first post-operative day were removed from the study. All rats were sacrificed by an overdose of sodium pentobarbital (Euthanyl).

2.3. Test meals and lymph collections

2.3.1. Test Meal Compositions (Table 2.1)

2.3.1.1. Control test meal

A lipid emulsion test meal was introduced into the duodenum via the duodenal cannula. The test meal consisted of 40 mM oleic acid (Sigma) labelled with 15 mCi/mol[1-^{14}C] oleic acid (Amersham), 20 mM 1-monoolein and 28.5 mM sodium taurocholate in 0.5 mL phosphate-buffered solution. Solvents were evaporated off to dryness under a light stream of nitrogen gas. The phosphate-buffered solution was composed of 6.75 mM Na_2HPO_4; 16.5 mM NaH_2PO_4; 115 mM NaCl and 5 mM KCl (pH 6.4). Emulsification of the test meal was achieved by vortexing and by 10 minute sonication in a Cole Parmer sonicating bath. The temperature of the test meal remained below 34 ° C after sonication. An aliquot of test meal was removed for scintillation counting.

2.3.1.2. Polycyclic aromatic hydrocarbon test meal

All polycyclic aromatic hydrocarbons were of the highest purity available and were used without further purification. The test meal described in section 2.3.1.1. was supplemented with 10mM of either benzo(a)pyrene, 1,2-benzanthracene, or anthracene (Sigma Chemicals Company). The PAHs were dissolved in an organic solvent prior to being added to the test meal. As above,

TABLE 2-1 : COMPOSITION OF TEST MEALS CONTAINING POLYCYCLIC AROMATIC HYDROCARBONS.*

Components	Control	PAHs	DMBA	DMBA (Low Dose)
Oleic Acid	40mM	40mM	40mM	40mM
1-Monoolein	20mM	20mM	20mM	20mM
Sodium Taurocholate	28.5mM	28.5mM	28.5mM	28.5mM
Lipid Inclusion	—	10mM B(a)P or 10mM A or 10mM 1,2-BA	10mM DMBA	1mM DMBA
^{14}C	15mCi/mol oleic acid	15mCi/mol oleic acid	150mCi/mol oleic acid	15mCi/mol oleic acid
^{3}H	—	—	6Ci/mol DMBA	—

* All test meals were administered in 0.5 mL phosphate buffered saline, pH 6.4

the solvents were evaporated to dryness under a gentle stream of nitrogen. The structures of the compounds used in this study are shown in Figure 2.2.

2.3.1.3. ^{3}H-DMBA

To determine the appearance of the first DMBA molecules in the lymph, 10 mM DMBA (Sigma) labelled with 6 Ci/mol ^{3}H-DMBA (Amersham) was combined with the test meal described in section 2.3.1.1. The oleic acid label was increased to 150 mCi/mol for this experiment. The test meal was made into an emulsion and administered via the duodenal cannula. An aliquot was removed for scintillation counting.

2.3.1.4. Cholesterol

10 mM cholesterol (99+ % pure, Sigma Chemical Company) was added to the test meal described in section 2.3.1.1. The test meal was made into an emulsion and administered as a bolus dose via the duodenum cannula.

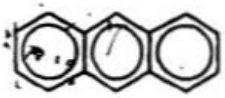
2.3.1.5. Pluronic L-81

The hydrophobic surfactant, Pluronic L-81 (BASF Wyandotte, Wyandotte, MI.), was added to the test meal described in section 2.3.1.1. at a concentration of 0.25 mg/mL. The test meal was then emulsified and an aliquot was taken for scintillation counting.

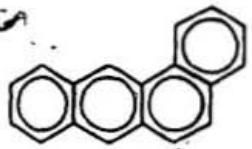
2.3.1.6. α-Glycerophosphate pathway efficiency

Two different test meals were used to test the efficiency of the α-glycerophosphate pathway. A test meal was prepared as outlined in section 2.3.1.1. except that 2-monoolein was used in place of 1-monoolein. Another test meal was prepared as described in section 2.3.1.1. except that the 1-monoolein was omitted from the preparation leaving the test meal with no monoglyceride.

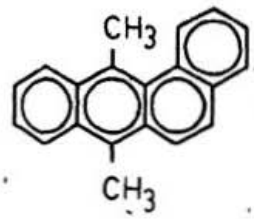
Figure 2-2: Polycyclic aromatic hydrocarbons used in this study



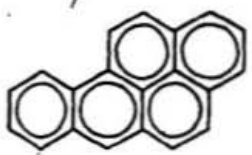
ANTHRACENE



1,2-BENZANTHRACENE



7,12-DIMETHYLBENZANTHRACENE



BENZO(a)PYRENE

TABLE 2-2 : COMPOSITION OF TEST MEALS CONTAINING VARIOUS LIPIDS AND PLURONIC L-81*

Components	Control	Cholesterol	Pluronic L-81	2-Monoolein	Monoglyceride Absent
Oleic Acid	40mM	40mM	40mM	40mM	40mM
1-Monoolein	20mM	20mM	20mM	—	—
Sodium Taurocholate	28.5mM	28.5mM	28.5mM	28.5mM	28.5mM
Lipid Inclusion	—	10mM Cholesterol	0.25 mg/mL Pluronic L-81	20mM 2-Monoolein	—
^{14}C	15mCi/mol oleic acid	15mCi/mol oleic acid	15mCi/mol oleic acid	15mCi/mol oleic acid	150mCi/mol oleic acid
^{3}H	—	—	—	—	—

* All meals were administered in 0.5 mL phosphate buffered saline, pH 6.4

The oleic acid label was increased to 150 mCi/mol for this experiment. An aliquot was removed for scintillation counting.

2.3.2. Test meal administration

Experiments were performed on the first post-operative day. At time zero, the glucose infusion was briefly interrupted and the test meal was administered as a pulse through the duodenum cannula. Test meals were administered directly into the duodenum to avoid variations in gastric emptying. 0.5 mL physiological saline was used to clear the cannula thereby ensuring the test meal had been delivered into the duodenum. The rat was immediately returned to the 5% glucose-saline infusion at a rate of 2.4 mL/hr.

2.3.2.1. Pluronic L-81

Test meal administration was carried out as above with one exception. Two hours prior to the test meal administration the 5% glucose-saline solution infusion was replaced with a 5% glucose-saline solution containing either 28.5 mM sodium taurocholate in the control animals or 28.5 mM sodium taurocholate and 0.25 mg/mL Pluronic L-81 in the experimental animals. The rats remained on this modified infusion over the entire experimental period.

For this set of experiments, the rate of infusion of the 5% glucose-saline solutions (including the modified solutions) was 3.6 mL/hr.

2.3.3. Lymph sample collection

Lymph was collected in preweighed, test tubes. To avoid clotting, 0.068 mg heparin was added to each tube prior to weighing. Lymph was collected continuously over the experimentation time at designated intervals. Lymph samples were collected in an automatic fraction collector (LKB 7000 Ultrarac) thus resulting in fewer disturbances for the rats.

Test tubes containing lymph were reweighed to determine lymph weight. Using a specific density of 1.00, the lymph volume was calculated to be equivalent to the lymph weight per unit time (Barrowman, 1966).

2.3.4. Liquid scintillation counting

100 μL of lymph was combined with 10 mL of liquid scintillation cocktail for direct scintillation counting by a Beckman LS 8100 scintillation counter. Each sample was counted for 10 minutes or to the 2% two sigma error.

The lymph samples were analysed to determine the proportion of oleic acid and DMBA in the chylomicron fraction of the lymph. To isolate the chylomicron fraction, pooled lymph was diluted 1:1 with 0.9% NaCl solution. Ultracentrifugation at 30,000 rpm for 30 minutes separated the mixture into an opaque supernatant containing the chylomicra and a clear infranatant phase. The opaque phase was separated and 100μL of the clear infranatant was taken for scintillation counting. By comparing the cpm of the whole lymph and that of the infranatant, the percentage of label in the chylomicron fraction can be determined.

2.4. Experimental procedures

2.4.1. Chylomicron appearance time determination

To determine the chylomicron appearance time the control test meal was administered via the duodenum cannula. Prior to experimentation, lymph samples were collected at 2 minutes intervals for a period of 8 min. After test meal administration, lymph samples were collected for 30 minutes at 2 minute intervals, and at 30 minute intervals for the next 150 minutes.

2.4.2. Effect of PAHs on chylomicron appearance time

In animals given a PAH test meal, lymph samples were collected at 2 minutes intervals for 60 minutes followed by 30 minutes interval collections for the next 120 min.

2.4.3. Dose effect of DMBA

The test meal described in section 2.4.2. above containing DMBA was modified such that the concentration of DMBA given was one tenth of the original meal. Samples were collected as above and the chylomicron appearance time was determined.

2.4.4. Effect of other hydrophobic xenobiotics on chylomicron appearance time

In rats given a cholesterol-supplemented test meal, lymph samples were taken at 2 minute intervals for 60 minutes and at 30 minute intervals for the next 120 minutes.

In rats given the Pluronic L-81 test meal, thoracic lymph samples were taken at 2 minute intervals for 60 minutes.

2.4.5. Enzyme induction experiment

Two randomly chosen groups of rats were used in this experiment. Group A was given 20mM DMBA dissolved in 0.5 mL ethanol as a bolus dose approximately 5 hours prior to test meal administration. Group B was given 0.5 mL ethanol as a bolus dose at the same time as the previous group. After the DMBA test meal was administered to both groups, lymph samples were collected at 2 minute intervals for one hour and at 30 minute intervals for the next 2 hours. Aliquots were taken for scintillation counting. Animals were returned to the 5% glucose saline infusion immediately after administration of both the induction dose and test meal.

2.4.6. Triglyceride Flux after administration of a test meal containing DMBA

In this experiment the total triglyceride flux over a 3 hour period was determined in rats given either a control test meal or one containing DMBA; 0.5 mL of the control or DMBA test meal was administered as a bolus dose. Lymph samples were collected at 15 minute intervals over the 3 hour period. Two 15 minute collections were taken prior to administration of the dose.

The triglyceride in μmol/L was quantified by a UV-test using an enzymatic process on a Hitachi 705 Automatic Analyzer (c.v.; 3%) as described below.

$$\text{triglycerides} + 3H_2O \xrightarrow{lipase} \text{glycerol} + 3\,RCOOH$$

glycerol + ATP $\xrightarrow{glycerol\ kinase}$ glycerol-3-phosphate + ADP

ADP + phosphoenolpyruvate $\xrightarrow{pyruvate\ kinase}$ pyruvate + ATP

pyruvate + NADH + H^+ $\xrightarrow{LDH}$ lactate + NAD^+

Triglyceride concentrations were converted to total triglyceride per unit time and the flux over the 3 hour period was calculated.

2.4.7. Chylomicron appearance time in rats given a test meal without monoglyceride or with 2-monoglyceride

The chylomicron appearance time was determined for rats given the test meal without monoglyceride or with 2-monoglyceride described in section 2.3.1.5. Lymph samples were taken at 2 minute intervals for one hour and at 30 minute intervals for the next 2 hours.

2.4.8. Triglyceride flux after administration of a test meal without monoglyceride

The efficiency by which the α-glycerophosphate pathway can resynthesize triglyceride can be further determined by examining the triglyceride flux over a 3 hour period. For this experiment, three groups of rats were randomly chosen.

