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INTESTINAL CYTOCHROME P-450: REGULATION BY GASTROINTESTINAL HORMONES AND DIETARY NUTRIENTS AND XENOBIOTICS

by

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DISSERTATION

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ABSTRACT

Intestinal cytochrome P-450 (I-P-450)-dependent mixed function oxidase (MFO) system is regulated to a remarkable extent by various ingested xenobiotics, as well as dietary nutrients. Accordingly, acute dietary iron deprivation results in a marked and reversible decrease in I-P-450 content and its dependent MFO activity. On examination of the mechanistic basis for such acute reduction, iron was found not only to be a cosubstrate for I-P-450 heme formation, but its dietary deprivation resulted in decreased activities of two potentially rate limiting enzymes of heme biosynthesis, δ-aminolevulinic acid synthetase and ferrochelatase (FC).

More significantly, acute dietary selenium deprivation reduced I-P-450 content and its dependent MFO activity, reflecting impaired I-P-450 heme formation subsequent to lowered FC activity. This decrease was not accounted for by altered heme degradation, nor as a consequence of potentially enhanced intracellular peroxidation.

Availability of the constitutive intestinal apocytochrome P-450 moiety was found to be dependent on concomitant synthesis of its heme moiety. Furthermore, dietary selenium was also found to directly modulate the availability of this apocytochrome, independent of intracellular heme status. Dietary β-naphthoflavone resulted in induction of the specific isozyme I-P-448, identified as a physico-chemically and functionally distinct form of I-P-450. Formation of this isozyme was also dependent on dietary iron. However, its apocytochrome is apparently independent of concomitant synthesis of its heme moiety.

The potential role of the gastrointestinal peptide hormone, gastrin, in the regulation of the I-P-450 MFO system and heme synthesis, was also examined by infusion of its analogue, pentagastrin, to unrestrained rats,

maintained by parenteral hyperalimentation, i.e., complete absence of intraluminal substances. Pentagastrin was found to be ineffective in increasing I-P-450 content, MFO activity, or heme synthesis, above "basal" levels, indicating that under these conditions it was not a regulator of the intestinal cytochrome P-450-dependent MFO system.

These studies indicate that, in addition to iron, dietary selenium is a critical modulator of cytochrome P-450-dependent MFO activity. Dietary deprivation of either iron or selenium may thus compromise the intestinal first pass metabolism of numerous orally ingested drugs and carcinogens, and alter their pharmacodynamics.

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ABBREVIATIONS

P-450, cytochrome P-450

MFO, mixed function oxidase

NADPH, reduced pyridine adenine dinucleotide phosphate

NADH, reduced adenine dinucleotide

EROD, 7-ethoxyresorufin O-deethylase

ECOD, 7-ethoxycoumarin O-deethylase

AHH, aryl hydrocarbon hydroxylase

CYTO P-450 RED, cytochrome P-450 reductase

CYTO b_5, cytochrome b_5

ALA, δ-aminolevulinic acid

ALAS, ALA synthetase

ALAD, ALA dehydratase

FC, ferrochelatase

MHO, microsomal heme oxygenase

EH, epoxide hydratase

DTE, dithioerythritol

GSH, reduced glutathione

GSH Px, glutathione peroxidase

HSA/BSA, serum albumin, human/bovine

β-NF, β-naphthoflavone

3-MC, 3-methylcholanthrene

BaP, benzo(a)pyrene

1.0 INTRODUCTION

Xenobiotics are chemicals foreign to the biological system, possessing no nutritional value. They include extraneous natural food chemicals, plant and fungal toxins, environmental pollutants, and synthetic chemicals such as drugs, food additives, and industrial, agricultural, and household chemicals (Epstein, 1972). A large number of xenobiotics are lipid soluble, weak organic acids or bases and as such are well absorbed but poorly eliminated from the body (Chhabra, 1979). They therefore must first be biotransformed, primarily in the liver but also in such extrahepatic organs as the lung, skin, and small intestine (Remmer, 1970; Gram, 1980, and references therein), into more polar metabolites. Biotransformation thus promotes the elimination of water soluble products, which, being less lipophilic, also bind less extensively to plasma and tissue proteins than parent compounds that would otherwise accumulate in body tissues. Such metabolic conversion generally renders a biologically active compound less toxic or inactive; this process thus serves as the major mechanism of bodily defense against foreign compounds.

The major route of exposure of the human population to xenobiotics is by oral ingestion. Thus, the first tissue that most xenobiotics contact is the mucosal epithelium of the gastrointestinal tract, which is responsible for their absorption. As the portal of entry for such compounds, the small intestine serves as the body's first and major line of defense against their potential toxicity. The morphological architecture of the mucosal epithelium, as well as the intrinsic biochemistry of the epithelial cells, suitably adapt this organ for its role of bodily defense.

The intestinal mucosa is capable of biotransforming xenobiotics

(BACKGROUND, Section 2.2) and as such is important in the biodisposition of orally ingested compounds. This process partly entails oxidative metabolism of foreign compounds, predominantly by the cytochrome P-450 system, generally resulting in detoxification of drugs and toxins and the conversion of chemical carcinogens to inactive products. However, it is also capable of converting carcinogens and/or toxins to their ultimate carcinogenic/toxic species. Thus, in the intestinal mucosa as in other organs capable of xenobiotic biotransformation, a tenuous balance exists between metabolic detoxification and activation of carcinogens and toxins. Thus, factors which modulate the activity of either or both of these processes may be of considerable pathophysiological significance. Intestinal cytochrome P-450-dependent biotransformations are known to be affected by dietary inducers of the cytochrome, as well as acute dietary iron deprivation. The subject of this dissertation is to explore whether endogenous factors such as intestinal hormones or exogenous nutritional factors modulate cytochrome P-450-dependent MFO activity in the intestinal mucosa by regulating its cytochrome P-450 content.

2.0 BACKGROUND

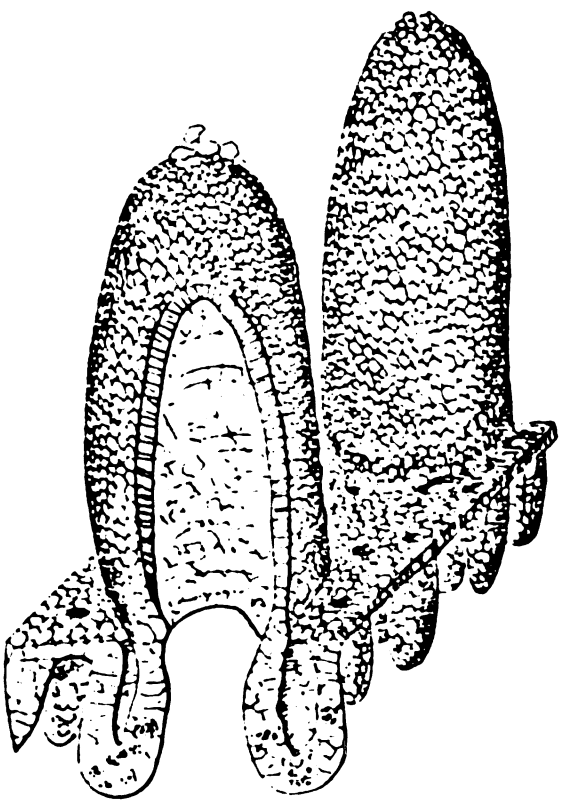
2.1 ARCHITECTURE AND CELL TURNOVER OF SMALL INTESTINAL MUCOSA:

Small intestinal mucosa is a unique tissue, characterized by a dynamic cellular turnover and structural specialization for absorption and metabolism of ingested foodstuffs and xenobiotics.

The mucosa of the small intestinal tract consists of a variety of cell types, whose primary functions include the absorption of intraluminal substances and mucous secretion into the lumen. Among these cell types, two classes of epithelial cells, differentiated by their mucosal location and specialized function, may be identified: the crypts of Lieberkuhn, consisting of highly proliferative and primarily secretory epithelial cells (Trier, 1967), and the villi, which are composed of highly differentiated absorptive columnar epithelial cells.

The epithelial cells of the intestinal mucosa originate in the crypts and progressively mature as they migrate up the villi to be eventually sloughed at the extrusion zone, located at the apex of the villi (Leblond & Messier, 1958), into the intestinal lumen (Figure 1). This unique feature of the intestinal mucosa may contribute to the physical elimination of foreign substances. That is, following uptake from the lumen, xenobiotics which escape absorption into blood, and are therefore trapped in the villous tip cells, are thus extruded back into the lumen, from where they may be either reabsorbed or travel with other cellular constituents along the intestinal tract to the colon for eventual excretion in the feces. The complete turnover of the mucosal epithelium occurs in 2-3 days in the rat (5-6 days in the human), a highly dynamic process when compared to the "resting" state of cells of

Figure 1. Spatial scheme of intestinal mucosa, showing two villi and several crypts. Representative spacing between villi and crypts is slighty greater than actual (from Quastler & Sherman, 1959).



most other organs.

During migration from the crypts to the villous tips, the epithelial cell undergoes dramatic changes in structure and function. The highly proliferative crypt cell, as it migrates through the transition zone between crypt and villus, begins to differentiate into an absorptive and metabolic cell. Characteristic of highly proliferative cells, crypt cells contain high activity of thymidine kinase (Fortin-Magana et al, 1970), and rapidly incorporate radiolabelled thymidine into DNA (Leblond & Messier, 1958), and uridine into RNA (Shorter & Creamer, 1962). The activity of these cellular processes progressively decreases as the cells migrate past the crypt/villus junction onto the mucosal villi. During the junctional migration, the cells acquire an increased ability to absorb amino acids and sugars (Kinter, 1961), and nutrients such as iron (Dowdle et al, 1960; Conrad et al, 1966a). Upon further migration to the villous tip they show an increased capacity to metabolize lactose intracellularly (Fortin-Magana et al, 1970) and disaccharides in the microvilli (Weiser, 1973). In a similar fashion, the development of mitochondrial enzymes such as cytochrome c oxidase and succinate dehydrogenase follows a progressive gradient from the crypt to the villous tip cells (Iemhoff & Hulsmann, 1971). This differentiation of cellular function during upward migration along the mucosal villi underscores the divergent roles of crypt and villous tip cells in absorption and metabolism of ingested dietary substances.

2.2 MUCOSAL METABOLISM OF XENOBIOTICS:

Biotransformation of xenobiotics involves one or more enzymatic conversions to polar and more readily excretable metabolites. The two

main processes may be categorized into phase I, functionalization and phase II, conjugation reactions. Frequently, but not necessarily always, xenobiotics after initial oxidations (Phase I) will undergo subsequent conjugation (Phase II) at the oxidized functionality with glucuronic acid (glucuronidation), sulfate (sulfation), or the tripeptide glutathione. These metabolic conversions are catalyzed by specific enzymes, such as cytochrome P-450-dependent mixed function oxidases (MFO), UDP-glucuronyl transferases, sulphotransferases, or glutathione S-transferase, all of which have been identified in the intestinal mucosa (Wattenberg et al, 1962; Stohs et al, 1976a; Uotila & Marniemi, 1976; Hartiala, 1954; Dollery et al, 1971; Dawson & Bridges, 1981a; Clifton & Kaplowitz, 1977; Pinkus et al, 1977). A number of drugs and environmental toxins are known to undergo extensive metabolic conversion in the intestinal mucosa (Table 1). Such a feature might be important in the clinical evaluation and dose titration of certain drugs such as warfarin, tolbutamide, and phenytoin, which have a low therapeutic index. Their therapeutic effectiveness may be greatly compromised should they undergo substantial metabolism in the intestine following oral administration. This phenomenon has been termed the "intestinal first pass effect" (Gibaldi & Perrier, 1974), to distinguish it from the first pass metabolic clearance by the liver, or intestinal/colonic microflora (Scheline, 1973). Because of its importance in clinical pharmocodynamics, the intestinal first pass metabolism of drugs is receiving increasing attention (Rowland, 1972; Routledge & Shand, 1979; Klippert et al, 1982).

Biotransformation of a lipophilic xenobiotic to more water soluble metabolites generally decreases the pharmacological activity or toxicity of the compound. However, examples exist of increased toxicity or

Table 1

METABOLISM OF XENOBIOTICS BY INTESTINAL MUCOSA

Compound	Reaction	% Metabolized	Reference
Flurazepam	N-Dealkylation	90	Mahon et al, 1977
Phenacetin	O-Deethylation[ad]	50	Klippert et al, 1982 Pantuck et al, 1974
Buprenorphin	N-Dealkylation Glucuronidation[ad]	75	Rance & Shillingford, 1976
Salicylamide	Glucuronidation[ac] Sulfation	50	Barr & Riegelman, 1970
Isopreterenol	Sulfation[d] O-Methylation	90	Conway et al, 1968 Connolly et al, 1972
Chlorpromazine	Demethylation[c] Sulfoxidation	--	Curry et al, 1970 & 1971 Knoll et al, 1977
Pentobarbital	Oxidation[d]	60	Knodell et al, 1980
Morphine	Oxidation[d] Glucuronidation	60	Iwamoto & Klassen, 1977a
Nalorphine	Dealkylation[d] Glucuronidation	30	Iwamoto & Klassen, 1977b
Proscillaridin	Glucuronidation[d]	75	Andersson et al, 1975
Oxamniquine	Oxidation[a]	30	Kaye & Roberts, 1980
Stilbesterol	Glucuronidation[a]	90	Fischer & Millburn, 1970
Androstenedione	Oxidation[a]	50	Kreek et al, 1963
Testosterone	Oxidation[a]	--	" "
Thyroxine	Glucuronidation	--	Herz et al, 1961
Ethanol	Oxidation	--	Seitz et al, 1979
Tetrahydro-cannabinol	Hydroxylation[bd]	50	Green & Saunders, 1974
1-Naphthol	Glucuronidation[c]	70	Pekas, 1974 Bock & Winne, 1975
Benzo(a)pyrene	Oxidation[a]	10	Hietanen, 1980

Table 1 (cont.)

Propranolol	--	60	Shand & Rangno, 1970
Clonazepam	--	40	Colburn et al, 1980
Isoetharine	--	--	Williams et al, 1974
Levodopa	--	--	Cheng & Fung, 1976
Methyldigoxin	--	--	Hinderling et al, 1977
Terbutaline	--	--	Conway et al, 1973

a- everted sacs; b- isolated gut; c- in situ perfusion; d- in vivo sampling

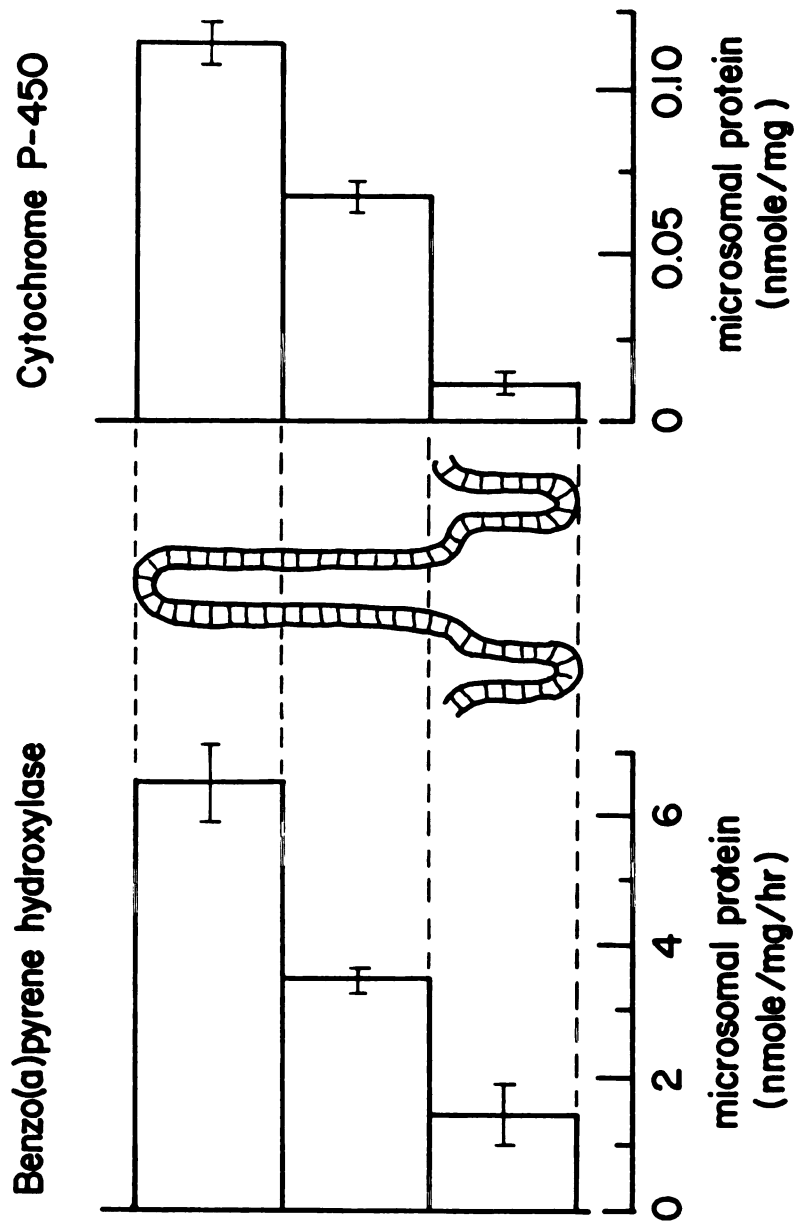
carcinogenicity of a compound following its cytochrome P-450-dependent oxidative metabolism. In these cases, such metabolism generates an active metabolite which covalently binds to tissue macromolecules, resulting in its toxicity or carcinogenicity (Mitchell et al, 1973; Mitchell et al, 1974; Jollow et al, 1974). Numerous environmental toxins and procarcinogens which could be rendered toxic or carcinogenic by cytochrome P-450-dependent oxidations have been shown to undergo biotransformation in the intestinal mucosa. For example, benzo(a)pyrene (BaP), the procarcinogen generated as a pyrolitic product of organic material, has been shown to be sequentially oxidized by the intestinal cytochrome P-450 dependent MFO system and converted by epoxide hydratase (EH) to its 7,8-dihydrodiol (Stohs et al, 1976a). This metabolite is then presumed to be further epoxidized by the MFO system to its 7,8-diol-9,10-epoxide. These metabolites have been identified as the proximate and ultimate carcinogenic species, respectively, of hepatic cytochrome P-450 dependent oxidation of the otherwise inert parent compound, BaP (Conney et al, 1978). However, in comparison to hepatic tissue, the intestinal mucosa exhibits low EH activity (Clifton & Kaplowitz, 1977; Stohs et al, 1977). Metabolic activation of BaP incurs initial epoxidation by the MFO system and subsequent conversion to the 7,8-dihydrodiol by EH. This metabolite is then readily converted by a second MFO reaction to the 7,8-diol-9,10-epoxide, which has not yet been determined to be a substrate for further detoxification by EH (Oesch, 1979; Oesch, 1982). However, due to the low EH activity in intestinal mucosa, BaP-7,8-dihydrodiol is very slowly produced by intestinal microsomes (Stohs et al, 1976a). It does however accumulate over time in isolated intestinal cells (Stohs et al, 1977), and thus it could be further converted to its

diol epoxide by the intestinal MFO system. Whether the low activity of intestinal EH is a beneficial attribute depends on the affinities of the different BaP epoxides for intestinal EH, and the extent of accumulation of the various epoxides in intestinal cells, both of which are presently unknown. Thus, after initial oxidation by cytochrome P-450, whether the carcinogenic potential of BaP in the intestinal mucosa is similar to that in the liver remains to be determined.

2.3 CELLULAR LOCALIZATION OF THE INTESTINAL CYTOCHROME P-450-DEPENDENT MFO SYSTEM:

The intestinal cytochrome P-450-dependent MFO system has been identified in the mucosa of small intestine of both humans and animals (Wattenberg et al, 1962; Welch et al, 1972; Zampaglione & Mannering, 1973; Hoensch et al, 1976; Hoensch et al, 1979). Starting from the pyloric junction, it has been shown to follow a decreasing gradient in content and activity along the length of the intestine to the colon. It is highest in the first 15 centimeters of the duodenum of the rat (Wattenberg et al, 1962; Ullrich & Weber, 1974; Hoensch et al, 1976). In addition, a decreasing gradient in cytochrome P-450 content and MFO activity (Figure 2) has been observed along the height of the mucosal villus from the villous tip to the crypt cells (Hoensch et al, 1976; Dawson & Bridges, 1981b; Hartman et al, 1982). Such a gradient quantitatively parallels the gradient in the functional differentiation, i.e., absorption and metabolism of food, of the mucosal cells along the height of the villus. Thus, the metabolic inactivation/activation of drugs, carcinogens, and toxins is apparently most active in the cells primarily responsible for the absorption of such compounds. It is therefore

Figure 2. Representative localization of content of cytochrome P-450 and specific activity of benzo(a)pyrene hydroxylase (aryl hydrocarbon hydroxylase, AHH), in three cell populations of duodenal mucosa of rats fed standard chow diet (Hoensch et al, 1976). Upper bar, villous tip cells; middle bar, intermediate cells; lower bar, crypt cells. Values are mean $\pm$ SEM.

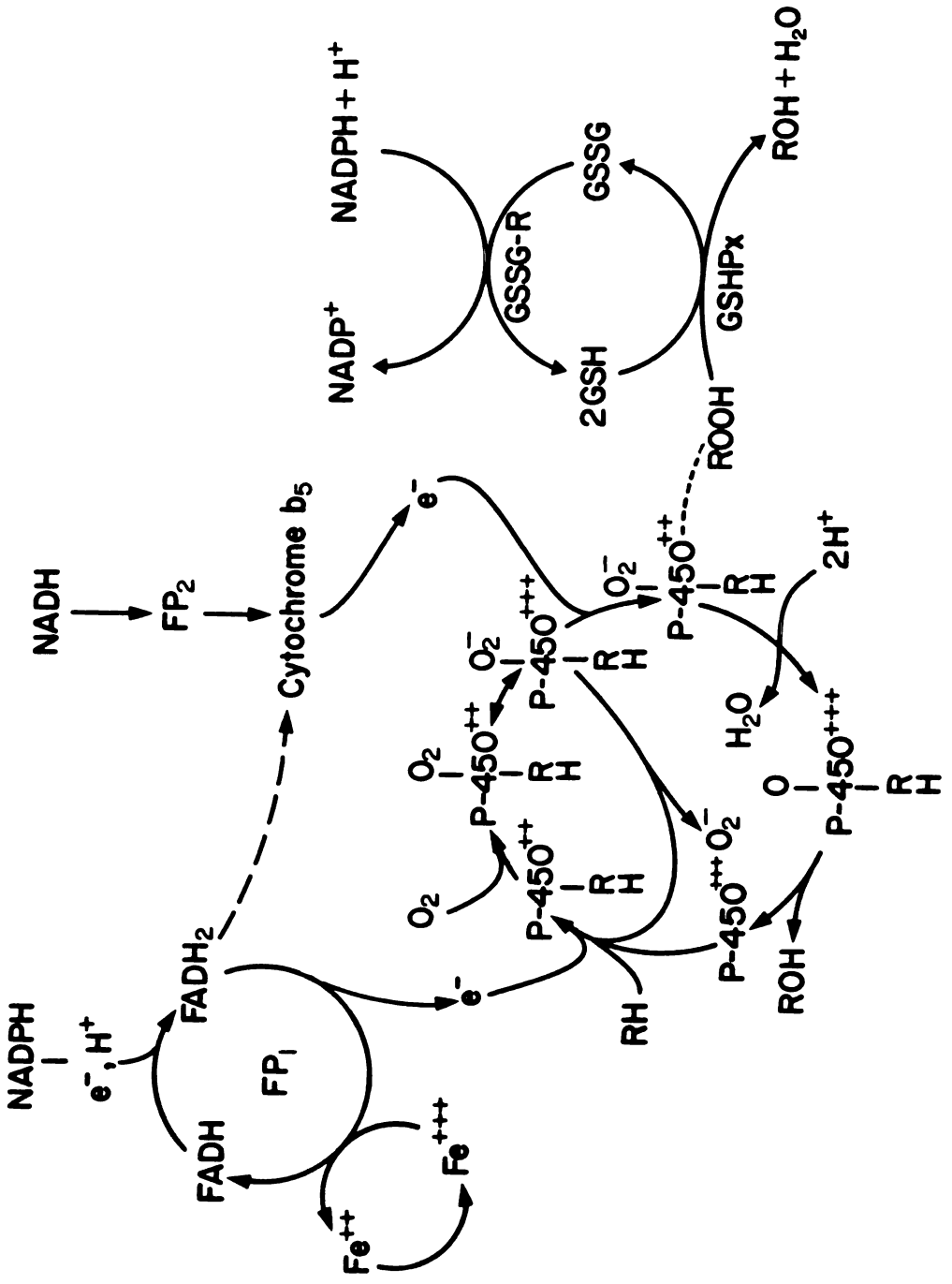

Cytochrome P-450
0
0.05
0.10
microsomal protein
(nmole/mg)
Benzo(a)pyrene hydroxylase
0
2
4
6
microsomal protein
(nmole/mg/hr)

reasonable to assume that the intestinal cytochrome P-450 system largely evolved in order to detoxify absorbed xenobiotics. Thus, examination of the regulation of the enzyme system within each of the two epithelial cell types of the intestinal mucosa may be important for understanding the role of the intestinal mucosa in the toxicity/carcinogenicity of foreign compounds in the gastrointestinal tract and colon. In addition, passage of xenobiotics through the intestinal mucosa may regulate their delivery to other organs and thus modulate their pharmacological/toxicological effects.

2.4 BIOCHEMICAL MECHANISM OF INTESTINAL CYTOCHROME P-450-DEPENDENT MIXED FUNCTION OXIDATIONS:

The mechanism of intestinal cytochrome P-450-dependent oxidation of subtrates is believed to be qualitatively similar to that of the hepatic cytochrome P-450 system (Welch et al, 1972; Lehrmann et al, 1973; Pantuck et al, 1975; Chhabra & Fouts, 1975; Hietanen et al, 1975; Hoensch et al, 1976, Stohs et al, 1976a; Ichihara et al, 1980, 1981). The hepatic cytochrome P-450 MFO system has been extensively characterized in mammals. In the liver it is localized primarily in the microsomal fraction, an artifact of cell fractionation, which consists of fragments of endoplasmic reticulum (Gillette et al, 1972). Isolation and purification of this system have resolved two main components: a hemoprotein, cytochrome P-450, and a flavoprotein, NADPH-cytochrome P-450 reductase (Lu et al, 1969a). Optimal functional reconstitution of the enzyme system however, in addition to the two components, requires lipid in the form of phosphatidylcholine (Lu et al, 1969b). The catalytic oxidative cycle (Figure 3; Estabrook & Werringloer, 1977) initially involves

Figure 3. Reaction scheme of microsomal cytochrome P-450-dependent mixed function oxidations and peroxide reduction by cytosolic glutathione peroxidase. NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; $FADH_2$, reduced flavine adenine dinucleotide; FP_1, flavoprotein (cytochrome P-450 reductase); GSH, reduced glutathione; GSSG-R, glutathione reductase; GSHPx, glutathione peroxidase; ROOH, lipid hyroperoxide or hydrogen peroxide.


NADPH
e⁻, H⁺
FADH
FADH₂
FP₁
Fe⁺⁺⁺
Fe⁺⁺
NADH
FP₂
Cytochrome b₅
e⁻
e⁻
O₂
P-450⁺⁺
R
H
O₂⁻
P-450⁺⁺⁺
RH
P-450⁺⁺O₂⁻
P-450⁺⁺-ROOH
2H⁺
H₂O
O
P-450⁺⁺⁺
ROH
NADPH + H⁺
NADP⁺
GSSG-R
GSSG
2GSH
GSHPx
ROOH
ROH + H₂O

binding of substrate to oxidized ferric cytochrome P-450, which is then reduced by an electron from NADPH transferred via cytochrome P-450 reductase. The ferrous cytochrome P-450 substrate complex binds molecular oxygen to form oxycytochrome P-450 which is then reduced by a second electron derived either from NADPH or from NADH via cytochrome b_5, to form a peroxide anion complex: a triplex of substrate, cytochrome P-450, and O_2^-. One atom of the activated oxygen (O_2^-) is then reduced to water while the other is transferred to the substrate, resulting in an oxidized product. The enzyme system is hence termed "mixed function oxidase".

Cytochrome P-450, the terminal oxidase of the system, is a group of hemoproteins, characterized by spectral absorption maxima around 450 nm when complexed with CO in the reduced state (Omura & Sato, 1964a). Although existence of multiple species of hepatic cytochrome P-450 have been described for quite some time (Lu & Levin, 1974; Guengerich, 1978; Johnson & Muller-Eberhard, 1977), intestinal cytochrome P-450 has only recently been resolved into three separate isozymes. These were identified by specific molecular weights of their apoprotein moities, their pattern of electrophoretic mobility on SDS polyacrylamide slab gels, and variable substrate specificities (Ichihara et al, 1980; Ichihara et al, 1981). Before the enzyme was successfully purified, the presence of multiple intestinal cytochrome P-450's was inferred from differences in substrate specificities and effects of specific inhibitors following enzyme induction by various organic compounds (Stohs et al, 1976b; Shirkey et al, 1979b). The phenomenon of cytochrome P-450 induction by xenobiotics and its relation to enzyme regulation and multiplicity will be further discussed in section 2.6.2.

2.5 STRUCTURAL COMPONENTS OF CYTOCHROME P-450:

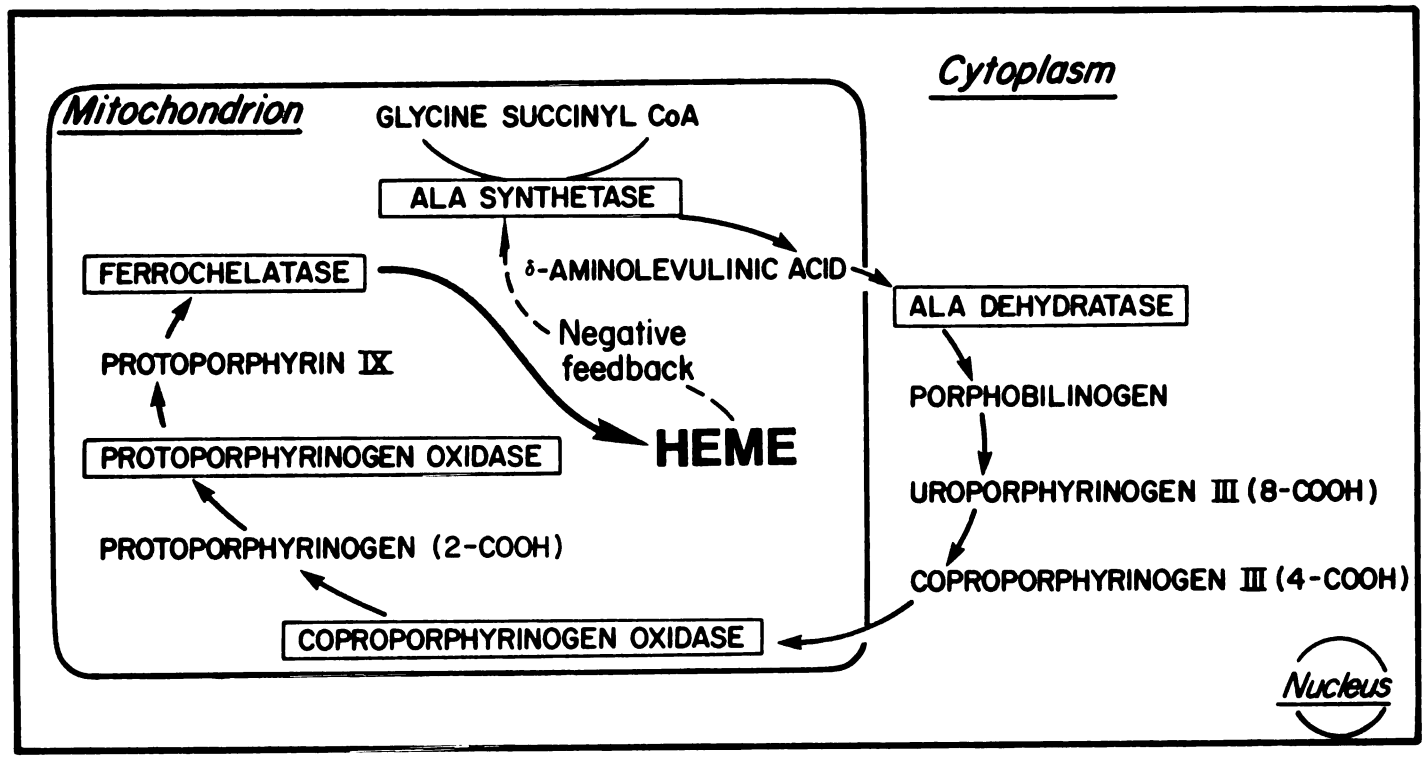
Cytochrome P-450 is a hemoprotein consisting of heme (protoporphyrin IX) and apocytochrome in a 1:1 molar ratio. The prosthetic heme of the cytochrome is the active site of the enzyme. Molecular oxygen binds to the iron moiety after its reduction with an NADPH-donated electron. Model studies have demonstrated that the presence of an axial thiolate ligand of the heme (Collman et al, 1975; Ullrich, 1971), which electronically enriches the iron center of the cytochrome, is essential for catalytic activity of the cytochrome. Destruction of the sulfur ligand results in irreversible conversion of the mammalian cytochrome to its enzymatically inactive product, cytochrome P-420 (Omura & Sato, 1964a). In contrast, cytochrome P-450 isolated from bacteria ($P\text{-}450_{cam}$) after similar conversion to cytochrome $P\text{-}420_{cam}$ can be readily reconverted by treatment with sulfhydryl compounds (Yu & Gunsalus, 1974b).

The apoprotein moiety of the cytochrome determines the unique substrate specificity, the particular ethylisocyanide binding spectra, and the characteristic electrophoretic behaviour of each of the known forms of hepatic cytochrome P-450 (Sladek & Mannering, 1969a,b; Alvares et al, 1967; Alvares & Siekevitz, 1973). Regulation of the turnover of the structural components of intestinal cytochrome P-450 will be discussed in section 2.6.

2.5.1 Heme Metabolism and Its Regulation by Intracellular Heme and Iron:

2.5.1.1 Heme Biosynthesis: The biosynthesis of heme has been extensively studied in mammalian erythrocytes and liver, though presumably all tissues possess this capability (Sassa, 1978). The following description of the heme synthetic pathway (Figure 4) is an encapsulated

Figure 4. Biosynthesis of heme.