Group A received a control test meal as described in section 2.3.1.1. and Group B received a test meal without monoglyceride as described in section 2.3.1.5. Group C received a test meal containing 2-monoglyceride. Lymph samples were taken at 4 minute intervals for one hour and at 30 minute intervals for the next 2 hours and analysed for triglyceride on the Hitachi 705 Automatic Analyzer.

2.4.9. Statistics

All chylomicron appearance times are expressed as mean $\pm$ standard error of the mean (SE)

Chylomicron appearance times are compared using Student's T test at a significance level of $p < 0.01$ unless otherwise noted.

Chapter 3

RESULTS

3.1. Effect of polycyclic aromatic hydrocarbons on lipid absorption and handling

3.1.1. Chylomicron appearance times (Table 3-1)

3.1.1.1. Control chylomicron appearance time

In order to determine the true appearance time of the radiolabel in the lymph, it was necessary to correct for the thoracic lymph duct cannula dead space. This was calculated from the lymph flow and cannula volume as shown in the following formulae.

$$\text{Cannula Dead Space Time} = \frac{\text{cannula volume}}{\text{average lymph flow per min.}}$$

This dead space time was then subtracted from the observed appearance time to determine the true appearance time.

The chylomicron appearance time as measured by ^{14}C appearance in the thoracic lymph duct was 10.81 $\pm$ 1.03 minutes as shown in Table 3-1. This value represents the control appearance time.

TABLE 3-1 : CHYLOMICRON APPEARANCE TIMES AFTER ADMINISTRATION OF TEST MEALS CONTAINING POLYCYCLIC AROMATIC HYDROCARBONS

Compound	n	Chylomicron Appearance Time Mean ± SE	Significance*
Control	8	10.81 ± 1.03 min	
Anthracene	8	14.81 ± 1.66 min	NS
1,2-Benzanthracene	8	12.11 ± 0.70 min	NS
Benzo(a)pyrene	8	12.20 ± 1.76 min	NS
7,12-Dimethylbenzanthracene (10mM)	8	23.08 ± 2.10 min	$p < 0.001$
7,12-Dimethylbenzanthracene (1mM)	8	11.28 ± 0.89 min	NS

* NS = not significant

3.1.1.2. Polycyclic aromatic hydrocarbons (Table 3-1, Figure 3-1)

The chylomicron appearance times in those rats given a test meal containing either anthracene, 1,2-benzanthracene or benzo(a)pyrene are shown in Table 3-1. None of these values differ significantly from that of the control.

3.1.1.3. ^{3}H-DMBA

Upon addition of the DMBA to the test meal, the chylomicron appearance time was 23.08 $\pm$ 2.10 minutes (Figure 3-2).

The appearance time of DMBA in the lymph (20.4 $\pm$ 1.98 minutes) coincides with the chylomicron appearance time (Figure 3-3).

The chylomicron appearance time in this experiment was significantly different from the chylomicron appearance time of the control group.

3.1.1.4. Low dose DMBA

When the concentration of DMBA added to the test meal was reduced to one tenth the concentration in section 3.1.1.3., no change was observed in the chylomicron appearance time as compared to the control (Figure 3-4). Here, the chylomicron appearance time was 11.28 $\pm$ 0.89 minutes.

3.1.2. Triglyceride flux

The three hour triglyceride flux in animals given a control test meal or one containing DMBA is shown in Figure 3-5.

The baseline triglyceride output was calculated and subtracted from the total output to determine the triglyceride output due to the test meal. Multivariate analysis (ANOVA) was used to compare the two groups. No

Figure 3-1: Chylomicron Appearance Time in animals given a control or PAH test meal

Each bar represents the mean [± SE].

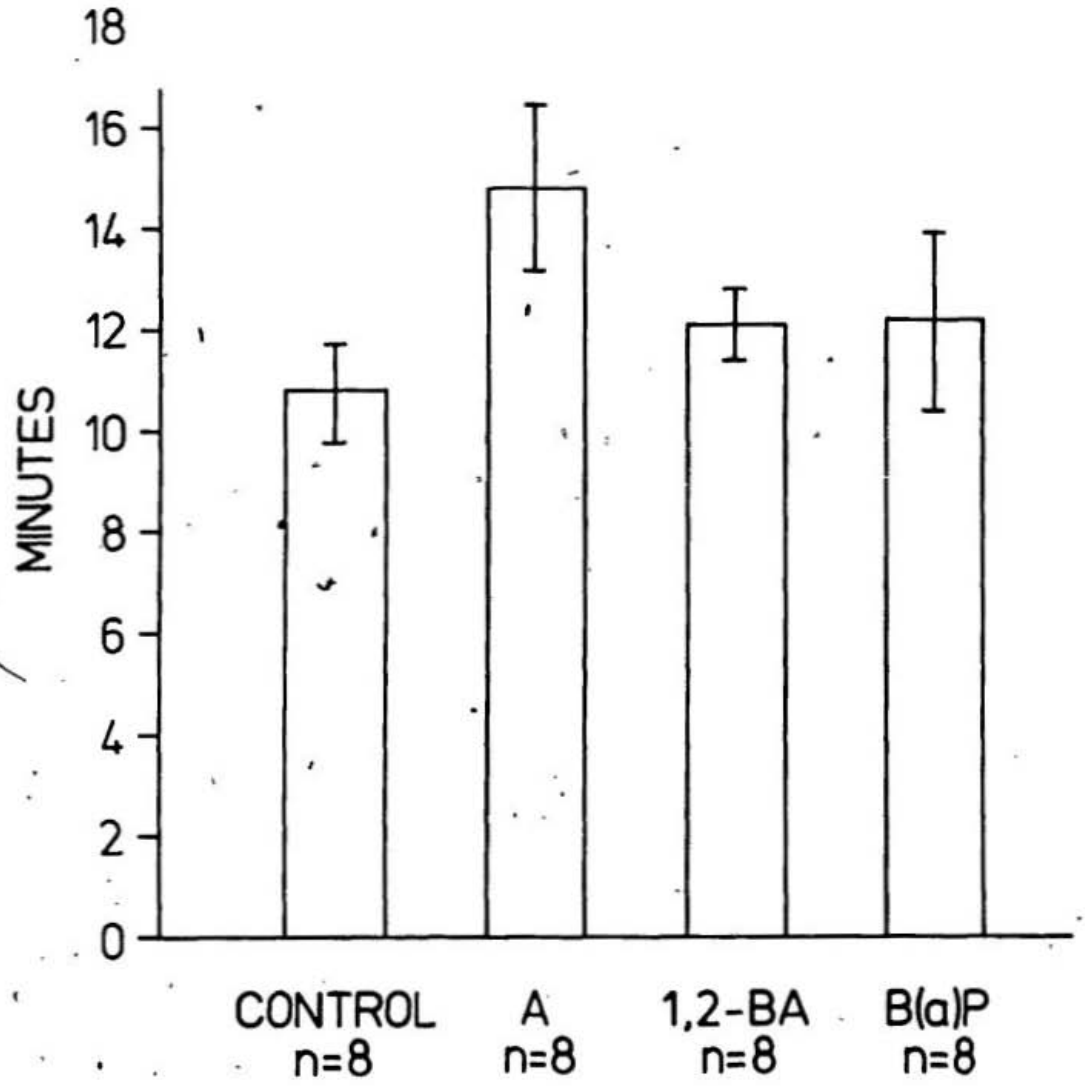
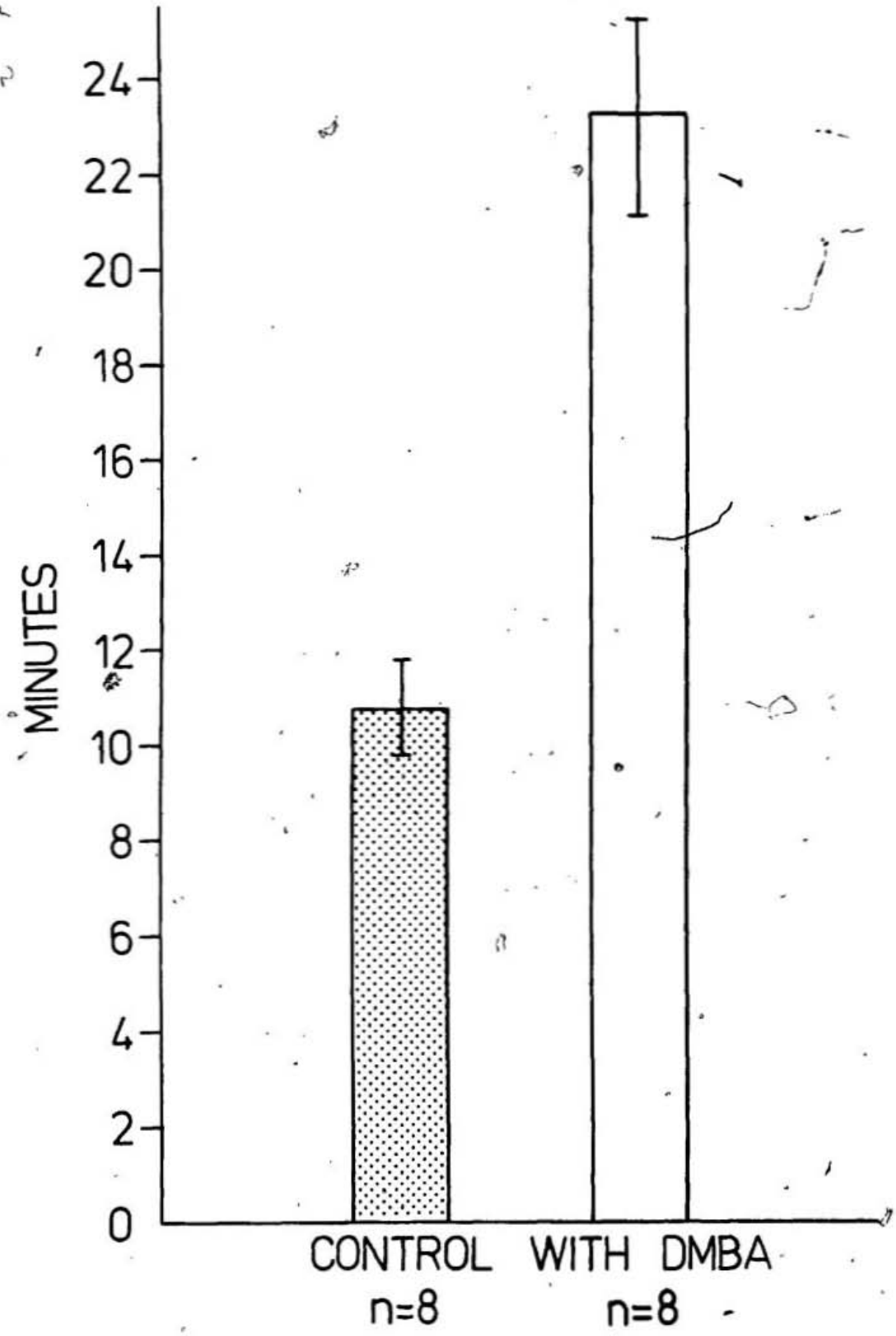

18
16
14
12
10
8
6
4
2
0
MINUTES
CONTROL
n=8
A
n=8
1,2-BA
n=8
B(a)P
n=8

Figure 3-2: Chylomicron Appearance Time in animals given a control or DMBA test meal

Each bar represents the mean [$\pm$ SE].


MINUTES
24
22
20
18
16
14
12
10
8
6
4
2
0
CONTROL
n=8
WITH DMBA
n=8

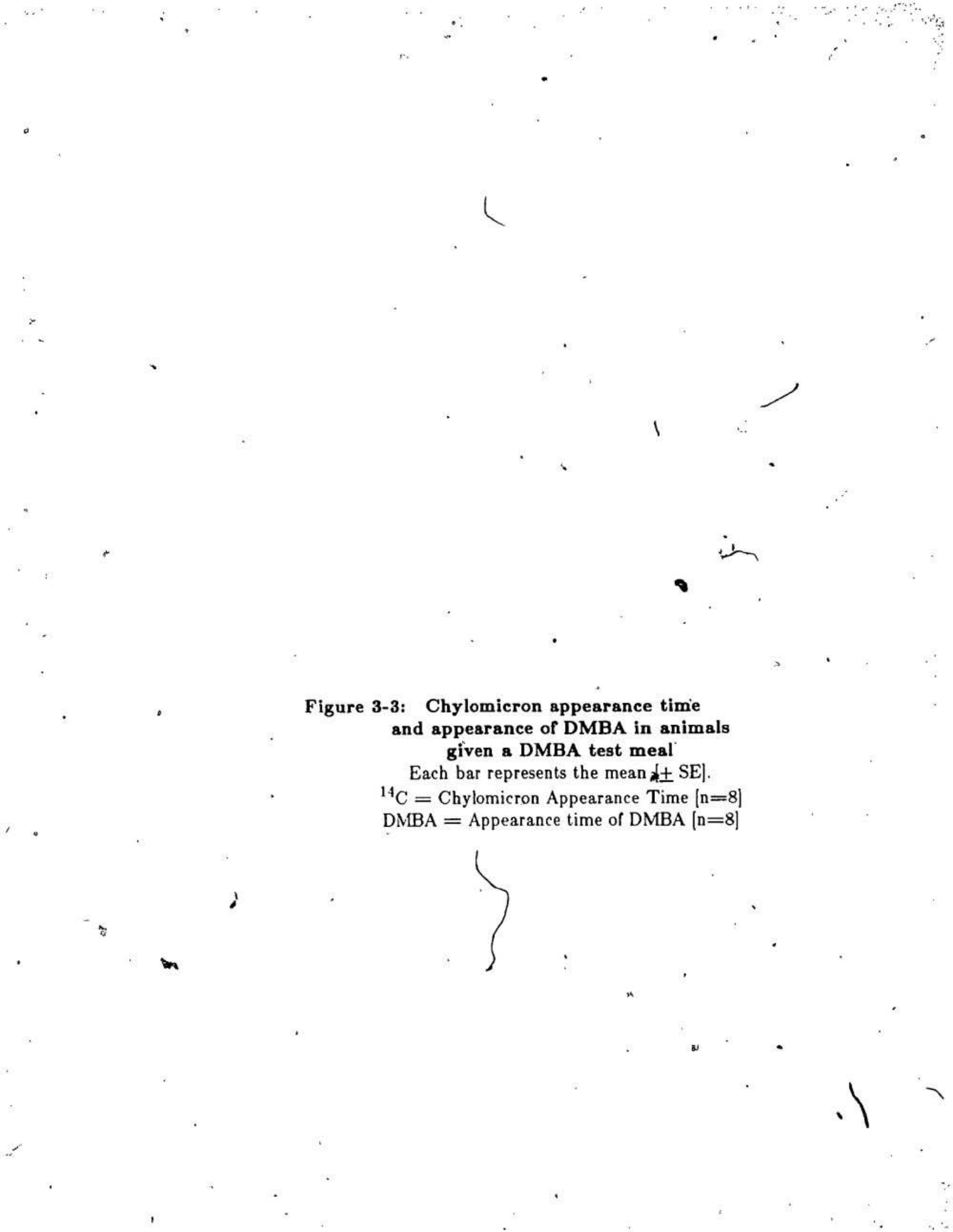


Figure 3-3: Chylomicron appearance time and appearance of DMBA in animals given a DMBA test meal

Each bar represents the mean [± SE].

^{14}C = Chylomicron Appearance Time [n=8]

DMBA = Appearance time of DMBA [n=8]

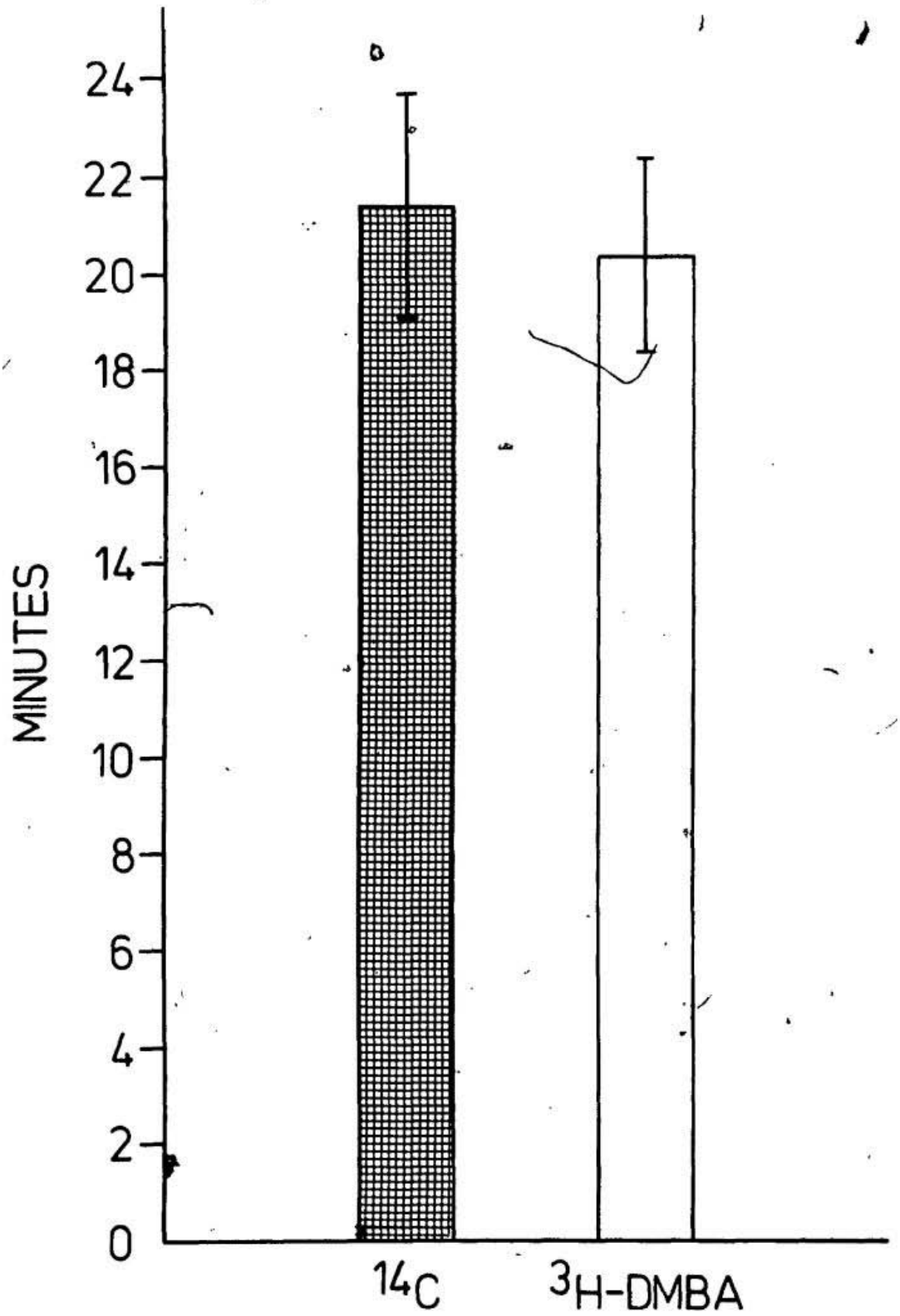

MINUTES
0
2
4
6
8
10
12
14
16
18
20
22
24
14C
3H-DMBA

Figure 3-4: Chylomicron appearance time in animals given 1 mM DMBA
Each bar represents the mean [± SE].