Cytoplasm
Mitochondrion
GLYCINE SUCCINYL CoA
ALA SYNTHETASE
δ-AMINOLEVULINIC ACID
ALA DEHYDRATASE
FERROCHELATASE
Negative feedback
PROTOPORPHYRIN IX
HEME
PORPHOBILINOGEN
PROTOPORPHYRINOGEN OXIDASE
UROPORPHYRINOGEN III (8-COOH)
PROTOPORPHYRINOGEN (2-COOH)
COPROPORPHYRINOGEN III (4-COOH)
COPROPORPHYRINOGEN OXIDASE
Nucleus

version of a review (Tait, 1978). The first step in heme synthesis is the condensation of succinyl CoA and glycine to form δ-aminolevulinic acid (ALA), catalyzed by the primary rate limiting enzyme, mitochondrial ALA synthetase (ALAS)(Granick, 1966). This reaction requires pyridoxal-5'-phosphate as cofactor (Jordan & Shemin, 1972). The activity of ALAS is regulated by negative feedback inhibition by the end product, heme (Granick, 1966). This control mechanism serves to maintain a very adaptable supply of heme during increased heme demand, a characteristic feature of increased hemoprotein synthesis. In addition, hepatic ALAS also appears to be influenced by iron availability. Depletion of tissue iron by chronic feeding of an iron deficient diet coupled with *in vivo* iron chelation, was shown to result in a slight diminution of hepatic ALAS activity (Liem *et al*, 1979). This is surprising since heme content would be expected to decrease during severe iron deficiency, leading to removal of feedback inhibition of ALAS with consequent stimulation of ALAS activity. The mechanism of hepatic ALAS regulation by heme is not fully understood and is further complicated by the unexpected finding that iron deprivation failed to derepress the enzyme.

Newly formed ALA is transferred to the cytoplasm where two molecules of ALA are condensed to form the monopyrrole porphobilinogen (PBG), a reaction catalyzed by the cytoplasmic ALA dehydratase (ALAD) (Shemin, 1972). Four molecules of PBG are then condensed by two cytosolic enzymes, URO-I synthetase and URO-III cosynthetase, to form uroporphyrin III (URO-III). Acetic acid side chains of URO III are sequentially decarboxylated to form coproporphyrinogen III (COPRO-III) by uroporphyrinogen decarboxylase. COPRO-III enters the mitochondria where two propionic acid side chains are decarboxylated to vinyl groups by coproporphyrinogen

oxidase to form protoporphyrinogen IX. Protoporphyrinogen IX is then believed to be enzymatically converted to protoporphyrin IX by protoporphyrinogen oxidase. The final but potentially a secondary rate limiting step in heme biosynthesis, is the catalytic insertion of iron into protoporphyrin by the mitochondrial ferrochelatase to form protoheme (heme) (Jones & Jones, 1969). In common with ALAS activity, hepatic ferrochelatase activity also significantly decreases in rats fed an iron deficient diet. Dependency of ferrochelatase activity on dietary iron is much more sensitive than that of ALAS activity since additional iron chelation *in vivo* was not necessary to demonstrate the effect of dietary iron deprivation on its activity (Liem *et al*, 1979).

2.5.1.2 Heme Degradation: The first step in heme degradation is catalyzed by the rate limiting enzyme microsomal heme oxygenase (MHO), which requires NADPH and oxygen (Tenhunen *et al*, 1968; 1969; Raffin *et al*, 1974). Cleavage of heme occurs at the α-methene bridge to yield equimolar amounts of biliverdin IX, iron, and carbon monoxide. Biliverdin is then converted to bilirubin by the cytosolic enzyme biliverdin reductase, also in the presence of NADPH as cofactor. MHO is induced by its substrate heme (Tenhunen *et al*, 1970), and thus serves as an important regulator of the intracellular pool of heme (Section 2.5.2).

2.5.2 Heme Dynamics- "Free" Heme Pool: A pool of "uncommitted" heme has been postulated to exist in the liver. This pool may contain protein-"free" heme in equilibrium with protein bound heme (Marver, 1969). This pool is apparently in communication with plasma heme (Correia *et al*, 1979) and cell-free hemoproteins, including hemoglobin (Wyman *et al*, 1981). Heme administered *in vivo* to rats is taken up by the liver and

transported into the hepatocyte, as evidenced by its ability to induce MHO (Tenhunen et al, 1970), its subsequent conversion to bilirubin in the hepatocyte (Landaw et al, 1970), and its ability to bind available apocytochrome of the unsaturated cytoplasmic hemoprotein, tryptophan pyrrolase (Badawy, 1978), as well as hepatic cytochrome P-450 (Correia et al, 1979). Together this evidence suggests that exogenously administered heme is incorporated into a dynamic "free" heme pool in hepatocytes and is available for formation of hepatic hemoproteins (Marver, 1969; Tschudy & Bonkowsky, 1972; Granick et al, 1975; Bissell & Hammaker, 1976). In addition, "free" apocytochrome experimentally generated by stripping cytochrome P-450 of its heme can be reassembled with exogenous heme in vivo to result in not only increased cytochrome content (Correia et al, 1979) but in functional restoration of the enzyme's ability to oxidize drugs in vivo (Farrell et al, 1979) and in vitro (Farrell & Correia, 1980).

After dissociation from holocytochrome P-450, the heme moiety is believed to be transferred to the "free" heme pool and thence to carrier proteins (Maines & Anders, 1973) possibly for utilization by other cytochromes (Ycas & Drabkin, 1957), or degradation to bilirubin by MHO (Maines & Anders, 1973). In addition, after dissociation from cytochrome P-450, but before its degradation by MHO, cytochrome P-450 heme may exchange with heme in the "free" heme pool. This in turn results in inhibition of ALAS activity through negative feedback (Bissell & Hammaker, 1976). Thus, hepatic cytochrome P-450 heme appears to be in dynamic equilibrium with a reservoir of "free" heme, which contains not only newly synthesized heme, and thus acts as a precursor depot for membrane bound cytochrome P-450 and other cellular hemoproteins, but also heme

discharged by turnover of the cytochrome.

Whether a "free" heme pool also exists in the enterocyte is not known. Heme is absorbed intact across the intestinal epithelium, after proteolytic cleavage of ingested cytochromes (myoglobin) or hemoglobin (Wheby et al, 1970; Conrad et al, 1966b). Some of the absorbed heme is cleaved in the mucosa by MHO (Raffin et al, 1974), while a portion is sequestered and cleaved in the liver (Conrad et al, 1966b). That fraction metabolized in the intestine releases inorganic iron which is transported by carriers to blood or storage proteins (Wheby et al, 1970). Absorption of intraluminal heme (Raffin et al, 1974), as well as inorganic and organic iron (Conrad et al, 1966a) is highest in the duodenum and progressively decreases along the length of the intestine. This is reflected by a parallel intestinal gradient in MHO activity (Raffin et al, 1974). In addition, a specific, high affinity binding-receptor has been described and found to display maximal binding of heme in the duodenum (Tenhunen et al, 1980), the site of maximal absorption not only of heme but of ingested xenobiotics. Thus intraluminal heme is absorbed intact into the duodenal mucosa where a substantial amount is cleaved for bodily iron storage or iron metabolism. Whether this absorbed heme can be reutilized for formation of hemoproteins, such as cytochrome P-450, in the intestinal mucosa has not been investigated. The possible existence and the regulation of the intracellular heme pool in the intestinal mucosa, its association with heme absorbed and/or synthesized in situ, and its utilization for the formation of intestinal cytochrome P-450, have never been elucidated.

2.5.3 Apocytochrome Biosynthesis and Its Assembly with Heme to form Holocytochrome P-450: Little is known about the synthesis of the apoprotein of cytochrome P-450 in the intestinal mucosa. Attempts to localize the subcellular site of apoprotein synthesis and its assembly with heme to form the holocytochrome have been restricted to the liver. Presumably subcellular synthesis of apocytochrome P-450 in the intestinal mucosa occurs in a manner similar to that in the liver. Studies in the liver suggest that the synthesis of the apocytochrome occurs at the rough endoplasmic reticulum (RER), following which assembly of the newly synthesized apoprotein with mitochondrial heme proceeds through mitochondrial-RER interactions to yield the holocytochrome (Correia & Meyer, 1975). Thus, the smooth endoplasmic reticulum (Levin et al, 1970), contains primarily fully or almost fully heme-saturated apocytochrome P-450 (Brown & Kupfer, 1975; Correia & Meyer, 1975; Bhat et al, 1977; Omiecinski et al, 1978).

This is in sharp contrast to reports of numerous extrahepatic cytochrome P-450 isozymes which apparently coexist with an unsaturated pool of free apocytochrome(s) in certain tissues. Incubation of subcellular fractions from extrahepatic tissues such as kidney, testes, lung (Omiecinski et al, 1978), aorta (Bond et al, 1979), and brain (Omiecinski et al, 1980b) of both adult and fetal rats (Omiecinski et al, 1980a) with exogenous heme in vitro for prolonged periods has been reported to result in marked (up to 40 fold) stimulation of MFO activity. Initial rates of oxidation were not monitored in those studies, stimulation of MFO activity was observed only after prolonged hemin-incubations, and spectral increases in cytochrome P-450 content were not observed. Nevertheless, it was concluded that hemin-mediated constitution

of holocytochrome P-450 was responsible for the observed stimulation of cytochrome P-450-dependent oxidative activities. It was further suggested that non-hepatic tissues contain pools of free apocytochrome with low affinity for the heme prosthetic group, and thus do not exhibit stimulated MFO activity after short incubations with hemin. After prolonged incubation with hemin, the apocytochromes were believed to undergo conformational changes to increase their relative affinities for heme to form holocytochromes. The hemin-constituted holocytochrome state was postulated to have a high oxidative turnover rate, responsible for the observed 40 fold increase in MFO activity (Omiecinski et al, 1980b). The conclusion that free apocytochrome is present in extrahepatic tissues and is available for holocytochrome formation is consonant with the findings of excess unsaturated apocytochrome b_5 in liver microsomes (Negishi & Omura, 1970), where apparently heme supply is limiting for holocytochrome b_5 assembly. But the data presented in the above studies (Omiecinski et al, 1978; 1980a; 1980b; Bond et al, 1979) do not offer convincing evidence that incubation of extrahepatic apocytochromes with hemin had structurally constituted cytochrome P-450. Similar experiments to detect free apocytochrome in intestinal tissue have not been conducted.

2.6 REGULATION OF INTESTINAL CYTOCHROME P-450 SYNTHESIS:

2.6.1 Introduction: Regulation of multi-component enzymes appears to operate through a finely tuned balance of synthesis and degradation of individual components as well as their assembly and dissociation. Regulation of cytochrome P-450 synthesis can be exerted through regulation of synthesis of the apoprotein and/or the prosthetic heme (Section

2.5). On the other hand, one component may regulate the synthesis of the other in a coordinated fashion, such that levels of one may determine the availability of the other moiety. Various external and internal factors may therefore influence cytochrome P-450 content and function by regulating the metabolic turnover of its moieties and/or by influencing their assembly.

In addition, regulation of intestinal cytochrome P-450 synthesis may be very different from that of the hepatic isozyme because of the unique features of the intestinal mucosa: rapid cell turnover, alterations in cellular function during the spatial migration along the mucosal villi, and exposure to potential enteral and parenteral regulators of enzyme synthesis.

2.6.2 Induction of Intestinal Drug, Hormone, and Xenobiotic Metabolism: A unique feature of the cytochrome P-450-dependent MFO system is its inducibility by substrates. There are numerous examples of cytochrome P-450 substrates which stimulate either their own metabolism or the metabolism of other drugs or endogenous steroids (McElnay & D'Arcy, 1980). It is believed by some investigators that induction of drug metabolism in man does not play a significant role in human drug therapy since its demonstration frequently necessitates administration of high doses (Hunter & Chasseaud, 1976; Remmer et al, 1979). However, numerous examples exist to the contrary, in which enhanced oxidation of drugs by the liver, and their accelerated clearance from the body, have been demonstrated after administration of cytochrome P-450 inducers. For example, in vivo treatment with phenobarbital stimulates the metabolism of phenytoin (Cucinell et al, 1965) and oral estrogens (Janz & Schmidt, 1975). In humans, alcohol intake has also been shown to enhance the

metabolism of phenytoin, tolbutamide and warfarin, three drugs with a relatively narrow margin of safety (Kater et al, 1969). Since both ethanol and phenobarbital moderately stimulate intestinal MFO activity of rats (Remmer et al, 1979; Lehrman et al, 1973; Stohs et al, 1976b), their enhancement of drug clearance due to induction of intestinal enzymes may be significant. The contribution of the intestinal MFO system to the first pass metabolism of warfarin, tolbutamide, and phenytoin has not been documented though (Andreasen & Vesell, 1974). Significantly stimulated oxidation of both warfarin (O'Reilly, 1975) and digitoxin (Peters et al, 1974), another orally administered drug with a low margin of safety, have also been observed clinically during oral rifampin intake. From this evidence, it may be inferred that stimulation of intestinal drug oxidations by concurrent administration of inducers of intestinal cytochrome P-450 could lead to clinically significant alterations in drug disposition.

Induction by orally administered therapeutic agents such as phenobarbital, rifampin, or phenytoin may alter not only the disposition of drugs, but the metabolism of endogenous compounds such as estrogens (Levin et al, 1974), cortisol (Werk et al, 1971), and vitamin D_3 (Hunter, 1976) as well. Moreover, stimulation of hepatic oxidative metabolism by these agents has been shown to alter the physiological actions of hormones such as estrogens. Both estrogens and cortisol undergo cytochrome P-450-dependent oxidation in the intestinal mucosa (Hartiala, 1973), but the physiological importance of this is unclear. Thus, induction of intestinal cytochrome P-450 may lead to significantly enhanced oxidation and subsequent inactivation of endogenous steroids.

Metabolism and clearance of orally ingested drugs in the intestinal

mucosa are stimulated not only by co-administered drug-substrates of intestinal cytochrome P-450, but orally ingested and inhaled environmental xenobiotics. For example, cigarette smoke, which contains up to 500 polycyclic aromatic hydrocarbons (PAH), including benzo(a)pyrene (BaP), a known inducer of intestinal cytochrome P-450 (Wattenberg et al, 1962; Lake et al, 1973), markedly stimulates cytochrome P-450-dependent MFO oxidation of phenacetin (Uotila & Marniemi, 1976; Kuntzman et al, 1977), pentazocine (Kerri-Szanto & Pomeroy, 1971), acetaminophen, and the procarcinogen BaP (Welch et al, 1972), in the intestinal mucosa of humans and animals. Cigarette smoking has also been reported to diminish the therapeutic effectiveness of chlorpromazine, diazepam, and chlordiazepoxide in humans (Miller, 1977), presumably by stimulation of their hepatic and/or intestinal metabolism by cytochrome P-450 (Table 1). The ingestion of charcoal broiled beef, which also contains BaP, has been shown to stimulate the first pass oxidation of orally ingested phenacetin, antipyrine, and theophylline in man in vivo (Pantuck et al, 1976a; Kappus et al, 1978). Moreover, ingestion of charcoal broiled beef likewise stimulates cytochrome P-450-dependent oxidation of phenacetin in vitro in intestinal mucosa of rats, which suggests that the enhanced first pass metabolism of these drugs in man is mediated primarily by the intestinal mucosa (Pantuck et al, 1975). In addition, the PAH's 2,3,7,8-tetrachlorodibenzo-ρ-dioxin and 3-methylcholanthrene have been shown to enhance the in vitro intestinal oxidation of BaP (Hook et al, 1975; Gelboin & Blackburn, 1964), thereby potentially altering its carcinogenicity (Section 2.6.2.4).

The quantitative contribution of intestinal metabolism to the in vivo clearance of many of the compounds mentioned above, including

BaP, has not been completely evaluated, though reliable estimates may be derived from in vitro studies (Table 1). There are numerous differences between the above studies in duration, and route of drug administration, which may lead to inaccurate estimates of the contribution of the intestinal mucosa to the metabolism of these compounds. Furthermore, the extent of induction of the intestinal MFO system is dependent upon the route of xenobiotic administration, the oral route permitting a greater inductive capacity than the parenteral one (Hietanen et al, 1980).

Thus, the evidence above suggests that the extent of intestinal cytochrome P-450 dependent oxidative metabolism of orally ingested drugs, carcinogens, and endogenous steroids may be substantial, and further increased by cytochrome P-450 induction. This might be of consequence in drug therapy and in carcinogenesis induced by orally ingested chemicals.

2.6.2.1 Biochemistry of Cytochrome P-450 Induction: Enzyme induction may be defined as an adaptive increase in the number of molecules of a specific enzyme secondary to either an increase in its rate of synthesis or to a decrease in its rate of degradation (Gelehrter, 1976). The mechanism of cytochrome P-450 induction involves many additional processes in the liver, besides induction of the cytochrome, which are not yet fully understood (Bock & Remmer, 1978). Cytochrome P-450 induction is initiated essentially by the intracellular presence of a lipophilic substrate of the enzyme, though glucagon and cyclic AMP have been implicated as secondary messengers in the process (Weiner et al, 1972). Inducers of cytochrome P-450 generally fall into 2 major classes, distinguished by the spectral absorption maximum of the major form of the specific isozyme induced, 450 nm and 448 nm (Lu & West, 1980). In

the case of PAH's, which induce the "P-448" class of cytochrome P-450 isozymes, the mechanism of induction apparently involves binding of the substrate to a cytoplasmic receptor, followed by its internalization into the nucleus. A high affinity cytoplasmic binding protein/receptor for PAH has been demonstrated in livers of "PAH-inducible" mice (Nebert et al, 1975; Poland & Glover, 1975). Genetically "noninducible" mice only exhibit low affinity binding of PAH's to the receptor. Phenobarbital, the prototype inducer of the other major class of cytochrome P-450 isozymes, shows no such receptor binding, nor is there a genetically determined differential response of the enzyme in these mice to phenobarbital induction, as there is to PAH induction. Whether a separate receptor exists for the phenobarbital class of cytochrome P-450 inducers is presently unknown. Following receptor binding of substrate, in general the process of cytochrome P-450 induction involves stimulation of nuclear RNA synthesis and increased apoprotein synthesis. Regulation of the process is believed to occur at either the level of transcription or post-transcription-pre-translation (Gelboin & Whitlock, 1979).

In addition to increased protein synthesis, induction of cytochrome P-450 usually also involves increased synthesis of its prosthetic heme group. Cytochrome P-450 heme synthesis during enzyme induction has been elucidated primarily by studies in livers of phenobarbital-treated animals. Treatment of rats with phenobarbital, or many other lipophilic drugs, results in increased activity of the first and rate limiting enzyme in heme biosynthesis, ALAS (Marver, 1969; De Matteis, 1975), and consequently increased hepatic heme content. However, stimulation of ALAS activity does not appear to be a prerequisite to induction of cytochrome by all drugs (De Matteis & Gibbs, 1972). The mechanism of

stimulated ALAS activity during cytochrome P-450 induction is believed to result from the removal of negative feedback inhibition of heme on ALAS (Section 2.5.1.1). Substrates of cytochrome P-450 that stimulate its synthesis deplete the intracellular free heme pool as the heme is consumed by the newly formed apoprotein to form the holoenzyme de novo. This depletion removes the feedback inhibition of heme on ALAS, thereby enhancing its activity (Granick, 1966). Similarly, agents which cause the loss or breakdown of cytochrome P-450 heme, such as allylisopropylacetamide (Ortiz de Montellano & Mico, 1981), 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (Ortiz de Montellano et al, 1981), or numerous other allyl containing compounds (Levin et al, 1972; Ortiz de Montellano & Kunze, 1980), also result in marked stimulation of ALAS activity and heme synthesis as the heme pool is depleted (De Matteis, 1970; Taub et al, 1976; Granick & Sassa, 1971).

The two enzymes catalyzing the first and last steps in hepatic heme biosynthesis, respectively, possess a number of similarities. Stimulation of ALAS activity by the treatment of animals with phenobarbital begins within 2 hours, and is associated with increased incorporation of glycine into microsomal heme (Baron & Tephly, 1970). The last enzyme in the heme biosynthetic pathway, ferrochelatase, is similarly induced by phenobarbital administration, but follows a slower time course of induction than that of ALAS (Hasegawa et al, 1970). Both of these enzymes are located on the inner membrane of the mitochondrion. ALAS is considered to be the primary rate limiting enzyme in heme biosynthesis, while ferrochelatase may be a secondary limiting enzyme, since depletion of the substrate iron in the ferrochelatase-catalyzed step in protoheme formation apparently limits the enzyme's functional activity (Liem et al,

al, 1969). Since ALAS activity may also be dependent on availability of dietary iron (Liem et al, 1969), it is suggested that both the enzymes are under similar regulatory mechanisms. Regulation of heme synthetic enzymes of the intestinal mucosa have not been investigated. However, on the basis of the evidence that the heme moiety of intestinal cytochrome P-450, in contrast to that of the hepatic system, may be made rapidly limiting by intraluminal iron deprivation (Hoensch et al, 1976), and coupled with the fact that intraluminal heme is absorbed intact into the mucosa (Wheby et al, 1970), it may be inferred that the intestinal cytochrome P-450 heme moiety has a rapid turnover. Therefore, heme synthesis in the intestinal mucosa may be more sensitive to potential regulators than that in the liver.

Not only is cytochrome P-450 heme synthesis stimulated during cytochrome P-450 induction, but the absorption of intact utilizable heme from the intestinal lumen might also be stimulated during phenobarbital-mediated enzyme induction (Thomas et al, 1972). This has not been investigated during enzyme induction by PAH's, the most potent inducers of intestinal cytochrome P-450 (Stohs et al, 1976b). Mucosal heme, absorbed due to phenobarbital treatment, is presumably used for intestinal or hepatic hemoprotein formation, though it may also be rapidly degraded in the mucosal cells to release its iron component for subsequent new intestinal or hepatic heme synthesis (Raffin et al, 1974).

It is not yet clear whether or how the processes of apocytochrome and heme synthesis in the liver are coordinated. Based on evidence of enhanced synthesis of both hepatic protein and heme during induction of the holocytochrome P-450 (Gelboin & Blackburn, 1963; Orrenius et al, 1965; Omura et al, 1967; Jacob et al, 1974), it has been postulated

that the synthesis of cytochrome P-450 apoprotein and heme in the liver might be concerted (Marver et al, 1966). Failure of administered heme or heme precursors, such as ALA, to increase the synthesis of hepatic cytochrome P-450 (Meyer & Marver, 1971b), suggested that increasing the heme content alone was not sufficient to increase holocytochrome levels. Additionally, the generation of a pool of "free" apocytochrome, created by cobalt-mediated inhibition of heme synthesis coupled with phenobarbital-mediated stimulation of apoprotein synthesis (Correia & Meyer, 1975), suggested that synthesis of the two moieties could be dissociated. Whether intestinal cytochrome P-450 synthesis behaves similarly to that of the liver is not known. However, as will be further discussed (Section 2.6.3.1), the small intestinal mucosa offers an excellent model to probe the interdependence of cytochrome P-450 heme and apocytochrome synthesis, since the synthesis of the two moieties may be dissociated by dietary deprivation of the prosthetic element iron to inhibit intestinal heme formation. Dissociation of the synthesis of the two moieties in this manner is difficult to institute in the liver (Liem et al, 1969).

2.6.2.2 Multiplicity of Intestinal Cytochrome P-450 During the Induced State: Studies on isolation, purification, and characterization of microsomal cytochrome P-450 in rat liver have demonstrated the multiplicity of the hepatic isozymes (Lu & West, 1980). Initial studies on the differential induction of intestinal cytochrome P-450 isozymes by 3-methylcholanthrene (3-MC) and phenobarbital, and the blue shift in the CO-difference spectra of reduced cytochrome from 450 nm to 448 nm ("P-448") after 3-MC induction, suggested the presence of at least 2 different cytochrome P-450 isozymes in the intestinal mucosa. Further-

more, substrate specificities and binding spectra of induced intestinal isozymes were markedly different from those of the induced hepatic isozymes (Stohs et al, 1976a; Shirkey et al, 1979b). Fractionation and purification of intestinal enzymes have recently revealed three isozymes of cytochrome P-450 in the rabbit intestine (Ichihara et al, 1980; 1981). Although they exhibit marked differences in their fractional content and in their individual rates of fatty acid oxidation and aromatic and aliphatic hydrocarbon hydroxylation, all three in the reduced form exhibited a CO-induced difference spectrum with an absorption maximum at 450 nm. Apparently, no cytochrome spectrally detectable at 448 nm was isolated by their techniques. The animal diets used in these studies were not specified, and since the fraction pertaining to each isozyme varies with the dietary content of cytochrome P-450 inducers, the isozyme fractions identified in that study may be the result of the specific diet used, and therefore may not be representative of the pattern of isozymes specific for the non-induced intestinal cytochrome.

2.6.2.3 Induction of Intestinal Cytochrome P-450 by Dietary Constituents: One of the most striking features of intestinal cytochrome P-450 induction is its sensitivity to dietary inducers. Subtle changes in dietary composition of different commercial chows is sufficient to alter intestinal MFO activity in rats (Hietanen & Hanninen, 1974). In contrast, the hepatic enzyme system does not respond appreciably to compositional changes in standard rat chow (Abbott et al, 1975), and in fact such response requires much larger doses of the inducers (Wattenberg & Leong, 1970; Remmer et al, 1979). For example, induction of hepatic cytochrome P-450 dependent AHH requires a 25 fold higher levels of orally administered PAH's than the intestinal isozyme (Aitio, 1974).

Marked decreases in cytochrome content and activity after the replacement of laboratory rat chow with a purified casein-based semi-synthetic diet (Wattenberg, 1971; Pantuck et al, 1975) suggest that the vegetable constituents of rat chow appear to be solely responsible for maintenance of intestinal cytochrome P-450 content and its dependent MFO activity in a continuously induced state. The original assumption that intestinal cytochrome P-450 existed solely as the P-448 isozyme (Zampaglione & Mannering, 1973) was based on spectral observations with cytochrome P-450 partially induced by vegetable components of the rat chow. That authentic constitutive cytochrome P-450 is indeed present in the absence of such vegetable-derived inducers has been demonstrated repeatedly in more recent reports (Hoensch et al, 1976; Stohs et al, 1976a). Standard rat chow contains alfalfa as the major vegetable product, which by itself stimulates intestinal MFO activity (Wattenberg, 1971). Numerous other vegetables have also been shown to stimulate the intestinal MFO system, including various members (Brussel sprouts, cabbage, etc.) of the Brassicaceae family (Pantuck et al, 1976b). Several indoles isolated from these plants were shown to be responsible for most of the inductive effect (Loub et al, 1975). Additional chemicals capable of inducing intestinal cytochrome P-450 have been isolated from dietary vegetable material. They include non-hydrogenated flavones, particularly tangeretin, nobiletin, and quercetin (Wattenberg et al, 1968; Wattenberg & Leong, 1970). Thus, it can be seen that intestinal cytochrome P-450 is not only much more sensitive to induction by PAH's than the hepatic isozyme, but is also very sensitive to naturally occuring chemicals in vegetable based diets. This is in contrast to the complete lack of sensitivity of the hepatic isozyme to natural dietary inducers.

The higher inducibility of the intestinal enzyme by xenobiotics in the intestinal lumen may reflect its role as a primary defense against orally ingested carcinogens and toxins. The hepatic enzyme system is then rallied as a secondary defense to cope with drugs and environmental xenobiotics that have escaped initial clearance by intestinal first pass metabolism.

2.6.2.4 Relation of the PAH-Induced Intestinal MFO System to Chemical Carcinogenicity: PAH's and flavones make up the primary class of cytochrome P-450 inducers to which the intestinal isozyme responds (Stohs et al, 1976b). The class of inducers of which phenobarbital is the prototype stimulates intestinal enzyme activity to a much lesser extent (Scharf & Ullrich, 1974; Stohs et al, 1976b). The synthetic flavone, β-naphthoflavone (β-NF), is the most potent inducer of intestinal cytochrome P-450 (Wattenberg et al, 1968). There is evidence to suggest that the flavone components of vegetable based diets, which stimulate intestinal MFO activity, act as inhibitors of chemical carcinogenesis in vivo; as such they have been implicated in the low incidence of colon cancer in vegetarians (Phillips, 1975). This may be of considerable importance since colon cancer incidence is presently rising in historically low risk populations (Berg et al, 1973). Additional evidence in support of anti-tumorigenesis of dietary flavones are the findings that feeding a β-NF-supplemented diet to mice inhibited by almost 100% and 50% respectively, the pulmonary adenomas and epidermal neoplasia induced by oral and topical administration of BaP (Wattenberg & Leong, 1970). Only flavones that possess MFO inducing ability demonstrate inhibition of chemical carcinogenesis. Furthermore, chemically induced neoplasia in other organs such as liver, breast, and lung have been prevented by

concurrent administration of β-NF or PAH inducers of intestinal MFO (Wattenberg, 1975). Natural (plant) indoles and sterols, inducers of intestinal MFO have also been shown to inhibit colon tumor formation in experimental animals (Wattenberg & Loub, 1978; Raicht et al, 1980). The inhibition of chemical-induced carcinogensis in vivo by treatment with cytochrome P-450 inducers is specific for the PAH class of inducers. In fact, phenobarbital-treatment has been found to have a "promoting" effect on chemical tumorigenesis (Peraino et al, 1977; Kitagawa et al, 1979).

In addition to detoxification, cytochrome P-450-dependent MFO is also capable of converting many chemical procarcinogens in vitro to highly reactive oxidative metabolites, their ultimate carcinogenic forms (Gelboin et al, 1972; Legraverand et al, 1980), which are capable of binding essential macromolecules such as DNA, RNA, or protein (Weinstein, 1978). For example, the procarcinogen BaP must first be converted to its oxidized ultimate carcinogenic form (Section 2.2) before its in vitro mutagenicity, high chemical reactivity, and consequent binding to tissue macromolecules, is observed (Conney et al, 1978). Lipophilic compounds which stimulate cytochrome P-450-dependent oxidation of BaP also increase its in vitro conversion to the ultimate carcinogen. However in vivo, additional mechanisms to detoxify oxidized metabolites occur, principally biotransformations mediated by conjugating enzymes such as glucuronyl, glutathione, and sulpho- transferases, and epoxide hydratase. Furthermore, reactive oxygen species formed as byproducts during metabolism of chemical carcinogens, which also have the potential to damage intracellular macromolecules, are scavenged by specific enzymes (superoxide dismutase, selenium-dependent glutathione peroxidase, catalase) or anti-

oxidants (vitamin E). It is believed that these reactions normally balance the activation reactions of the MFO system resulting in eventual detoxification of the parent compound and its metabolic byproducts (Wattenberg, 1978). Because dietary cytochrome P-450 inducers reduce the incidence of chemical carcinogenesis, it is believed that induction of intestinal MFO, by enhancing xenobiotic detoxification, may serve as a primary protective mechanism against chemical insult to man and animals. In addition, it is also proposed that intraluminal carcinogens that escape complete biotransformation and detoxification in the intestinal mucosa, may contribute to a potential carcinogenic scenario in the colon (Hoensch & Hartman, 1981). Biotransformation activity is low in the colon compared to that in the small intestine (Fang & Strobel, 1978), and transit time is markedly less, allowing greater residence time in the colon for unmetabolized or incompletely metabolized carcinogens.

2.6.3 Regulation of Intestinal Cytochrome P-450 by Dietary Nutrients: More surprising than the susceptibility of intestinal cytochrome P-450 to induction by natural dietary constituents, is the sensitivity of the basal constitutive (non-induced) enzyme to dietary nutrients. Nutritional status of animals is known to affect drug and carcinogen metabolism, specifically the hepatic and intestinal cytochrome P-450 system (Campbell & Hayes, 1974; Campbell, 1977; Conney et al, 1977a,b). Acute dietary deprivation of folic acid (Clement et al, 1981), fat (Edes et al, 1979; Laitinen, 1976), and amino acids (Wattenberg et al, 1962) have been shown to lower intestinal cytochrome P-450 dependent MFO activity. Although acute dietary deprivation of fat or protein will not elicit similar responses in the liver, they can be demonstrated by chronic deprivation (Campbell & Hayes, 1974). Thus, not only is the intestinal

enzyme system more sensitive to dietary nutrient deprivation than the hepatic system, but findings that chronic dietary deprivation of either vitamins A or C diminishes hepatic MFO activity but not that of the intestinal enzyme (Miranda et al, 1979; Kuenzig et al, 1977), further suggest that the mechanisms of regulation of the two systems by dietary nutrients are not necessarily similar.

2.6.3.1 Iron: The only reported dietary mineral dependency of the intestinal MFO system is that of iron. Cytochrome P-450 and its dependent MFO activity decrease markedly in the intestinal mucosa of rats fed an iron deficient diet for as short a time as 24 hours (Hoensch et al, 1975; 1976). This acute dependency on iron is restricted to intraluminal iron, since parenteral iron supplementation is without effect on the intestinal enzyme system. The effect is more prominently seen in villous tip cells than crypt cells (Hoensch et al, 1976), and reflects the dependence of the enzyme system in the cells primarily committed to absorption of xenobiotics from the intestinal lumen. Since iron is the prosthetic element of the heme moiety of cytochrome P-450, this finding indicates that the enzyme present at the site of xenobiotic absorption, and thus mainly responsible for their oxidation, critically depends on an exogenous source of its own prosthetic element, rather than on body iron stores. Because of this requirement, dietary iron deficiency may lead to alterations in intestinal metabolism of drugs and thus in their clinical effectiveness.

The intestinal MFO system is much more sensitive to dietary iron deficiency than is the hepatic system. Studies of chronic iron deficiency in rats have yielded equivocal findings of its effects on in vivo and in vitro hepatic drug metabolism; both increases and decreases in

drug metabolism have been reported, though hepatic cytochrome P-450 content has generally remained unaltered (Catz et al, 1970; Becking, 1972; Sweeney et al, 1979). Dependency of hepatic cytochrome P-450 on iron has been experimentally elicited in rats only by chronic dietary iron deprivation coupled with in vivo iron chelation with desferrioxamine (White et al, 1978b). Inability of chronic dietary iron deficiency to alter hepatic cytochrome P-450 without additional chelation indicates the conservativeness of hepatic microsomal iron stores (Dallman & Goodman, 1971). This conservation is in sharp contrast to the immediate diminution of intestinal cytochrome P-450 content and its dependent MFO activity provoked by acute dietary iron deficiency (Hoensch et al, 1976).

Most studies on drugs extensively metabolized by the hepatic MFO system have failed to detect in vivo alterations in their metabolism in humans during iron deficiency (O'Malley & Stevenson, 1973). Iron deficiency does appear to adversely affect in vivo drug metabolism in humans since the plasma elimination half life of warfarin was significantly increased in an iron deficient patient (Nicholls & Shepard, 1978). Though it is not known whether warfarin undergoes intestinal first pass metabolism, it is both extensively oxidized by hepatic cytochrome P-450 and orally administered therapeutically (O'Reilly & Aggeler, 1970). It is therefore possible that, during such administration, warfarin is oxidized by the intestinal cytochrome P-450 MFO system during its first pass. During severe iron deficiency, alterations in the intestinal cytochrome P-450-dependent metabolism of warfarin may contribute to, or be responsible for, its altered disposition (Nicholls & Shepard, 1978). Indeed, such alterations may be critical for drugs which undergo extensive intestinal first pass metabolism (Table 1). Such drugs would be

expected to exhibit increased plasma concentrations and/or pharmacological responsiveness if their intestinal metabolism was impaired by dietary iron deficiency. Together this evidence indicates that dietary iron deprivation results in alterations of the intestinal cytochrome P-450-dependent drug oxidations both *in vitro* and *in vivo*, and that iron deficiency may result in observable alterations in intestinal cytochrome P-450-dependent drug metabolism in humans. It may be of further interest to note that the Plummer-Vinson syndrome, an iron deficiency anemia seen in women (Chisholm *et al*, 1971), is associated with cancer of the esophageal mucosa (Wynder *et al*, 1957). Altered metabolism of carcinogens in the esophageal mucosa by iron deficiency has been implicated as an etiological factor (Hoensch & Hartman, 1981).

The sensitivity of intestinal cytochrome P-450 to dietary iron has been postulated to be intimately related to the intestinal control of dietary iron absorption (Hegenauer *et al*, 1977). The absorption of dietary iron is physiologically related to body iron need for maintenance of internal iron homeostasis, and is adaptively increased during dietary iron deficiency in both man and animals (Conrad *et al*, 1966a, 1967; Wheby, 1970). It is generally agreed that dietary iron absorption is regulated by a mucosal factor directly related to the content of *mucosal* iron (Pearson *et al*, 1967), rather than to whole body iron content (Pollack *et al*, 1964; Turnbull, 1974). It is of further interest to note that cytochrome P-450-dependent MFO activity and mucosal absorption of both iron and heme are maximal in the proximal one-third of the small intestine (Hoensch *et al*, 1976; Manis & Kim, 1979a; Raffin *et al*, 1974). In addition, induction of intestinal cytochrome P-450 by PAH's and phenobarbital is paralleled by simultaneous stimulation of iron

absorption in the duodenal mucosa (Manis & Kim, 1979a,b, 1980; Thomas et al, 1972). Such a finding lends support to the hypothesis that intestinal cytochrome P-450 may be involved in the control of dietary iron absorption (Hegenauer et al, 1977).

2.6.3.2 Selenium: To date, no reports of the interaction of the intestinal cytochrome P-450-dependent MFO system and dietary selenium are available. Selenium was first suspected to be involved in the hepatic microsomal MFO system as part of a non-heme iron protein, analogous to adrenodoxin of the adrenal mitochondrial MFO system (Diplock & Lucy, 1973). It was believed to function in the transfer of electrons from NADPH-cytochrome P-450 reductase to cytochrome P-450 (Estabrook, 1971). But no evidence for non-heme iron protein in microsomes other than ferritin has been found (Masters et al, 1971), and recent reconstitution studies of hepatic cytochrome P-450 MFO using highly purified NADPH-cytochrome P-450 reductase and cytochrome P-450 show no such requirement for an additional factor for cytochrome P-450-mediated oxidation (van der Hoeven et al, 1974). Since the first report of impaired phenobarbital-mediated induction of hepatic cytochrome P-450 and cytochrome b_5 in selenium deficient rats (Burk et al, 1974), numerous reports have attempted to decifer the biochemical function of selenium in the cytochrome P-450 MFO system. Subsequently, neither cytochrome b_5 nor cytochrome P-448 induction by 3-MC have been shown to be affected by chronic dietary selenium deprivation (Burk & Masters, 1975), indicating that the effect of selenium deficiency is specific for the phenobarbital-inducible hepatic isozyme. Although incorporation of the heme precursor ALA into hepatic microsomal heme has been reported to be impaired by dietary selenium deprivation after phenobarbital treatment (MacKinnon & Simon, 1976),

dietary selenium deprivation was not found to result in impairment of heme synthesis upon further investigation (Correia & Burk, 1976; Burk & Correia, 1977). In addition, the activity of two potentially rate limiting enzymes in cytochrome P-450 heme biosynthesis, ALAS and ferrochelatase, were also reported not to be impaired by chronic dietary selenium deprivation after phenobarbital induction of cytochrome P-450 (Burk & Correia, 1977). However, these authors did report a possible, albeit non-significant (27%), decrease in hepatic ferrochelatase activity in untreated selenium deficient rats, relative to that in selenium-adequate rats. Furthermore significant increases in urinary coproporphyrin excretion in untreated and phenobarbital-treated selenium-deficient rats were observed, suggesting a block at or beyond coproporphyrinogen oxidase in the heme synthetic pathway. The variability in the data of the porphyrin excretion products precluded any conclusions about the involvement of selenium in heme biosynthesis though (Burk & Correia, 1977). The impairment of hepatic cytochrome P-450 induction during chronic selenium deprivation has been hypothesized to be due to a lack or deficiency of a selenium-dependent factor involved in the utilization of heme for holocytochrome P-450 formation during phenobarbital induction. It has been shown that hepatic heme, rapidly synthesized during phenobarbital-mediated induction of cytochrome P-450, fails to associate with the induced apocytochrome P-450 in selenium-deficient rats, and subsequently stimulates its own degradation by inducing MHO (Correia & Burk, 1978).

In addition to phenobarbital-induced cytochrome P-450, the cytoplasmic hemoprotein tryptophan pyrrolase is also dependent on selenium for its maximal assembly (Correia & Burk, 1978). This dependency on

selenium may thus be specific for hemoproteins with rapid turnover characteristics, such as phenobarbital-induced hepatic cytochrome P-450 (Levin & Kuntzman, 1969a) and tryptophan pyrrolase (Badawy & Evans, 1975). Similarly, nonproliferating cultured rat hepatocytes have been shown to require selenium in the culture media for de novo synthesis of phenobarbital-induced cytochrome P-450 apoprotein (Newman & Guzelian, 1982). The slower turning over 3-MC-inducible form of hepatic cytochrome P-448 (Levin & Kuntzman, 1969b) shows no such impaired formation in selenium-deficient rats (Correia & Burk, 1978). Moreover, the slow turnover constitutive form of hepatic cytochrome P-450 was not dependent on selenium either (Burk & Masters, 1975; Newman & Guzelian, 1982). These findings indicate that cells or tissues with rapidly turning over cytochrome P-450, are critically dependent on intracellular selenium for maintenance of the enzyme. Since intestinal cytochrome P-450 possesses rapid turnover characteristics, (Hoensch et al, 1976), it is possible that it too might exhibit similar requirements for dietary or intracellular selenium.

2.6.3.3 Relation of Selenium to Lipid Peroxidation and Cytochrome P-450 Stability: Assessment of the effects of selenium deficiency on membrane bound enzymes, such as cytochrome P-450, must include examination of the role of selenium in protection of such membranes. Selenium is the prosthetic element of the cytosolic enzyme, glutathione (GSH) peroxidase (Oh et al, 1974; Hafeman et al, 1974). Selenium-dependent GSH peroxidase is believed to play a role in the destruction and removal of hydrogen peroxides and lipid hydroperoxides generated in vivo (Figure 3), while non-selenium-dependent GSH peroxidase is apparently responsible for the breakdown of only lipid hydroperoxides (Tappel, 1965; Hoekstra,

1975; Lawrence & Burk, 1976). Hydrogen peroxide has the potential to initiate formation of lipid hydroperoxides, so it is possible that in the absence of selenium-dependent GSH peroxidase, such hydroperoxides generated in vivo could accumulate. Lipid hydroperoxides have been shown to destroy membrane bound hepatic cytochrome P-450 both in vitro and in vivo (Hrycay & O'Brien, 1971; Levin et al, 1973; Schacter et al, 1973; De Matteis & Sparks, 1973). But the impairment of phenobarbital-mediated induction of hepatic cytochrome P-450 by chronic selenium deprivation was dissociated from the concomitant reduction of hepatic selenium-dependent GSH peroxidase activity (Correia & Burk, 1978), thus excluding the possibility that lipid peroxidation was responsible for the impaired cytochrome P-450 induction (Correia & Burk, 1978).

Selenium-dependent GSH peroxidase in the intestine has received far less attention. Its activity in rat intestinal mucosa markedly decreases after a 2 week feeding of a selenium-deficient diet (Reddy & Tappel, 1974). This is in contrast to the much more stringent and prolonged (8-12 weeks post-weaning) dietary restriction of selenium required to produce comparable decreases in hepatic GSH peroxidase activity (Correia & Burk, 1978). From this, it may be inferred that the dependence of the enterocyte on dietary selenium is much more acute than that of hepatocytes. Intestinal selenium-dependent GSH peroxidase, in contrast to the hepatic enzyme, may thus derive its selenium requiremnt directly from the diet rather than from bodily stores.

2.6.3.4 Selenium-Dependent Inhibition of Chemical Carcinogenesis: Numerous reports exist that dietary selenium resupplementation inhibits chemically induced neoplasias, including dimethylhydrazine (DMH)-induced colon cancer and dimethybenz(a)anthracene-induced breast cancer in sele-

nium-deprived rats (Gariola & Chow, 1982; Thompson & Becci, 1979; Ip, 1981; Jacobs, 1977a). Additionally, selenium supplementation in S-9 fractions of the Ames bioassay for 2-acetylaminofluorene (2-AAF)-induced mutagenicity has resulted in inhibition of the in vitro mutagenic activation. More importantly, dietary selenium supplementation to rats during tumor induction with 2-AAF also inhibits the incidence of tumor formation (Jacobs et al, 1977b; Marshall et al, 1979). The mechanism of such selenium-dependent inhibition of chemical carcinogenicity is presently unknown. Leading hypotheses contend either a) the involvement of selenium-dependent alterations in cytochrome P-450-dependent oxidation of carcinogens, since both DMH and 2-AAF are metabolicly activated to their proximate or ultimate carcinogenic species by the hepatic cytochrome P-450-dependent MFO system (Malejka-Giganti et al, 1978); or b) enhanced intracellular oxidative damage of macromolecules, such as single strand DNA breaks, by accumulated peroxides or hydroxy radicals, secondary to diminished selenium-dependent GSH peroxidase activity following dietary selenium deprivation (Wortzman et al, 1980). Moreover, lipoperoxides accumulated in the epithelial cells of small intestinal mucosa of rats deprived of dietary selenium may, along with the cells, shed into the intestinal lumen and become lodged in the colon. The slow transit time of the colon may in turn increase their residence time, and promote a carcinogenic scenario similar to that postulated for incompletely metabolized chemical carcinogens (Hoensch & Hartman, 1981).

2.6.4 Endogenous Regulation of Intestinal Cytochrome P-450: Intestinal cytochrome P-450 and its dependent MFO activity are maintained at low albeit detectable levels in the absence of dietary inducers and enzyme regulators such as dietary iron or fat (Section 2.6.3). Possibly, since

cytochrome P-450 is a substrate-inducible enzyme, the basal level of the constitutive form of the intestinal enzyme is maintained by endogenous substrates such as fatty acids, prostaglandins (Ichihara et al, 1979; Craven & De Rubertis, 1980), or steroid hormones (Hartiala, 1973; Al-Turk et al, 1980b), as indeed proposed for that of the liver (Kupfer, 1980; Hall, 1982).

In addition, the constitutive form of intestinal cytochrome P-450 and its basal MFO activity may be under the regulation of endogenous peptide hormones. For example, insulin treatment of streptozotocin-induced diabetic rats reverses a noticeable increase in intestinal microsomal cytochrome P-450 content and activity resulting from the diabetic state (Al-Turk et al, 1980a; Stohs et al, 1979). In contrast, in vitro studies with the liver enzyme from diabetic rats indicated equivocal changes in rates of drug metabolism (Dixon et al, 1961; Kato & Gillette, 1965; Ackerman & Liebman, 1977). Evidence that insulin may modulate hepatic cytochrome P-450 heme synthesis is equally less striking in that insulin has been described as having a "permissive" effect on the stimulation of hepatic ALAS activity, following steroid induction or following allylisopropylacetamide-induced heme depletion in hepatocyte cultures (Sassa & Kappas, 1977; Sassa et al, 1979). The significance of these findings is at present unclear.

The only truly unequivocal example of peptide hormone regulation of cytochrome P-450 and its dependent MFO activity is that of the adrenocorticotropic hormone (ACTH) in rat adrenal. Both microsomal and mitochondrial cytochrome P-450 and their dependent MFO activities in rat adrenal are maintained by ACTH administration after hypophysectomy (Purvis et al, 1973; Brownie et al, 1973). Furthermore, the activity

of ALAS, the rate limiting enzyme in cytochrome P-450 heme biosynthesis, is stimulated in rat adrenals by ACTH (Condie et al, 1976). Since no action in other organs was noted, this effect is apparently specific to the target tissue for the trophic action of the hormone (Idelman, 1970).

In analogy to the trophic action of ACTH in the adrenals, the structural and functional integrity of the small intestinal mucosa is maintained by the trophic action of the peptide hormone gastrin (Johnson, 1976). Both gastrin and its pentapeptide analogue, pentagastrin (Barrett, 1966), stimulate cell growth (Mayston & Barrowman, 1971; Mak & Chang, 1976) and exert a "pleiotypic response" in the intestinal mucosa, which includes the stimulation of DNA, RNA, and protein synthesis (Johnson & Chandler, 1973; Lichtenberger et al, 1973; Johnson & Guthrie, 1974; Johnson & Guthrie, 1976; Chandler & Johnson, 1972; Johnson et al, 1969a,b; Enochs & Johnson, 1974; Walsh & Grossman, 1975). This trophic role for gastrin is supported by the fact that in experiments to control for the deleterious effects of starvation on intestinal mucosa (Steiner et al, 1968), replacement of oral feeding, known to stimulate gastrin release, with intravenous nutritional hyperalimentation markedly decreased not only gastrin release from the gastric antrum, but duodenal mucosal weight, and mucosal disaccharidase activities as well (Johnson et al, 1975a). These effects were partially reversed by pentagastrin infusion (Johnson et al, 1975b), proving not only that gastrin is the sole direct mediator of the trophic action of the foodstuffs in the intestinal lumen, but also indicating that the synthetic pentapeptide could be substituted for gastrin. This trophic effect was specific for the duodenal mucosa; no action was noted in liver, skeletal muscle, kidney, spleen, or testes (Johnson et al, 1969b; Mayston & Barrowman, 1971; Johnson & Guthrie,

1974; Chandler & Johnson, 1972; Johnson et al, 1975b). These findings indicate that endogenous gastrin serves to finely regulate intestinal structure and function through maintenance of structural protein and enzyme activities. Since other intestinal peptide hormones such as cholecystokinin, secretin, V.I.P., and motilin show no such trophism for the intestinal mucosa, it could be expected that, in analogy to the actions of ACTH on the adrenal cytochrome P-450 MFO system, gastrin/pentagastrin could in addition modulate the cytochrome P-450 MFO system in its target organ, the small intestinal mucosa. This has not yet been investigated.

3.0 PROPOSAL

The small intestinal mucosa appears to be involved in the disposition of numerous exogenous and endogenous substances. For example, the intestinal mixed function oxidase (MFO) system, plays a significant role in the biotransformation of many drugs, exogenous toxins and carcinogens (Table 1), as well as in the metabolism of certain endogenous lipophilic compounds (e.g. steroids, fatty acids).

The role of the intestinal MFO system in xenobiotic oxidation has been identified and characterized to some extent (BACKGROUND, Section 2.2). More recent work has been focused however, on the regulation of such metabolism by a variety of exogenous factors and their impact on the metabolic profile and biologic effects of xenobiotics. Changes in the "first pass" metabolism of a particular xenobiotic during its absorption across the intestinal mucosa can apparently alter the relative concentrations of the parent compound and its metabolites in the blood, and can consequently affect their biological activity. For example, the presence of natural dietary inducers of intestinal cytochrome P-450, or absence or deficiency of certain dietary nutrients, have been shown to alter the metabolic profile of a number of drugs and carcinogens (BACKGROUND, Section 2.6). This alteration in metabolism of any given xenobiotic may or may not be beneficial to the host. Thus, the type of modification, e.g., induction or repression of a particular cytochrome P-450 isozyme, and the specific alterations inflicted metabolically on the chemical structure of the xenobiotic, determine to a large extent whether the xenobiotic is detoxified or activated (BACKGROUND, Sections 2.2 and 2.6.2). An understanding of the biochemical mechanisms underlying such regulation of the intestinal enzyme system thus appears

essential for prediction of the metabolic fate of ingested xenobiotics, and for modulation of their biological response.

Stimulation of intestinal cytochrome P-450-dependent MFO activity by exogenous chemical inducers and natural dietary inducers has been amply documented (BACKGROUND, Section 2.6.2). On the other hand, the effects of dietary nutrients (essential minerals and vitamins) on the intestinal MFO system has been explored to a much lesser extent (BACKGROUND, Section 2.6.3). The only nutritional mineral deficiency reported to affect the intestinal cytochrome P-450 system is that of iron. Its dietary deprivation results in a marked diminution of cytochrome P-450 content and its dependent MFO activity in villous tip cells (Hoensch et al, 1976). The mechanism underlying such regulation by dietary iron has not been elucidated.

It thus appears that intestinal oxidations of xenobiotics by the MFO system may be largely regulated by modulating the content of cytochrome P-450, the terminal oxidase of the MFO system. This is largely brought about by altering its turnover, i.e., synthesis and/or degradation (BACKGROUND, Section 2.4 and 2.6.1). However, the turnover characteristics of intestinal cytochrome P-450, in contrast to that of the hepatic isozyme, have not been previously examined.

The constitutive form of intestinal cytochrome P-450 appears to exhibit rapid turnover characteristics, since it rapidly declines during dietary iron deprivation and is equally rapidly restored upon dietary iron resupplementation (Hoensch et al, 1976). In this respect, intestinal cytochrome P-450 resembles the rapidly turning over phenobarbital-induced hepatic isozyme, which is dependent on dietary selenium (Correia & Burk, 1978). Moreover, in primary non-proliferating cultures of rat

hepatocytes, it has been shown that the phenobarbital-induced apocytochrome is dependent on selenium content of the media (Newman & Guzelian, 1982). Since the enterocyte, because of its dependence on the intraluminal environment, ostensibly resembles a cell in culture, it remains to be determined whether the formation of the intestinal isozyme, or the synthesis of its heme or apoprotein moieties, exhibits a similar dependency on dietary selenium.

In addition, since cytochrome P-450 occurs, albeit to a quantitatively different extent, in both villous tip and crypt cells, it remains to be determined where specifically the synthesis, assembly, and degradation of its structural components, heme and apocytochrome P-450, occur, and what factors modulate enzyme turnover in the individual cell populations. Since the two cell types are structurally and functionally different, and are exposed to different pericellular environments, it is possible that cytochrome P-450 turnover may be regulated differently in each of these cell types.

The observation that the intestinal enzyme system is maintained at detectable levels in the absence of dietary inducers and nutrients suggests that its activity is regulated by endogenous factors which may include the gastrointestinal peptide hormones. This possibility is further suggested by the fact that ACTH regulates microsomal cytochrome P-450 content and activity in its target tissue, the adrenal. Whether intestinal cytochrome P-450 MFO activity is similarly regulated by gastrin, the trophic hormone of the gastrointestinal tract (BACKGROUND, Section 2.6.4), is not known.

Thus, the specific objectives of this investigation are to

1) Characterize the cellular location and quantitate the synthesis

of the heme moiety of intestinal cytochrome P-450; and to determine the cellular location of its assembly with the apocytochrome moiety to form the holoenzyme. This will be examined by assaying the activity of key enzymes involved in intestinal heme synthesis and degradation in the two cell populations. Cytochrome P-450 content and its MFO activity will also be monitored in parallel. Such studies are expected to reveal whether heme required for cytochrome P-450 is synthesized in the villous tip cells which are highly enriched in the cytochrome, or whether it is first synthesized in the crypt cells and assembled to the holoenzyme during their migration to the villous tip.

2) Determine whether the reduction of intestinal cytochrome P-450 content, elicited by dietary iron deprivation, is due to impaired synthesis or accelerated degradation of its heme moiety.

3) Determine whether the apocytochrome moiety of the enzyme is formed in the same mucosal cells where the heme moiety is synthesized, or whether its synthesis is independent of heme synthesis and therefore occurs in a different cell population. This will be examined directly, by monitoring its de novo synthesis, i.e., incorporation of amino acid precursors into the apoprotein; and indirectly, by monitoring holocytochrome increase after the complexation with exogenous heme of "free" apocytochrome generated by the deliberate restriction of heme synthesis.

4) Investigate the effect of dietary iron deprivation on the synthesis of intestinal apocytochrome P-450, using the protocol outlined in 3). This should elucidate whether in addition to affecting the heme moiety, dietary iron deprivation also affects the apoprotein moiety of cytochrome P-450, which together with decreased heme, would thus account for the observed impairment of in vitro intestinal drug metabolism. Moreover,

it will indicate whether, following impairment of heme synthesis, the apocytochrome is formed independently of heme in the intestinal mucosa, or whether the syntheses of the two moieties are coordinated events.

5) Investigate whether the intestinal MFO system is regulated by additional dietary nutrients such as selenium. The effect of selenium deprivation on the synthesis and degradation of the heme moiety of the enzyme will be examined. Similarly, the apocytochrome moiety will be examined for its dependency on dietary selenium, utilizing the protocol outlined in 3). In addition, since selenium deprivation would also lower intestinal GSH peroxidase activity, and consequently raise the intracellular concentration of pernicious peroxides capable of destroying cytochrome P-450, its role in intestinal cytochrome P-450 turnover if any will be assessed.

6) Examine whether intestinal peptide hormones affect intestinal cytochrome P-450 MFO activity, by monitoring the the effect of gastrin on intestinal cytochrome P-450 content and activity.

In summary, the specific cellular localization of intestinal cytochrome P-450 assembly and the biosynthesis of its constitutive moieties will be examined. The dependence of these processes on dietary iron will be scrutinized and the role of dietary selenium in intestinal cytochrome P-450 turnover if any will be investigated. Finally, the potential role of the gastrointestinal peptide hormone gastrin in the regulation of intestinal cytochrome P-450 will also be explored. These studies are expected to elucidate the hitherto unexplored features of intestinal cytochrome P-450 structure and function, and to determine whether an established endogenous peptide regulator of intestinal structure and function is also a regulator of intestinal cytochrome P-450.

4.0 MATERIALS

4.1 CHEMICALS:

Adenosine triphosphate, δ-aminolevulinic acid, 3,4-benzo(a)pyrene, coenzyme A, cysteine, cytochrome c, dithioerythritol, EDTA, glutamine, glutathione, GSH reductase, hemin, histidine, isocitrate, isocitrate dehydrogenase, NADPH, NADH, pyridoxal-5-phosphate, serum albumin (human & bovine), succinate, succinyl thiokinase, Tris buffers, trypsin inhibitor, and umbelliferone were obtained from Sigma Chemical Co., St. Louis, MO. [1,3,6-^{3}H]-Benzo(a)pyrene (NET-572, 50 Ci/mmol), [1,4-^{14}C]-succinic acid (NEC-099, 35.5 mCi/mmol), and [methyl-^{3}H]-thymidine (NET-027, 6.7 Ci/mmol) were obtained from New England Nuclear Co., Boston, Mass. β-Naphthoflavone and 7-ethoxycoumarin were purchased from Aldrich Chem. Co., Milwaukee, WI. Sodium selenite was purchased from ICN Biochemicals, Cleveland, OH; resorufin from Matheson, Coleman, & Bell, Norwood, OH.; 7-ethoxyresorufin from Pierce Chemical Co, Rockford, Ill; and 3-OH benzo(a)pyrene from IIT Research Institute, Chicago, Ill. Custom packed ion exchange columns for δ-aminolevulinic acid sythetase assay, electrophoresis purity acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetra-methylethylenediamine, and ammonium persulfate, were obtained from Bio-Rad Laboratories, Richmond, CA. Fetal calf serum was purchased from Grand Island Biological Co., Grand Island, N.Y. Deuteroporphyrin was obtained from Porphyrin Products, Logan, Utah. Pentagastrin (Peptavlon^{R}, 250μg/ml in 0.85% NaCl) was obtained from Ayerst Laboratories, Inc., N.Y., N.Y. 8.5% Travasol (amino acid) Injection without Electrolytes was obtained from Travenol Laboratories, Inc., Deerfield, Ill. M.V.I. Concentrate (multiple vitamins) was purchased from U.S.V. Laboratories, Tuckahoe, N.Y. All other laboratory reagents, salts, and sugars, were

of analytical grade and were purchased from Mallincrodt Chemical Works, St. Louis, MO., or Fisher Scientific, St. Louis, MO.

4.2 ANIMALS:

All experiments were performed with adult male Sprague-Dawley rats (200-240g) from Simonsen Labs (Gilroy, Cal.). The animals were housed in hanging stainless steel cages in rooms maintained at 25°C with controlled 12 hour diurnal lighting, and protected from contact with pesticides and cigarette smoke, which are known to influence cytochrome P-450-dependent MFO activity (Welch et al, 1972; Uotila & Marniemi, 1976).

Preliminary investigations were carried out using guinea pigs as the animal model, using the dietary regimen described (Section 4.3). Although intestinal cytochrome P-450 content in the guinea pig is much greater and more stable than in the rat (Dawson & Bridges, 1981; Chhabra et al, 1974; Stohs et al, 1976a; Chhabra et al, 1974), it exhibits different characteristics of enzyme inhibition and induction from the rat enzyme (Miranda & Chhabra, 1980), precluding direct interspecies comparison. In addition, the guinea pig was also found to be an inadequate animal model in feeding experiments because of the animals refusal to ingest semi-synthetic diets, either in the powdered or pelleted form, with resultant severe anorexia.

4.3 DIETS:

Animals were routinely weaned from the standard laboratory chow diet onto the specific dietary regimen by feeding the semi-synthetic diet deficient in the experimental variable for two days. This regimen was instituted to permit sufficient time for a complete crypt to villous

tip cell transformation, sloughing of tip cells from the mucosa, and consequent expurgation of natural cytochrome P-450 inducers normally present in the laboratory chow (Wattenberg et al, 1962; Pantuck et al, 1975; Loub et al, 1975). The animals were then fed the specific purified diets, deficient in or supplemented with iron, selenium, and/or the cytochrome P-448 inducer, β-naphthoflavone (β-NF), 0.1%. Higher dietary concentrations of β-NF did not further increase the extent of cytochrome P-450 induction.

The following diets were routinely used: i) semi-synthetic iron and selenium deficient (-Fe -Se), consisting of vitamin-free casein, 27%; corn starch, 56%; hydrogenated vegetable oil, 14%; salt mixture without added ferrous sulfate or sodium selenite, 3%; and ICN vitamin diet fortification mixture, 1% (ICN Nutritional Biochemicals, Cleveland, OH); ii) semi-synthetic iron supplemented, selenium deficient (+Fe -Se) consisting of the above diet with 10 mg iron as ferrous sulfate added per 100 g diet; iii) semi-synthetic iron deficient, selenium supplemented diet (-Fe +Se) consisting of -Fe -Se diet supplemented with 0.05 mg selenium as sodium selenite per 100 g diet; iv) semi-synthetic iron and selenium supplemented diet (+Fe +Se) consisting of -Fe -Se diet with iron and selenium supplements as in diets (ii) and (iii); v) semi-synthetic iron deficient, selenium supplemented, β-NF supplemented diet (-Fe +β-NF) consisting of -Fe +Se diet (iii) supplemented with β-NF, 0.1 g per 100 g of diet; vi) semi-synthetic iron, selenium, and β-NF supplemented diet (+Fe +β-NF) consisting of the +Fe +Se diet (iv) supplemented with β-NF, 0.1 g per 100 g of diet. Following weaning, animals were divided into experimental groups, each fed ad libitum one of the above diets and given free access to water for four days unless specified otherwise.

Regardless of the dietary regimen, all animals gained weight at approximately equal rates for periods up to 7 days. No decrease in food consumption was observed in rats fed the -Fe diets, as reported during much longer periods (10 weeks) of iron deprivation (Lee et al, 1981).

Iron content of the iron deficient diet was determined to be below 0.01 mg per 100 g diet by the o-phenanthroline method (Pringle, 1946) following extraction of aliquots of the diet with 25% nitric acid at 90°C for 1 hr, neutralization with 10N NaOH, and filtration. Selenium content of the diets was kindly determined by Dr. Ivan Palmer, South Dakota State University.

The intracellular selenium status of the intestinal mucosa of rats fed the above diets was assessed by determining the activity of the selenium-dependent glutathione peroxidase (Lawrence & Burk, 1976); the intracellular iron status was assessed by monitoring the activity of the mitochondrial hemoprotein cytochrome oxidase (Smith, 1955), known to reflect cellular iron content (Richmond et al, 1972).

5.0 METHODS

5.1 PREPARATION OF INTESTINAL CELL POPULATIONS:

Rats were stunned by a blow to the head and exsanguinated by decapitation. The abdomen was opened by a midline incision, the intestinal tract was excised from the stomach at the pyloric junction and gently pulled away from the mesentery. The small intestine was then sectioned 20 cm distal to the pylorus, flushed with ice cold saline followed by ice cold 0.05 M Tris buffer (pH 7.8) containing 20% glycerol and 1.15% KCl (Stohs et al, 1976a) to wash off intestinal contents (20ml), and placed in the same buffer at 4°C. All subsequent procedures were conducted at 4°C.

Mucosa from intestinal segments was separated from the underlying connective tissue by the differential scraping technique (Dietschy & Siperstein, 1965; Hoensch et al, 1976). The intestine was laid on a cold glass plate and split longitudinally along the mesenteric axis. Villous cells were removed by light hand scraping with the edge of a metal spatula and transferred to the homogenizing buffer, while crypt cells when used, were harvested by heavier scraping.

The effectiveness of this method for isolating individual cell populations was determined by following the in vivo incorporation of ^{3}H-thymidine into isolated nuclear DNA as described (Hoensch et al, 1976). ^{3}H-Thymidine (0.5mCi, specific activity 6.7mCi/mmol, Net-027, New England Nuclear, Boston, Mass.) was administered intraperitoneally to 12 rats and 6 animals sacrificed at either 1 or 48 hr later. Individual cell fractions from 2 rats were pooled and homogenized in 0.25M sucrose, the cell nuclei were sedimented at 900 x g for 10 min, and resuspended in 3 ml of water. Radioactivity of 0.2 ml nuclear fractions

was measured by liquid scintillation counting in Aquasol after dissolution with soluene. DNA was precipitated from 1 ml nuclear fractions with ice cold 10% trichloroacetic acid (TCA), pelleted by centrifugation, washed once with 10% TCA, and twice with cold 100% ethanol to remove lipid. The pellet was then mixed with 10% TCA and heated at 90°C for 15 min, repelleted and the supernatant assayed for DNA by the following method of Dische (1955). Diphenylamine reagent (1.0%, in glacial acetic acid : concentrated sulfuric acid, 100:2.75) was added to aliquots of supernantant (2:1, respectively) and heated for 10 min in boiling water. Absorption was measured at 600 nm against a standard curve of calf thymus DNA (30-300µg). After one hour, crypt cell DNA fraction exhibited the highest radioactivity both on basis of µg DNA and mg nuclear protein, while at 48 hours villous tip cell DNA showed maximal radioactivity (Table 4). This is in agreement with previous reports (Imondi et al, 1969; Hoensch et al, 1976), and illustrates the 2-3 day migration of the highly proliferative crypt cells along the villi and their transformation into non-proliferative villous tip cells (Leblond & Messier, 1958).

5.2 PREPARATION OF CELL FRACTIONS:

Whole homogenate, 9000 x g supernatant, cytosol (105,000 x g supernatant), and microsomal fractions were prepared by differential centrifugation at 4°C. The mitochondrial fraction was prepared from a different cellular homogenate regimen (Section 5.6.4). Whole mucosa or individual cell fractions from rats fed the semi-synthetic diets were pooled from 2-4 animals, while mucosa or villous cells from rats fed the β-NF-supplemented diet were used without pooling. Tissue was homogenized in a glass-Teflon Potter-Elvenhjem homogenizer with 20 strokes at 200 rpm,

in 2-7 volumes of 0.05M Tris/20% glycerol (v/v)/1.15% KCl (w/v) buffer (pH 7.8), supplemented with trypsin inhibitor (5mg/g wet weight of small intestine) and heparin (3U/ml) to decrease degradation and agglutination of the MFO system (Stohs et al, 1976). For studies of cytochrome P-450 constitution with exogenous hemin in subcellular fractions (Section 5.3.2.1), histidine HCl, 0.1M, pH 7.8 (Wagner et al, 1981) was added to the homogenate.

Homogenates were used for ALAS activity determination (Section 5.6.2), or for in vitro hemin-incubation studies (Section 5.3.2.1), and/or sedimented at 9000 x g for 20 min at 4°C. The supernatant was collected for MFO activity determination, or for in vitro hemin incubation studies, and/or centrifuged at 105,000 x g for 60 min at 4°C for final separation of microsomes from cytosol. The microsomal pellet was collected and dispersed in 0.05M Tris/20% glycerol/1.15% KCl (pH 7.8) by 10 passes through a 16 gauge needle, and repelleted by centrifugation at 105,000 x g (60min) at 4°C. This procedure usually eliminated contaminating hemoglobin, in the absence of exogenously added heme, as indicated by the absence of the 420 nm peak in the CO-reduced minus reduced difference spectrum of the final microsomal suspension. In attempts to remove excess heme non-specifically bound to microsomes, additional studies were conducted in which human serum albumin (HSA, 60μM), with or without dithioerythritol (DTE, 1mM), in 0.05M Tris buffer (pH 7.8) was added to the microsomal suspension and incubated at 37°C for 4 min (White et al, 1978a), and the microsomes resedimented by centrifugation at 105,000 x g for 60 min at 4°C. The final microsomal pellet was resuspended and homogenized in the 0.05M Tris/20% glycerol/1.15% KCl buffer with 10 strokes by hand in a glass-Teflon homogenizer, to yield

approximately 2 mg protein per ml buffer and 1 mg protein per ml for microsomes from non-inducer fed-rats and β-NF-fed rats, respectively.

5.3 INTESTINAL APOCYTOCHROME P-450 DETERMINATION:

5.3.1 Electrophoretic Identification of Apocytochrome: Control (+Fe +Se, diet iv) and β-NF-fed (+Fe +β-NF, diet vi) rats received intragastrically 200 μCi of L-[4,5-^{3}H]-leucine (5mCi/mmol, NEC-460, New England Nuclear) and 50 μCi of L-[U-^{14}C]-leucine (0.25mCi/mmol, NEC-279, New England Nuclear), respectively. Pairs of rats from each of the two experimental groups were killed 1 or 24 hours later, intestines were removed, and homogenates prepared as described (Section 5.2). Equal volumes of homogenates from the two members of both of the control and β-NF-fed groups were combined (total 4 mucosal scrapings) and microsomal fractions prepared. Portions of the microsomal fractions containing 250 μg protein were electrophoresed by the sodium dodecyl sulphate/polyacrylamide-slab-gel discontinuous buffer method of Neville (1971), as modified (Siekevitz, 1973). The gels, stained with Coomassie Blue, were sliced to separate the distinctly stained protein bands. The slices were digested in protosol/NH_3/Aquasol (New England Nuclear) at 37°C for 24 hr (Ward et al, 1970). Radioactivity (^{14}C and ^{3}H) was monitored in a Beckman liquid scintillation spectrometer.

5.3.2 Protocol for Hemin-Mediated Holocytochrome Assembly: The in vitro assembly of intestinal apocytochrome P-450 and hemin was examined in two systems: mucosal cell fractions and everted intestinal sacs.