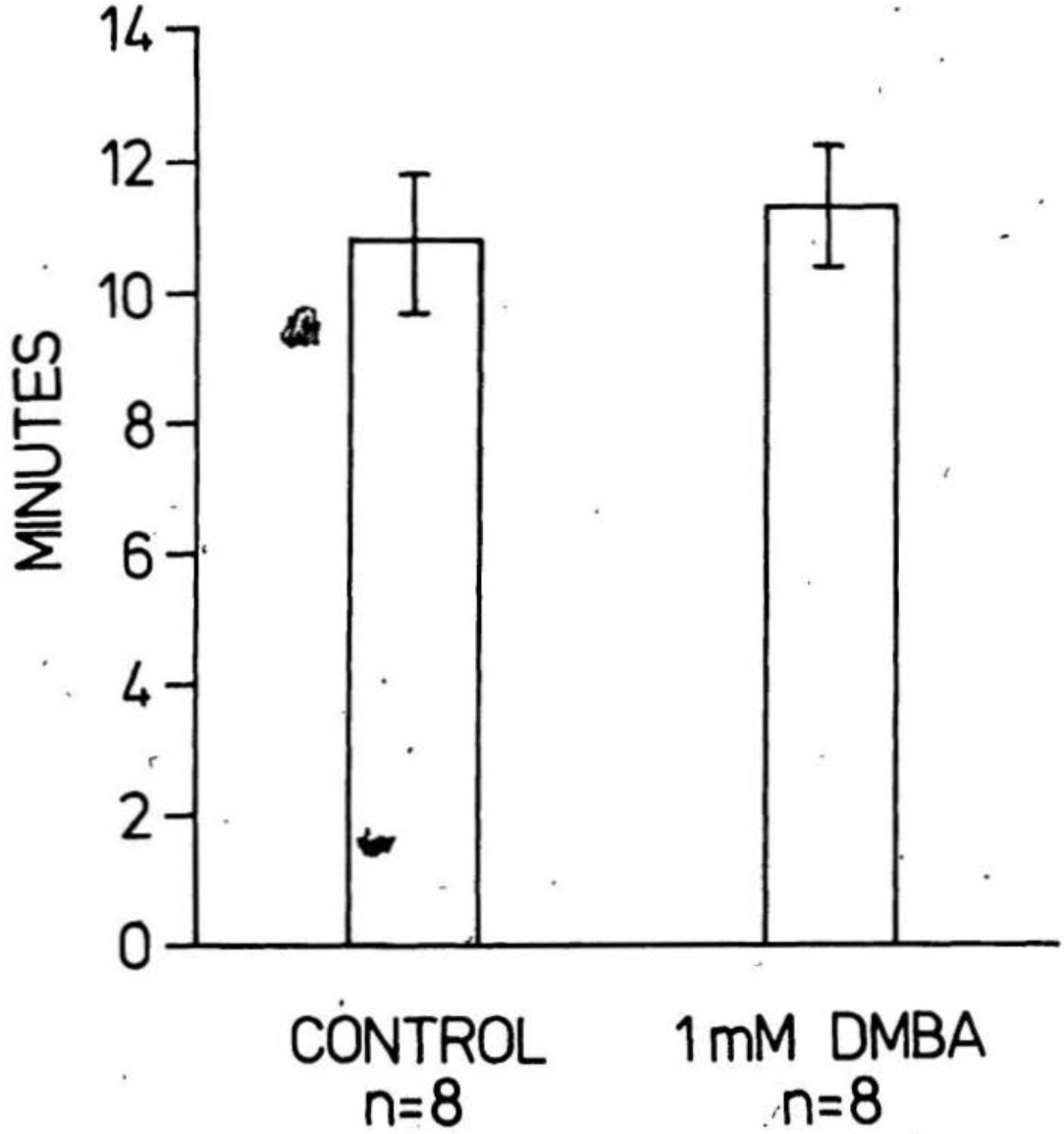
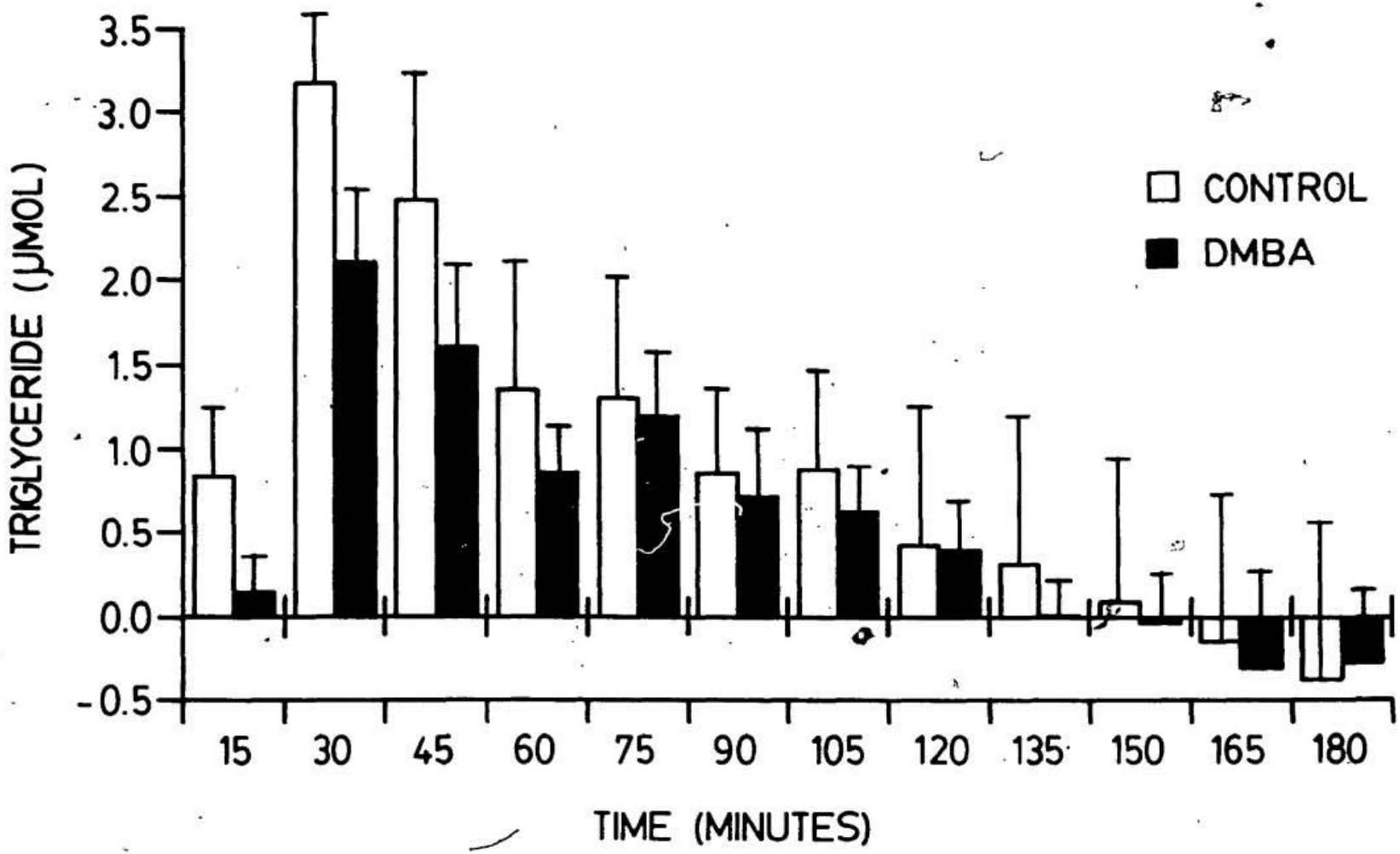

14
12
10
8
6
4
2
0
MINUTES
CONTROL
n=8
1mM DMBA
n=8

Figure 3-5: Triglyceride flux over a three hour period after administration of a control or DMBA test meal

Each bar represents the mean [± SE] for 8 animals.


3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
TRIGLYCERIDE (μMOL)
CONTROL
DMBA
15
30
45
60
75
90
105
120
135
150
165
180
TIME (MINUTES)

significant difference was found between the two groups with respect to triglyceride output over the time period. The total triglyceride output over this time period for the control animals is 11.15 $\pm$ 7.25 μmol. This does not differ significantly from 7.01 $\pm$ 2.99 μmol; the total triglyceride output in the DMBA animals.

It appears that the triglyceride output for both the control and experimental groups peak at approximately 30 minutes. It must be noted that the times are not corrected for cannula dead space. Because of the large time intervals, though, alteration of the time scale by approximately 6 minutes would not modify the results since the cannula dead space does not differ significantly between the control and experimental groups.

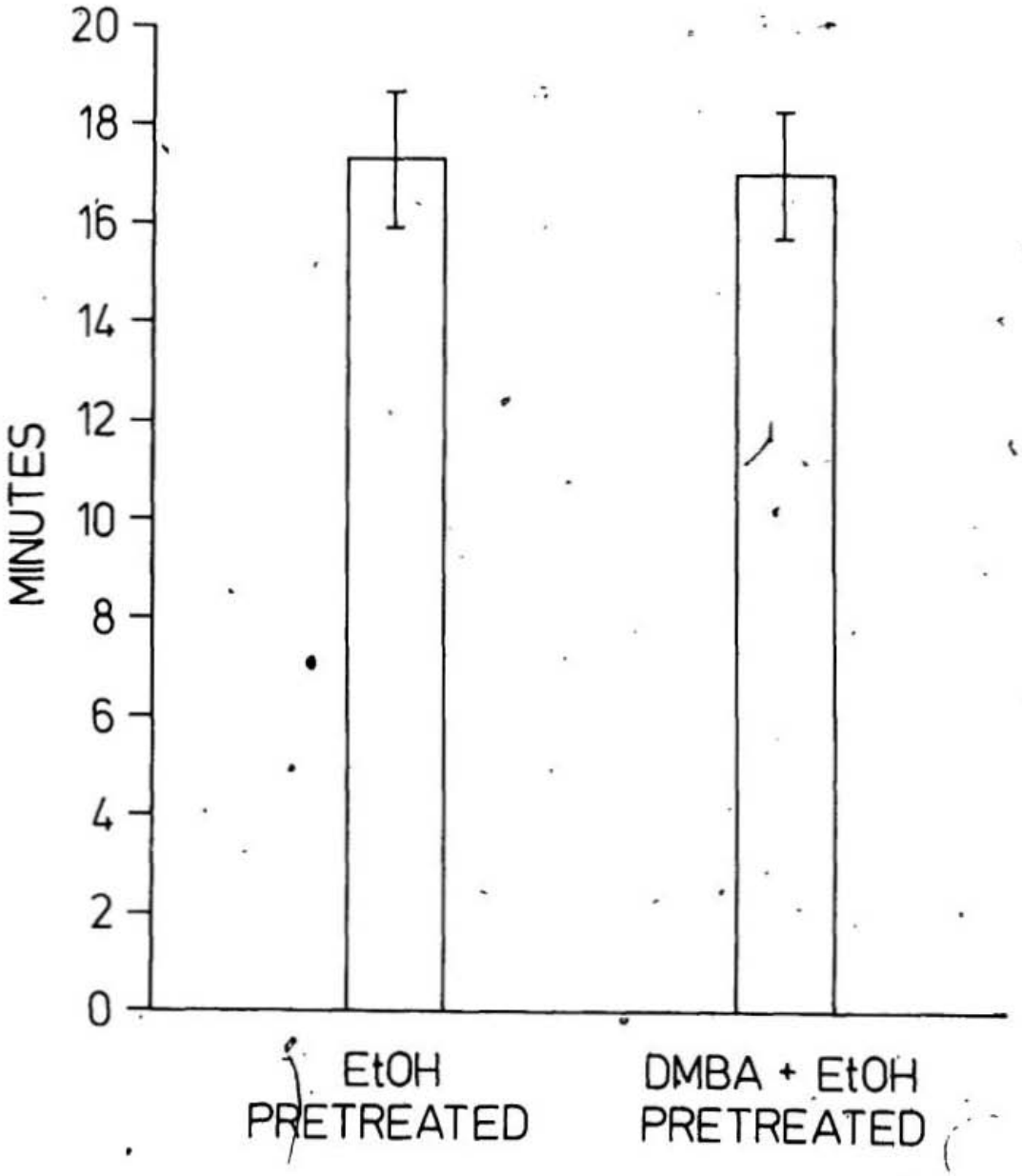
3.1.3. Enzyme induction experiment

The chylomicron appearance time was compared in rats given a pretreatment of 0.5 mL ethanol 4 hours prior to test meal administration to those given a pretreatment that included DMBA. No difference was found between the two groups (Figure 3-6).

3.2. Effect of other hydrophobic xenobiotics on lipid absorption and handling

Figure 3-6: Chylomicron appearance time in polycyclic aromatic hydrocarbon hydroxylase induced animals

Each bar represents the mean [± SE]
EtOH PRETREATED rats [n=6] are given 0.5mL EtOH
4 hr prior to test meal administration
DMBA + EtOH PRETREATED rats [n=8] are given
40 mM DMBA in 0.5 mL EtOH
4 hr prior to test meal administration


20
18
16
14
12
10
8
6
4
2
0
MINUTES
EtOH
PRETREATED
DMBA + EtOH
PRETREATED

3.2.1. Chylomicron appearance times (Table 3-2)

3.2.1.1. Cholesterol

The addition of the cholesterol to the test meal, did not significantly alter the chylomicron appearance time (Figure 3-7).

3.2.1.2. Pluronic L-81

Because the 5% glucose-saline infusion rate was increased to 3 mL/hr and sodium taurocholate was added to the glucose-saline solution for this experiment, a new set of control chylomicron appearance time values were determined and was found to be 10.82 $\pm$ 0.09 minutes. This does not differ from the previously determined controls.

Upon addition of Pluronic L-81 to the test meal, the chylomicron appearance time was 16.60 $\pm$ 0.77 minutes. This differs significantly from the control (Figure 3-8).

3.3. Alpha-glycerophosphate pathway efficiency

3.3.1. Chylomicron appearance time

3.3.1.1. Monoglyceride absent

The absence of 1-monoolein in the test meal resulted in no significant difference in the chylomicron appearance time in the experimental group as compared with the control group. The chylomicron appearance time for the experimental group in this experiment was 9.41 $\pm$ 0.61 minutes (Figure 3-9.).

TABLE 3-2 : CHYLOMICRON APPEARANCE TIMES AFTER ADMINISTRATION OF TEST MEALS CONTAINING VARIOUS XENOBIOTICS.

Compound	n	Chylomicron Appearance Time Mean ± SE	Significance*
Control	8	10.81 ± 1.03 min	
Cholesterol	8	11.85 ± 1.01 min	NS
Pluronic L-81	7	16.60 ± 0.77 min	$p < 0.01$
2-Monoolein	9	12.92 ± 0.76 min	NS
Monoglyceride absent	8	9.41 ± 0.61 min	NS

* NS = not significant

Figure 3-7: Chylomicron appearance time in animals given a cholesterol-supplemented test meal

Each bar represents the mean [± SE].