In preliminary experiments, incubation of heme with intestinal preparations resulted in a proportionately higher contamination of intestinal microsomes with non-prosthetically bound heme than that with liver

microsomes. In order to avoid interference by non-prosthetically bound heme in substrate oxidation or during subsequent product analysis from such hemin-constituted intestinal preparations, MFO assays had to be prescreened for the effect of hemin. Assays of MFO activity in the 9000 x g supernatant fraction were conducted with a radiometric AHH assay using [^{3}H]-BaP as substrate (Van Cantfort et al, 1977). The radiometric AHH assay was originally designed to avoid heme mediated quenching of 3-OH BaP fluorescence (Brown & Kupfer, 1975). EROD activity was chosen for monitoring MFO activity in the microsomal fraction because its basal values in the intestinal mucosa are sufficiently high for easy detection and it was most markedly stimulated following induction by dietary inducers (Table 7). In preliminary studies, heme was found to quench the fluorescence of resorufin (0.4% decrease in fluorescence per nmol hemin per ml); however in the EROD assay, heme was not detected in the supernatant obtained following protein precipitation of incubations of 9000 x g supernatant or microsomes with hemin.

5.3.2.1 Cellular Fractions: Incubation mixtures contained whole homogenate or 9000 x g supernatant of villous cells in 10 ml final volume, or microsomes in 2 ml final volume, at 4-5 mg protein per ml. Hemin (ferriprotoporphyrin IX hydrochloride) was dissolved in 0.1N NaOH and buffered with 0.05M Tris buffer, pH 7.8. Aliquots of the hemin solution were added to the cellular fractions and incubated at 37°C at various concentrations and for different time periods (RESULTS, Section 6.4.2.1). At the end of incubation, the homogenates or 9000 x g supernatant fractions were either mixed with an equal volume of ice cold homogenizing buffer and centrifuged to obtain microsomes (Section 5.2), or directly assayed for MFO activity as soon as possible. On the other hand,

microsomes were immediately assayed for MFO activity after incubation.

Preliminary Findings: In an attempt to maximize the hemin-mediated constitution of cytochrome P-448 in 9000 x g supernatants from β-NF-treated rats (RESULTS, Section 6.4.3.1), hemin-incubation was performed in the presence of various sulfhydryl compounds. This procedure has been found to maximize the reconstitution or reactivation of cytochrome $P\text{-}450_{cam}$ from cytochrome $P\text{-}420_{cam}$ (Yu et al, 1974; Yu & Gunsalus, 1974, a,b). In contrast to those studies with bacterial cytochrome P-450, and in contrast to similar results of enhanced hepatic cytochrome P-450 reconstitution with sulfhydryl agents (Correia & Meyer, 1975), supplementation of incubations of intestinal 9000 x g supernatant from -Fe +β-NF fed rats with the sulfhydryl compounds cysteine, glutathione (GSH), or DTE, not only inhibited the hemin-mediated assembly of the intestinal holocytochrome, but in fact substantially reduced the cytochrome content and activity (Table 2). Incubation of intestinal microsomes from +Fe +β-NF fed rats with cysteine also decreased cytochrome P-448 content by 27%. Presumably, the sulfhydryl agents replace the sulfur to heme-iron linkage at the fifth ligand of the heme moiety of the cytochrome. Because of these findings, all incubations of 9000 x g supernatants with hemin were conducted without added sulfhydryl agents.

In attempts to diminish the interference of non-specifically bound microsomal heme in the spectral assay of intestinal cytochrome P-450 (Section 5.5.1), excess heme was removed by incubation of the microsomal suspension at 37°C for 4 min with HSA (60μM), with or without DTE (1mM) in 0.05M Tris buffer (pH 7.8), (White et al, 1978). This procedure was found to eliminate the increases in intestinal microsomal cytochrome P-448 content and EROD activity observed in hemin incubations of 9000 x g

TABLE 2

EFFECT OF SULFHYDRYL COMPOUNDS ON HEMIN-MEDIATED INCREASE IN MICROSOMAL CYTOCHROME P-450 AND MFO ACTIVITY IN MUCOSA OF RATS FED THE -Fe +β-NF DIET

SH-compound added to 9000xg Supernatant*	Cytochrome P-450 pmol/mg microsomal protein			
	-SH-compound		+SH-compound	
	-Hemin Control	+Hemin	+Hemin	% Change from +Hemin -SH-cmpound
cysteine[a]	76.3	146.3	57.1 ± 16.7	- 61%
GSH[b]	133.3	169.3	109.5 ± 13.3	- 36%
	EROD pmol/mg microsomal protein/min			
cysteine[a]	8.2	15.9	4.3 ± 1.0	- 73%
GSH[b]	13.7	18.3	11.5 ± 1.8	- 37%
DTE[c]	12.3	16.1	9.3 ± 0.5	- 42%

* All samples contained 9000xg supernatant (2mg protein/ml) in a final vol of 10 ml. Hemin (15μM) was incubated for 20 min at 37°C, reactions were stopped by cooling and centrifugation of microsomes.
Each sulfhydryl compound was dissolved in buffer and pH adjusted to 7.4 before addition to the incubation mix.
Values are individual representative samples, or mean ± SEM, n=3.

a --- cysteine, 80mM
b --- GSH, glutathione, 3.5mM
c --- DTE, dithioerythritol, 20mM

supernatants from rats fed the iron deficient diet supplemented with β-NF (Table 3). Moreover, cytochrome P-448 of microsomes from non-hemin incubations of 9000 x g supernatants from rats fed the iron supplemented (+Fe +β-NF) diet also decreased to levels congruent with -Fe +β-NF levels. The maximal removal of cytochrome P-448 heme by HSA occured within 3-4 min of incubation with HSA, at which time the levels of cytochrome content and MFO activity apparently "stablized". Further incubation up to 7 min produced no additional effect on the enzyme system.

From these findings it can be suggested that this "stable" level of the cytochrome content and activity during β-NF induction is representative of a pool of intestinal apocytochrome P-448 with a relatively greater affinity for heme than HSA, while the difference between this stable "high" affinity apocytochrome and the maximal +Fe +β-NF or hemin constituted levels of the enzyme system is representative of a pool of apocytochrome P-448 with weaker affinity for heme than HSA. This "low" affinity pool would thus be subject to heme deficiency during iron deprivation, resulting in "free" hemin-constitutible "low" affinity apocytochrome P-448, while the "high" affinity apocytochrome would be unaffected by heme deficiency induced by dietary iron deprivation. The finding that cytochrome b_5 is similarly affected by HSA incubation (Table 3) alternatively suggests that the phenomenon may be intrinsic to the intestinal microsomal preparation.

Incubation of hepatic microsomes with either sulfhydryl agents or HSA does not affect their basal cytochrome P-450 content (Correia & Meyer, 1975; White et al, 1978). In contrast, the findings reported here indicate that the intestinal cytochrome is adversely affected by such incubations and thus may not be as stable as the hepatic isozyme.

TABLE 3

EFFECT OF HSA OR HSA/DTE INCUBATIONS OF MICROSOMES DERIVED FROM HEMIN-INCUBATED 9000xg SUPERNATANT ON INTESTINAL HEMOPROTEIN CONTENT AND MFO ACTIVITY

Diet	Hemin	Incubation*		Cytochrome P-450 pmol/mg protein	EROD nmol/mg protein/min	Cytochrome b_5 pmol/mg protein
-Fe +β-NF	-	-	n=3	57.2 ± 2.5	6.1 ± 1.0	--
"	+	-	n=3	118.1 ± 13.9[a]	11.2 ± 1.8[a]	--
"	+	+ HSA	n=2	54.2 ± 8.8	4.5 ± 0.8	--
"	+	+ HSA/DTE	n=1	54.0	3.4	--
+Fe +β-NF	-	-	n=3	155.8 ± 23.3[a]	14.1 ± 1.8[a]	28.2 ± 2.1
"	-	+ HSA	n=1	61.4	5.1	17.9 ± 2.3[b]

* Microsomes (0.2mg protein/ml) obtained from 9000xg supernatant incubations with hemin (15μM), were incubated at 37°C for 4 min, with or without HSA (human serum albumin, 60μM) or or HSA (60μM)/DTE (dithioerythritol, 1mM).

Values are either mean ± SEM, or values of individual animals.

a --- $p < 0.02$ vs -Fe -Hemin or -Fe +Hemin +HSA values, student's "t" test.
b --- $p < 0.02$ vs value obtained from incubations without added HSA, student's "t" test, n=3.

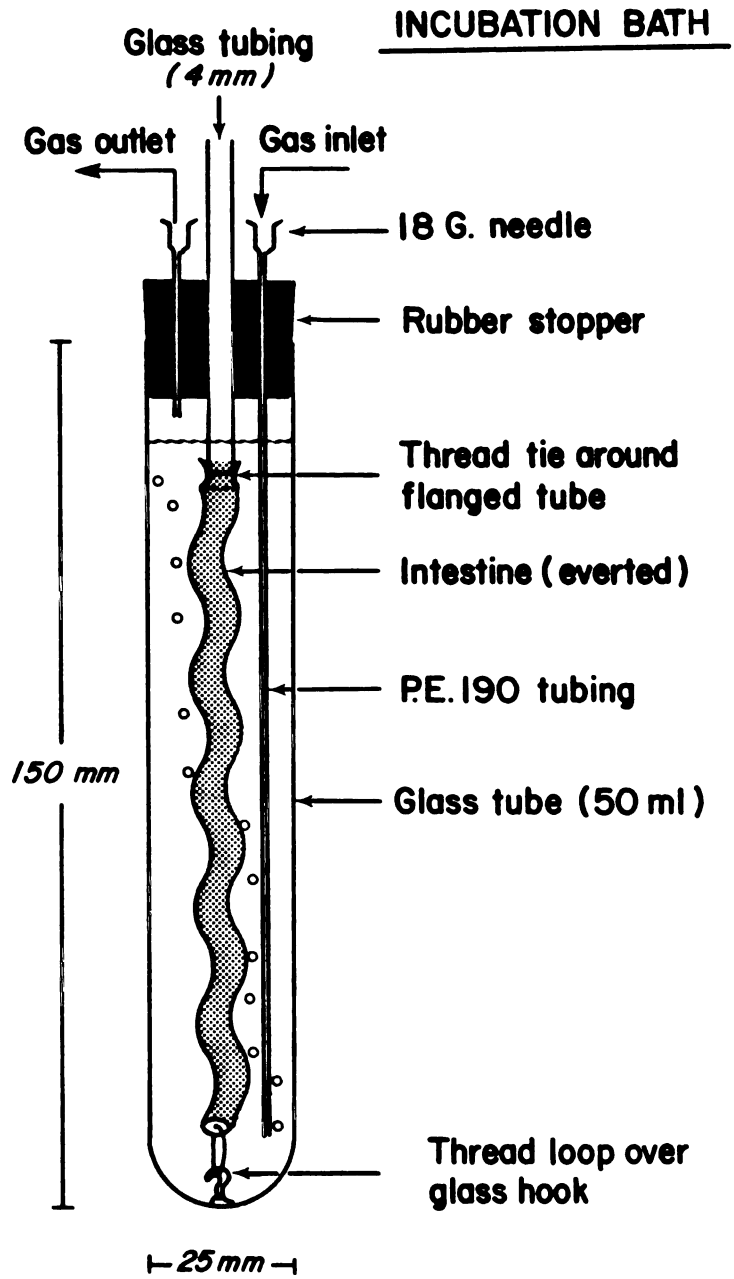
It remains to be determined whether these results indicate that the hepatic and intestinal apocytochromes have different affinities for their prosthetic hemes.

5.3.2.2 Everted Intestinal Sacs: In order to circumvent any loss of cytochrome P-450 viability after prolonged incubations of subcellular fractions at 37°C, the everted intestinal sac was used as an *in vitro* model to examine the assembly of constitutive holocytochrome P-450 in intestinal mucosa of rats fed inducer-free diets. This particular *in vitro* technique has been used infrequently for the examination of intestinal xenobiotic oxidation (Hietanen, 1980), or even to scrutinize the biochemical characteristics of the MFO system itself. More frequently it has been used for quantitative analysis of phase II biotransformations (Kreek *et al*, 1962; Fischer & Millburn, 1970), and extensively exploited in the study of mucosal transfer of drugs and nutrients (Wilson & Wiseman, 1954; Benet *et al*, 1971; Kaplan & Cotler, 1972; Chowhan & Amaro, 1977; Hurwitz & Gutman, 1979; Dowdle *et al*, 1960).

Everted sacs were prepared by modification of the methods of Wilson and Wiseman (1954) and Chowhan and Amaro (1977). Intestinal segments (12cm) were excised, placed in carbogen (95% O_2, 5% CO_2)-gassed ice cold incubation buffer, and everted over a glass rod at 4°C. The intestinal segments were attached at the pyloric end to a flared glass tube which was passed through a rubber stopper. They were tied off at the distal end, placed in ice cold carbogen-gassed incubation media in 50 ml tubes, and distally anchored to the bottom of the tube (Figure 5). Carbogen inlet and gas outlet consisted of 18 gauge needles passed through the rubber stopper, with PE 190 tubing on the inlet.

Everted sacs were incubated by gentle shaking at 37°C in a water

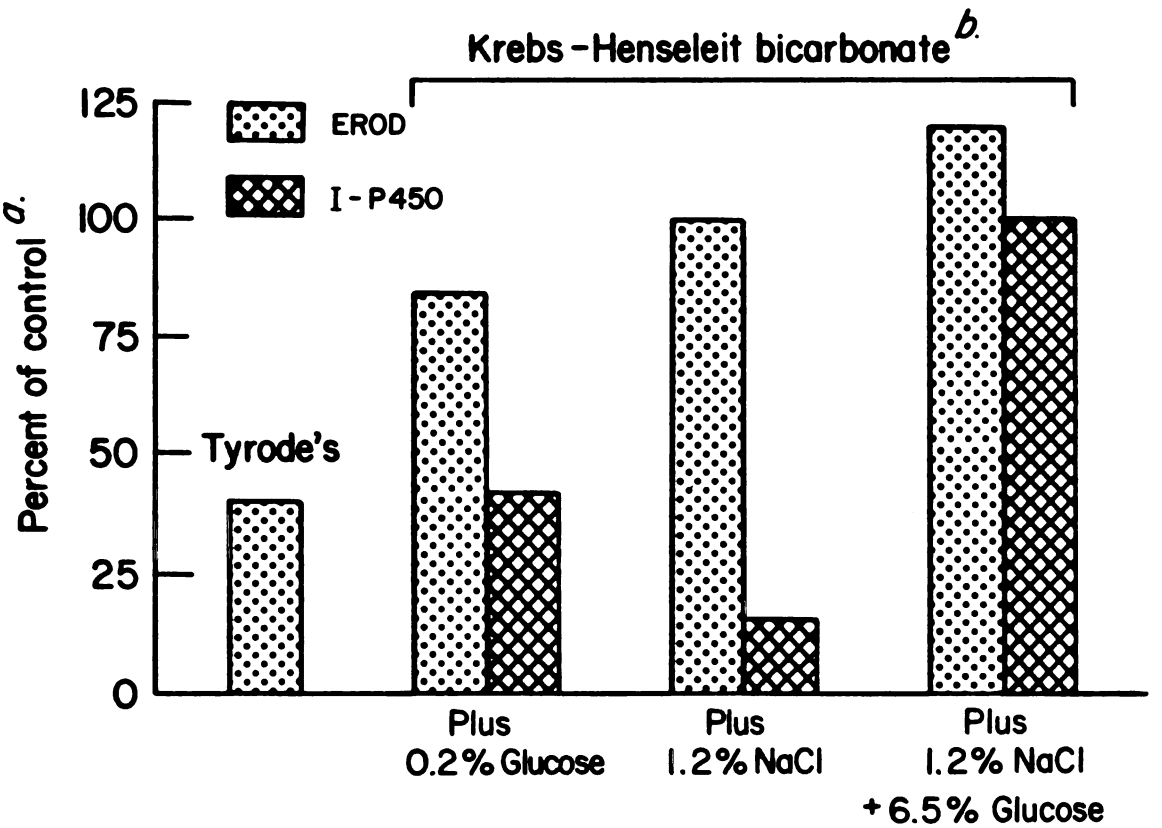
Figure 5. Diagram of the incubation apparatus used in everted intestinal sac studies.


INCUBATION BATH
Glass tubing
(4 mm)
Gas outlet
Gas inlet
18 G. needle
Rubber stopper
Thread tie around
flanged tube
Intestine (everted)
P.E. 190 tubing
Glass tube (50 ml)
150 mm
Thread loop over
glass hook
25 mm

bath with constant gentle bubbling of carbogen in one of the following media: i) Tyrode's buffer (Tyrode, 1910), containing Na^+ (0.67M), K^+ (0.15M), Mg^{2+} (0.15M), Ca^{2+} (0.11M), Cl^- (1.17M), $H_2PO_4^-$ (0.15M), and HCO_3^- (24.9mM), supplemented with 0.2% glucose; ii) modified Krebs-Henseleit (K-H) bicarbonate buffer, pH 7.8 (Krebs & Henseleit, 1932), consisting of Na^+ (0.143M), K^+ (5.9mM), Cl^- (0.128M), Mg^{2+} (1.2mM), Ca^{2+} (2.6mM), $H_2PO_4^-$ (2.2mM), HCO_3^- (24.9mM), and SO_4^{2-} (1.2mM), supplemented with 0.2% glucose; iii) the above K-H bicarbonate buffer supplemented with 1.2% NaCl (0.205M); iv) the above K-H bicarbonate buffer supplemented with 1.2% NaCl and 6.5% glucose. The addition of NaCl and glucose to the incubation media was estimated from Kitazawa et al (1975) to sufficiently change the media osmolality in order to prevent net fluid movement across the mucosa. This was anticipated to overcome the loss of mucosal cellular membrane integrity (Levine et al, 1970) and subsequent loss of mucosal transfer function (Gibaldi & Grundhofer, 1972) observed during everted sac incubations, with the subsequent stabilization of cellular membranes and the membrane-bound component, cytochrome P-450. The stability of microsomal cytochrome P-450 and MFO activity was therefore examined following 1 hr incubation of everted sacs at 37°C in one of the above buffers. K-H bicarbonate buffer supplemented with 1.2% NaCl and 5% glucose was the only one capable of maintaining a fully functional enzyme system (Figure 6), presumably by protecting intracellular membranes from hypertonic insult (Kitazawa et al, 1975).

During hemin-incubations, the everted sac media was supplemented with glutamine (15mM), the major mucosal respiratory "fuel" (Windmueller & Spaeth, 1978), and cysteine (6mM) for protection of sulfhydryl groups

Figure 6. Effects of various incubation media on intestinal cytochrome P-450 (I-P450) and ethoxyresorufin O-deethylase (EROD) activity in everted intestinal sac preparations. Everted intestinal sacs were prepared from rats fed +Fe +Se diet for 5 days, and incubated for 1 hr in various media. Controls consisted of everted intestinal segments (METHODS, Section 5.3.2.2) immediately used for mucosal cell separation without incubation. Values represent mean of two experiments, obtained from tissues pooled from 2 animals. a) Control value for I-P-450: 23.9 $\pm$ 2.8 pmol/mg microsomal protein; EROD: 24.3 $\pm$ 3.6 pmol/mg microsomal protein/ min. Values are mean $\pm$ SD, n=3. b) Krebs-Henseleit bicarbonate buffer supplemented with glucose or NaCl (METHODS, Section 5.3.2.2).


Krebs-Henseleit bicarbonate b.
125
100
75
50
25
0
Percent of control a.
EROD
I-P450
Tyrode's
Plus
0.2% Glucose
Plus
1.2% NaCl
Plus
1.2% NaCl
+6.5% Glucose

(Yu & Gunsalus, 1974a). Hemin was dissolved in 0.1N NaOH, adjusted to pH 7.8 with 0.1N HCl, buffered in 0.1M Na^+ K^+ PO_4^{2-}, and added to 50 ml incubation media after a 5 min preincubation at 37°C. Incubations were carried out for 20 min unless stated otherwise, with and without added hemin (15μM), unless stated otherwise. In order to remove excess heme in the media and non-specifically bound to the mucosal cells (White et al, 1978a), at the end of 20 min, HSA (0.1mM, final) and DTE (1.0mM, final) were added in 5 ml volumes to the incubation media and further incubated for 4 min.

At the end of all incubations, the sacs were removed from the apparatus, rinsed twice in 50 ml ice cold K-H buffer (pH 7.8), and whole mucosa separated from the submucosa and prepared as described (Section 5.1). Individual cell populations were not used because an increased tendency for sloughing occured post incubation of the intact tissue, making cell separation difficult with this technique.

5.4 STUDIES ON THE IN VIVO EFFECTS OF PENTAGASTRIN ON THE INTESTINAL CYTOCHROME P-450-DEPENDENT MFO SYSTEM:

Animals were routinely weaned from the standard laboratory chow diet by feeding the +Fe +Se semi-synthetic diet for two days prior to surgery. All studies were conducted in unrestrained rats infused continuously via intrajugular vein with drug or placebo. Animals were supplemented with or without intravenous nutrients, as modified from Steiger et al (1972).

5.4.1 Infusion Harness and Catheter Attachment: Animal harnesses consisted of a medium sized rat jacket (Alice King Chatham, Medical Arts, Los Angeles, Ca.) with a 40 cm spring-type catheter sleeve passing

through the back panel. At the proximal end of the spring sleeve a 1 cm circular steel panel, with equi-distant holes for suturing the panel to the midscapular skin area, was attached. The distal end was glued to a 30 gauge plastic needle casing in which a 'window' was cut to serve as a reservoir for an extra 5-10 cm length of catheter looped through it. The catheter consisted of 55 cm length of PE 10 tubing, mounted on the 30 gauge needle, which was fixed into its plastic housing.

5.4.2 Animal Preparation: Each rat was anesthetized with ether, its neck and midscapular area shaved, and restrained in supine position under continuous light ether anesthesia. A small transverse incision was made in the neck lateral to the midline and 0.25 cm above the clavicle. The jugular vein was then isolated as it courses over the clavicle. A 16 gauge needle was passed through the incision subcutaneously around the neck, to exit at the midscapular area. The infusion catheter was passed in the counter direction through the needle to exit at the incision site, and trimmed to proper length. The jugular vein was then ligated 0.5 cm above the clavicle. A second suture was placed around the vein immediately above the clavicle and was left untied until the catheter had been inserted. Heparinized saline (40U/ml) was first flushed through the catheter, then the catheter tip was inserted through a phlebotomy in the jugular vein between the sutures, and advanced into the superior vena cava or right atrium (2.4cm from vessel entrance point). The catheter was then secured with the two sutures. Periodic flushing of the catheter with 0.1 ml heparinized saline prevented obstruction of the catheter flow. The neck incision was then sutured closed, and the catheter sleeve plate sewn to the skin of the midscapular area, circling the catheter exit site. The harness was then strapped around the rat.

Each animal was placed in an individual cage fitted with a plexiglass lid cut centrally with a 3 inch groove through which the catheter sleeve passed. A teflon ball, slightly larger than the groove, was secured to the sleeve to maintain proper sleeve length in the cage. The catheter 30 gauge needle casing was attached to PE 190 tubing (4-5ft), the other end of which was fit snugly inside silicon rubber tubing (18 cm length, I.D. 1.3mm, O.D. 3mm, LKB-Produkter, Sweden). The silicon rubber tubing was passed through the squeeze plate of a Harvard peristaltic infusion pump with a capacity of 8 simultaneous infusions. The speed was adjusted for an initial flow rate of 0.5 ml/hr, and increased at 24 hr at the begining of drug infusion to 2.0 ml/hr.

Animals were warmed by heating lamps throughout the study. In order to adequately maintain intestinal cytochrome P-450 content in the absence of intraluminal nutrients (Table 8), iron (10mg Fe/100ml as $FeSO_4$) and selenium (50µg Se/100ml as $NaSeO_3$) were added to the drinking water.

5.4.3 Intravenous Nutrient Solution: Unrestrained animals were infused with either saline (0.9% NaCl), supplemented to 5% glucose, or a specially formulated hypertonic glucose-amino acid solution (Steiger et al, 1972) containing balanced essential electrolytes (Seltzer et al, 1978). The hyperalimentation solution consisted of an 8.5% solution of amino acids (8.5% Travasol Injection without Electrolytes), supplemented to 32% glucose. Each liter was made to contain 100 mEq sodium, 60 mEq potassium, 90 mEq chloride, 0.2 g calcium, 0.01 g phosphorous, and 0.02 g magnesium (added as sodium chloride, sodium acetate, potassium acetate, KH_2PO_4, calcium gluconate, and magnesium sulfate). Five ml of a multiple vitamin solution (M.V.I., USV Pharmaceutical Corp., NY) were added per liter of hyperalimentation solution. The complete solution was passed

through a 0.22 μ membrane filter (Millipore Corp., Bedford, Mass.), stored at 4°C in the dark, and used fresh each evening of infusion.

With this hyperalimentation regimen, rats were found to gain normal weight throughout the studies (approximately 20g per 8 days). Average daily caloric intake was estimated to be 50 kcal, comparable to standard rat chow (Johnson et al, 1975b). Unhealthy appearing animals (edema, severe sluggishness, loss of weight, gastric ulcer, or black duodenal contents) were rejected from further study. Experiments were run with 8 paired animals, half of which received pentagastrin and half serving as controls.

5.4.4 Pentagastrin Administration: Fresh pentagastrin was mixed daily in the infusion solution to give appropriate doses at the infusion rate of 2 ml/hr. Maximal doses used were deliberately kept below those known to produce gastric acid-induced mucosal ulceration (Robert et al, 1970).

5.5 ASSAYS OF THE INTESTINAL MFO SYSTEM:

5.5.1 Cytochrome P-450: Cytochrome P-450 content of uninduced intestinal microsomes was determined by the method of Omura and Sato (1964b) which employs CO-difference spectra of reduced microsomes. This was monitored in 0.5 ml matched cuvettes in an Aminco Dual Beam DW-2 spectrophotometer, using an extinction coefficient of $91 mM^{-1}cm^{-1}$. In studies of hemin-mediated constitution of intestinal cytochrome P-450 in subcellular fractions from β-NF fed rats, the method of Johannesen and DePierre (1978), which employs the reduced difference spectrum of CO-bound microsomes (extinction coefficient of $105 mM^{-1}cm^{-1}$), was used to determine cytochrome content. This procedure was not used in assays of the non-induced cytochrome due to spectral interference at 450 nm from the much

greater absorption of cytochrome b_5 at 426 nm. The amount of sodium dithionite required for reducing the pigment was carefully controlled since excess dithionite appears to cause microsomal degradation with consequent loss of spectrally detectable cytochrome P-450. CO was passed through a deoxygenating solution, consisting of 0.5% sodium dithionite/ 0.05% sodium anthraquinone-2-sulfonate in 0.1N NaOH (Gigon et al, 1969). The difference spectrum of the highly turbid microsomal suspension frequently resulted in an ascending baseline, detectable after bubbling the reduced microsomal suspension with CO. The baseline was then graphically corrected and cytochrome concentration was determined from the absorption peak to the new baseline.

Occasional occurrence of an absorption peak in the CO-difference spectrum at 420 nm, due to inefficient removal of contaminating hemoglobin or heme, interfered with the absorption peak of basal cytochrome P-450 at 452 nm (RESULTS, Section 6.2). This was corrected graphically by a modification of the method of Kowal et al (1970). Various concentrations of hemin were added to intestinal microsomal suspensions and the red shift produced in the 452 nm absorption peak in the CO-difference spectrum of reduced microsomes was correlated to the decrease in the 452 nm peak height. From this correlation, a formula was derived for conversion of the "shifted" cytochrome P-450 absorption to actual absorption at 452 nm. Such mathematical corrections for cytochrome concentrations were made for all subsequent cytochrome P-450 determinations in the presence of contaminating heme or hemoglobin.

5.5.2 Aryl Hydrocarbon Hydroxylase (AHH, EC 1.14.14.2): Activity of the enzyme was determined by either a fluorescent or radiometric assay.

Fluorescence Assay: Generally, AHH activity was determined by the

fluorescence assay of Alvares et al (1967). The reaction mixture contained NADPH (10mM) and BSA (5mg/ml) in 0.05M Tris buffer (pH 7.4). Assays were carried out with 0.5 ml of 9000 x g supernatant fraction in the dark (final vol, 1ml) at 37°C in a shaking water bath. After 3 min pre-incubation, reactions were started with benzo(a)pyrene (BaP, 40mM) in 20 µl acetone, and terminated after 15 min with 1 ml of ice cold acetone. Substrates and metabolites were extracted by shaking with 3.25 ml of hexane for 10 minutes. Polar metabolites were then extracted from 1 ml of the organic phase into 3 ml of 1N NaOH by vortexing in 12 ml centrifuge tubes. After centrifugation at 2000 rpm, the fluorescence of the aqueous phase was monitored in an Aminco-Bowman spectrofluorimeter (excitation 396nm, emission 522nm) and the concentration of fluorescent product was determined from a standard curve obtained with 3-OH BaP, one of the major oxidative metabolites of BaP in the intestine (Stohs et al, 1977). Activity is expressed as pmol 3-OH BaP/mg 9000 x g supernatant protein/15 min. The addition of BSA was found to increase the linearity of the reaction, presumably by either dispersing hydrophobic BaP (Hansen & Fouts, 1972), or by sequestering polyvalent cationic inhibitors, as suggested (Pohl & Fouts, 1980).

Radiometric Assay: In order to prevent the interference of hemin with the fluorescence of 3-OH BaP (Brown & Kupfer, 1974), AHH activity in subcellular fractions incubated with hemin was determined by a modification of the radiometric assay of Van Cantfort et al (1977). [^{3}H]-BaP was first purified by drying 0.2-0.5 ml of an acetone solution under N_2, redissolving in 5 ml hexane, and extracting 3 times by vortexing with 2.5 ml aqueous 0.1M KOH/57% DMSO. Organic and aqueous phases were separated by centrifugation at 2000 rpm for 2 min, the hexane layer was

removed, dried under N_2 and the residue was stored at -20°C for a maximum of 3 days. Immediately before use, the residue was dissolved in acetone to yield 1.4 mCi/ml.

Assays were carried out in scintillation vials containing 0.2-0.5 mg 9000 x g supernatant or microsomal protein in 0.05M Tris buffer (pH 7.4), containing NADPH (0.5mM), NADH (0.5mM), and $MgCl_2$ (5mM), with the addition of $MnCl_2$ (5μM), isocitrate (6mM), isocitrate dehydrogenase (0.36U/ml), and BSA (1.8mg/ml) when microsomal protein was used (DePierre et al, 1975), in a final volume of 0.5 ml. Preliminary studies on substrate saturation kinetics were conducted with 9000 x g supernatant containing either non-induced or β-NF-induced cytochrome P-450. AHH activity as determined from the percent of radiolabelled substrate converted to polar products was maximal at a substrate concentration of 80μM BaP for β-NF-induced enzyme. Similar values were found for the liver isozymes (DePierre et al, 1975). In contrast, saturation with radiolabelled substrate was not observed with the non-induced enzyme when examined over a reasonable substrate concentration range (Figure 7). Affinity of the substrate for the non-induced enzyme, characterized by the apparent Km derived from Lineweaver-Burk plot of the substrate saturation data (Figure 8), was 1.1×10^{-3}M, which contrasts with the much higher affinity of the substrate for β-NF-induced enzyme with an apparent Km of 2.4×10^{-5}M. Substrate concentrations used in the assays of both induced and non-induced enzyme were routinely 80 μM.

Reactions were started with BaP in 20 μl of acetone, spiked with 150,000-200,000 cpm [^{3}H]-BaP, and after pre-incubation for 3 min, carried out at 37°C for 15 min with vigorous shaking. Reactions were terminated with 1 ml of 0.15M KOH in 85% DMSO, and transferred to 12 ml glass

Figure 7. Substrate saturation kinetics of aryl hydrocarbon hydroxylase (AHH) in 9000xg supernatant of intestinal mucosa. Reactions were carried out with varying concentrations of substrate, as described (METHODS, Section 5.5.2). a) 9000xg supernatant protein from rats fed the +Fe +β-NF diet, n=2; b) 9000xg supernatant protein from rats fed control (+Fe) diet, n=6.

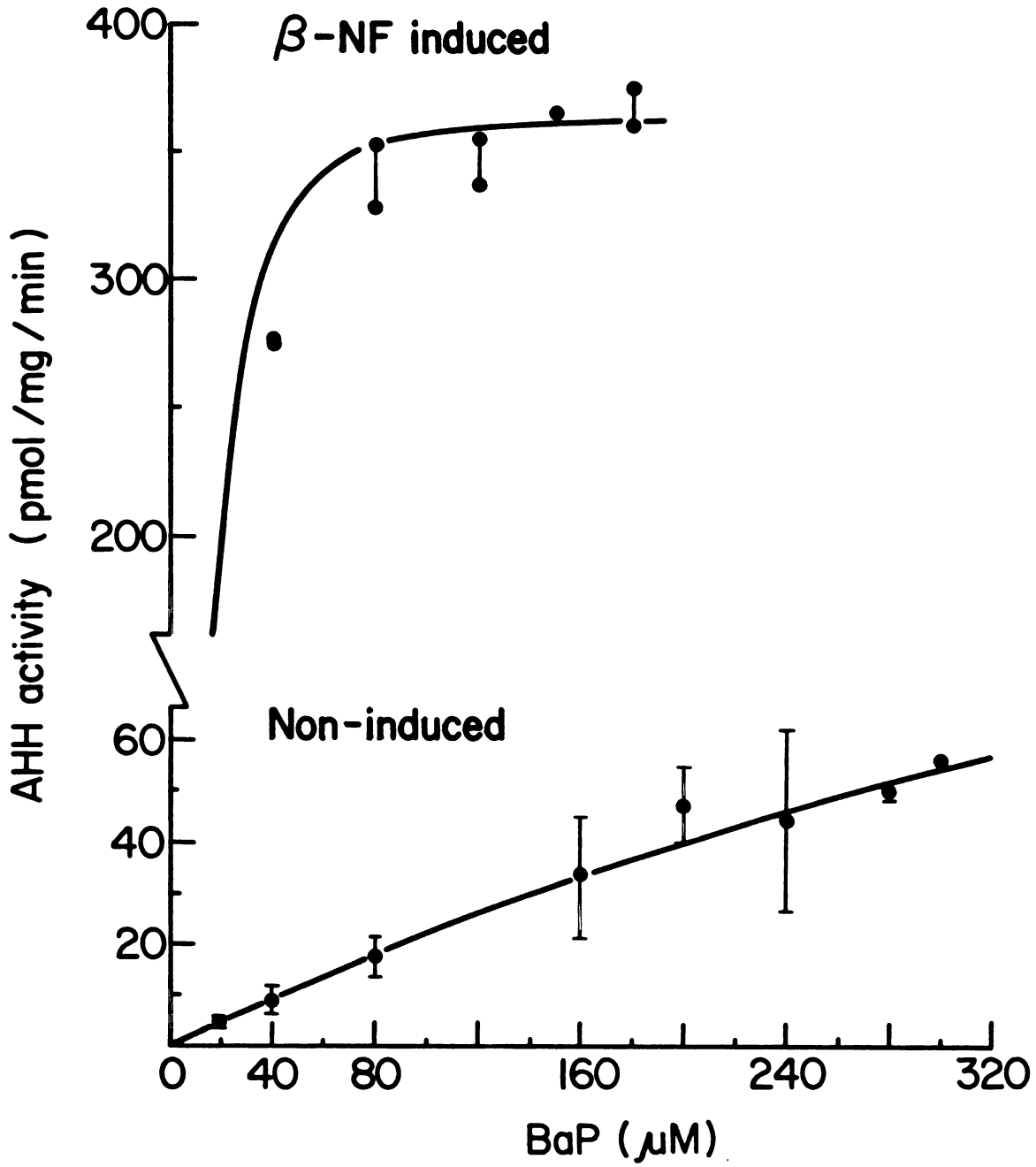
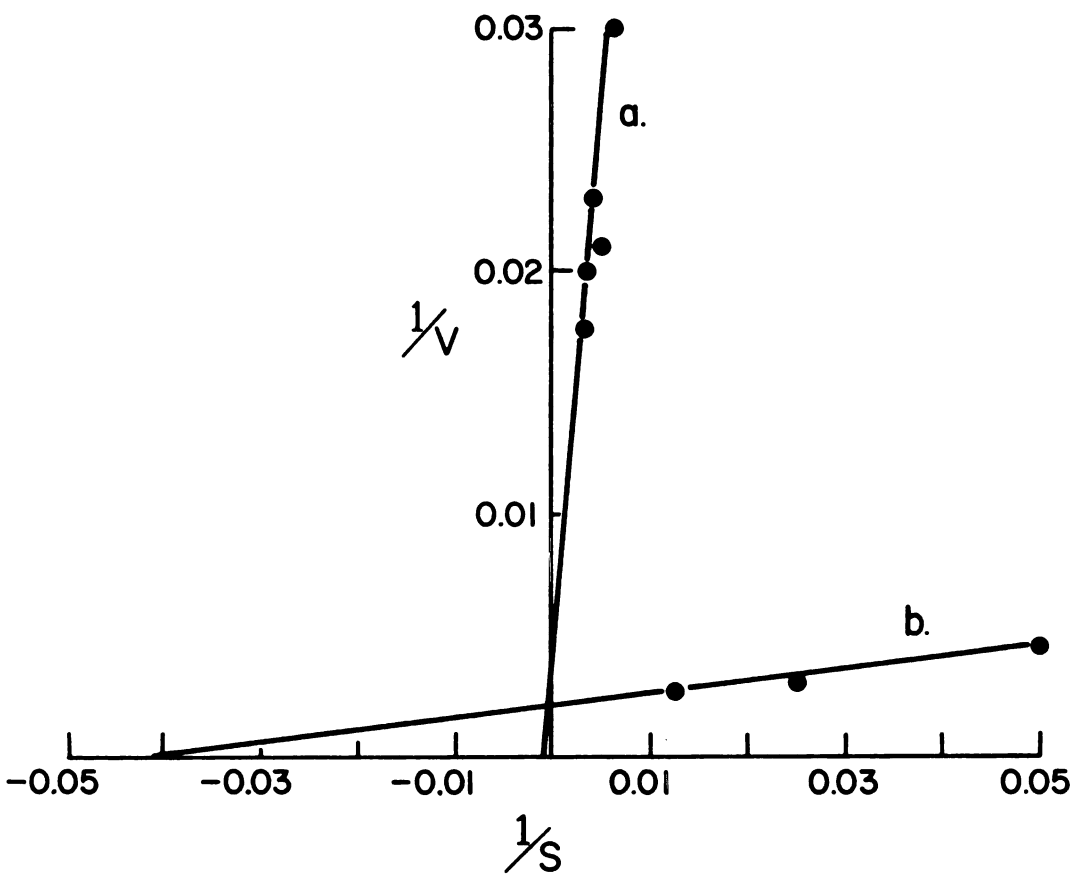

β-NF induced
Non-induced
AHH activity (pmol/mg/min)
400
300
200
60
40
20
0
40
80
160
240
320
BaP (μM)

Figure 8. Lineweaver-Burk plot of intestinal aryl hydrocarbon hydroxylase (AHH). Reciprocals of the velocities are expressed as pmol products formed/mg 9000xg supernatant protein/min and of substrate concentrations as μM. a) non-induced AHH: Km $1.1x10^{-3}$M; Vmax 250 pmol/mg protein/min. Each data point is the mean of 5 experiments, with tissue pooled from two rats per experiment. b) β-NF-induced AHH: Km $2.4x10^{-5}$M; Vmax 476 pmol/mg protein/min. Each data point is the mean of 2 experiments, with tissue used from individual rats per experiment.


0.03
0.02
0.01
1/V
a.
b.
-0.05
-0.03
-0.01
0.01
0.03
0.05
1/S

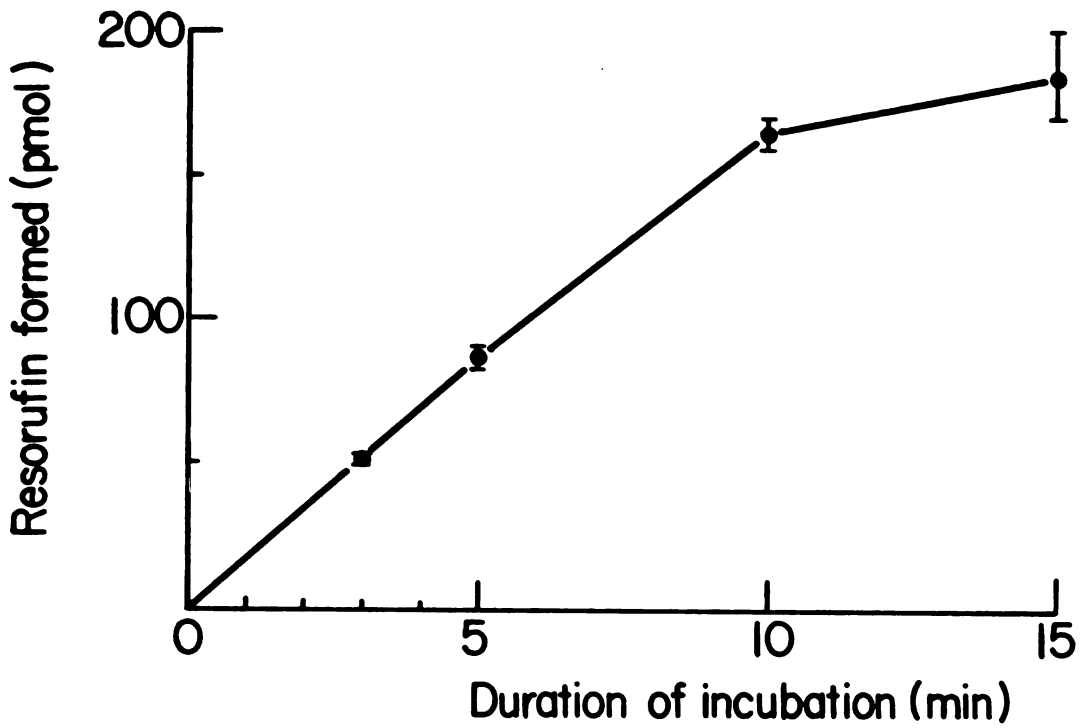
centrifuge tubes. Assay blanks consisted of the same incubation mixture, with the addition of the KOH/DMSO mixture before incubation. Products were extracted twice into 5 ml of hexane by vortexing for 1 min and centrifuging for 2 min at 2000 rpm. Aliquots (0.5ml) of the final aqueous phase were counted in 15 ml of Dimilume without neutralization. Percent of [^{3}H]-BaP metabolized to extractable product was determined, and enzyme activity calculated as pmol BaP hydroxylated per mg protein per 15 min.

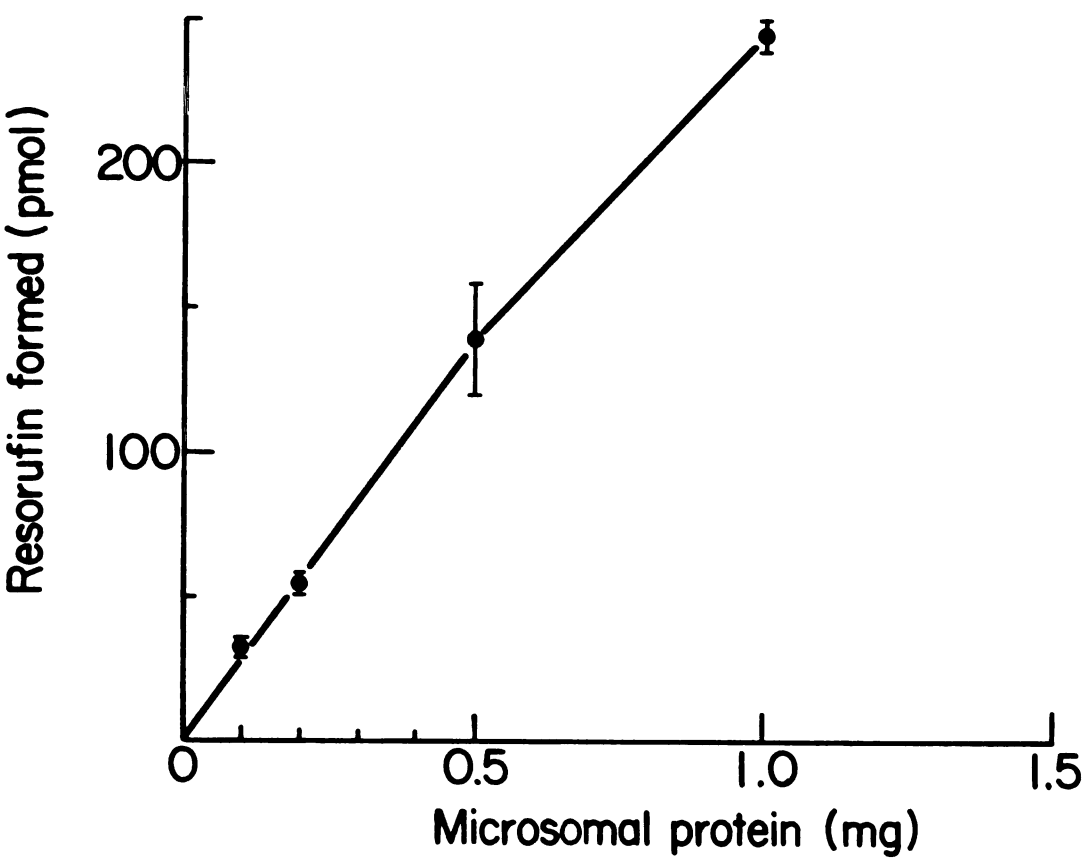
5.5.3 7-Ethoxyresorufin O-Deethylase (EROD): Activity was determined by a modification of the fluorimetric assays described originally by Burke and Mayer (1974) and by McCormack et al (1979). Reactions were carried out in 1 ml volumes in 12 ml glass conical centrifuge tubes. The reaction mixture consisted of Tris buffer (0.05M, pH 7.4 at 37°C) containing $MgCl_2$ (5mM) and $MnCl_2$ (5μM), to which was added fresh NADPH (0.5mM), NADH (0.5mM), isocitrate (6mM), isocitrate dehydrogenase (0.02 U/ml), and BSA (0.8mg/ml). This mixture was previously used to attain maximal microsomal oxidative activity (DePierre et al, 1975). Reactions with uninduced microsomes contained 0.5-1.0 mg of protein, 1 nmol of 7-ethoxyresorufin (0.33mM in DMSO) for substrate saturation of the enzyme (Pohl & Fouts, 1980; Warner & Neims, 1979), and were carried out for 10 min; reactions with β-NF-induced microsmes contained 0.1-0.2 mg protein, 7-ethoxyresorufin (5nmol, unless stated otherwise), and were carried out for 2 min. Reactions were started with substrate after a 3 min preincubation at 37°C and terminated with 1.0 ml ice cold acetone (McCormack et al, 1979). Assay blanks consisted of incubation mixtures in which the reaction was stopped immediately after substrate addition, followed by 10 min incubation at 37°C. The presence of microsomes in the

reaction mixture reduced the background fluorescence of the 7-ethoxyresorufin, apparently by binding the substrate; therefore a standard curve (10pmol to 10nmol resorufin in 0.05M Tris buffer) was run in the presence of microsomes and 7-ethoxyresorufin and the mixtures were subsequently treated exactly like assay blanks. After acetone addition, samples were vortexed, centrifuged for 15 min at 2000 rpm in an ICN centrifuge, and the supernatant was read in an Aminco-Bowman spectrofluorimeter, with an excitation wavelength at 530 nm and emission wavelength at 586 nm. Reaction rates were expressed as pmol of resorufin formed/mg microsomal protein/min. Using this procedure, reactions were found to be linear for up to 10 min and with up to 1.0 mg protein (Figure 9).

5.5.4 7-Ethoxycoumarin O-Deethylase (ECOD): Activity was determined by the method of Greenlee and Poland (1978) with the modifications of Guengerich (1978). The assay was performed in scintillation vials, in a volume of 1 ml containing NADPH (0.5mM), NADH (0.5mM), $MgCl_2$ (5mM), and BSA (1mg/ml) in 0.05M Tris buffer (pH 7.4). Reactions contained 0.1 ml of 9000 x g supernatant and started with 7-ethoxycoumarin (0.5 nmol in 0.025 ml of 50% aqueous methanol). After a 3 min preincubation, reactions were carried out at 37°C for 10 min with vigorous shaking. Reactions were stopped with 0.1 ml of 2N HCl and 3 ml of chloroform, transferred to 12 ml centrifuge tubes, vortexed and centrifuged for 1 min at 2000 rpm to separate the layers. A 1.5 ml aliquot of the organic phase was extracted with 2.5 ml sodium borate (30mM, pH 9.2) by vortexing (1min) and centrifugation. Fluorescence of the aqueous phase was monitored in an Aminco-Bowman spectrofluorimeter (excitation at 368nm and emission at 456nm) and compared with a standard curve obtained with 10 pmol to 5 nmol 7-OH coumarin. Assay blanks consisted of the complete

Figure 9. 7-Ethoxyresorufin O-deethylase (EROD) linearity. a) Time course of EROD reaction. The reaction was carried out for the time intervals indicated, as described (METHODS, Section 5.5.3), except that the microsomal protein concentration was 0.7 mg in 1 ml. Values are mean of 2 experiments with range bars. b) Effect of variation of microsomal protein concentration on EROD activity. Reactions were carried out for 10 min on varying amounts of microsomal protein in a volume of 1 ml. Values are the mean of 2 experiments with range bars.


200
100
Resorufin formed (pmol)
0
5
10
15
Duration of incubation (min)


200
100
Resorufin formed (pmol)
0
0.5
1.0
1.5
Microsomal protein (mg)

reaction mixture to which substrate was added after addition of HCl and chloroform. Product recovery was determined by monitoring an internal standard of 7-OH coumarin, and ranged from 80-95%.

5.5.5 Cytochrome b_5: Content was determined by the NADH-reduced minus oxidized difference spectrum between 424 nm and 409 nm in an Aminco DW-2 spectrophotometer, using an extinction coefficient of $185 mM^{-1} cm^{-1}$ (Omura & Sato, 1964a).

5.5.6 NADPH-Cytochrome P-450 Reductase (EC 1.6.2.4): Activity was determined by the method of Williams and Kamin (1962) using cytochrome c as the external electron acceptor. Reactions contained equal volumes of KCN (3mM), oxidized cytochrome c (0.15mM), and Tris buffer (0.1M, pH 7.7), to which was added 1-2 mg of microsomal protein. After a 3 min preincubation in cuvettes at 37°C, reactions were initiated by the addition of 50 µl NADPH (0.5mM) to the sample cuvette, and were followed at 550 nm for the first minute of reduction in an Aminco DW-2 spectrophotometer equipped with a 37°C circulating water bath. Activity was calculated from an extinction coefficient of $21 mM^{-1} cm^{-1}$, and expressed as nmol cytochrome c reduced/mg microsomal protein/min.

5.6 DETERMINATION OF HEME AND THE HEME METABOLIC PATHWAY:

5.6.1 Microsomal Heme: Content was measured by the reduced minus oxidized difference (557-575nm) spectrum of the pyridine hemochromogen method (Paul et al, 1953). Microsomes (0.5ml) were diluted with 1 ml of 0.1N NaOH/20% pyridine, split into 2 cuvettes, and the baseline obtained. The sample cuvette was mixed with sodium dithionite, and the spectrum was again recorded by an Aminco DW-2 spectrophotometer. Heme content

was determined using an extinction coefficient of $34.7 mM^{-1} cm^{-1}$.

5.6.2 δ-Aminolevulinic Acid Synthetase (ALAS, EC 2.3.1.37):

Enzyme activity: This was determined essentially by the method of Strand et al (1972). The assay was performed on 0.1 ml of intestinal homogenates (50% v/v in 1:1 0.05M Tris buffer/20% glycerol/1.15% KCl) diluted 1:1 (v:v) with Tris buffer (0.1M)/pyridoxal phosphate (0.1mM)/ 0.9% NaCl (pH 7.4) as modified by Yoda et al (1974). Mix I, stable at -20°C for 3 months, contained arsenite (10mM), antimycin A (0.0005%, 0.1% ethanol solution made up to 50ml with 0.1% BSA), malonate (20mM), DL-malic acid (10mM), EDTA (2mM), glycine (0.2M), $MgCl_2$ (40mM), and pyridoxal-5'-phosphate (2mM), in 0.1M Tris-HCl buffer (pH 7.4). Mix II was prepared fresh prior to use, and contained ATP (62.5M), GSH (5mM), coenzyme A (1.06mM), succinate (25mM), [^{14}C]-succinate (6.25μCi), and succinyl thiokinase (2.5U per sample). Reactions were carried out in a volume of 0.5 ml with 0.05 ml homogenate, 0.25 ml Mix I (1:2 final dilution), and 0.2 ml Mix II (1:2.5 final dilution), at 37°C for 30 min in a shaking water bath. Reactions were terminated with 0.1 ml ice cold 25% trichloroacetic acid, supplemented with ALA-sodium succinate carrier (12.5μmol and 125nmol, respectively), and made up to 1.0 ml.

Product isolation: Enzymatically synthesized radiolabelled ALA was separated from succinate and other metabolic products by a three step sequential ion-exchange column. The pH of the reaction mix was adjusted to 7.0 and passed through a Dowex-acetate form column, 100-200 mesh, equilibrated to pH 7.0 with Na acetate (25mM), which was placed in tandem on a column of Dowex-hydrogen form, 100-200 mesh, equilibrated to pH 2.0 with 0.01N HCl. After a 20 ml wash with water, the hydrogen column was washed with 20 ml of 0.1N HCl followed by 1 ml of 1M Na acetate.

ALA was eluted with 7 ml of 1M Na acetate into 100 ml glass tubes. The ALA was converted to 2-methyl-3-acetyl-4-(3-propionic acid) pyrrole by boiling for 20 min with 0.2 ml acetylacetone at pH 4.5. This pyrrole was applied to a Dowex-acetate form column, mesh 200-400, equilibrated to pH 4.6 with Na acetate (0.05M), washed with 15 ml of water, and eluted with 4.5 ml of methanol/glacial acetic acid (2:1), without the acetic acid/methanol wash as described (Strand et al, 1972). Quantitation of the [^{14}C]-ALA formed from [^{14}C]-succinate in Mix II was determined by counting 3 ml aliquots of final eluate and aliquots of Mix II in Aquasol. Recovery of ALA was 80-90% as determined by comparison of the recovery of carrier ALA to fresh ALA after reacting aliquots of the elute with Ehrlich's reagent (1:1, v:v) for 30-45 min and determining the absorbance at 556 nm (Mauzerall & Granick, 1956).

5.6.3 δ-Aminolevulinic Acid Dehydratase (ALAD, EC 4.2.1.24): Activity was measured in intestinal cytosolic fractions (105,000xg supernatant) obtained from 20% standard homogenates (v/v) (Finelli et al, 1974). 10-20 mg of protein were incubated with ALA (0.01M) in a volume of 2.5 ml of 0.05M Tris buffer (pH 6.8) for 60 min at 37°C. Reactions were terminated with 2.5 ml of 10% ice cold TCA, centrifuged for 10 min at 2000 rpm, and the supernatant mixed 1:1 (v:v) with Ehrlich's reagent. After 30-45 min, absorption of the pyrrole formed was monitored at 556 nm against a standard curve of porphobilinogen (3-30nmol).

5.6.4 Ferrochelatase (FC, EC 4.99.1.1):

Enzyme solubilization: Intestinal mucosal cell fractions (30cm distal to the pylorus), were pooled from 4 rats and homogenized in 7 vol of 0.05M Tris/0.25M sucrose/1mM EDTA (pH 8.2) supplemented with trypsin

inhibitor and heparin (Section 5.2), by 10 strokes at 250 rpm in a glass-Teflon homogenizer. Additional homogenizing tended to cause gelling of the crypt cell homogenate upon centrifugation, presumably due to mucus present in the crypt cell population. Isolation of mitochondria and solubilization of the ferrochelatase enzyme was performed as described (Cole et al, 1979; Porra et al, 1967). The homogenate was centrifuged at 1200 x g for 10 min to remove nuclei and cell membrane debris. The supernantant was centrifuged at 11,000 x g for 10 min and the resulting mitochondrial pellet was washed twice with Tris buffer (0.02M, pH 8.2). The washed mitochondria were suspended in Tris buffer (0.1M, pH 10.4, 0.5ml/g intestine) and stirred vigorously in scintillation vials, while 0.1 vol of aqueous 10% (w/v) Tween 20 solution, followed by 0.1 vol of ethanol, were added dropwise. After 2.5 hr of gentle stirring, the mixture was centrifuged for 10 min at 20,000 x g. The supernatant fraction was dialyzed overnight (16hr) against 2 changes of 30 vol of 0.02M Tris buffer (pH 8.2). The dialysate was centrifuged at 105,000 x g for 60 min and the supernatant fraction stored at -80°C. Final protein concentrations were 0.7-1.0 mg/ml. Ferrochelatase activity was assayed within 5 days.

Activity Determination: Enzyme activity was assayed by a modification of the method of Cole et al (1979) for chick embryo liver. A deuteroporphyrin solution was used instead of mesoporphyrin since the amount of heme formation during this assay is three fold greater with deuteroporphyrin (Porra & Jones, 1963). Deuteroporphyrin dihydrochloride was dissolved in 0.5 ml of 2N NH_4OH in a dark tube, diluted 10-20 x with water and the pH adjusted to 8.2 with 1N HCl. To this was added 0.05 vol of 0.2M Tris buffer (pH 8.2) and 0.2 vol of 1% aqueous Tween 80 (Porra et

al, 1967). A 10 µl aliquot of the solution was diluted 300 fold with 0.1N HCl and the substrate concentration was determined by its absorption at 398 nm, using an extinction coefficient of $433mM^{-1}cm^{-1}$ (Furhop & Smith, 1975). Reagents were added to 12 ml round bottom tubes in the following order: deuteroporphyrin (120nmol), 1% (w/v) Tween 80 (0.3ml), ethanol (0.3ml), 0.2M Tris buffer (pH 8.2, 1.5ml), 0.2M DTE (60µl), 10mM sodium succinate (100µl) to insure anaerobosis (Jones & Jones, 1969), and ferrochelatase (1-2mg protein). The tubes were bubbled with N_2, capped with rubber caps, and continuously gassed with N_2 through 23 gauge needles during the assay. Control reactions included both iron and enzyme blanks. After preincubation at 37°C with gassing for 5 min, reactions were started with 120 µl of 1mM $FeSO_4$ by injection through the rubber caps. The reactions were incubated at 37°C anaerobically for 20 min in a shaking water bath and terminated by the addition of 0.4M iodoacetamide (0.5ml) and vortexing. Pyridine (1ml) and 1N NaOH (0.5ml) were added, the tubes vortexed, and within 5 min the absorption spectrum of sodium dithionite-reduced minus oxidized pyridine hemochromogen was recorded on an Aminco DW-2 spectrophotometer. The content of the pyridine hemochromogen was calculated from the peak-trough spectral difference obtained between 545 and 530.5 nm using an extinction coefficient of $15.3mM^{-1}cm^{-1}$ (Porra & Jones, 1963).

5.6.5 Microsomal Heme Oxygenase (MHO, EC 1.14.99.3): Intestinal mucosal cells were homogenized by the "standard" homogenizing procedure (Section 5.2) with the addition of 5% (v/v) fetal calf serum to the buffer. The enzyme activity was monitored by a modification of the procedure described by Raffin et al (1974) for intestinal mucosa. Incubation mixtures (3ml) contained microsomes (1.5-2.0mg protein), liver cytosol

(1.5mg protein as source of biliverdin reductase), and 1.9 ml of a methemalbumin stock solution (32μM, final) containing heme (2.6mM) in Tris buffer (0.1M, pH 7.4), NaCl (5mM), and 0.67% BSA, as previously reported (Correia & Schmid, 1975). Reactions were started with 0.5 μmol of NADPH in 50 μl of 0.05M Tris buffer (pH 7.4), carried out for 5 min in cuvettes maintained at 37°C, and monitored at 468 nm in an Aminco DW-2A spectrophotometer. An extinction coefficient of $60mM^{-1}cm^{-1}$ was used to calculate bilirubin (pmol) formed/mg microsomal protein/min (Raffin et al 1974).

5.7 OTHER ASSAYS:

5.7.1 Glutathione Peroxidase (GSHPx, EC 1.11.1.9): Activity was monitored in the cytosolic fraction by a coupled reaction (Paglia & Valentine, 1967) as modified (Lawrence & Burk, 1976). The reaction mixture consisted of NADPH (0.2mM), GSH (1mM), GSSG-reductase (1U/ml), in a total vol of 1 ml in 0.05M Tris buffer (pH 7.0) containing EDTA (1mM) and NaN_3 (1mM). Intestinal cytosol (0.1ml) was added to 0.8 ml of the above mixture and incubated at 25°C for 5 min before initiation of the reaction by addition to the sample cuvette of 0.1 ml of H_2O_2 (0.25mM, final) as the substrate for the selenium-dependent enzyme, or cumene hydoperoxide (1.5mM, final, in 25μl DMSO) to determine total GSHPx activity. The change in absorbance of NADPH was monitored at 340 nm for 1-2 min, and its rate of oxidation per mg protein per min was determined using an extinction coefficient of $6.2mM^{-1}cm^{-1}$ (Lu et al, 1969b). Non-selenium-dependent GSHPx activity was estimated as the difference between total GSHPx activity and the activity of selenium-GSHPx (Lee et al, 1981).

5.7.2 Cytochrome Oxidase: Activity was determined in solubilized enzyme from isolated mitochondria (Smith, 1955), extracted as described (Section 5.6.4). Sample and reference cuvettes each contained 0.1 ml of $K^+PO_4^{2-}$ buffer (pH 7.0), 0.76 ml of H_2O, and 0.04 ml of ferrocytochrome c (1% in $K^+PO_4^{2-}$ buffer, pH 7.0), reduced with potassium ascorbate and dialyzed overnight against three changes of 0.01 M $K^+PO_4^{2-}$ (pH 7.0). The blank cuvette was oxidized with 0.01 ml of potassium ferricyanide (0.1M) and after pre-incubation at 38°C for 3 min, the reaction was started with 0.1 ml enzyme added to both cuvettes. The rate of oxidation of ferrocytochrome c per mg protein per 10 min, monitored at 550 nm, was determined using an extinction coefficient of $21.1 mM^{-1}cm^{-1}$ (Van Gelder & Slater, 1962).

5.7.3 Protein Determination: Preliminary protein analyses were occasionally performed using the Coomassie Brilliant blue binding assay of Bradford (1976). All final protein determinations utilized the Folin phenol reagent method of Lowry et al (1951).

5.8 STATISTICAL ANALYSIS:

All experiments utilized pair fed animals, of identical weight, from the same commercial shipment. In the studies of dietary effects on cytochrome P-450 heme, comparisons were made between rats fed diets supplemented with iron, selenium, or both, and the corresponding diets deficient in one or both of these dietary variables. Statistical analysis employed the student's paired "t" test for differences between paired data. Percent values for the results of the variable dietary regimens in the figures are expressed as percent of control values; the standard errors of the means are similarly expressed as percent of

control means in these figures. During the studies on hemin-mediated cytochrome P-450 constitution, animals were pair fed the specific diets of each experiment, deficient in, or supplemented with, iron, selenium, or β-NF; each group of experimental diet-fed animals were then divided into two groups, one for in vitro hemin treatment and one to serve as non-hemin treated controls. During pentagastrin infusion studies, animals were similarly divided into two groups, one receiving pentagastrin, the other serving as control. Statistical analysis in these studies and all other data employed the student's paired "t" test for differences between means of the paired samples, or when indicated in the data tables, the student's "t" test for differences between sample means with unequal sample population.

6.0 RESULTS

6.1 PRELIMINARY STUDIES AND FINDINGS:

6.1.1 Separation of Intestinal Cell Populations: The two major cell types of the mucosal epithelium, the highly differentiated villous tip cells and the rapidly proliferating crypt cells (Figure 1; BACKGROUND, Section 2.2), were separated by the differential scraping technique (METHODS, Section 5.1), which yields crude fractions of each cell type. The validity of this technique for cell separation was confirmed by following the time dependent incorporation of ^{3}H-thymidine into nuclear DNA of the individual cell. This approach permits differentiation of the proliferative crypt cells from the mature villous tip cells. In confirmation of previous reports (Imondi et al, 1969; Hoensch et al, 1976), 1 hr after administration, most of the labelled thymidine (expressed as cpm per mg protein or cpm per μg DNA) was incorporated into isolated crypt cell nuclear DNA whereas at 48 hr it was detectable in isolated villous tip cell DNA (Table 4). These results indicate that the differential scraping technique is a valid method for separating villous tip and crypt cells. In addition, the specific microsomal cytochrome P-450 content of each cell population (Table 8) separated by this technique, is not only similar to values previously reported by others using the same technique (Hoensch et al, 1976), but to those obtained in cells isolated by mechanical vibration (Shirkey et al, 1979a) or collagenase digestion (Hartman et al, 1982).

6.1.2 Confirmation of the Biological Effectiveness of the Test Diets: Samples of the diets were chemically assayed for iron and selenium to verify their dietary content, as described (MATERIALS, Section 4.3).

TABLE 4

CONSECUTIVE INCORPORATION OF ^{3}H-THYMIDINE INTO CELL FRACTIONS OF DUODENAL MUCOSA*

Cell Fraction	Time after Administration 1 hr	48 hr
	(% of total cpm/mg nuclear protein), n=4	
Villous tip	21 ± 3	42 ± 3
Intermediate	21 ± 4	28 ± 2
Crypt	58 ± 6	30 ± 2
	100%	100%
	(% of total cpm/μg nuclear DNA), n=6	
Villous tip	27 ± 5	50 ± 5
Intermediate	36 ± 5	25 ± 4
Crypt	38 ± 5	26 ± 3
	100%	100%

Values are mean ± SEM of percents of total cpm for each time of administration.
Rats were given i.v. injections of ^{3}H-thymidine at time zero and killed after the intervals indicated.

From time to time, the biological effectiveness of the diets specifically stated to be deficient in iron and or selenium were verified by assaying the functional activity of key marker enzymes, as follows:

6.1.2.1 Iron Deficiency and Cytochrome Oxidase Activity: Iron status of the diets was confirmed by monitoring the activity of the intestinal mitochondrial hemoprotein cytochrome oxidase, which was previously found to be reduced within 3 months of dietary iron deprivation to 20% of iron-fed rats (Richmond et al, 1972). Cytochrome oxidase activity was indeed decreased to 64% of control values in rats fed diets deficient in iron for merely 4 days (Table 5). These findings thus demonstrate that cytochrome oxidase is in fact acutely dependent on iron.

Moreover, in parallel, the intestinal content of the microsomal hemoprotein cytochrome P-450 was also markedly reduced by dietary iron deprivation (Section 6.3.2), thereby indicating that the diet under investigation was indeed reliably iron deficient.

6.1.2.2 Selenium Deficiency and Glutathione Peroxidase Activity: The selenium status of the diets was routinely monitored by assaying the activity of the seleno-enzyme glutathione (GSH) peroxidase in intestinal cytosolic fractions of rats fed diets supplemented (+Se) or deficient (-Se) in selenium. GSH peroxidase activity was decreased to 63% in villous tip cells of rats fed a selenium deficient (+Fe -Se) diet for 4 days (Table 6, Figure 10), confirming earlier reports of similar findings (Reddy & Tappel, 1974).

GSH peroxidase activity in crypt cells was significantly greater than that in villous tip cells in all experimental groups (Table 6).

TABLE 5

EFFECT OF DIETARY IRON ON INTESTINAL
VILLOUS TIP CYTOCHROME OXIDASE ACTIVITY

Diet	Cytochrome Oxidase (nmol ferrocytochrome c oxidized/mg protein/min)[a]
+Fe +Se	10.25 ± 1.2
-Fe +Se	6.51 ± 2.1[b]

Values are mean ± SEM; n=3, each n represents a pool of tissue from 3 animals.

a --- solubilized mitochondria
b --- $p < 0.02$.

TABLE 6

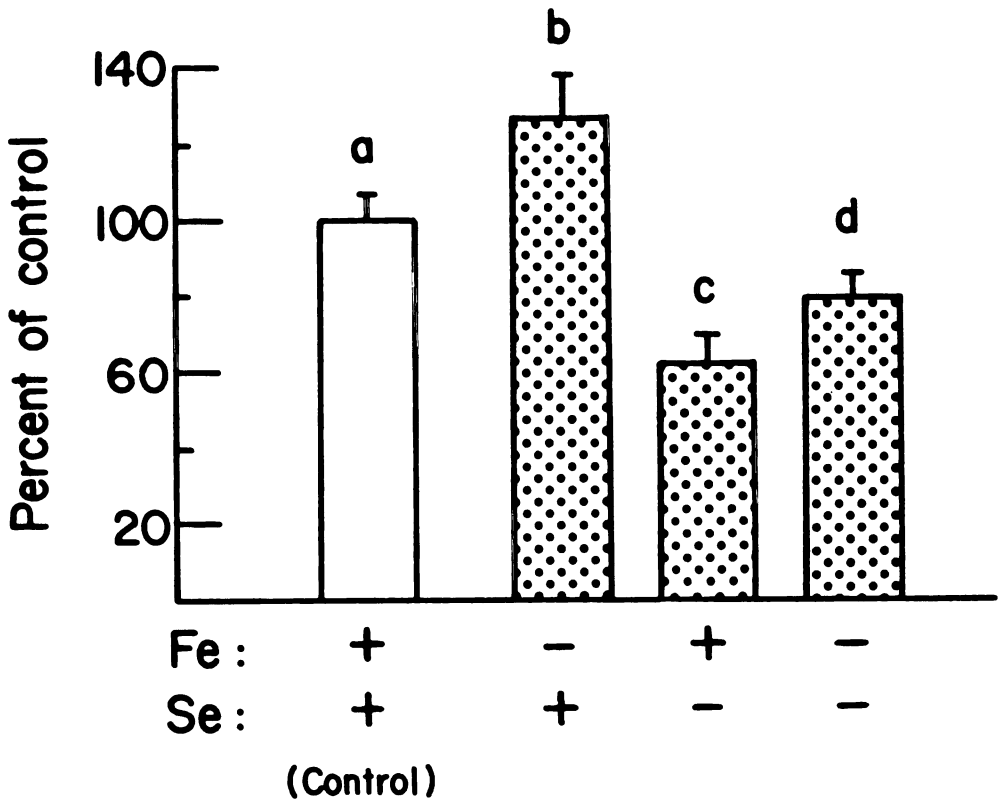
DEPENDENCE OF INTESTINAL GLUTATHIONE PEROXIDASE
ON DIETARY SELENIUM AND IRON

		Selenium-Dependent GSHPx (nmol NADPH/mg protein/min)[a]	Non-Selenium-Dependent GSHPx (nmol NADPH/mg protein/min)[a]
+Fe +Se			
	tips	9.49 ± 0.4	10.34 ± 1.4
	crypts	20.47 ± 1.1[b]	4.92 ± 0.7[c]
-Fe +Se			
	tips	12.15 ± 0.8[d]	---
	crypts	22.93 ± 1.1[b]	---
+Fe -Se			
	tips	6.10 ± 0.6[d]	7.67 ± 0.8[e]
	crypts	13.70 ± 1.5[bf]	7.26 ± 1.0

All values are mean ± SEM; n=6, each n represents tissue pooled from 3 animals.

a --- 105,000 x g supernatant
b --- $p < 0.01$ vs corresponding tips
c --- $p < 0.05$ vs corresponding tips
d --- $p < 0.02$ vs +Fe +Se tips
e --- $p < 0.05$ vs +Fe +Se tips
f --- $p < 0.001$ vs +Fe +Se crypts

Figure 10. Effect of dietary iron and selenium on intestinal selenium-dependent glutathione peroxidase of villous tip cells. a) Control value from +Fe +Se fed rats: 9.49 $\pm$ 0.4 nmol NADPH oxidized/mg cytosolic protein/min; H_2O_2 substrate. b) $p < 0.02$ vs +Fe +Se. c) $p < 0.001$ vs +Fe +Se. d) $p < 0.05$ vs +Fe -Se and -Fe +Se. Values are mean $\pm$ SEM, n=6.