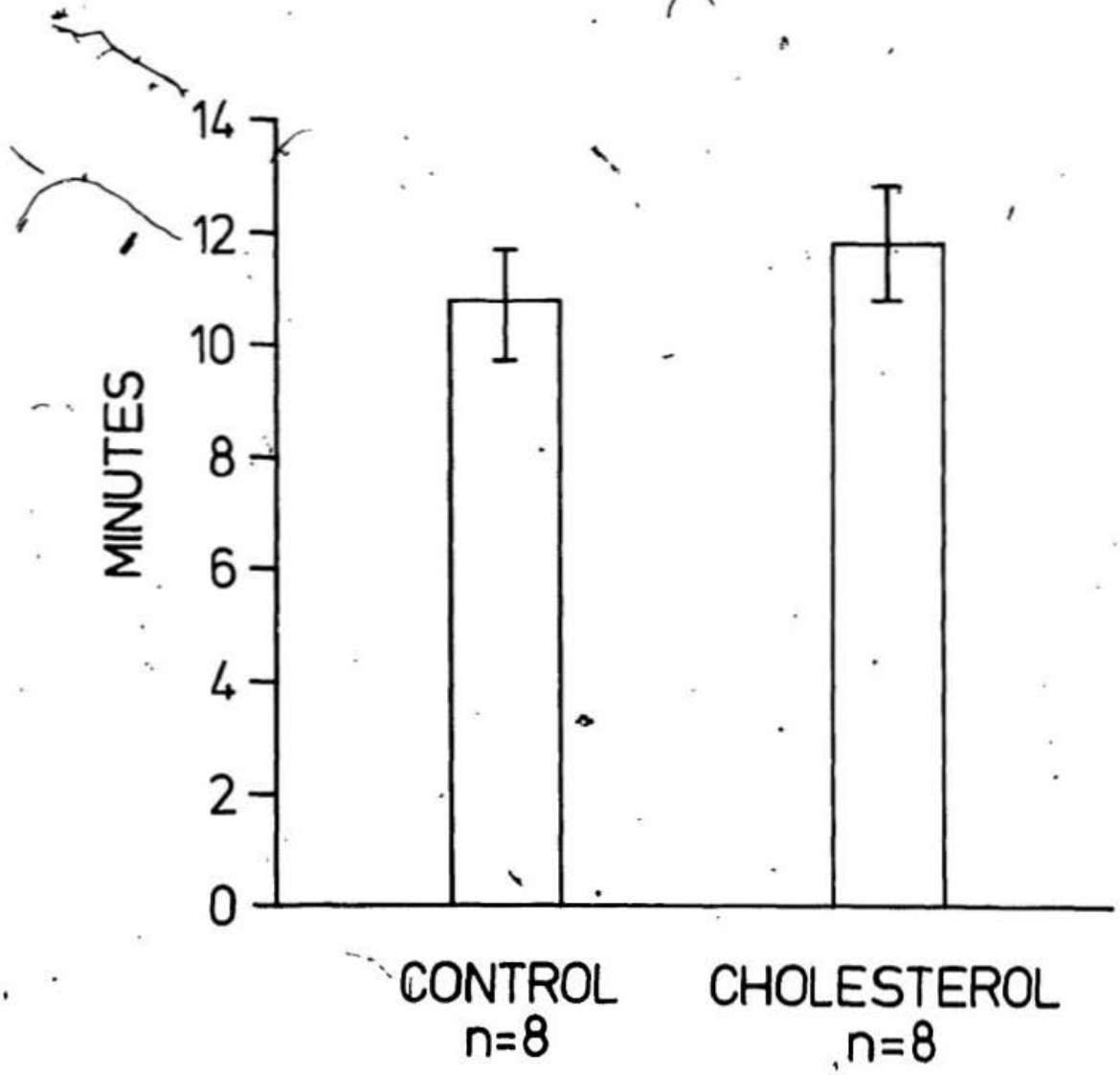

MINUTES
0
2
4
6
8
10
12
14
CONTROL
n=8
CHOLESTEROL
n=8

Figure 3-8: Chylomicron appearance time in animals given a test meal containing Pluronic L-81
Each bar represents the mean [± SE].

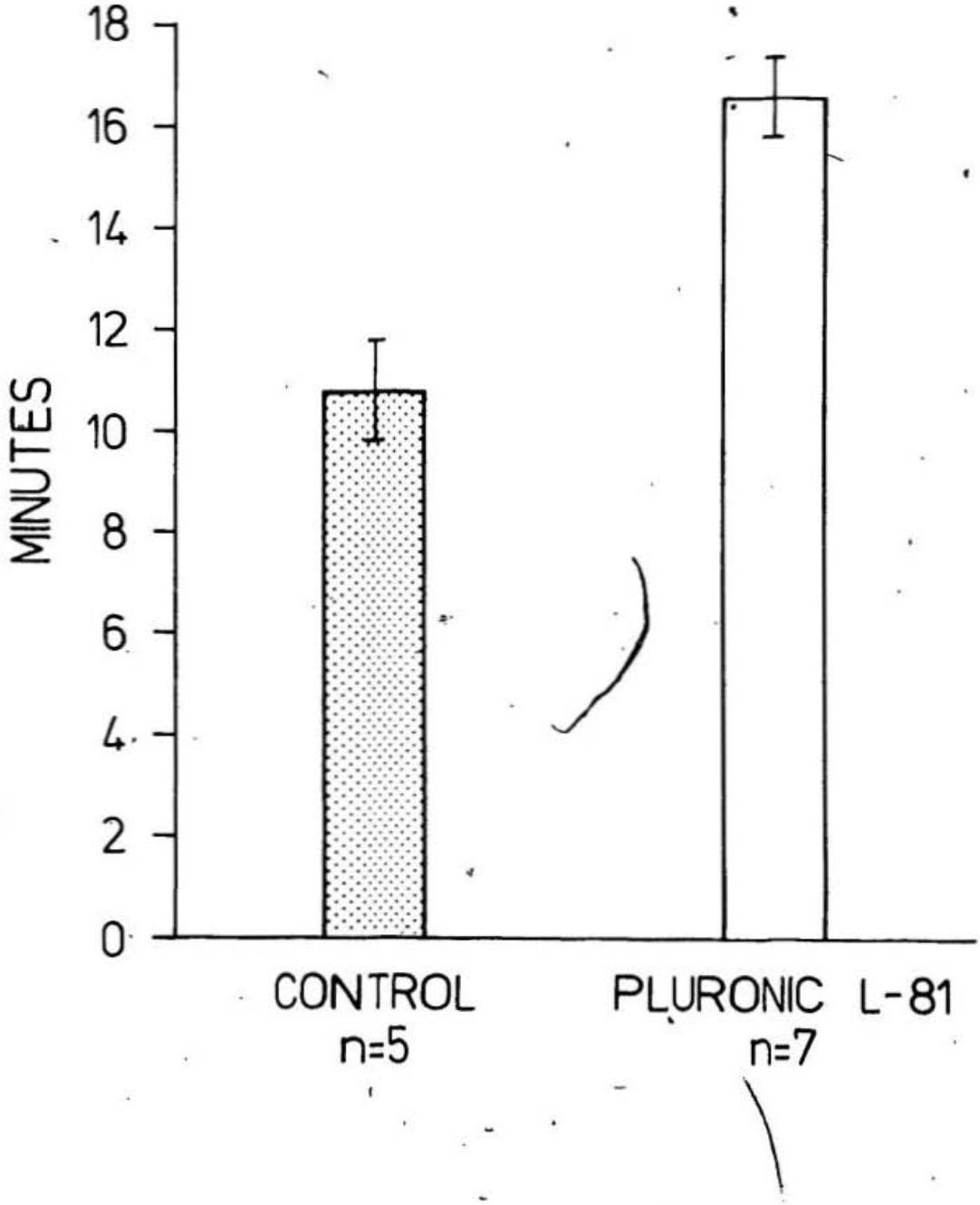
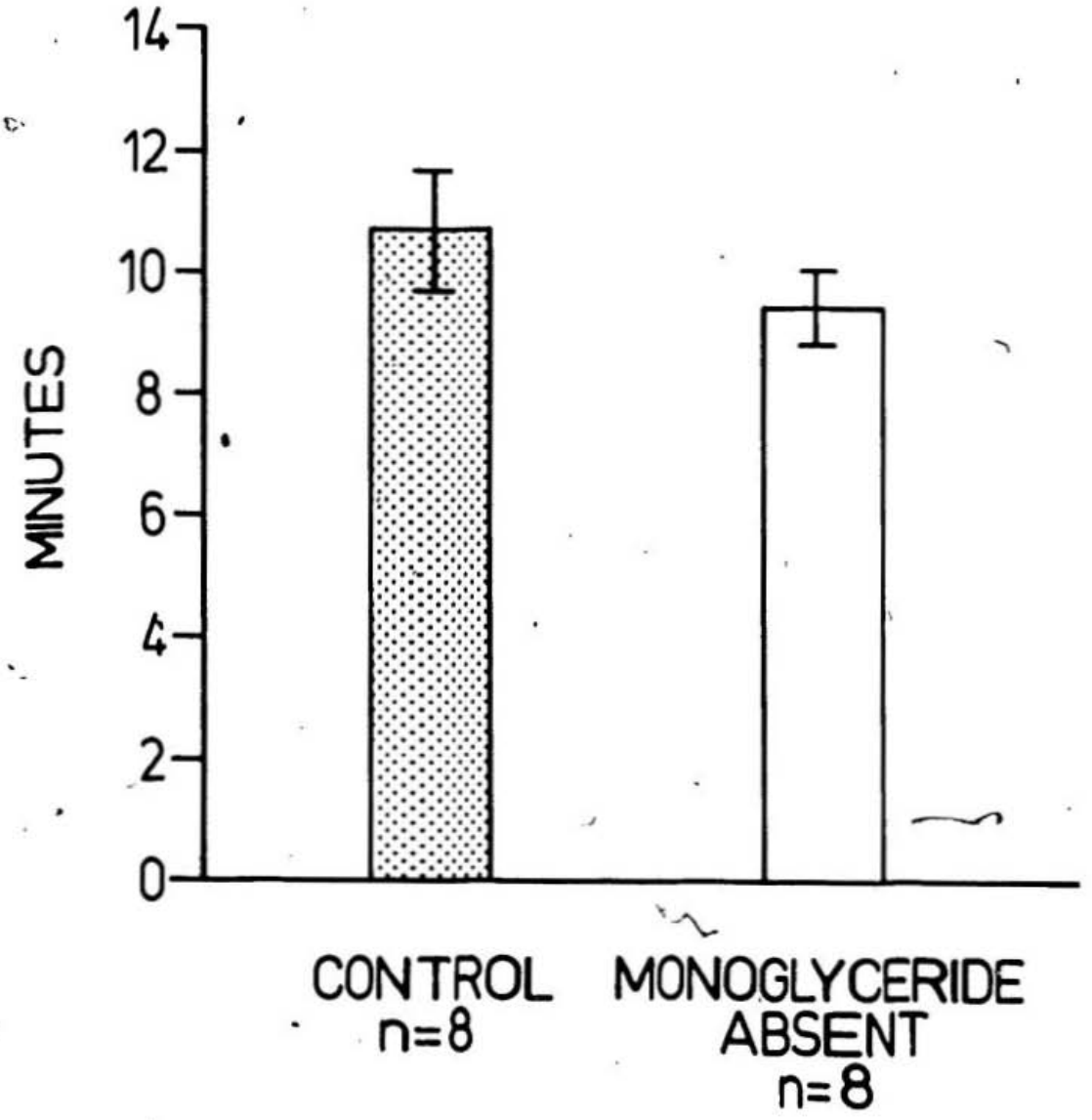

18
16
14
12
10
8
6
4
2
0
MINUTES
CONTROL
n=5
PLURONIC L-81
n=7

Figure 3-9: Chylomicron appearance time in animals given a test meal without monoglyceride.
Each bar represents the mean [± SE].