Percent of control
140
100
60
20
a
b
c
d
Fe :
Se :
+
+
−
+
+
−
+
−
−
(Control)

Dietary selenium deficiency produced a significant decrease in crypt and tip cell GSH peroxidase activity.

Dietary iron deprivation (-Fe +Se), in contrast, produced a substantial increase in GSH peroxidase activity (Table 6). Similarly, in selenium-deprived rats, the additional deprivation of dietary iron (-Fe -Se) resulted in increased GSH peroxidase activity, subsequently raising it to near control (+Fe +Se) values (Figure 10). The finding that iron deficiency results in increased intestinal enzyme activity is in disagreement with reports of decreased erythrocyte and hepatic GSH peroxidase activity in iron-deficient rats (Rodvien et al, 1974; Perona et al, 1977; Lee et al, 1981). The significance of this apparent discrepancy is presently unclear.

On the other hand, non-selenium GSH peroxidase activity was not only detectable in intestinal mucosal tip cells, but was significantly greater than that of selenium-dependent GSH peroxidase (Table 6), in contradiction of previous reports (Lawrence & Burk, 1978). Similarly, in contradiction of previous reports (Lee et al, 1981), the activity of non-selenium-dependent GSH peroxidase was decreased rather than increased by dietary selenium deprivation (Table 6). The reasons for this discrepancy may be that, in contrast to procedures utilized herein (METHODS, Section 5.2), the tissue preparation conditions used in those studies failed to adequately protect against proteolytic degradation of the enzyme during subcellular fractionation.

6.2 SPECTRAL AND FUNCTIONAL CHARACTERISTICS OF RAT INTESTINAL CYTOCHROME P-450 ISOZYMES:

The basal "constitutive" species of intestinal cytochrome P-450 exhibited an absorption maximum of the CO-bound reduced form at 452.5 nm

in rats fed the inducer-free diet (Figure 11). β-NF supplementation of the diet resulted in a blue shift of this absorption maximum to 448.5-449 nm with a concomitant 6 fold increase in the chromophore content (Figure 11). Furthermore, this absorption maximum was consistently observed in the complete absence of any intraluminal dietary substances in rats intravenously hyperalimented for periods up to 8 days, indicating that it truly reflected the spectral characteristics of the constitutive form of intestinal cytochrome P-450.

Basal cytochrome P-450 content and MFO activity of intestinal microsomes from rats fed a purified semi-synthetic diet, devoid of the natural inducers normally found in standard rat chow (Wattenberg et al, 1962; Pantuck et al, 1976), are shown in table 7. In these rats, the rate of ethoxycoumarin O-deethylation (ECOD) was found to be substantially lower than that of ethoxyresorufin O-deethylation (EROD). However, when these rats were fed the β-NF-supplemented semi-synthetic diet for 4 days, EROD activity rose 1000 fold, a rise strikingly greater than the corresponding 300 fold increase in ECOD activity, but both in parallel with the 6 fold increase in cytochrome P-450 content. The increases in O-deethylation activities were 200 and 50 fold, respectively, when expressed per pmol cytochrome P-450.

Such increases in the activities of the two intestinal dealkylases (1000 and 300 fold) are indeed dramatic and have not been previously reported. A 50 fold increase in intestinal AHH activity has been observed after oral β-NF treatment of rats (Wattenberg & Leong, 1970). A 20 fold increase has also been observed in intestinal microsomes of rats orally infused with 3-MC (Stohs et al, 1976b), a PAH inducer of cytochrome P-448 which is believed to induce by a mechanism similar to

that of β-NF (Boobis et al, 1977). The relatively greater stimulation of intestinal EROD as compared with that of ECOD after β-NF induction (Table 7) has also not been observed following 3-MC induction (Stohs et al, 1977). Since the rats in those studies were fed crude rat chow, instead of the semi-synthetic diet free of cytochrome P-448 inducers (MATERIALS, Section 4.3), they exhibited significantly higher basal values for dealkylation, which largely masked the extent of cytochrome P-450 induction by β-NF or 3-MC as well as any possible differences between stimulated activities of EROD and ECOD.

Feeding of rats with β-NF also greatly stimulated intestinal aryl hydrocarbon hydroxylase (AHH) activity (Figure 8). With benzo(a)pyrene (BaP) as substrate, β-NF-induced AHH exhibited a Km of 2.4×10^{-5}M and a Vmax of 476 pmol/mg protein/min, whereas the non-induced enzyme exhibited a corresponding Km of 1.1×10^{-3}M, and a Vmax of 250 pmol/mg protein/min. These results thus contradict previous reports that Km values for the 3-MC-induced and non-induced intestinal enzymes are identical (Zampaglione & Mannering, 1973), though the Km value for the 3-MC-induced enzyme is similar to the one reported here for the β-NF-inducible enzyme (Figure 8). Again, the most likely reason for this discrepancy is that feeding of standard rat chow containing natural cytochrome P-448 inducers to "control" non-induced animals apparently resulted in partial induction of a cytochrome P-450 isozyme in control rat intestine (Zampaglione & Mannering, 1973). If this presumably "constitutive" isozyme should be homologous to that induced by 3-MC, then it would also exhibit similar substrate affinities, and consequently identical Km values.

Figure 11. CO-difference spectrum of reduced cytochrome P-450 in intestinal microsomes from a) β-NF-treated rats, cytochrome P-450 content: 150 pmol/mg protein, λ_{max} 448.5-449 nm; b) rats fed the control diet, cytochrome P-450 content: 25 pmol/mg protein, λ_{max} 452.5 nm. Spectra were obtained with 1 mg microsomal protein/1 ml 0.05M Tris/20% glycerol buffer (pH 7.4).

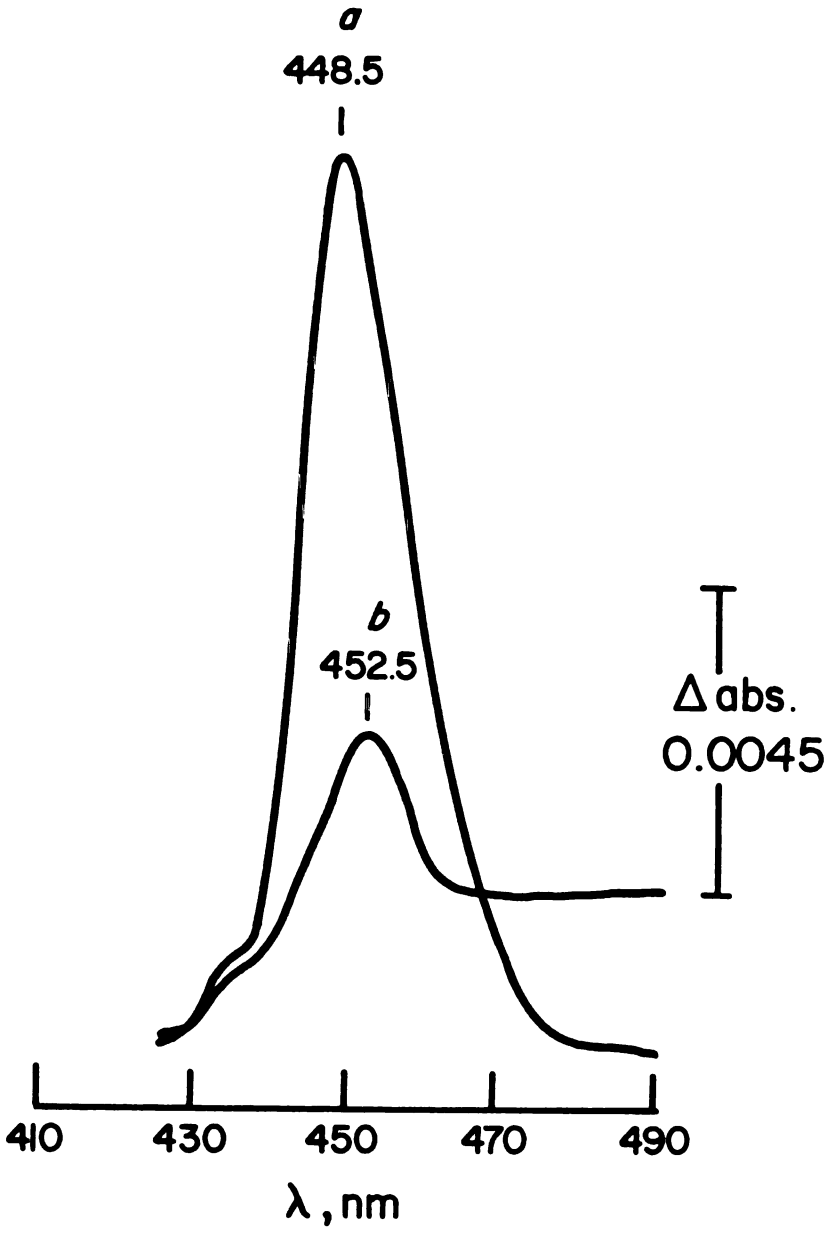

a
448.5
b
452.5
Δ abs.
0.0045
410
430
450
470
490
λ, nm

TABLE 7

EFFECTS OF β-NAPHTHOFLAVONE (β-NF) ON INTESTINAL MICROSOMAL CYTOCHROME P-450 AND RELATED MFO ACTIVITY

	Cytochrome P-450	Ethoxyresorufin O-deethylase (EROD)		Ethoxycoumarin O-deethylase (ECOD)	
β-NF*	pmol/mg protein	pmol/mg protein/min	pmol/pmol P-450/min	pmol/mg protein/min	pmol/pmol P-450/min
-	25.0 ± 5.0	21.0 ± 4.4	0.75 ± 0.2	8.5 ± 1.2	0.34 ± 0.1
+	150.0 ± 25.0	23,110 ± 1950	154.1 ± 12.5	2,450 ± 250	16.3 ± 1.6

* 0.1% supplemented in the semi-synthetic diet, replete in iron and selenium (MATERIALS).

Values are mean ± SEM, n=4; each n represents a pool of tissue from 2 non-induced animals, or individual tissue samples from β-NF-treated animals.

6.3 REGULATION OF INTESTINAL CYTOCHROME P-450 AND ITS HEME MOIETY BY DIETARY NUTRIENTS:

6.3.1 Cytochrome P-450 and MFO Gradients in the Intestinal Mucosa: A tip to crypt descending gradient in the content of cytochromes P-450 and b_5, NADPH-cytochrome P-450 reductase activity and cytochrome P-450-dependent AHH and EROD activities is observed along the height of the intestinal mucosal villus of rats (Tables 8 & 9), in accordance with previous findings (Hoensch et al, 1976; Dawson & Bridges, 1981b; Hartman et al, 1982). Although the data (Table 10) indicate that microsomal heme content did not differ from tip to crypt cells, the actual heme content of crypt cell microsomes may be lower than that measured, due to variable contamination of crypt cell preparations with heme of hemoglobin and/or myoglobin derived from the underlying tissue layers. Such contaminating heme is apparently resistant to removal by the routine washing procedures (METHODS, Section 5.2). Consistent with this possibility, spectral measurements revealed a large 420 nm peak in crypt cell microsomes following CO binding. Thus, microsomal heme content probably follows a descending gradient from tip to crypt cells along the mucosal villus.

6.3.2 Dependence of Cytochrome P-450 Content and Oxidative Function on Dietary Iron and/or Selenium: Deprivation of intraluminal iron by feeding rats a purified semi-synthetic iron-deficient diet significantly lowered intestinal cytochrome P-450 content and its associated AHH and EROD activities (Table 8), in confirmation of previous findings (Hoensch et al, 1976). This reduction was selectively observed in the mature epithelial cells situated at the villous tip.

That dietary selenium was critically required to maintain cytochrome

P-450 content and activity in these cells, was indicated by the finding that its acute deprivation for a period as short as one day (Table 8, Figure 12) also lowered cytochrome P-450 content and activity, albeit to a lesser extent than that observed following iron deprivation, and in spite of adequate dietary iron. Due to the high variability routinely encountered in assay of intestinal enzymes, only alterations in EROD activity following acute selenium deprivation were found to be statistically significant (Table 8). That intestinal AHH and EROD activities are critically dependent on both iron and selenium is further confirmed by their dramatic reduction following combined dietary deprivation of both elements (Table 8). These findings not only confirm the critical requirement of iron for intestinal cytochrome P-450-dependent MFO's but reveal a hereto unknown acute dependence on dietary selenium for their structural maintenance and function.

In addition, acute dietary deprivation of either iron or selenium failed to affect villous tip microsomal cytochrome b_5 content and the activity of the usually rate-limiting enzyme in microsomal cytochrome P-450-dependent oxidations, NADPH-cytochrome P-450 (c) reductase (Figure 13). Essentially similar findings were obtained in crypt cells (Table 9). Moreover, combined dietary deficiency of both elements produced no observable changes in these microsomal components in either cell population. This indicated that the observed impairment of intestinal MFO activity was primarily due to decreased cytochrome P-450 content.

6.3.3 Effects of Acute Dietary Iron Deprivation on Intestinal Heme and Heme Metabolism: To investigate whether the observed lowering of intestinal cytochrome P-450 content and activity in rats acutely deprived of intraluminal iron was due to impaired synthesis of the prosthetic heme

(ferriprotoporphyrin IX) moiety of the cytochrome, intestinal microsomal heme content and activity of three enzymes in the heme synthetic pathway were examined: mitochondrial δ-aminolevulinic acid synthetase (ALAS), the rate limiting enzyme; cytoplasmic δ-aminolevulinic acid dehydratase (ALAD); and mitochondrial ferrochelatase (FC), the terminal enzyme which actually inserts the iron into the porphyrin moiety and often constitutes a secondary rate limiting step. Furthermore, to exclude the possibility that accelerated heme degradation was responsible for the observed microsomal cytochrome P-450 reduction in the intestinal mucosa, the activity of microsomal heme oxygenase (MHO), the rate limiting enzyme in heme degradation, was also determined.

As expected, dietary deprivation of its prosthetic element substantially decreased microsomal heme and cytochrome P-450 content in mucosal villous tip and crypt cells of rats fed the iron deficient (-Fe +Se) diet compared to rats fed the control (+Fe +Se) diet (Tables 8 and 10). Crypt cells in control rats (+Fe +Se) were found to exhibit greater ALAS activity than that in the corresponding tip cells (Table 10). In parallel with the reduction of heme and cytochrome P-450 content, acute dietary iron deprivation significantly lowered ALAS activity in both intestinal tip and crypt cells. ALAD activity followed a similar decreasing gradient from crypt cells to villous tip cells in control rats, but acute iron deficiency failed to lower it (Table 10). Similarly, a decreasing gradient was also observed for ferrochelatase activity from crypt to tip cells. Acute iron deficiency lowered ferrochelatase activity in villous tip cells (Table 10). Since this occured in spite of deliberately optimized iron concentrations in the assay mixture, it suggests that iron may be necessary for maintenance of this enzyme.

In contrast to the effects of prolonged iron deficiency (Raffin et al, 1974), acute iron deficiency only minimally stimulated MHO activity in villous tip cells, but apparently did not affect it in crypt cells (Table 11).

6.3.4 Effects of Acute Dietary Selenium Deprivation on Intestinal Heme and Heme Metabolism: Given the novel finding that dietary selenium is critically required for maintenance of cytochrome P-450 and its dependent MFO activity (Table 8), its role in intestinal heme metabolism was investigated. Microsomal heme of villous tip cells moderately but significantly decreased in rats fed the selenium deficient (+Fe -Se) diet. Microsomal heme content of crypt cells also decreased in rats fed the selenium deficient diet, irrespective of its iron content. Acute dietary selenium deficiency per se had no effect on ALAS and ALAD activities in either cell type, but significantly reduced their ferrochelatase activity (Table 10). Thus, impairment of this particular enzyme might be responsible for the observed decrease in microsomal heme content (Table 10) and consequent reduction in intestinal cytochrome P-450 content and its associated MFO activity in rats deprived of iron and/or selenium (Table 8).

Dietary selenium deprivation apparently lowered MHO activity in villous tip cells (Table 11), reflecting decreased heme catabolism, however, this finding was not statistically significant. On the other hand, it did significantly lower MHO activity in crypt cells (Table 11). Reduction of MHO activity may thus reflect decreased microsomal heme content resulting from acute selenium deficiency in these cells.

TABLE 8

EFFECTS OF DIETARY IRON AND SELENIUM
ON THE INTESTINAL CYTOCHROME P-450 MFO SYSTEM

Diet		Cytochrome P-450[a] pmol/mg protein	AHH[b] pmol/mg protein/15 min	EROD[a] pmol/mg protein/min
+Fe +Se				
	tips	23.18 ± 9.5	11.68 ± 3.7	21.00 ± 4.4
	crypts	2.21 ± 0.4[c]	2.56 ± 0.5[c]	3.20 ± 0.8[c]
-Fe +Se				
	tips	9.13 ± 5.8[d]	3.17 ± 0.7[d]	5.16 ± 1.2[d]
	crypts	2.63 ± 1.3[e]	1.91 ± 0.6[cf]	1.35 ± 0.1[cf]
+Fe -Se				
	tips	16.90 ± 5.1	6.20 ± 1.0	5.95 ± 1.1[d]
	crypts	4.00 ± 0.7[c]	1.60 ± 0.3[c]	1.17 ± 0.4[ce]
-Fe -Se				
	tips	8.40 ± 4.5[dg]	2.60 ± 0.3[dg]	1.64 ± 0.1[dh]
	crypts	2.30 ± 0.5[e]	1.11 ± 0.3[cf]	0.80 ± 0.2[ci]

All values are mean ± SEM, each n represents pool of mucosal tissue from 4 rats.

a --- microsomes, n=4-5
b --- 9000 x g supernatant, n=4-5
c --- $p < 0.05$ vs corresponding tips
d --- $p < 0.05$ vs +Fe +Se control tips
e --- $p < 0.1$ vs corresponding tips
f --- $p < 0.05$ vs +Fe +Se crypts
g --- $p < 0.05$ vs +Fe -Se tips
h --- $p < 0.02$ vs +Fe -Se and -Fe +Se tips
i --- $p < 0.05$ vs -Fe +Se crypts

TABLE 9

EFFECT OF DIETARY IRON AND SELENIUM
ON MFO SYSTEM COMPONENTS

Diet		NADPH-Dependent Cytochrome P-450 Reductase[a] nmol/mg protein/min	Cytochrome b_5[a] pmol/mg protein
+Fe +Se			
	tips	32.03 ± 2.9	25.03 ± 5.0
	crypts	23.71 ± 3.8[b]	19.63 ± 2.4[b]
-Fe +Se			
	tips	32.02 ± 4.0	26.27 ± 4.6
	crypts	23.05 ± 6.3[b]	18.73 ± 5.9[b]
+Fe -Se			
	tips	41.82 ± 4.3[c]	25.26 ± 0.8
	crypts	25.07 ± 4.0[b]	16.50 ± 3.4[b]
-Fe -Se			
	tips	63.1 ± 3.6[d]	22.40 ± 2.1
	crypts	38.2 ± 4.2[b]	15.07 ± 4.0[b]
+Fe -Se			
	tips	63.6 ± 6.4	
	crypts	31.9 ± 7.1[b]	

All values are mean ± SEM, n=4.

a --- microsomes
b --- $p < 0.05$ vs corresponding tips
c --- (vs +Fe +Se or -Fe +Se tips --- NS)
d --- pair fed with +Fe -Se group below

TABLE 10

EFFECT OF DIETARY IRON AND SELENIUM ON INTESTINAL HEME SYNTHESIS

Diet	Heme[a] pmol/mg protein	ALAS[b] pmol/mg protein/30 min	ALAD[c] nmol/mg protein/hr	FC[d] nmol/mg protein/20 min
+Fe +Se				
tips	128.0 ± 9.3	83.47 ± 12.1	3.38 ± 0.5	9.02 ± 1.1
crypts	125.0 ± 14.6	238.50 ± 65.1[e]	4.32 ± 0.4[e]	20.79 ± 3.7[f]
-Fe +Se				
tips	105.0 ± 5.5[g]	45.41 ± 12.1[g]	4.23 ± 0.5[h]	4.69 ± 0.6[g]
crypts	114.1 ± 15.5[i]	152.99 ± 46.4[ej]	4.02 ± 0.3	19.43 ± 2.5[f]
+Fe -Se				
tips	99.2 ± 6.5[g]	86.48 ± 9.9	4.19 ± 0.5	5.65 ± 1.1[g]
crypts	69.1 ± 10.2[ej]	195.58 ± 19.0[e]	6.25 ± 0.9[e]	12.92 ± 2.6[ej]
-Fe -Se				
tips	93.3 ± 5.7[g]	56.01 ± 8.1[gk]	6.09 ± 0.3[gk]	--
crypts	76.9 ± 8.3[ej]	166.78 ± 19.2[ejl]	6.69 ± 0.2	--

All values are mean ± SEM, each n represents a pool of mucosal tissue from 4 animals.

a --- microsomes, n=5
b --- whole homogenate, n=8
c --- 105,000 x g supernatant, n=4
d --- solubilized mitochondria, n=5
e --- $p < 0.05$ vs corresponding tips
f --- $p < 0.005$ vs corresponding tips
g --- $p < 0.05$ vs +Fe +Se tips
h --- $p < 0.005$ vs +Fe +Se tips
i --- $p < 0.1$ vs +Fe +Se crypts
j --- $p < 0.05$ vs +Fe +Se crypts
k --- $p < 0.05$ vs +Fe -Se tips
l --- $p < 0.005$ vs +Fe -Se crypts

Figure 12. Time course of the effect of dietary selenium deficiency on intestinal villous tip EROD (○) and GSHPx (△) activities. Rats were fed the control (+Fe +Se) semisynthetic diet for 5 days prior to switching to the selenium deficient (+Fe -Se) diet. In each separate experiment, mucosal tissue pooled from 3 pair fed rats was prepared as described (METHODS). Results are the mean ± S.E. from 3-4 experiments and are expressed as % of the values from control fed (+Fe +Se) rats. Control values for EROD (●) and GSHPx (▲) activities were 21.9 ± 0.8 pmol/mg microsomal protein/min (n=4) and 9.4 ± 0.4 nmol/mg cytosolic protein/min (n=4), respectively.

*Values significantly different ($p<0.05$) from pair fed control (+Fe +Se) values.

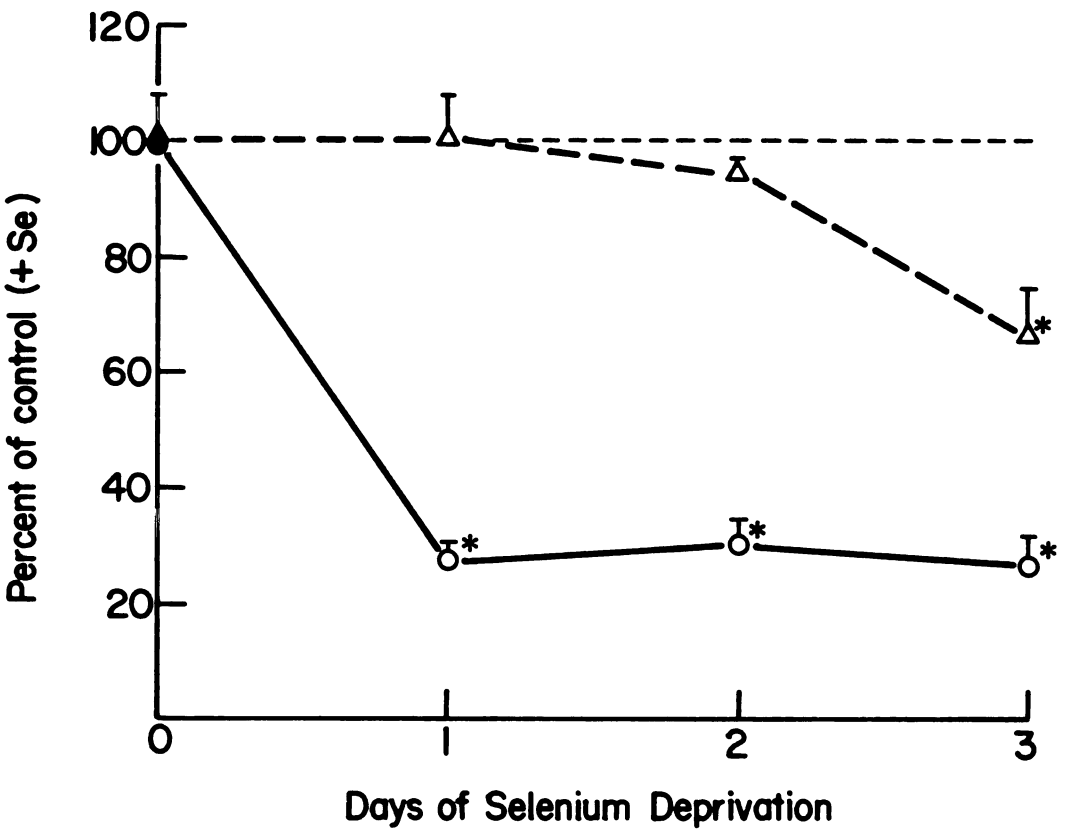

120
100
80
60
40
20
0
1
2
3
Percent of control (+Se)
Days of Selenium Deprivation
*
*
*
*

Figure 13. Effect of dietary iron and selenium deficiencies on microsomal NADPH cytochrome P-450 (c) reductase (CYT c RED) activity and cytochrome b_5 (CYT b_5) content of villous tip cells. Results are the mean $\pm$ S.E. and expressed as % of values from pair fed control (+Fe +Se) rats. Control values for P-450 reductase activity and cytochrome b_5 content are 32.0 $\pm$ 2.9 nmol cytochrome c reduced/mg protein/min and 25.0 $\pm$ 5.0 pmol/mg protein, respectively. No statistically significant differences were found.

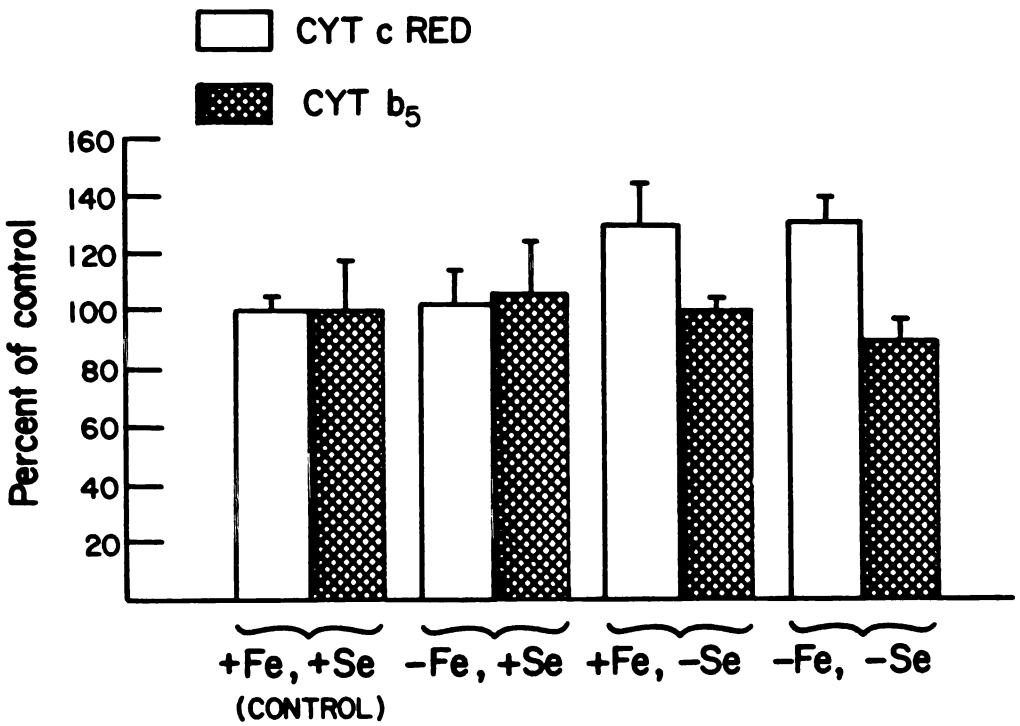

CYT c RED
CYT b_5
Percent of control
160
140
120
100
80
60
40
20
+Fe, +Se
(CONTROL)
-Fe, +Se
+Fe, -Se
-Fe, -Se

TABLE 11

EFFECT OF DIETARY IRON AND SELENIUM
ON INTESTINAL HEME CATABOLISM

Diet		MHO[a] pmol/mg protein/min
+Fe +Se		
	tips	332.3 ± 46.9
	crypts	153.7 ± 24.2[b]
-Fe +Se		
	tips	379.8 ± 1 8.5[c]
	crypts	152.7 ± 25.5[d]
+Fe -Se		
	tips	209.9 ± 43.8
	crypts	98.2 ± 13.7[d]
-Fe -Se		
	tips	339.4 ± 107.2
	crypts	81.3 ± 25.1[d]

Values are mean ± SEM, each n represents a pool of tissue from 4 animals.

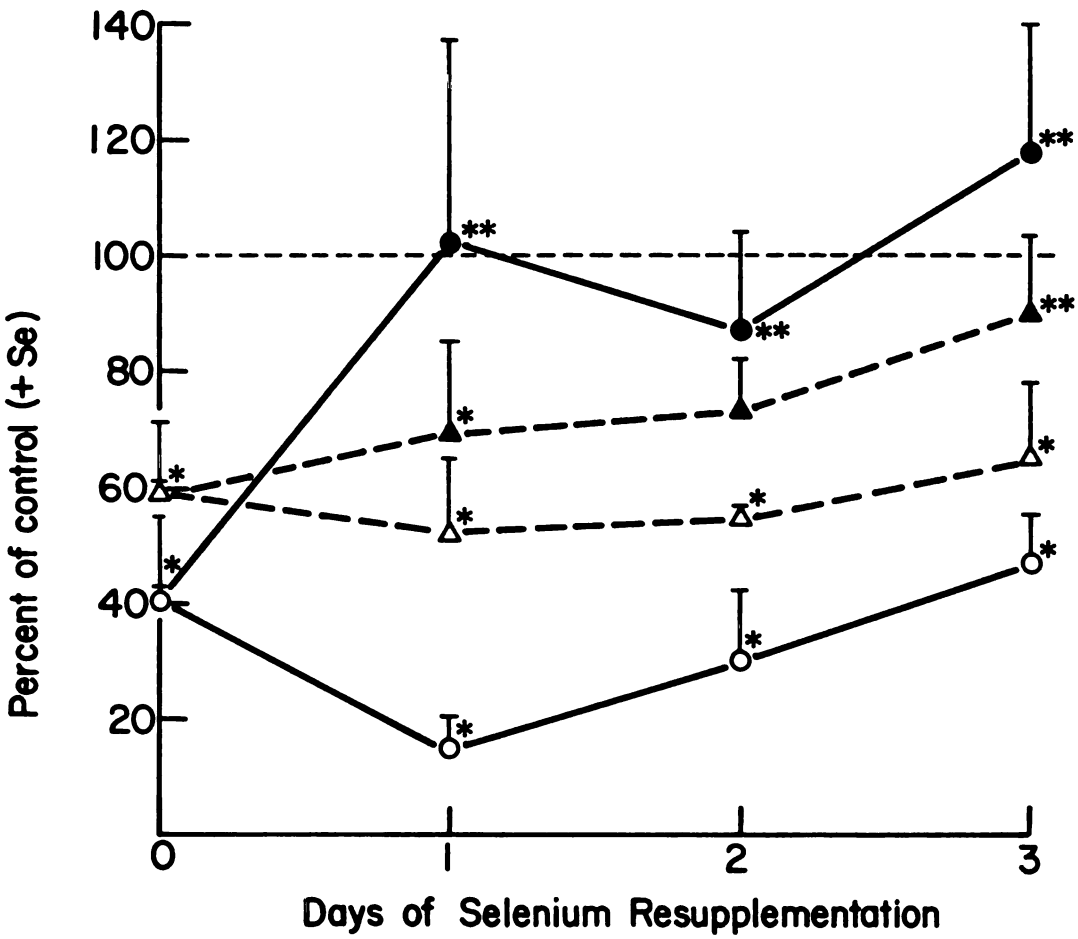
a --- microsomes, n=5
b --- $p < 0.005$ vs corresponding tips
c --- $p < 0.05$ vs +Fe +Se tips
d --- $p < 0.05$ vs corresponding tips
e --- $p < 0.05$ vs +Fe +Se crypts

6.3.5 Role of Dietary Selenium in Regulation of Intestinal Cytochrome P-450-Dependent MFO Activity: Selenium is the prosthetic moiety of GSH peroxidase (Oh et al, 1974; Hafeman et al, 1974), an enzyme critical in detoxification of organic and inorganic peroxides (Lawrence & Burk, 1976; Tappel, 1965; Hoekstra, 1975). It was conceivable therefore that acute dietary selenium deprivation, by impairing the functional activity of intestinal GSH peroxidase, might result in accumulation of toxic peroxides, capable of intracellular membrane damage and hence cytochrome P-450 inactivation (Hrycay & O'Brien, 1971; Tenhunen et al, 1970). Indeed, such acute selenium deprivation significantly reduced the selenium-dependent GSH peroxidase activity of rat villous tip cells to 63% of basal values (Table 6). Feeding of rats with a selenium deficient diet for 2 weeks has been previously reported to reduce their intestinal GSH peroxidase activity (Reddy & Tappel, 1978). The present findings indicate that this reduction occurs much more rapidly, i.e., within just 3 days of dietary selenium deprivation. To determine whether reduced GSH peroxidase activity was responsible for the observed impairment of cytochrome P-450-dependent MFO activity (Table 8), rats acutely deprived of dietary selenium were re-fed the selenium supplemented diet (+Fe +Se) for 0,1,2 or 3 days, and the restoration of GSH peroxidase activity was correlated with reversal of impairment of EROD activity in villous tip cells (Figure 14). Selenium resupplementation for even a single day reversed villous tip EROD activity to control values without significant restoration of GSH peroxidase activity (Figure 14). These findings, coupled with the results in figure 12, indicate that the critical role of dietary selenium in maintenance of intestinal cytochrome P-450 structure and function is independent of its function as the prosthetic moiety of GSH peroxidase.

Figure 14. Time course of dietary selenium resupplementation effects on intestinal villous tip EROD (——) and GSHPx (----) activities. For each experiment, rats were fed the selenium deficient (+Fe -Se) semisynthetic diet (open symbols) for 4 days, at which time rats were switched to the selenium supplemented (+Fe +Se) diet (closed symbols); additional rats were pair fed as controls (+Fe +Se, 100% levels) throughout. In each separate experiment, mucosal tissue pooled from 3 rats was prepared as described (METHODS). Results are the mean ± S.E. from 3 experiments. o - Mucosal EROD activity of rats fed the selenium deficient or ● - selenium resupplemented diets. Δ - Mucosal GSHPx activity of rats fed the selenium deficient or ▲ - selenium resupplemented diets. Control (+Fe +Se) values for EROD and GSHPx activities are 10.6 ± 1.1 pmol/mg microsomal protein/min and 13.3 ± 1.3 nmol/mg cytosolic protein/min, respectively.

* Values significantly different from control (+Fe +Se) fed values ($p<0.05$).

** Values significantly different from selenium deficient (+Fe -Se) fed values ($p<0.05$).


140
120
100
80
60
40
20
Percent of control (+Se)
0
1
2
3
Days of Selenium Resupplementation

6.4 REGULATION OF APOCYTOCHROME P-450 BY DIETARY NUTRIENTS AND INTRACELLULAR HEME:

6.4.1 Examination of Apocytochrome P-450 Synthesis by SDS-Slab Gel Electrophoresis: Preliminary attempts to monitor alterations in intestinal apocytochrome P-450 content and/or synthesis included measuring the incorporation of radiolabelled amino acids into mucosal apocytochrome separated from microsomal proteins by sodium dodecyl sulfate (SDS)/ polyacrylamide slab gel electrophoresis. This technique has been successfully used to monitor changes in phenobarbital-mediated apocytochrome induction in the liver of selenium-deficient rats (Burk & Correia, 1977).

Rats were fed the semi-synthetic diet either free of cytochrome P-450 inducers or supplemented with β-NF. Control rats were administered ^{3}H-leucine while β-NF-fed rats received ^{14}C-leucine, and the cellular homogenates of their intestinal mucosa were pooled (METHODS, Section 5.3.1). Intestinal microsomal cytochrome P-450 apoprotein, separated by electrophoresis on SDS polyacrylamide gels and identified by co-migration with purified hepatic apocytochrome P-450 (gift of Dr. W. Levin), showed only slight stimulation of ^{14}C-leucine incorporation into the apocytochrome after induction by β-NF. On slicing the SDS-slab gels, the band which corresponded to purified hepatic apocytochrome repeatedly showed only a slight increase (1.2 fold) in the $^{14}C/^{3}H$ ratio, reflecting very little induced apoprotein. Thus, this technique did not appear sufficiently promising for further examination of the effects of dietary iron deprivation on intestinal apocytochrome P-450 formation.

6.4.2 Determination of "Free" Intestinal Apocytochrome P-450 by Monitoring the In Vitro Assembly of Holoenzyme with Hemin: In view of the failure to monitor intestinal apocytochrome P-450 synthesis by the dual labelling technique, an indirect approach was employed to determine intestinal apocytochrome P-450 content. In this approach, "heme-free" apocytochrome was generated by deliberate impairment of cytochrome P-450 heme synthesis by dietary iron deprivation. It was expected that such apocytochrome could be spectrally quantitated after its assembly to the holocytochrome following incubation with hemin in vitro. This in vitro approach, used successfully to reconstitute the hepatic holoenzyme in rat liver preparations deliberately enriched in apocytochrome P-450 (Correia & Meyer, 1975), thus permits indirect quantitation of the constitutible apocytochrome content. The function of newly assembled holocytochrome could then be examined by monitoring its MFO activity.

6.4.2.1 Assembly of Cytochrome P-450 with Hemin in Cellular Fractions: Initial attempts to reconstitute intestinal apocytochrome P-450 from rats fed the -Fe or +Fe selenium-supplemented diets by incubating mucosal homogenates (20% v/v) with hemin, resulted in complete inactivation of the enzyme (Table 12). Although the homogenizing buffer contained sufficient glycerol and trypsin inhibitor to stabilize intestinal cytochrome P-450 and protect it from tryptic degradation (Stohs et al, 1976a), the intestinal enzyme appeared to be inherently unstable under incubation conditions found suitable for successful cytochrome P-450 reconstitution in liver homogenates in vitro (Correia & Meyer, 1975).

Cytochrome P-450 assembly in incubations of heme with mucosal microsomes from rats fed the same diets yielded erratic results. For example, EROD activity was slightly decreased in hemin-incubated micro-

TABLE 12

EFFECT OF HEMIN-INCUBATION ON MICROSOMAL CYTOCHROME P-450 AND MFO ACTIVITY IN CELLULAR FRACTIONS

Cellular Fraction	Diet	Cytochrome P-450 pmol/mg protein	EROD pmol/mg protein/min		AHH pmol/mg protein/min	
		---	-Hemin	+Hemin	-Hemin	+Hemin
Homogenate*	-Fe	ND	ND	ND	ND	ND
	+Fe	ND	ND	ND	ND	ND
Microsomes*	-Fe	--	7.0 (1.2-12.8)	3.6 (ND-3.6)	8.0 (6.8-9.2)	15.0 (11.8-18.2)
	+Fe	--	16.3 (9.3-23.3)	14.6 (3.1-26.0)	11.2 (9.1-13.23)	18.8 (18.4-19.2)

* Hemin (4 μM) was incubated at 37°C for 10 min as described (METHODS).
ND --- not detectable

Values are mean of range in parentheses.
Each value represents a pool of tissue from 2 animals.
Differences between hemin treatment and control values are not significant, or inconsistent between MFO assays.

somes from both -Fe and +Fe fed rats, while substantial increases were observed in AHH activity in the same preparations (Table 12). Moreover, varying hemin concentrations (1-15μM) and/or incubation times (5 to 20min) in systems containing intestinal homogenates or microsomes gave similar results. Since in the EROD assay no heme was detected in the supernatant after microsomal protein precipitation, fluorescence quenching by hemin was excluded as the explanation for the apparent discrepancies between the observed findings of each of the assays. Moreover, product inhibition of EROD activity (Pohl & Fouts, 1980) was also excluded, since it was not confirmed even after inclusion of 20-fold greater concentrations of resorufin than those reported to be inhibitory. It is possible however that the EROD-specific cytochrome P-450 is selectively inactivated during the preincubation. In fact EROD activity of non-hemin incubated microsomes begins to deviate from linearity after incubation for 10 min at 37°C (Figure 9).

In contrast to the variability in values for MFO activity encountered in the presence of hemin in assay systems containing intestinal microsomes, incubation of the 9000 x g supernatant for 20 min with hemin, in which AHH activity was assayed during the final 15 min, gave reproducible results of hemin-stimulated MFO activity (Table 13). As with incubations of homogenates though, prolonged incubation of this subcellular fraction resulted in significant inactivation of the enzyme. Microsomes prepared after incubation of 9000 x g supernatants contained only 20% of the MFO activity present in microsomes from corresponding non-incubated controls. This inactivation is also reflected by the abolition of differences in AHH activity between rats fed -Fe and +Fe diets (Table 13).

TABLE 13

HEMIN STIMULATION OF AHH ACTIVITY
IN 9000xg SUPERNATANTS* OF INTESTINAL MUCOSA

	AHH pmol/mg supernatant protein/min	
Diet	-Hemin	+Hemin
-Fe +Se	1.4 ± 0.8[a]	7.2 ± 1.2[b]
+Fe +Se	1.6 ± 0.8	7.9 ± 1.1[c]

* 9000xg supernatant (10 mg protein/ml) was preincubated with hemin (15μM) for 5 min before assay, using an aliquot of the preincubated supernatant.

All values are mean ± SEM, n=5, each n represents a pool of tissue from 2 animals. Note: Hemin-incubated +Fe +Se value is only 20% of non-incubated +Fe +Se (7.5 pmol/mg protein/min).

a --- No significant differences between values of -Fe and +Fe AHH activity.
b --- $p < 0.001$ vs -Hemin value.
c --- $p < 0.005$ vs -Hemin value.

6.4.2.2 Assembly of Cytochrome P-450 with Hemin in Everted Intestinal Sacs: Since *in vitro* incubation of homogenates or subcellular fractions with hemin did not permit detection of significant assembly of cytochrome P-450, the everted intestinal sac was employed as a model for the intact organ. Incubations of everted intestinal sacs from rats fed either -Fe or +Fe diet with hemin (15μM) for 30 min in NaCl and glucose-supplemented K-H bicarbonate buffer (pH 7.4), resulted in significant increases in microsomal cytochrome P-450 content and EROD activity (Table 14). The intestinal cytochrome content in iron-deprived (-Fe) rats was in fact raised to levels found in rats fed the +Fe diet. In parallel, EROD activity was also increased significantly although the magnitude of the increase was not as great as that of cytochrome P-450 (Table 14).

Moreover, that heme was indeed taken up by the enterocytes was indicated by the small but consistent absorption peak at 420 nm, observed in the CO-difference spectrum of reduced microsomes obtained from hemin-incubated intestinal sacs. This is indicative of non-specifically bound heme and is generally not seen in adequately washed microsomes (METHODS, Section 5.2) and/or in the absence of hemin incubation. It may be noted here that heme has been previously shown to be absorbed intact and metabolized to release its iron in the mucosa (Wheby *et al*, 1970; Raffin *et al*, 1974). Moreover, the recent demonstration of a mucosal receptor for intraluminal heme further evidences that heme is indeed absorbed intact from the lumen into the enterocyte (Tenhunen *et al*, 1980).

The findings in Table 14 indicate that regardless of the dietary iron status of the rats, some "heme-free" apocytochrome P-450 appears to exist in the intestinal mucosa and can be constituted with exogenous heme. Furthermore, iron deficiency apparently produces no additional

"free" apocytochrome that is constitutible by this procedure. Thus, the significantly lower heme-constituted cytochrome P-450 values in rats deprived of dietary iron versus those in rats fed iron, suggest that iron deprivation, and consequently impaired heme formation, may have also limited the availability of intestinal apocytochrome P-450 for assembly to the holocytochrome. Alternatively, "free" apocytochrome deprived of intracellular heme might be unstable and undergo accelerated degradation and/or inactivation, thereby accounting for the lower heme-constitution of intestinal cytochrome P-450 in rats deprived of dietary iron.

6.4.2.3 Requirement for Dietary Selenium in Hemin-Mediated Cytochrome P-450 Assembly: Everted intestinal sacs from rats deprived of both dietary iron and selenium exhibit lower cytochrome P-450 content and EROD activity than those from rats deprived of only selenium (Table 15). However, when the basal values of the two parameters in +Fe -Se fed rats (Table 15) are compared with corresponding values in +Fe +Se fed rats (Table 14), the variability in cytochrome P-450 content is too great to permit noticeable differences of selenium deprivation between the two groups. This lack of significant difference is in contrast to findings in pair fed animals (Table 8) and probably arises from the fact that the animals in table 14 were not pair fed with those in table 15. Even so, the values for EROD activity, the more sensitive parameter, exhibit significant differences between +Fe +Se and +Fe -Se fed rats (Tables 14 and 15).

Since intestinal cytochrome P-450 content and EROD activity decrease in rats deprived of dietary selenium (Table 8), and since synthesis of the apocytochrome moiety of the rapidly turning over phenobarbital-inducible isozyme of cultured hepatocytes was reported to require sele-

nium (Newman & Guzelian, 1982), it was investigated whether dietary selenium was required for intestinal apocytochrome P-450 synthesis.

Incubation of everted intestinal sacs from -Fe -Se fed rats with hemin resulted in substantial increases in both cytochrome P-450 content and EROD activity (Table 15). However, in +Fe -Se fed rats no such increases are apparent, indicating the absence of constitutable "free" apocytochrome in mucosa of these rats. This is in sharp contrast to the observed hemin-mediated increase in this parameter in +Fe +Se fed rats (Table 14). Thus, selenium deprivation appears to reduce the extent of constitutable microsomal "heme-free" apocytochrome, which normally occurs in rats fed an iron-supplemented diet adequate in selenium (Table 14). In addition, although partial structural and functional assembly of microsomal cytochrome P-450 with exogenous hemin is observed in intestinal sacs from rats deprived of both iron and selenium (Table 15), it occurs to a much lesser extent than that seen in intestinal sacs from rats deprived of iron but supplemented with selenium (Table 14). This partial assembly thus reflects reduced availability of constitutable apocytochrome P-450 which may result from its impaired formation or lack of viability. Thus, in addition to regulating heme synthesis, intraluminal iron as well as selenium may modulate intestinal apocytochrome P-450 availability. It is unclear whether such modulation is exerted by direct effects of these nutritional elements on the apoprotein moiety; or indirectly, via impairment of heme synthesis. In the second event, it could be inferred that formation of intestinal heme and "constitutive" apocytochrome P-450 are coordinated, interdependent processes.

Because of these findings, all the remaining experiments were carried out in rats fed diets supplemented with selenium.

TABLE 14

EFFECT OF EXOGENOUS HEMIN ON MICROSOMAL CYTOCHROME P-450 AND MFO ACTIVITY IN EVERTED INTESTINAL SACS*

	Cytochrome P-450 pmol/mg protein		EROD pmol/mg protein/min	
Diet**	- Hemin	+ Hemin	- Hemin	+ Hemin
-Fe +Se	15.7 ± 4.7	25.1 ± 5.0[a]	6.2 ± 2.0	10.1 ± 2.9[b]
+Fe +Se	20.9 ± 2.7[c]	33.2 ± 10.3[bd]	21.8 ± 3.3[c]	25.5 ± 9.1[e]

*Everted intestinal sacs were incubated with hemin (15μM) for 20 min at 37°C, as described (METHODS).
**Rats were fed either -Fe or +Fe, Se supplemented diet (diet iii or iv).
Values are mean ± SEM, n=4, each n represents a pool of tissue from 2 animals.

a --- $p < 0.1$ vs -Hemin value.
b --- $p < 0.05$ vs -Hemin value.
c --- $p < 0.02$ vs -Fe -Hemin value.
d --- $p < 0.05$ vs -Fe +Hemin value.
e --- $p < 0.1$ vs -Fe +Hemin value.

TABLE 15

EFFECT OF EXOGENOUS HEMIN ON MICROSOMAL CYTOCHROME P-450 AND MFO ACTIVITY IN EVERTED SACS* FROM RATS DEPRIVED OF DIETARY SELENIUM

		Cytochrome P-450 pmol/mg protein		EROD pmol/mg protein/min	
Diet**		- Hemin	+ Hemin	- Hemin	+ Hemin
-Fe -Se	n=7	9.53 ± 1.6	13.45 ± 2.1[a]	1.59 ± 0.2	2.63 ± 0.3[b]
+Fe -Se	n=5	22.58 ± 6.2[c]	23.86 ± 9.1	2.01 ± 0.2[d]	3.02 ± 0.5[e]

*Incubations with Hemin (15μM) were carried out as described (METHODS).
**Rats were pair fed either -Fe or +Fe, Se deficient diet (diet i or ii).
Values are mean ± SEM, each n represents a pool of tissue from 2 animals.

a --- $p < 0.05$ vs - Hemin value, not statistically different (N.S.) from corresponding +Fe value.
b --- $p < 0.001$ vs -Hemin value.
c --- $p < 0.02$ vs -Fe -Hemin value.
d --- $p < 0.1$ vs -Fe -Hemin value.
e --- N.S. vs -Hemin value.

6.4.3 Effect of Heme Deficiency on Apocytochrome P-448 Formation: To test whether intestinal apocytochrome P-448 formation is similarly affected by the absence of heme, a relative excess of "free" apocytochrome was deliberately generated as follows: Rats were deprived of dietary iron in order to restrict intracellular heme formation, while they were concomitantly fed β-NF, a cytochrome P-448 inducer which was expected to increase apocytochrome synthesis. Under such dual dietary regimen, "heme-free" apocytochrome P-448 generated in excess would be expected to be spectrally quantifiable after complexation with exogenous hemin.

6.4.3.1 Incubation of Cellular Fractions with Hemin: Attempts to constitute intestinal holocytochrome P-448 by incubations of intestinal homogenates or microsomes from iron-deprived rats with hemin yielded erratic results (Table 16), similar to those found after hemin-incubation of cellular fractions from rats fed inducer-free diets (Table 12). In contrast to non-induced intestinal cytochrome P-450 however, cytochrome P-448 from β-NF fed rats was stable after incubation of 9000 x g supernatant with hemin for 20 min at 37°C. Therefore, subsequent hemin-mediated assembly of intestinal holocytochrome P-448 was carried out in 9000 x g supernatants, in addition to everted intestinal sacs.

Iron deprivation reduced intestinal microsomal cytochrome P-448 content and its dependent MFO activity in β-NF fed rats (Tables 17 and 18). Incubation of 9000 x g supernatant of intestinal mucosa of these rats with hemin markedly reversed the reduction in MFO activity, but it failed to fully restore MFO activity to levels found in rats fed the +Fe +β-NF diet (Table 17). The increase in AHH activity following hemin incubation of 9000 x g supernatant from rats fed the +Fe +β-NF diet was not significant. In order to eliminate possible hemin interference

TABLE 16

EFFECT OF HEMIN-INCUBATION ON MICROSOMAL CYTOCHROME P-450 AND MFO ACTIVITY IN CELLULAR FRACTIONS FROM β-NF-FED RATS

Cellular Fraction	Diet	EROD nmol/mg protein/min -Hemin	EROD nmol/mg protein/min +Hemin	AHH nmol/mg protein/min -Hemin	AHH nmol/mg protein/min +Hemin
Homogenate*	-Fe +β-NF	0.23	0.26	--	--
	+Fe +β-NF	0.18	0.10	--	--
Microsomes*	-Fe +β-NF	1.4 (1.3-1.5)	2.1 (1.2-3.0)	0.32 (0.21-0.42)	0.33 (0.28-0.39)
	+Fe +β-NF	2.4 (2.4,2.4)	2.1 (1.2-3.0)	0.40 (0.31-0.49)	0.39 (0.28-0.49)

* Hemin (10μM) was incubated at 37°C for 10 min as described (METHODS).

Values are individual samples or mean of range in parentheses.
Note: Values for EROD activity are below non-incubated values (Table 5); no differences were observed between values from the -Fe and +Fe fed rats.

TABLE 17

HEMIN STIMULATION OF AHH ACTIVITY
IN MUCOSAL 9000xg SUPERNATANTS* OF β-NF-FED RATS

Diet	AHH pmol/mg supernatant protein/min -Hemin	+Hemin
-Fe +β-NF	117.9 ± 19.3	191.6 ± 34.4[a]
+Fe +β-NF	210.1 ± 28.5[b]	254.9 ± 16.7[c]

* 9000xg supernatant (10 mg protein/ml) was preincubated with hemin (15μM) for 5 min before assay.
Values are mean ± SEM, n=3 individual animals.

a --- $p < 0.05$ vs -Hemin value.
b --- $p < 0.05$ vs -Fe -Hemin value.
c --- $p < 0.1$ vs -Fe +Hemin value.

TABLE 18

EFFECT OF INCUBATION OF 9000xg SUPERNATANT WITH EXOGENOUS HEMIN* ON MICROSOMAL CYTOCHROME P-450 AND MFO ACTIVITY IN β-NF-FED RATS

Diet	Cytochrome P-450 pmol/mg protein		EROD nmol/mg protein/min	
	-Hemin	+Hemin	-Hemin	+Hemin
-Fe +β-NF	76.3 ± 18.0	132.6 ± 19.2[a]	8.2 ± 1.9	11.3 ± 1.2[b]
+Fe +β-NF	155.8 ± 20.2[c]	147.3 ± 24.1	14.1 ± 1.9[c]	15.6 ± 1.9

* Reaction mixtures contained 9000xg supernatant (2 mg protein/ml) in a final vol of 10 ml, and were incubated with or without hemin (15 μM) for 20 min at 37°C. Reactions were stopped by cooling and centrifugation of microsomes.

Values are mean ± SEM; n= at least 5 individual animals.

Statistical differences were determined with the student's paired "t" test, unless stated otherwise.

a --- $p < 0.001$ vs -Hemin value.
Not significant vs +Fe values.

b --- $p < 0.02$ vs -Hemin value.
$p < 0.05$ vs +Fe values, student's "t" test.

c --- $p < 0.05$ vs -Fe -Hemin value.

in the AHH assay, determinations of cytochrome content and MFO activity were performed on microsomes isolated from the hemin-incubated 9000 x g supernatant fractions. Incubations with hemin of intestinal 9000 x g supernatant from rats fed the -Fe +β-NF diet resulted in increases in both microsomal cytochrome P-448 content and EROD activity, to levels observed in animals fed the +Fe +β-NF diet (Table 18). Such hemin-mediated increases were not observed in intestinal microsomes from the +Fe +β-NF fed rats (Table 18).

From these findings, it can be inferred that "free" intestinal apocytochrome P-448 exists in rats deprived of dietary iron and therefore deficient in intestinal heme. Furthermore, such apocytochrome may be complexed with heme to restore holocytochrome P-448 content to levels found in iron-supplemented animals. Thus, in contrast to the constitutive apocytochrome P-450, apocytochrome P-448 formation and/or existence appear(s) to be independent of heme synthesis and/or availability.

6.4.3.2 Incubation of Everted Intestinal Sacs with Hemin: If "free" intestinal apocytochrome P-450 is generated during dietary iron deprivation in intact rats, it should be possible to assemble it to its holocytochrome by exogenous heme administration. This possibility was examined by incubation of everted intestinal sacs from β-NF-treated rats with hemin. Hemin-incubations of everted intestinal sacs from rats fed a β-NF-containing diet deficient in iron (-Fe +β-NF) increased both cytochrome P-450 content and EROD activity (Table 19). However, the relative magnitude of the hemin-mediated increases was lower in this system than in that containing intestinal 9000 x g supernatant. This discrepancy is largely due to the fact that values for cytochrome P-450 content and MFO

TABLE 19

EFFECT OF EXOGENOUS HEMIN ON MICROSOMAL CYTOCHROME P-450 AND MFO ACTIVITY IN EVERTED INTESTINAL SACS* FROM β-NF-FED RATS

Diet	Cytochrome P-450 pmol/mg protein		EROD nmol/mg protein/min	
	- Hemin	+ Hemin	- Hemin	+ Hemin
-Fe +β-NF n=5	66.9 ± 7.1	82.2 ± 19.2[ab]	3.7 ± 0.8	6.4 ± 1.7[ac]
+Fe +β-NF n=7	115.1 ± 9.1[d]	102.8 ± 16.0	18.7 ± 1.4[d]	12.8 ± 1.1[a]

*Everted sacs were incubated with hemin (15μM) at 37°C for 20 min, as described (METHODS).

Values are mean ± SEM, obtained from individual animals.

a --- $p < 0.05$ vs -Hemin value, student's paired "t" test.
b --- $p < 0.05$ vs +Fe -Hemin, not significant vs +Fe +Hemin, student's "t" test.
c --- $p < 0.01$ vs +Fe values, student's "t" test.
d --- $p < 0.005$ vs -Fe -Hemin value, " " " .

activity of intestinal sacs reflect a mixed pool of microsomes from villous tip and crypt cells, whereas the 9000 x g supernatants were obtained from villous tip cells, which are relatively enriched in cytochrome P-450 and MFO activity. To a lesser extent, such a discrepancy may also be due to failure of hemin absorption into the enterocytes to meet the increased requirement for intracellular heme for structural assembly of the cytochrome following its induction by β-NF. Although stimulation of intestinal absorption of iron has been demonstrated after PAH induction of rat intestinal cytochrome P-448 (Manis & Kim, 1979a,b; Manis & Kim, 1980), stimulated absorption of intact heme across the intestinal epithelium of rats has only been demonstrated after phenobarbital administration (Thomas et al, 1972).

6.5 REGULATION OF THE INTESTINAL CYTOCHROME P-450-DEPENDENT MFO SYSTEM BY THE INTESTINAL PEPTIDE HORMONE, GASTRIN:

The intestinal cytochrome P-450-dependent MFO system is functional albeit at a low level in the absence of dietary inducers and nutrients such as iron and selenium (Table 8). Moreover, preliminary results indicated that cytochrome P-450 content and ECOD activity were detectable in the intestinal mucosa of food-deprived rats after 48 hr of intravenous glucose, or even after 8 days of intravenous hyperalimentation. Thus, cytochrome P-450 content and MFO activity are stable during complete absence of intraluminal substances such as amino acids, fats, vitamins and minerals, considered to be essential for maintenance of the constitutive form of the enzyme (BACKGROUND, Section 2.6.3). Therefore, the content and activity of the intestinal enzyme system during this nutritional regimen may be considered to truly represent the "basal" state.

It was investigated whether under these conditions intestinal cytochrome P-450 is maintained by endogenous intestinal peptide hormones. In the gastrointestinal tissue, gastrin, and its pentapeptide analogue pentagastrin, demonstrate a "pleiotypic response", i.e., organspecific growth modulation, which includes stimulation of DNA, RNA, and protein synthesis (Johnson & Chandler, 1973; Johnson & Guthrie, 1976; Enochs & Johnson, 1974; Walsh & Grossman, 1975). This trophic action of the hormone suggests that it might serve as a potential endogenous regulator of the mucosal cytochrome P-450 system (BACKGROUND, Section 2.6.4).

Rough comparisons of the "basal" levels of the enzyme system in parenterally fed rats (Table 20, "Control" values) with values obtained in rats fed a properly supplemented (+Fe +Se, diet iv, MATERIALS, Section 4.3), semi-synthetic diet (Table 8) indicate a substantial decrease in cytochrome P-450 content and MFO activity in the absence of intraluminal nutrients. The addition of iron and selenium to the drinking water (METHODS, Section 5.4.2) failed to raise these parameters to levels found in orally fed rats. Thus, the low levels of the intestinal cytochrome P-450 MFO system observed after intravenous nutritional supplementation appear to result from lack of food substances in the intestinal lumen. Since the absence of intragastric substances lower both antral release and serum levels of gastrin (Johnson et al, 1975a,b), the observed low level of cytochrome P-450 MFO activity (Table 20, "Control" value) might in fact have resulted from reduced serum gastrin.

To test this possibility, experiments were conducted in unrestrained rats maintained intravenously with either 5% glucose infusion or complete parenteral nutrition, with or without added pentagastrin in the infusate. This experimental approach not only prevented the adverse effects of

starvation on the intestinal cytochrome P-450 system of rats, but at the same time avoided stimulation of antral gastrin release (Johnson et al, 1975a) and potential alteration of cytochrome P-450-dependent MFO activity by intraluminal substances (Wattenberg, 1975). Additionally, continuous pentagastrin infusion was expected to compensate for its rapid inactivation by the liver (Walsh & Grossman, 1975), and thus maintain relatively constant serum pentagastrin levels.

6.5.1 Effects of Pentagastrin Infusion on the Intestinal Cytochrome P-450-Dependent MFO System and Heme Synthesis: Initial experiments were conducted to determine whether short term intravenous infusion of pentagastrin in 5% glucose would maintain cytochrome P-450 content at greater than "basal" levels during the absence of intragastric and intraluminal food substances. Infusion of pentagastrin for 48 hr at a dose of 18 μg/kg-hr, estimated to be one-third of its maximal non-ulcerogenic dose (Robert et al, 1970) but three times the dose required to maintain mucosal structure and function on a chronic basis (Johnson et al, 1975b), failed to alter microsomal cytochrome P-450 content or ECOD activity of 9000 x g supernatant (Table 20). When these data were expressed per length of mucosa rather than per mg protein, slight, highly variable but non-significant increases in both cytochrome P-450 content and ECOD activity were observed. Furthermore, raising the dose of pentagastrin to 60 μg/kg-hr failed to elicit any changes in MFO activity after 48 hr infusion.

In order to determine whether this lack of observable effect on intestinal cytochrome P-450 MFO system was due to caloric deprivation of the animals during glucose infusion, further experiments were conducted in rats nutritionally maintained by parenteral hyperalimentation. After

TABLE 20

EFFECT OF ACUTE PENTAGASTRIN INFUSION
ON CYTOCHROME P-450 AND RELATED MFO ACTIVITY

	Cytochrome P-450		Ethoxycoumarin O-deethylase (ECOD)	
	pmol/mg microsomal protein	pmol/15 cm mucosa	pmol/mg 9000xg supernatant protein/min	pmol/15 cm mucosa/min
Experiment I*				
Control	10.1 ± 1.3	28.0 ± 1.3	2.6 ± 0.6	124.0 ± 1.9
+ Pentagastrin	16.6 ± 6.6	46.9 ± 15.5	1.9 ± 0.3	163.0 ± 12.3[a]
Experiment II**				
Control	10.5 ± 3.5	17.8 ± 9.6	10.7 ± 4.2	151.4 ± 65
+ Pentagastrin	10.5 ± 0.6	15.6 ± 5.8	10.2 ± 6.8	150.0 ± 104

* 18 µg/kg-hr. Unrestrained rats were infused with saline containing 5% glucose.

**30 µg/kg-hr. Unrestrained rats were maintained by intravenous hyperalimentation as described (METHODS).

Values are mean ± SEM; n=3, each n represents tissue pooled from 2 animals.

a --- $p < 0.2$ vs control.

48 hr infusion, no differences were found in either intestinal cytochrome P-450 content or ECOD activity, expressed either per mg protein or length of intestine, between control and pentagastrin (30μg/kg-hr)-treated rats (Table 20).

Since the cytochrome P-450 isozyme responsible for ECOD activity differs from that responsible for AHH activity in certain tissues (Jacobson et al, 1974), AHH activity was also monitored after pentagastrin infusion at 90 μg/kg-hr for 48 hr in hyperalimented rats. Pentagastrin also failed to increase intestinal AHH activity (Table 21).

Since induction of hepatic cytochrome P-450 is often preceeded by stimulation of ALAS (Marver, 1969), the primary rate limiting enzyme in heme biosynthesis, its activity in the intestinal mucosa was monitored as a possible predictor of cytochrome P-450 induction. Infusion with the maximal dose of pentagastrin (90μg/kg-hr) for 48 hr similarly failed to increase ALAS activity when expressed either per mg homogenate protein or per length of intestine (Table 22).

Since the above experiments were carried out over 48 hr, it was possible that such periods were too short to observe any measurable differences in cytochrome content or MFO activity. This notion was strengthened by the report that reversal of mucosal structural and functional changes observed after extended hyperalimentation (Johnson et al, 1975a) required continuous infusions of low doses of pentagastrin over a prolonged period (8days) (Johnson et al, 1975b). Pentagastrin infusions (6μg/kg-hr) for 8 days to intravenously hyperalimented rats produced no observable changes in intestinal cytochrome P-450 content nor ECOD activity when compared to corresponding control values (Table 23). Thus, regardless of dose or duration of administration, pentagastrin

failed to affect the intestinal cytochrome P-450-dependent MFO system or the synthesis of the cytochrome heme moiety.

TABLE 21

EFFECT OF ACUTE PENTAGASTRIN INFUSION ON CYTOCHROME P-450 AND ARYL HYDROCARBON HYDROXYLASE (AHH) ACTIVITY

	Cytochrome P-450		AHH	
	pmol/mg microsomal protein n=2	pmol/15 cm mucosa n=1	pmol/mg 9000xg supernatant protein/min	pmol/15 cm mucosa/min
Control	23.9 ± 9.9	32.4	3.8 ± 1.4	66.6 ± 1.3
+ Pentagastrin*	26.3 ± 1.3	23.6	4.4 ± 0.9	78.5 ± 10.6

*90 μg/kg-hr. Unrestrained rats were maintained by intravenous hyperalimentation.

Values are mean ± SEM; n=4 unless stated, each n represents a pool of tissue from 2 animals.

TABLE 22

EFFECT OF ACUTE PENTAGASTRIN INFUSION*
ON MUCOSAL HEME SYNTHESIS

δ-Aminolevulinic Acid Synthetase	
pmol/mg homogenate protein/30 min	pmol/15 cm/30 min
141.2 ± 28.6	2580 ± 300
141.0 ± 26.8	2355 ± 207

*90 μg/kg-hr. Unrestrained rats were maintained by intravenous hyperalimentation.

Values are mean ± SEM; n=4, each represents a pool of tissue from 2 animals.

TABLE 23

EFFECT OF PROLONGED PENTAGASTRIN INFUSION ON
CYTOCHROME P-450 AND RELATED MFO ACTIVITY

	Cytochrome P-450		ECOD	
	pmol/mg microsomal protein	pmol/15 cm mucosa	pmol/mg 9000xg supernatant protein/min	pmol/15 cm mucosa/min
Control	26.6 ± 2.7	46.5 ± 2.5	2.9 ± 0.6	135.4 ± 44.0
+ Pentagastrin*	20.2 ± 3.2	44.4 ± 4.7	2.6 ± 0.6	122.0 ± 45.2

*6 μg/kg-hr. Unrestrained rats were maintained by intravenous hyperalimentation.

Values are mean ± SEM; n=5, each n represents a pool of tissue from 2 animals.

7.0 DISCUSSION

7.1 SPECTRAL AND KINETIC CHARACTERISTICS OF INTESTINAL CYTOCHROME P-450:

Studies with intestinal microsomes (RESULTS, Section 6.2) demonstrate that the "constitutive" intestinal isozyme, from rats fed the inducer-free semi-synthetic diet, and the β-NF-inducible form of cytochrome P-450 are distinct isozymes that exhibit specific and unique spectral and kinetic characteristics. Constitutive intestinal cytochrome P-450 exhibited a CO-difference spectrum of the reduced form with a maximal absorption at 452.5 nm. This finding has not been previously reported. The two spectrally distinct intestinal cytochrome P-450 isozymes, detectable in non-inducer and 3-MC-treated rats, have been previously reported to exhibit a CO-induced absorption maxima at 450 and 448 nm respectively, regardless of whether the animals were fed standard rat chow or semi-synthetic diet free of natural enzyme inducers (Stohs et al, 1977; Hoensch et al, 1976). Similarly, the three recently isolated and purified forms of rabbit intestinal cytochrome P-450 all exhibited CO-induced spectral absorption maxima at 450 nm; however the animals in that study were not fed a diet free of natural inducers (Ichihara et al, 1980, 1981). Definitive characterization of rat intestinal cytochrome P-450 isozymes awaits raising of specific antibodies to highly purified isozyme preparations isolated from animals fed purified semi-synthetic diets supplemented with and without enzyme inducers.