MINUTES
14
12
10
8
6
4
2
0
CONTROL
n=8
MONOGLYCERIDE
ABSENT
n=8

3.3.1.2. 2-Monoolein

When 2-monoolein was substituted for 1-monoolein in the test meal, the chylomicron appearance time was found to be 12.92 $\pm$ 0.76 minutes. This was not significantly from the chylomicron appearance time in rats given a test meal containing 1-monoolein (Figure 3-10).

3.3.2. Triglyceride flux

3.3.2.1. Monoglyceride absent

Concurrent with the similarity in chylomicron appearance time, there is no significant difference in the triglyceride flux over a one hour period in those animals given a control test meal or one without monoglyceride as determined using multivariate statistics (ANOVA).

The total triglyceride output (over baseline) in the control group is 6.636 $\pm$ 0.986 μmol. The one hour total triglyceride (over baseline) in the experimental group is 5.461 $\pm$ 1.185 μmol. This triglyceride flux for the one hour period following test meal administration is shown in Figure 3-11.

It appears that in the experimental group the triglyceride output peaks at about 20 minutes and at 30 for the control group. As in the previous triglyceride study, these times are not corrected for cannula dead space. The dead space times do not differ significantly between the two groups and therefore the relative positions of the corrected curves would not change. Instead, both curves would shift to the left by approximately 6 minutes.

The triglyceride output peaks for both groups were compared. There is a

Figure 3-10: Chylomicron appearance time in animals given a test meal containing 1- or 2-monoolein
Each bar represents the mean [$\pm$ SE].

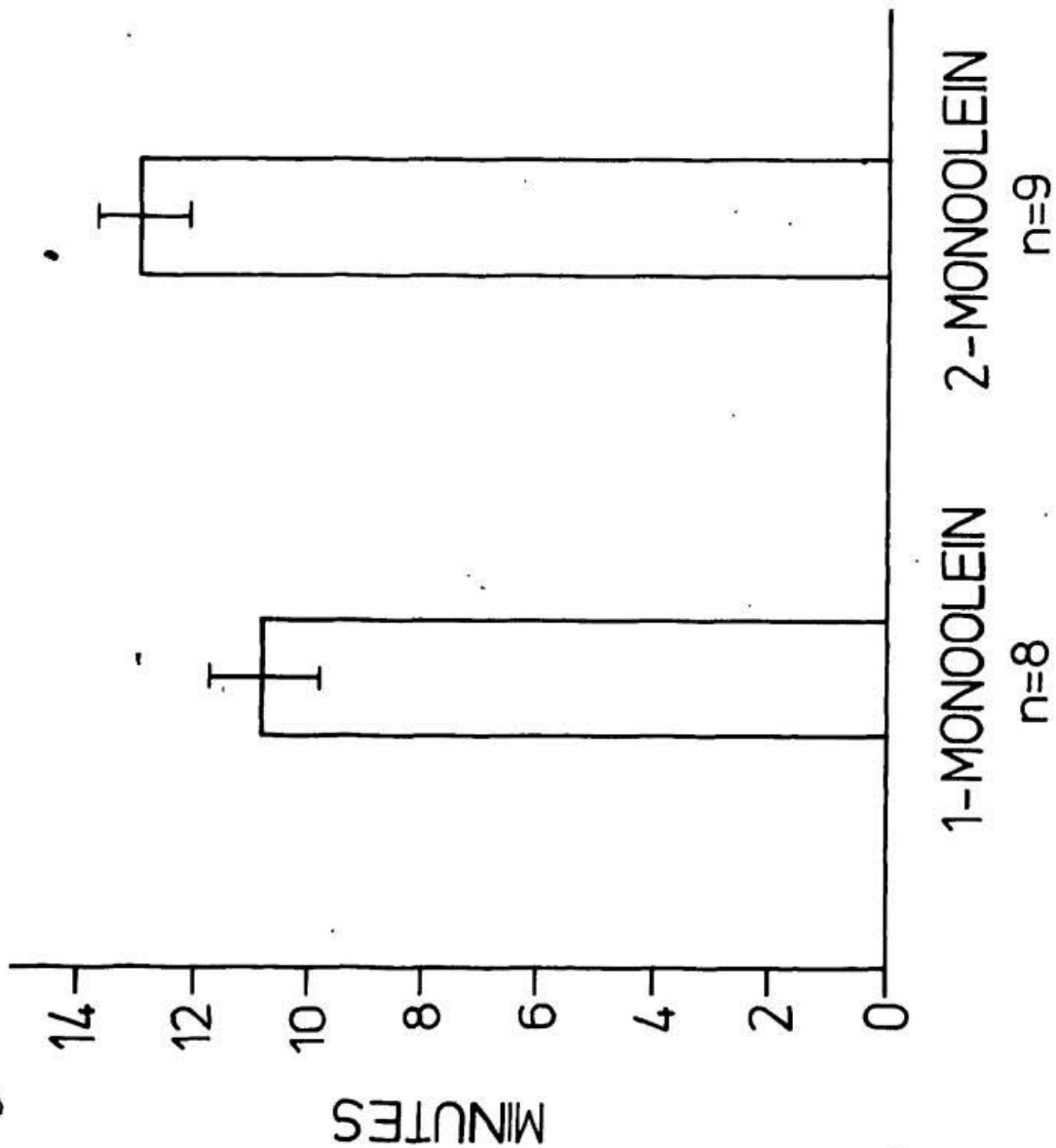
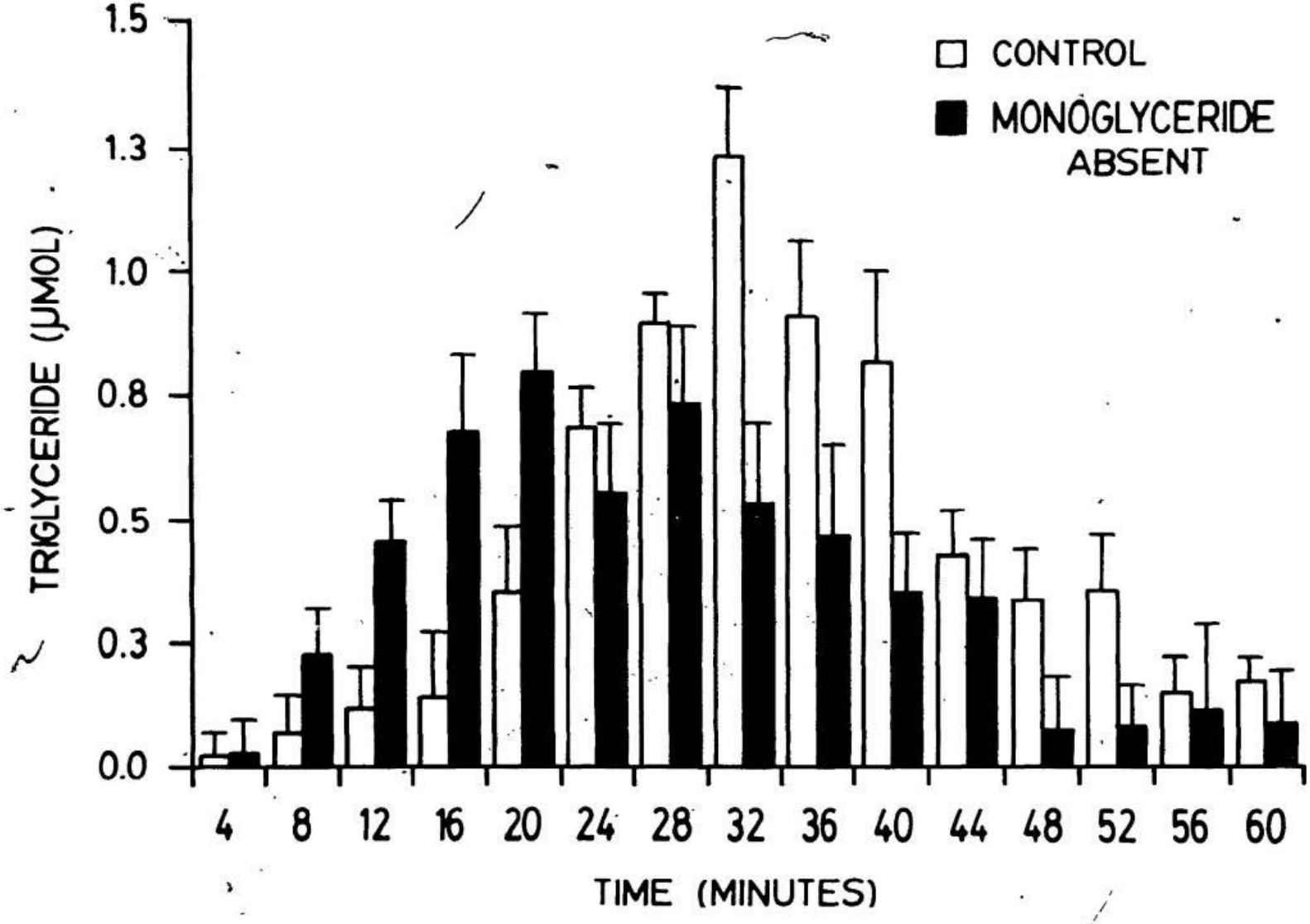

MINUTES
0
2
4
6
8
10
12
14
1-MONOOLEIN
n=8
2-MONOOLEIN
n=9

Figure 3-11: Triglyceride flux over a one hour period after administration of a test meal without monoglyceride

Each bar represents the mean [$\pm$ SE].

Control n=6

Monoglyceride Absent n=7


CONTROL
MONOGLYCERIDE ABSENT
TRIGLYCERIDE (μMOL)
1.5
1.3
1.0
0.8
0.5
0.3
0.0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
TIME (MINUTES)

significant difference ($p < 0.05$) between the control peak of 1.235 ± 0.139 at 32 minutes and the monoglyceride absent peak of 0.769 ± 0.128 at 20 minutes.