The remarkable inducibility of intestinal cytochrome P-450 reflects its role as a highly adaptable detoxification system for orally ingested dietary and environmental chemicals and carcinogens. In this respect, the hepatic system, which, when compared to the intestinal system, is only marginally stimulated by orally administered xenobiotics (Wattenberg

& Leong, 1970; Remmer et al, 1979), appears to play a backup role in the disposition of those lipophilic substances which escape intestinal metabolism and are absorbed into the portal blood.

7.2 DEPENDENCE OF INTESTINAL CYTOCHROME P-450 HEME METABOLISM ON DIETARY IRON:

The findings that intestinal cytochrome P-450 exhibits an acute dependence on dietary iron (Table 8) confirm previously reported observations that intraluminal iron deprivation for a period as short as 48 hours, results in a markedly reduced microsomal cytochrome P-450 content and its dependent MFO activity in the rat intestinal mucosa (Hoensch et al, 1976). This reduction is again observed to be most pronounced in the villous tip cells, i.e., the very site of iron absorption. Maintenance of intestinal villous cytochrome P-450 structure and function in rats thus is critically dependent on intraluminal iron. These findings also indicate that iron is essential for formation of the cytochrome P-450 heme moiety in intestinal villous tip cells, not only directly by providing the prosthetic moiety for insertion into protoporphyrin IX as suspected, but more importantly by modulating the intestinal activity of ferrochelatase (Table 10), the key enzyme catalyzing such insertion. Moreover, acute intraluminal iron deprivation also reduces intestinal ALAS activity (Table 10), indicating a regulatory role of iron at this rate-limiting step. Therefore, the dependency of intestinal cytochrome P-450 on dietary iron is merely a reflection of the critical requirement for iron in the enzymatic synthesis of the heme moiety of the cytochrome.

That dietary iron deprivation is not stimulating the breakdown of cytochrome P-450 heme is indicated by the lack of substantial stimulation of MHO activity (Table 11) and consequently of heme degradation in the

mucosa of rats fed the iron deficient diet. It is known that chronic dietary iron deprivation stimulates the mucosal absorption of hemoglobin or cytochrome heme in order to increase substrate (heme) availability for mucosal MHO and thus to immediately restore depleted iron stores (Wheby et al, 1970). Presumably, intestinal cytochrome P-450 heme, released after degradation of the cytochrome, is also a substrate of mucosal MHO. Although chronic dietary iron deprivation (minimum 2 weeks) results in a substantial elevation of mucosal MHO activity in rapidly growing weanling rats (Raffin et al, 1974), the minimally elevated MHO activity observed after acute (4 days) dietary iron deprivation indicates that minimal if any degradation of intestinal cytochrome P-450 occurs at that time.

Hepatic ALAS and ferrochelatase activities have similarly been reported to be reduced in rats fed a low iron diet for a 10-30 day period following weaning (White et al, 1978b; Liem et al, 1979). In agreement with those reports, the present findings of reduced intestinal ALAS and ferrochelatase activities also suggest that i) iron is possibly not a repressor of intestinal ALAS since its acute deprivation reduces rather than enhances ALAS activity; and ii) the lowering of intracellular heme in the intestinal mucosa produced by dietary iron deprivation does not appear to derepress the enzyme as would be expected normally, following heme depletion (De Matteis, 1975; Meyer & Marver, 1971a). Thus, the regulation of intestinal heme synthesizing enzymes appear to be mechanistically similar to that of the corresponding hepatic isozymes. However, in contrast to findings in the liver (White et al, 1978b; Liem et al, 1979), acute reduction of heme synthesizing activity in the intestinal mucosa is associated with a marked fall in villous tip

cytochrome P-450 content and activity. These findings indicate that the villous tip hemoprotein, unlike its hepatic isozyme, is critically dependent on heme synthesized de novo and in situ from intraluminal iron. This is not surprising since the enterocyte appears to enrich itself in cytochrome P-450 during its upward migration to the villous tip (Hoensch et al, 1976; Hartman et al, 1982). On the other hand, under the same conditions of acute dietary iron deprivation, crypt cells are capable of maintaining their cytochrome P-450 content, thus resembling the hepatocyte in this aspect. It is noteworthy that such iron-dependent modulation of intestinal villous tip heme and hemoprotein formation, in concert with intestinal cell turnover kinetics, is extremely rapid, unlike that observed in the liver.

7.2.1 Dependence of Mitochondrial Heme Utilization on Dietary Iron: The findings of acute iron dependency of ALAS, ferrochelatase (Table 10), and cytochrome oxidase (Table 5) illustrate the unusual sensitivity of the mitochondrion to iron deprivation. Presence of intestinal mitochondrial cytochrome P-450 in the rat, in contrast to that reported in the rabbit (Jones et al, 1980), was not detectable in these studies, and therefore the effects of dietary iron deprivation on such a mitochondrial cytochrome were not investigated. Morphological and biochemical changes in hepatic mitochondria of rats made chronically deficient in iron have been well characterized (Dallman & Goodman, 1971). In addition, numerous biochemical alterations have also been described in intestinal mitochondria after acute iron deprivation. For example, cytochrome c and cytochrome oxidase decrease in intestinal mitochondria after only a few days of dietary iron deprivation, whereas corresponding decreases in liver mitochondria are observed only after 8 weeks of dietary iron

restriction (Dallman, 1974). Moreover, this decrease of hepatic mitochondrial cytochromes is somewhat in contrast to that of hepatic microsomal cytochromes b_5 and P-450 which are unaffected by even severe iron deficiency (Dallman & Goodman, 1971; Liem et al, 1979). Additionally, the intestinal activities of the mitochondrial metaloflavoproteins, succinate dehydrogenase and monoamine oxidase, presumed to contain non-heme iron, are reduced by iron deficiency (Dallman, 1974). Thus not only are the mitochondrial cytochromes generally more sensitive to iron deficiency than microsomal cytochromes, but most iron containing enzymes of the intestinal mitochondria are extremely sensitive to iron deprivation. Furthermore, the deleterious effect of iron deprivation on mitochondrial heme synthesis appears to be responsible for the diminution of both microsomal and mitochondrial cytochrome heme. However, prolonged iron deprivation (30 days) failed to affect microsomal cytochrome b_5 heme content (Hoensch et al, 1976). This finding indicates that the reduction of microsomal cytochrome P-450 elicited by iron deprivation directly results from reduced mitochondrial heme synthesis and probably not from general morphological perturbation of the organelle. Both the metabolism and utilization of heme within the mitochondrion are therefore stringently governed by intracellular iron status.

7.3 DEPENDENCY OF INTESTINAL CYTOCHROME P-450 HEME ON DIETARY SELENIUM:

The finding that intestinal cytochrome P-450 is acutely dependent on dietary selenium (Table 8) has not been previously reported. Such acute dependence hinges on the fact that selenium is critically required for the synthesis of the heme moiety of the cytochrome (Table 10). Intestinal ferrochelatase activity is decreased after dietary selenium deprivation, indicating that ferrochelatase is regulated by selenium,

although the mechanism of such regulation is unclear. Also, chronic dietary selenium deprivation decreases hepatic ferrochelatase activity in untreated rats (BACKGROUND, Section 2.6.3.2). Furthermore, in both untreated and phenobarbital-treated selenium-deficient rats, significant increases in urinary coproporhyrin excretion (Burk & Correia, 1977) suggest a partial block in the hepatic heme synthetic pathway at or beyond coproporphyrinogen oxidase. Thus, this block may in fact be localized at ferrochelatase in selenium-deficient rats.

That the decrease in intestinal cytochrome P-450 content was not due to increased catabolism of its heme moiety was indicated by the finding that MHO activity slightly decreased after selenium deprivation in villous tip cells (Table 11). This indicated decreased heme catabolism in the selenium-deprived enterocyte. This is in contrast to findings in the liver of selenium-deficient rats given phenobarbital. In these animals, underutilization of heme results in excess heme, MHO induction, and acceleration of heme degradation (Correia & Burk, 1978). Since intestinal MHO is heme-inducible, but no such elevation in intestinal MHO activity was observed in rats acutely deprived of dietary selenium, it suggests that no excess heme was available in the enterocytes of rats deprived of dietary selenium.

The marked fall in intestinal cytochrome P-450-dependent EROD activity observed with acute deprivation of intraluminal selenium for a single day (Figure 12) is most probably associated with the observed reduction of cytochrome P-450 content, since the activity of intestinal NADPH-cytochrome P-450 (c) reductase was unaffected at that time (Figure 13). Since selenium-dependent GSH peroxidase is responsible for the degradation of intracellular hydroperoxides, known to peroxidize membrane

lipids and thus decrease membrane-bound cytochrome P-450 (BACKGROUND, Section 2.6.3.3), its possible role in the dependence of intestinal MFO activity on selenium was also investigated. Intestinal GSH peroxidase was similarly unaffected by selenium deprivation for a single day (Figure 12), also indicating its lack of involvement in the decrease in EROD activity during that time. Oral selenium deprivation for a further 2 day period lowered GSH peroxidase to 70% of its basal level, but did not further reduce EROD activity (Figure 12). Since resupplementation of dietary selenium for a single day dramatically restored EROD activity to normal levels without significant concurrent restoration of GSH peroxidase activity (Figure 14), this effect of selenium on intestinal cytochrome P-450-dependent EROD activity appears to be independent of its well recognized prosthetic function in GSH peroxidase (Oh et al, 1974; Hafeman et al, 1974). That is, selenium does not appear to maintain intestinal cytochrome P-450 by protecting it from inactivation by potentially pernicious intracellular peroxides that might accumulate as a consequence of impaired GSH peroxidase activity.

7.4 REGULATION OF INTESTINAL APOCYTOCHROME P-450 BY HEME AND DIETARY SELENIUM:

The findings that holocytochrome P-450 can be structurally and functionally assembled from experimentally generated "free" apocytochrome and heme in everted intestinal sacs (Tables 14 and 15) indicate that the reduction of intestinal cytochrome P-450 by dietary iron deprivation can be largely reversed by in vitro incubation of intestinal tissue with exogenous heme. This implies that ingestion of heme containing compounds could similarly reverse any impairment of cytochrome P-450-dependent MFO activity provoked by heme deficiency in the intestinal mucosa.

However, the incompleteness of the hemin-mediated reversibility of cytochrome P-450 impairment (Table 14) also suggests that in rats fed the inducer-free diet the formation and/or existence of the constitutive form of intestinal apocytochrome P-450 may also be dependent on heme availability. That is, that the synthesis of the two moieties may be either synchronously coordinated or interdependent, or else that the constitutive apocytochrome may be relatively unstable when not complexed with heme. Conversely, the affinity of the intestinal apocytochrome for its prosthetic heme might be quite low, and/or vary from one isozyme subspecies to another (Tables 2 and 3, METHODS, Section 5.3.2.1). As such, after its in vitro assembly, heme dissociation from the apocytochrome during subsequent methodological procedures may account for the decreased extent of holocytochrome assembly.

In mucosa of rats fed the control (+Fe +Se) diet, a pool of "free" apocytochrome apparently exists which may be assembled with exogenous heme to a structurally intact and functionally active holocytochrome (Table 14). Intracellular selenium apparently modulates this apoprotein population, since upon its dietary deprivation, the apocytochrome becomes unavailable for heme complexation (Table 15). Thus, selenium not only regulates the heme moiety of constitutive cytochrome P-450 in the intestinal mucosa, but regulates its apocytochrome moiety as well.

In contrast to the constitutive apocytochrome P-450, the β-NF-inducible apocytochrome P-448 in iron-deprived rats can be assembled with exogenous heme to almost fully restore cytochrome P-448 content and activity to levels found in +Fe +β-NF fed animals. This finding suggests that unlike the constitutive apocytochrome P-450, apocytochrome P-448, induced by β-NF, is independent of the presence of heme for its existence.

Together, these findings suggest that in rats fed inducer-free purified semi-synthetic diets, dietary iron deprivation limits intestinal cytochrome P-450 content and its associated MFO activity by impairing heme synthesis and apocytochrome availability. Thus, heme and apocytochrome moieties of the constitutive form of intestinal cytochrome P-450 appears to be interdependent. In contrast, synthesis of β-NF-inducible apocytochrome P-448 moiety is unaffected when dissociated from the synthesis of its heme moiety. This further attests to the inherent differences between the constitutive and inducible cytochrome P-450 isozymes.

7.5 SELENIUM REQUIREMENT OF CYTOCHROME P-450 ISOZYMES WITH RAPID TURNOVER CHARACTERISTICS:

The finding of a prompt fall in the functional activity of intestinal cytochrome P-450 following its acute dietary selenium deprivation (Figure 12), and its dramatic reversal on selenium resupplementation (Figure 14) in rats fed inducer-free diets, not only confirms that the constitutive form of intestinal cytochrome P-450 exhibits dynamic turnover characteristics [as indeed suggested by studies of dietary iron deprivation and resupplementation (Hoensch *et al*, 1976)], but is critically dependent on intracellular selenium. A similar requirement has been recently demonstrated for the *de novo* synthesis of a phenobarbital-inducible species of hepatic cytochrome P-450 in primary nonproliferating cultures of adult rat hepatocytes (Newman & Guzelian, 1982). In contrast, maintenance of the *constitutive* form of hepatic cytochrome P-450, with relatively slow turnover characteristics, apparently was not dependent on selenium (Burk & Masters, 1975; Newman & Guzelian, 1982). Parallel findings of intracellular selenium requirement in cultured hepatocytes and intestinal villous tip cells indicate that cells actively synthe-

sizing cytochrome P-450 are critically dependent on exogenously supplied selenium. Such acute dependence of intestinal cytochrome P-450 on exogenous selenium is in contrast to that of the hepatic isozymes and their dependent MFO activity in rats, which are unaffected by even more rigorous dietary deprivation, i.e., selenium-withdrawal instituted postweaning and subsequent maintenance on selenium deficient diet for 8-12 weeks (Burk & Correia, 1977; Correia & Burk, 1978). Thus, the intestinal enterocyte apparently derives its selenium supplement solely from the diet, whereas the liver may derive its supplement from the whole body stores of selenium. This is consistent with the widely recognized fact that induction of selenium deficiency in most tissues of the rat is an impossible feat (Burk, 1978).

These studies thus suggest that rapidly regenerating tissues such as the intestinal mucosa exhibit inordinately high selenium turnover characteristics. If selenium is also required for maintenance of cytochrome P-450 content of such tissues, then acute selenium deprivation may greatly impair their ability to detoxify potential carcinogen and other noxious xenobiotics. In addition, acute deprivation of selenium and subsequent reduction of GSH peroxidase activity may considerably reduce the potential of the intestinal mucosa (and possibly other rapidly turning over tissues) to detoxify notoriously toxic organic (lipid) peroxides and hydroperoxides. Since such hydroperoxides have been shown to be capable of bioactivating procarcinogens to their ultimate carcinogenic species (Floyd et al, 1976), the enhanced intracellular peroxidative potential of the selenium deprived tissue could indeed promote a scenario for chemical carcinogenesis (Wortzman et al, 1980).

7.6 INTESTINAL CYTOCHROME P-450 HEME AND APOPROTEIN SYNTHESIS: CELLULAR LOCATION AND REGULATION BY DIETARY IRON AND SELENIUM:

Holocytochrome P-450 formation in intestinal cells of rats fed the control (+Fe +Se) diet follows a gradient along the mucosal villus, with minimal formation in the crypt cells, and increasing as the cells migrate along the mucosal villi to result in the maximal expression of cytochrome content and its dependent MFO activity in the villous tip cells (Table 8). Although the activities of the heme synthetic enzymes ALAS, ALAD, and ferrochelatase are highest in the crypt cells (Table 10), microsomal heme content, in parallel with cytochrome P-450 content, may in fact be maximal in tip cells. Although microsomal heme content (Table 10, +Fe +Se) does not reflect such a gradient from tip to crypt cells, incidental hemoglobin-heme contamination of crypt cells, derived from the underlying mucosal vasculature (METHODS, Section 5.1), may mask the difference in heme content between the two cell populations. This excess heme is reflected by a substantial 420 nm peak in the CO-reduced minus reduced difference spectrum of washed crypt cell microsomes.

Possibly very little of the heme formed in crypt cells is utilized for cytochrome P-450 or mitochondrial cytochrome formation since these cytochromes are barely detectable in crypt cells (Table 8; Padykula, 1962). If this is so, the heme synthesized in the crypt cells may presumably be used primarily for other hemoproteins such as cytochrome b_5, which does not exhibit any quantitative gradient along the mucosal villus (Table 9). Since, unlike cytochrome P-450, cytochrome b_5 is a slow turnover hemoprotein (Bock & Siekevitz, 1970), most of cytochrome b_5 found in tip cells can be assumed to have been assembled while the cells were located in the crypts.

The heme synthetic enzymes in the tip cells on the other hand, are predominantely committed to synthesis of heme for microsomal cytochrome P-450 formation. Since the cytochrome has a relatively rapid turnover (Section 7.5), it has a high demand for heme. In addition, the heme synthetic enzymes of the tip cells would also be expected to provide heme for formation of mitochondrial cytochromes, which in the intestinal mucosa also apparently exhibit considerably short half lives (Dallman, 1974). This is consistent with the finding that these mitochondrial cytochromes are also under the influence of intracellular iron content (Dallman, 1974), as is microsomal cytochrome P-450 (Table 8). In contrast, the slower turning over microsomal hemoprotein, cytochrome b_5, is unaffected by iron deprivation (Table 9). The similarities in rapid turnover kinetics, responsiveness to intracellular iron content, and the parallel progressive gradient in their enzymatic activity along the mucosal villous, suggest that microsomal cytochrome P-450 and mitochondrial cytochromes, in contrast to cytochrome b_5, may either receive their heme from a common intramitochondrial heme pool, or that their heme utilization may be governed by similar regulatory processes.

The relative rate of apocytochrome synthesis in the two cell populations is presently not determinable, since available techniques are not sufficiently sensitive to detect alterations in apocytochrome P-450 synthesis. If the synthesis of heme and apocytochrome moieties of intestinal cytochrome P-450 are indeed interdependent as suggested (Section 7.4), then the apparently lower heme content of the crypt cells, compared to that of the villous tip cells, may be associated with decreased apocytochrome availability in crypt cells. Thus, the lower holocytochrome P-450 content of crypt cells versus that in tip cells may be due to both

lower heme content and apocytochrome availability in those cells. If apocytochrome is indeed substantially lower in crypt cells, it is suggested that intestinal apocytochrome may be considered to fall in the group of proteins whose synthesis is triggered after the migration of the epithelial cells through the transition zone between villous crypt and tip (Fortin-Magana et al, 1970), resulting in greater apocytochrome content and hence holocytochrome P-450 in the tip cells. Thus, the reduced holocytochrome P-450 content observed primarily in tip cells elicited by dietary selenium deprivation (Table 8), is due to diminished apocytochrome availability (Tables 14 and 15) and reduced microsomal heme content (Table 10).

7.7 REGULATION OF THE INTESTINAL CYTOCHROME P-450-DEPENDENT MFO SYSTEM BY GASTRIN:

In the complete absence of intraluminal substances, including iron and selenium, the content and activity of intestinal cytochrome P-450 is detectable, albeit at low "basal" levels (RESULTS, Section 6.5). In the absence of exogenous affectors of the intestinal cytochrome P-450 MFO system, it is possible that it is maintained at these basal levels by endogenous regulators, particularly local hormones such as gastrin.

The gastrointestinal peptide hormone, gastrin, has been shown to exert a trophic response specifically in the gastrointestinal mucosa, where it is responsible for maintenance of mucosal structure and function. This trophic action formed the basis for its consideration as a potential endogenous regulator of the intestinal MFO system. Evidence has been reported to show a stimulatory action of not only gastrin but the other non- or weakly trophic gastrointestinal peptides, cholecystokinin and secretin, on MFO activity of colonic mucosa and certain oxidative reac-

tions in the liver (Fang & Strobel, 1981). The finding of this hepatic response to these peptides was suggested to indicate a non-tissue-specific response of MFO systems, i.e., a common property of the intestinal peptides.

Experiments were therefore conducted in unrestrained rats, continuously infused with pentagstrin, the synthetic analogue of gastrin, to determine whether a regulatory role of gastrin could be detected in the intestinal mucosa. In contrast to the studies in colonic and hepatic tissue, the experimental protocol (METHODS, Section 5.4) ensured constant serum pentagastrin levelsby infusion, thus controlling for its rapid inactivation. In order to prevent antral gastrin release by intragstric substances, animals were also nutritionally maintained by parenteral hyperalimentation. The failure to observe increases in intestinal cytochrome P-450 content, its dependent MFO activity, or ALAS activity, the rate limiting enzyme in the biosynthesis of cytochrome P-450 heme moiety, after infusion of pentagastrin, regardless of the dosage or duration of administration (Tables 20 to 23), strongly suggest that gastrin is not an endogenous modulator of intestinal cytochrome P-450, nor its dependent MFO activity. Thus, in the duodenum gastrin is presently restricted to the role of regulation of mucosal growth and the activiy of enzymes in carbohydrate metabolism (Johnson et al, 1975a,b). The recent finding that ACTH modulates the cytochrome P-450 MFO system in the adrenal, the target organ of its trophic effects, only in immature rats (Hallberg et al, 1983), suggests the need for future investigation of the possible influence of animal maturity in gastrin-mediated regulation of the intestinal cytochrome P-450-dependent MFO system, for definitive assessment of its role.

7.8 SUMMARY AND CONCLUSIONS:

Intestinal cytochrome P-450-dependent MFO system is regulated to a remarkable extent by dietary xenobiotics and nutrients. Acute dietary deprivation of iron results in reduced intestinal cytochrome P-450 content and its dependent MFO activity. The mechanism underlying this reduction was examined. Dietary selenium, known to influence hepatic cytochrome P-450 formation, was similarly investigated for its potential role as a regulator of the intestinal cytochrome P-450-dependent MFO system. Deprivation of dietary iron and/or selenium was found to selectively lower cytochrome P-450 content since other components of the MFO system were not affected. In addition, such lowering by selenium deprivation was found to be independent of the activity of the seleno-enzyme GSH peroxidase.

Findings detailed in this dissertation indicate that both dietary iron and selenium were found to regulate intestinal MFO activity by affecting the turnover of the structural components of intestinal cytochrome P-450. Dietary deprivation of either iron or selenium reduced intestinal cytochrome P-450 heme content by decreasing heme synthesis, thereby revealing that key rate limiting enzymes in heme biosynthesis are regulated by these nutrients. A small pool of "free" apocytochrome was detected in intestinal microsomes of control, untreated rats, which was also dependent on dietary selenium for its existence and/or structural intactness. Reduction of cytochrome P-450 heme elicited by dietary iron deprivation, partially reduces this apocytochrome pool, indicating its interdependence on microsomal heme content.

Dietary β-NF supplementation induced a unique form of the cytochrome ("P-448"), which was shown to be distinct from the constitutive intestinal

cytochrome P-450 both spectrally and kinetically. In contrast to the constitutive apocytochrome, apocytochrome P-448 was not affected by diminished cytochrome heme content, thereby further attesting to the distinctiveness of this induced form of the enzyme. Furthermore, in the absence of any dietary inducers and/or nutrients, the intestinal cytochrome P-450-dependent MFO system is maintained at detectable, albeit low, levels by as yet undetermined endogenous regulators. Although the peptide gastrin, a trophic hormone in the gastrointestinal tract, appeared to be a potential candidate for such endogenous regulation, its role in modulating basal cytochrome P-450 levels in the intestinal mucosa could not be established under conditions in which its pleiotypic response is easily observed.

The finding that intestinal cytochrome P-450 content and its dependent MFO activity are reduced by acute, i.e., within 24-48 hr, dietary iron or selenium deprivation, underscores characteristic differences between the intestinal MFO system and that of the liver. Hepatic cytochrome P-450 is reduced only after chronic dietary iron deprivation coupled with in vivo iron chelation. Similarly, the selenium-dependency of phenobarbital-inducible hepatic cytochrome P-450 is observed only in rats chronically deprived of dietary selenium since weaning. Moreover, the activities of the heme synthetic enzymes, ALAS and FC, are reduced in hepatic tissue only after chronic iron deprivation, whereas their response to dietary iron was acutely manifested in the intestinal mucosa. Similarly, the reduction of intestinal FC activity, and hence mucosal heme synthesis, by dietary selenium deprivation is acutely manifested. Although evidence suggests a partial block in hepatic heme synthesis in chronically selenium-deprived rats, in fact hepatic MHO activity was

enhanced, indicating excess unutilizable heme in the liver. In contrast, acute dietary deprivation of selenium lowered intestinal microsomal heme and cytochrome P-450 content. This is consistent with the lack of increase in intestinal MHO activity after dietary selenium deprivation, in contrast to the marked stimulation of the hepatic enzyme. Thus, selenium appears to affect heme metabolism differently in the two tissues. However, when a rapidly turning over form of cytochrome P-450 is generated in the liver following phenobarbital-induction, such an isozyme then appears to resemble the rapidly turning over intestinal isozyme in its requirement of selenium.

The intestinal cytochrome P-450-dependent MFO system is not only extremely responsive to dietary nutrients but to dietary inducers as well. Feeding of animals with a semi-synthetic diet free of natural inducers lowered intestinal cytochrome P-450 content and MFO activity to their constitutive levels. Such dietary regimen is without effect on the hepatic isozyme or its MFO activity. Furthermore, the physicochemical and functional characteristics of the hepatic and intestinal constitutive enzymes also differ considerably (RESULTS, Section 6.2). Dietary supplementation with β-NF markedly increased intestinal cytochrome P-448 content and MFO activity. In contrast, dietary β-NF induces the hepatic isozyme and stimulates its dependent MFO activity to considerably less extent than the intestinal system. Thus, the intestinal MFO system may be primarily responsible for the detoxification of ingested PAH's and other xenobiotics, while the hepatic system appears to play a backup role in the biotransformation and disposition of those xenobiotics that escape intestinal metabolism during their absorption.

Thus, short term deficiencies of nutrients such as iron and selenium

rapidly and markedly affect intestinal cytochrome P-450 content and function. Of the two intestinal mucosal cell populations, the villous tips appear by far to be the more responsive and/or sensitive to such intraluminal influences than are the cells in the crypts of Lieberkuhn. As such, dietary nutrients significantly affect xenobiotic metabolizing enzymes in the very cells of the intestinal mucosa which are committed to their absorption. This finding is of profound clinical significance given the impressive growing list of commonly used drugs in the therapeutic arsenal whose biodisposition entails a predominant intestinal "first pass" (Table 1). Alteration of the intestinal "first pass" metabolism of any such drug might severely hamper its delivery to pharmacological sites and thereby modify its effectiveness. Iron deficiency, for example, has been implicated in the altered oxidation of a number of orally ingested drugs. On the other hand, impaired intestinal detoxification of other ingested xenobiotics, either intentionally supplemented (food additives) or naturally occuring (carcinogens) in the diet, may constitute a decisive risk factor in environmentally induced gastrointestinal carcinogenesis. The finding that xenobiotic metabolism by the intestinal mucosa may be significantly modified by dietary iron and/or selenium attains greater clinical importance in human health concerns with recent reports of increased incidence of iron deficiency anemia, and of a widespread potential for severe selenium deficiency, in certain segments of the human population (Burroughs & Huenemann, 1970; Kripke & Sanders, 1970; White, 1970; Reinhold, 1975; Burk, 1978).

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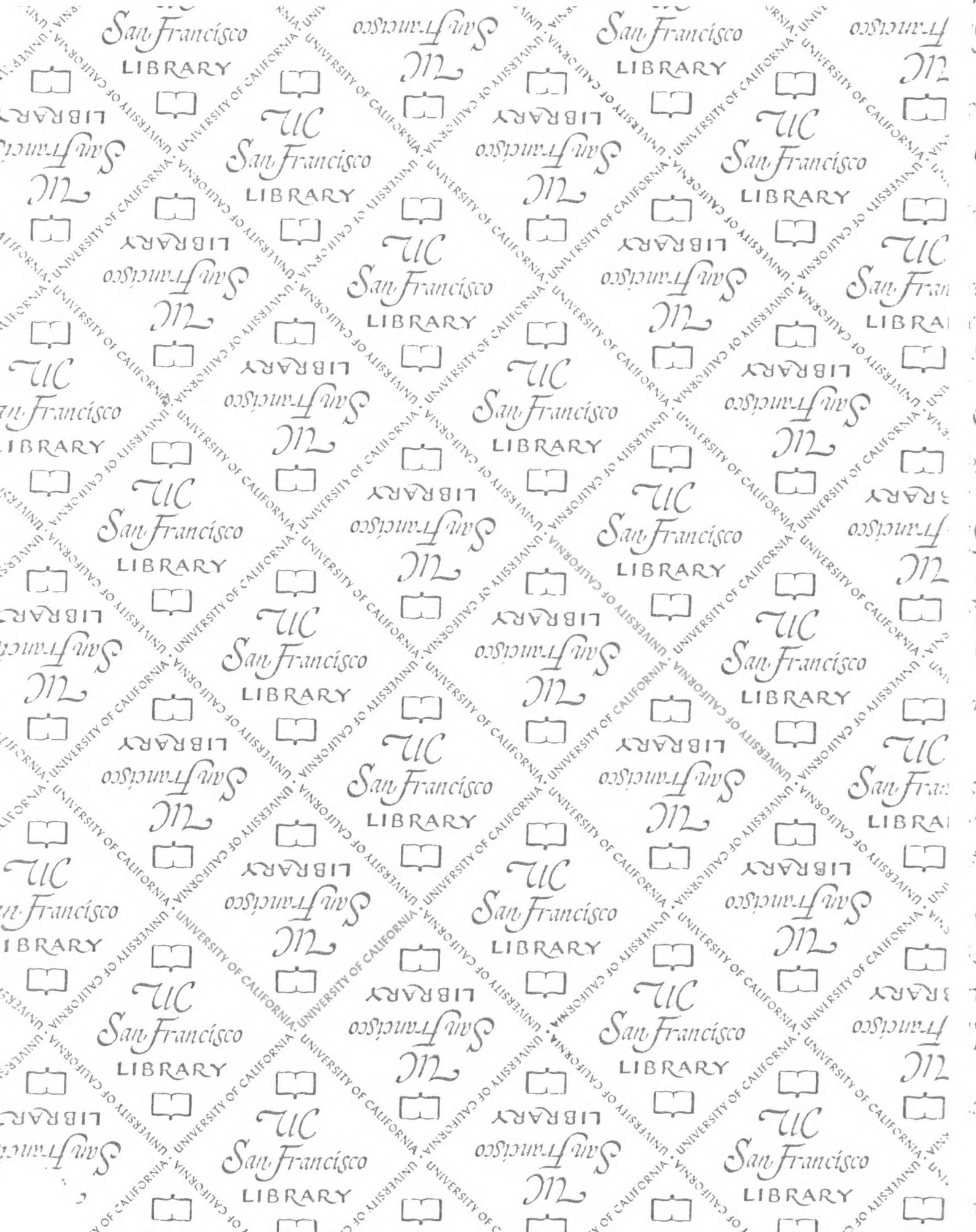
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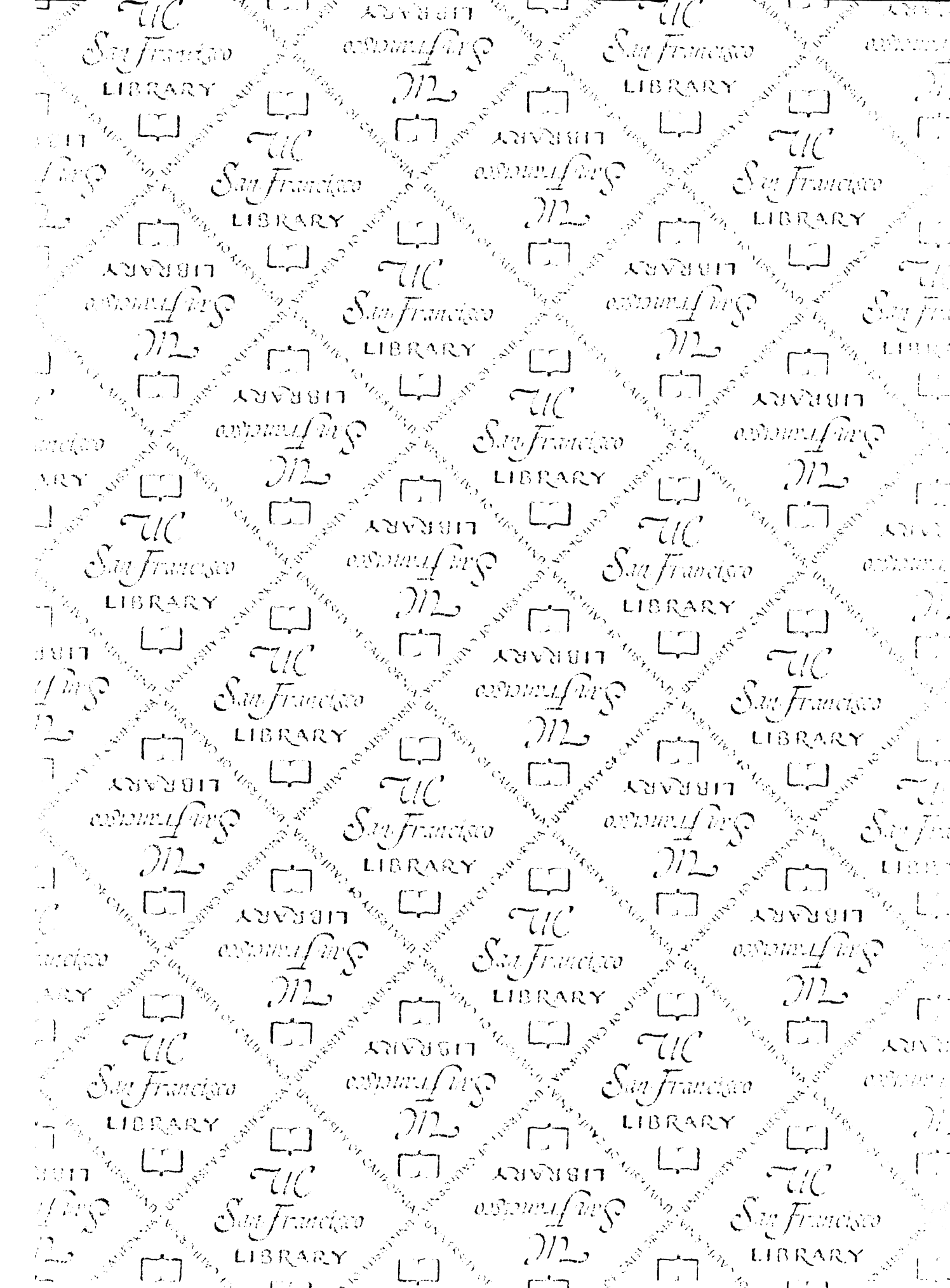
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