3.3.2.2. 2-Monoolein

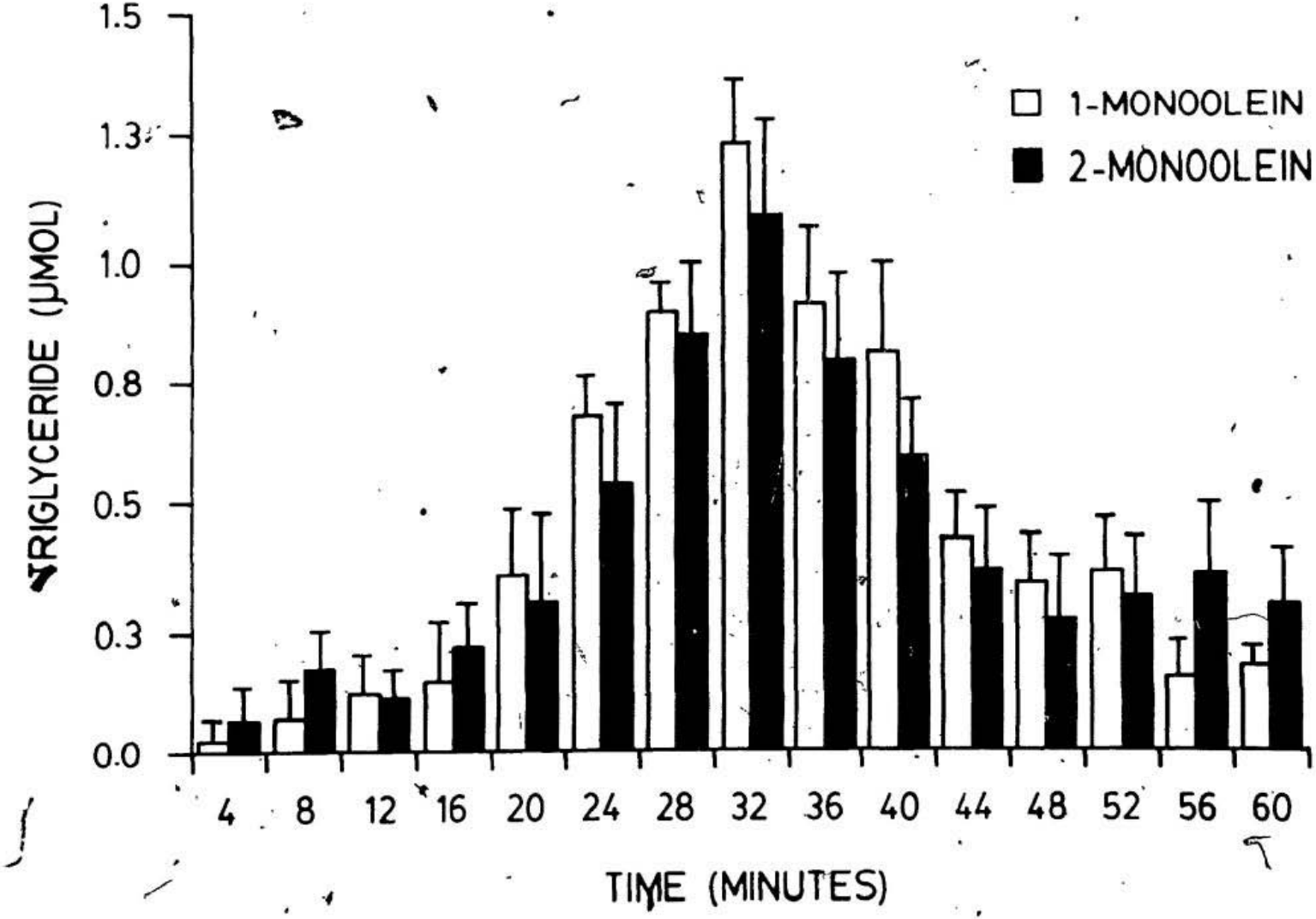
The triglyceride flux over a one hour period in rats given a test meal containing either 1- or 2-monoolein is shown in Figure 3-12. These curves were compared using multivariate statistics (ANOVA). No significant difference due to monoglyceride isomer was found over the time period. The total triglyceride output in the rats given the 2-monoolein (6.344 ± 1.20 μmol) is not significantly different from those rats given 1-monoolein (6.636 ± 0.99 μmol). The peak triglyceride outputs occur in the same time periods (32 minutes) and are not significantly different between the two groups.

Figure 3-12: Triglyceride flux over a one hour period after administration of a test meal containing 2-monoolein

Each bar represents the mean [± SE].

1-Monoolein n=6

2-Monoolein n=7


1.5
1.3
1.0
0.8
0.5
0.3
0.0
TRIGLYCERIDE (µMOL)
1-MONOOLEIN
2-MONOOLEIN
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
TIME (MINUTES)

Chapter 4

DISCUSSION

4.1. Chylomicron appearance time

The chylomicron appearance time is a good indicator of initial changes in lipid absorption. An appearance time of approximately 10.8 minutes in conscious restrained rats given a lipid emulsion test meal is a very reproducible result (Figure 3-1). Our results agree with those previously published (Tso et al, 1986). A good rate of lymph flow and sufficient matrix hydration is necessary in these studies as it has been shown that a decrease in either of these parameters results in a modification of chylomicron appearance time. Above a certain level of hydration, the chylomicron appearance time becomes a constant value. Therefore care was taken to insure that this level of hydration was attained.

The cannula dead space correction became very important in determining the true CAP. In some cases, the cannula dead space was quite large (6-8 minutes) owing in part to the large cannula diameter. Although decreasing the lumen diameter of the cannula would decrease the cannula dead space time, a large cannula is preferred as it better approximates the size of the lymphatic vessel. In thoracic lymph fistula surgeries performed by Tso et al, (1986a) the OD of the cannula was 0.8 mm. Despite this difference, the chylomicron appearance time in control animals were in agreement.

The sensitivity of the CAP enables it to be used as a method of monitoring changes in lipid absorption and handling. It is relatively safe to assume that any changes seen in the chylomicron appearance time as a result of test meal manipulation is an effect exerted either on absorption by the enterocyte or during intracellular processing. This assumption is based on the fact that other than the factors mentioned above, extracellular transport time of the chylomicrons is probably constant over the 50 μm distance from exocytosis to the intestinal lymphatic.

4.2. Influence of polycyclic aromatic hydrocarbons on lipid absorption

Polycyclic aromatic hydrocarbons are generally lipophilic compounds which associate with the lipid portion of the diet during digestion and absorption (Lo and Sandi, 1978). As discussed previously, transport to the enterocyte of these compounds is believed to be dependent on the hydrocarbon continuum proposed by Patton (1981). Studies have shown that these compounds dissolve in the mixed lipid-bile salt micelles required for lipid absorption (Laher and Barrowman, 1983) and are absorbed with the lipolytic products by the enterocyte of the small intestinal mucosa.

4.2.1. Chylomicron appearance time

In each chylomicron appearance time experiment, the chylomicron appearance time was determined after administration of a lipid emulsion test meal. The test meal components were chosen specifically to support PAH solubilization. Oleic acid and monoolein were used since long chain fatty acids and

monoglycerides are better able to promote solubilization of PAHs than are the medium chain fatty acids (Laher and Barrowman, 1983). In addition, the parent triglyceride of these lipolytic components, triolein is one of the most commonly consumed triglycerides (Guthrie, 1983).

The bile salt concentration in the test meals was relatively high. This high concentration was required since the addition of the various xenobiotics to the test meal exerted an additional lipid load. Also, the absorption of larger PAHs is largely dependent on the presence of bile (Rahman, Barrowman and Rahimtula, 1986). In general, the conditions of the test meal described above allow these compounds to be readily bioavailable for absorption by the enterocyte.

Our results showed that the presence of anthracene, 1,2-benzanthracene and benzo(a)pyrene did not have an effect on the chylomicron appearance time (Table 3-1, Figure 3-1) whereas DMBA did exert an effect (Table 3-1, Figure 3-2). These particular PAHs were chosen based largely on their structures and lipophilicity. The smaller, 3 ring compounds such as anthracene are the least lipophilic, with an aqueous solubility of 0.073 ± 0.005 whereas the larger 5 ring compounds such as B(a)P with an aqueous solubility of 0.0038 ± 0.00031 are highly lipophilic (MacKay and Shiau; 1977).

The delay in chylomicron appearance time observed following the administration of the 10 mM dose of DMBA may be a result of several factors. It is evident that the effect is dose related since 1 mM DMBA under the same conditions resulted in no effect.

It is doubtful that the presence of a large xenobiotic content of the test meal impedes absorption since an equal amount of each of the PAHs were used.

The metabolism of PAHs in the liver has been well documented as it is here that much of the detoxification of these compounds occurs. In the early 1960s it was shown that the intestinal mucosa also contained these enzymes capable of PAH biotransformation (Wattenberg et al, 1962).

In the intestinal tract, the Phase I reaction is mediated by the microsomal cytochrome P-448 resulting in the production of oxides, quinones and dihydrodiols (Hietanen, 1980). In addition, the Phase II conjugation enzyme glutathione-S-transferase has been identified in the small intestinal mucosa of the rat (Clifton and Kaplowitz, 1977). Since the identification of both detoxifying systems in the small intestine, it has been found that the PAH hydroxylase enzymes are readily inducible (Laitinen and Watkins, 1986).

With this background, we investigated the effect of an enzyme-inducing dose of DMBA on subsequent chylomicron appearance time. As shown in Figure 3-6, enzyme induction did not result in a change in the chylomicron appearance time as compared to the rats not given an induction dose. It is therefore probable that the observed effect of DMBA on lipid absorption is not a result of the parent compound if the effect is occurring after triglyceride resynthesis in the microsomes of the endoplasmic reticulum. Instead, the effect may be one exerted by DMBA metabolites.

As stated above, this does not rule out the possibility that parent DMBA

may be involved in the delay of the chylomicron appearance time. It is possible that parent DMBA interferes with the FABP required for the long chain fatty acids to be carried from the site of absorption by the enterocyte to the microsomes of the endoplasmic reticulum. As described previously, this step is critical in the transport of long chain fatty acids (Ockner and Manning, 1974).

We postulate that the specificity of the DMBA in delaying the CAP is due to the structure of the compound. A comparison between the DMBA molecule and the other PAHs used in the study may implicate certain portions of the molecule which contribute to the effect. The structures of the PAHs used are shown in Figure 2-2. Since DMBA is one of the larger PAHs, one would expect that if the effect was a function of the molecular weight of the xenobiotic then a compound such as benzo(a)pyrene would also cause a delay in the CAP. Figure 3-1 shows that this is not the case.

Comparison of the DMBA to compounds closely resembling it implies an important role for the two methyl groups. Anthracene, 1,2-benzanthracene and 7,12-dimethyibenz[a]anthracene closely resemble each other in structure (Figure 2-2). Neither anthracene, nor 1,2-benzanthracene delayed the chylomicron appearance time. From this comparison we postulate that one or both of the methyl groups of DMBA play a key role in the observed CAP delay. The logical next step would be to run the chylomicron appearance time studies with DMBA-related compounds which differ only in the methyl group sites. Such available compounds include the DMBA aldehydes, 7-formyl-12-methylbenzanthracene and 7-methyl-12-formylbenzanthracene isolated by Fried and Schumm in 1967, and 7-

methylbenzanthracene (Cavalieri and Rogan, 1985). All these compounds are powerful carcinogens.

As stated previously, it is possible that a DMBA metabolite is responsible for the delay in CAP. There are numerous sites where the metabolite could exert its effect. Lipoprotein synthesis may be affected resulting in incomplete chylomicron packaging. Microtubule formation may also be altered as is the case with colchicine administration (described below). Exocytosis of the chylomicra would be affected if microtubule formation was inhibited. Because of the numerous possible sites of action of the DMBA or a metabolite, we suggest that electron microscopic examination of the enterocytes at various time intervals after administration of the DMBA-containing test meal would help to pinpoint a site of action. Factors that would be important in such an examination would include the following: 1) examination of the lipid particles to determine if they were membrane bound. Membrane-bound particles that do not coalesce indicate proper assembly of the lipoprotein coat. 2) positioning of the lipid particles in either the smooth endoplasmic reticulum or Golgi complexes 3) lipid particle size determination.

As shown in the triglyceride output study (Figure 3-5) the total triglyceride output over a three hour time period does not differ from that of the control which indicates a transient effect. Because of this, electron micrographs should be taken at small time intervals over the 23-minute period after test meal administration.

4.3. Cholesterol

Cholesterol was included in this study to serve as a natural lipid inclusion in the test meal. Because cholesterol absorption and handling is closely tied in with the handling of other dietary lipids, its effect on this process was investigated. Since cholesterol is known to have no major effects on lipid absorption , the absence of an effect on the CAP is not surprising.

4.4. Pluronic L-81

The hypolipidemic properties of the compound Pluronic L-81 have been well documented (Tso et al, 1980, Tso et al, 1981; Bochenek, Slowinska, Kapuscinska and Mrukowicz, 1984). Further, it has been determined that the effect of this detergent is pathway-specific and only affects the formation and transport of chylomicrons but not VLDL.

Our studies examined the effect of Pluronic L-81 on chylomicron formation and transport by employing the chylomicron appearance time technique. If the production of chylomicra were affected by this compound then a delay in the radiolabel appearance time should be observed. Our studies indicate that there is indeed this delay in radiolabel appearance (Figure 3-8). It is interesting to note that this delay is not infinite. After approximately 16 minutes we do see the radiolabel in the thoracic lymph despite the continuous infusion of the Pluronic L-81. The transient nature of the delay therefore indicates a possible rerouting/repackaging of the lipid such that it is in a form capable of entering the lymph despite the Pluronic L-81 influence.

In general, the chylomicron appearance time is a useful tool in determining the initial effects of different compounds on the processing of lipid in the small intestinal mucosa. There are many other compounds that have been shown to affect lipid absorption. The chylomicron appearance time can be utilized to investigate two very specific questions. First, the effect of a compound on the handling of the initial few molecules of lipid that the enterocyte encounters can be examined by this technique. It is possible that a depression of total lipid absorption may be a result of a depression in overall transport rather than a delay in handling. This situation would be observed as a normal chylomicron appearance time with a depressed triglyceride output over a set period of time. Secondly, pathway specific effects can be examined as shown with the Pluronic L-81.

The compound colchicine, an inhibitor of microtubule polymerization, has been shown to impair lipoprotein secretion from intestinal cells (Glickman , Perrotto and Kirsch, 1976). In this set of experiments, the effect of colchicine on lymphatic absorption of ^{14}C-oleic acid was determined. It was found that in control animals (no colchicine given), 85% of absorbed radioactivity appeared in the lymph within the first 1 1/2 hrs after lipid administration whereas only 40% absorbed radioactivity appeared in the colchicine treated group in the same time period. Examination of the recovery in the first half hour indicates only a marginal amount in the colchicine treated group. What is not known is whether the appearance of the first few molecules is different between the control and colchicine-treated groups. Analysis of the chylomicron appearance time would answer this question.

The use of the chylomicron appearance time determination in the colchicine experiment is just an example where this technique could be used to gain information on lipid malabsorption. Investigation of the mechanism behind the lipid malabsorption due to zinc deficiency is yet another example of an area where this technique could be employed.

4.5. Alpha-glycerophosphate pathway efficiency

The α-glycerophosphate pathway has long been considered as the minor pathway for triglyceride resynthesis. This assumption is based on the fact that the digestion of dietary fat produces 2-monoglyceride and fatty acids; the chief components required in the monoglyceride pathway. In addition, the energy required for triglyceride resynthesis using the monoglyceride pathway is substantially less than that required in the α-glycerophosphate pathway. The former pathway requires 4 high energy phosphate bonds whereas the latter requires 10 (Shiau, 1987).

In our studies, we investigated another aspect of the efficiency of the α-glycerophosphate pathway; that being the efficiency by which the enterocyte can resynthesise the first few molecules of triglyceride when it is forced to utilize the α-glycerophosphate pathway.

It is well known that the 2-monoglyceride is the preferred isomer in the monoglyceride pathway (Brown and Johnston, 1964b; Shiau, 1987). This isomer cannot be further hydrolysed in the lumen or mucosa and therefore combines directly with activated fatty acids to form a diglyceride, and subsequently a triglyceride.

Interpretation of studies involving the handling of the 1-monoglyceride is more complicated. In studies by Johnston and Brown (1962, 1964a) using both the 1- and 2-isomer of monopalmitin, direct acylation of both isomers is documented using a cell free system. In the protocol followed by Johnston and Brown, dual labeled 1-monopalmitin was introduced into the system and the ratio of $^{3}H/^{14}C$ in the resulting di- and triglyceride was determined. Assuming direct acylation of the 1-monoglyceride occurred, this ratio should be very close to 1.00. The values obtained in that study were indeed close to 1.00 (Johnston and Brown, 1962, Johnston and Brown, 1964a). Polyoxyethylene sorbitan monolaurate (Tween 20) was added to this system. This compound inhibits glyceride formation by the glycerophosphate esterification pathway (Senior and Isselbacher, 1961). In subsequent studies, this group determined that there is a preference for the 2-isomer in the monoglyceride pathway even though both isomers could be used (Johnston and Brown, 1964b).

During the early 1960s, Mattson and Volpenhein (1964) studied the absorption of 1- and 2-monoolein in an *in vivo* system. In their studies, dual labeled 1-monoolein and free fatty acid were fed together to lymph-fistula rats. Analysis of lymph triglyceride from this experiment showed an almost equal distribution of the labeled fatty acid in all three positions. Since 1-monoglyceride does not readily isomerize, this distribution must arise from hydrolysis of the 1-monoolein to glycerol and fatty acid. The subsequent pooling of the labeled and free fatty acids prior to triglyceride resynthesis results in an even distribution of label in the triglyceride; in other words, utilization of the α-glycerophosphate pathway.

In our studies we compared the efficiency of the two pathways for triglyceride resynthesis in their initial response to a lipid load. For these studies, we chose to compare the CAP in rats given a test meal containing 2-monoolein, where the monoglyceride pathway is utilized, or containing 1-monoolein, where it is probable that the α-glycerophosphate pathway is used, or containing no monoglyceride, where the α-glycerophosphate pathway must be used. As shown in Figure 3-10 and Table 3-2, the chylomicron appearance time remains the same, irrespective of the monoolein isomer used. In addition, the absence of monoglyceride in the test meal, which necessitates the utilization of the α-glycerophosphate pathway, results in no change in the chylomicron appearance time (Figure 3-9). It is therefore apparent that both pathways can handle efficiently the first molecules of lipid encountered by the enterocyte producing the first few molecules of triglyceride equally as quickly.

An interesting comparison would be to determine the CAP in animals given 1-monoolein and forced to utilize the monoglyceride pathway by concomitant administration of Tween 20. Assuming the α-glycerophosphate pathway is the preferred route for 1-monoolein it is possible that the CAP may be delayed under the proposed conditions.

It is obvious that the CAP is not a sufficient measurement to allow discussion of the overall efficiency of the monoglyceride and α-glycerophosphate pathways. Our next step was to compare the triglyceride flux in animals given 1- or 2-monoglyceride (Figure 3-12) . The similarity of the curves over the one hour period after test meal administration indicates that the efficiency of the

α-glycerophosphate pathway goes beyond the handling of the first few molecules. At this lipid dose, the α-glycerophosphate pathway appears to be as equally efficient at handling the lipid load as the preferred monoglyceride pathway. It is possible though that a change in efficiency may be observed after administration of either a larger lipid load or a continuous lipid infusion.

The comparison between triglyceride outputs in animals given 1-monoolein and those given no monoglyceride at all again illustrates this point. With no monoglyceride present, the lipid must utilize the α-glycerophosphate pathway for triglyceride resynthesis. Here, there is no difference in total triglyceride output over the one hour time period. The difference in the peak outputs may be a result of different lipid loads. The group of rats given no monoglyceride would have a 20μmol less lipid due to the absence of the monoolein. This being the case, it is probable that a difference would be observed in the subsequent hour time period.

From these studies one can conclude that within a limited time period with a small lipid load, both the monoglyceride and α-glycerophosphate pathways for triglyceride resynthesis are equally efficient.

SUMMARY

We have studied the absorption of certain lipids and lipophilic xenobiotics by the enterocyte of the small intestinal mucosa.

The polycyclic aromatic hydrocarbons are an important group of lipophilic xenobiotics. Many of the members of this group are highly carcinogenic. The ubiquity of these compounds in the environment often leads to their introduction

into an organism via the enterocyte of the small intestinal mucosa. Because they are generally lipophilic, trace amounts of these compounds have been shown to dissolve readily in dietary fat and to travel passively into the enterocyte through the hydrocarbon continuum postulated to exist in the small intestine during fat digestion and absorption.

The close association between PAHs and dietary fat during absorption creates the potential for an interaction between them. Our studies show that 7,12-dimethylbenz[a]anthracene significantly delays the chylomicron appearance time (the time between introduction of radiolabeled fatty acid into the duodenum to the appearance of the label in the thoracic lymph) in rats given a lipid emulsion test meal containing 10mM DMBA [23.08 $\pm$ 2.10 minutes]. The chylomicron appearance time in control animals is 10.81 $\pm$ 1.03 minutes. Triglyceride output studies indicate that this effect is transient because the total triglyceride flux in the thoracic lymph is not depressed over a three hour time period after test meal administration. Previous exposure to a high dose of DMBA several hours before test meal administration does not change the effect exerted by the DMBA on the CAP. This effect may be dose dependent since 1mM DMBA does not result in a delay in CAP. Electron micrograph studies should be employed to help determine where this compound is exerting its effect. Other PAHs tested in our study did not delay the CAP.

Pluronic L-81, a hydrophobic surfactant, also delays the appearance of radiolabeled oleic acid in the thoracic lymph of rats given a test meal containing this compound [16.60 $\pm$ 0.77 minutes]. Previous evidence which indicates that

this compound interferes with chylomicron formation is substantiated by this result.

The chylomicron appearance time technique employed by these studies described above is a useful tool in the investigation of the effects of various compounds on lipid absorption. The CAP of 10.81 $\pm$ 1.03 minutes in rats given a lipid emulsion test meal is a very reproducible result. Although the technique does not give insight into the site or extent that a compound may have on lipid absorption, it is useful as a first step in examining any changes in lipid absorption.

The second part of this thesis concentrated on the comparison of the two pathways involved in triglyceride resynthesis in the enterocyte. Following lipid digestion in the intestinal lumen and absorption by the enterocyte, the lipolytic products travel to the microsomes of the endoplasmic reticulum where triglyceride resynthesis occurs. Here this process can occur through one of two pathways. The first is the monoglyceride pathway through which 2-monoglyceride and activated fatty acids are directly acylated to form first a diglyceride and then a triglyceride. This is the major pathway for triglyceride resynthesis. The alternative pathway is the α-glycerophosphate pathway which is utilized in the absence of monoglyceride or in the presence of 1-monoglyceride.

The monoglyceride pathway is the preferred pathway largely because 2-monoglyceride is one of the major lipolytic products of dietary lipid digestion and only four high energy phosphate bonds are required for this pathway as opposed to the ten required in the α-glycerophosphate pathway. In our studies, we

investigated the efficiency of each of the two pathways to respond to the first few molecules of lipid it encounters. To do this, the chylomicron appearance time was measured in rats given a lipid emulsion test meal containing either 1- or 2-monoglyceride or no monoglyceride at all. The CAP did not differ significantly among the three groups. Further, the triglyceride flux over the first hour after test meal administration did not differ among the three groups. These studies indicate that, at least in the initial response to lipid, both pathways for triglyceride resynthesis are equally efficient.

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