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Title: B-Naphthoflavone Induction and Its Effect on Hepatic Phospholipid Metabolism in Rainbow Trout (Salmo gairdneri)

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The induction of drug-metabolizing enzymes is a complex biochemical process manifested not only by increases in various cytochrome P-450 isozymes and related activities, but also by alterations in endoplasmic reticulum membranes. Little work has been done to examine how cytochrome P-450 inducing agents influence other cellular processes, such as phospholipid metabolism. Furthermore, characterization of the poikilotherm mixed-function oxidase (MFO) system has lagged considerably behind numerous sophisticated mammalian studies, although differences between the two species have been reported. With the proposal that fish be used as monitors of environmental pollution for man, the importance of characterizing fish MFO

induction and related cellular events becomes apparent. The present study was undertaken with this in mind in an effort to further evaluate the consequences of induction by polyaromatic hydrocarbons (PAH) in fish. This may be one of the first reports of the effects of PAH induction on liver phospholipid metabolism in poikilotherms.

B-Naphthoflavone (BNF) induction of microsomal cytochrome P-450 and its effects on various aspects of liver phospholipid (PL) metabolism were examined in rainbow trout 24 and 96 hours after intraperitoneal injection. Spectrophotometrically-measured microsomal cytochrome P-450 content from BNF-induced trout liver showed no significant elevation 24 hours after injection, but increased substantially at 96 hours to approximately double the P-450 content in control liver. This corresponds to results from computer-analyzed laser densitometry scans of LDS-polyacrylamide gels of 96-hour BNF-treated liver microsomal proteins, where a tentatively-identified cytochrome P-448 (P-450LM4) increased 90% relative to controls.

BNF-induced alterations in microsomal phospholipid (PL) composition of trout liver occurred

before and after the actual observed increase in cytochrome P-450 contents. A substantial elevation in liver microsomal PL/mg protein (34%) was noted at 24 hours after BNF injection, with a significant accumulation of choline, ethanolamine and inositol PLs. This was accompanied by a slight reduction in serine PLs when compared to 24-hour controls. At 96 hours much of the initial increase of individual PLs had subsided, although total PL content remained higher in BNF-treated microsomes. This was apparently due to a sustained elevation of ethanolamine PLs, which remained at the same level at 96 hours as at 24 hours. The most significant observation at 96 hours was the substantial depletion of serine PLs (33%). The observed alterations in PL composition and possible metabolic pathways responsible are discussed in relation to previous findings.

The fatty acid compositions of the two major microsomal lipid constituents, choline and ethanolamine PLs, were not significantly different from controls at either 24 or 96 hours after a single injection of BNF. In addition, the enzyme activities of de novo hepatic choline phospholipid synthesis were not significantly altered at 24 hours. Following 96 hours of exposure to BNF, some differences in enzyme activity were noted;

choline kinase and cytidylyltransferase activities were reduced, while cholinephosphotransferase activity was substantially increased. In light of the current information available on how induction affects liver microsomal PL metabolism in mammals, these results suggest that trout hepatic enzymes may not be able to respond with the same degree of complexity as mammalian systems to inducers of monooxygenase activity.

B-Naphthoflavone Induction and Its Effect on Hepatic Phospholipid Metabolism in Rainbow Trout (*Salmo gairdneri*)

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DEDICATION

This thesis is dedicated to my parents for their constant love and support, even from 3000 miles away, who have always been there when I needed them.

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LITERATURE REVIEW

Liver Phospholipid Metabolism

Phospholipids (PL) comprise the major lipid component of liver cell membranes. Phospholipid metabolism of all cells reflects the processes of membrane synthesis, degradation, modification, and repair. Most of the structural phospholipids are derived from the same precursor pools, and interconversions of one phospholipid into another are commonplace. Metabolic pools of prime biochemical interest in PL synthesis are those available to the endoplasmic reticulum (ER) for this is the primary site of membrane biogenesis. However, rapid exchange of PLs between microsomes and other functionally different membranes makes it impossible to view these as discrete pools (1).

Although 20 years have passed since the general pathways of phospholipid synthesis were described by Kennedy and his coworkers (2), the precise biosynthetic routes for the production of phospholipids in eukaryotic cells remain uncertain. Enzyme activity

measured *in vitro* is affected by such a variety of factors, e.g. substrate levels and accessibility, product acceptor sites, and cofactor concentrations, that a final resolution of these pathways is still a long way off. This review will not cover in detail each step of the individual biosynthetic and catabolic pathways as these have been elaborated elsewhere (3-5). Reviews that provide valuable information on the reactions of PLs have been written by a number of investigators (6-9). In addition, useful overviews are provided by the many contributors to the comprehensive book on phospholipids edited by Ansell *et al* (10).

Most of the investigations on phospholipid metabolism in poikilotherms have centered on temperature adaptation (11, 12). The most important finding of this research was that a decrease in environmental temperature results in increased unsaturation of acyl groups at the one and two positions of membrane phospholipids (13-15), with a resulting increase in membrane fluidity. Most of the published work on phospholipid metabolism in fish has gone by the assumption that pathways involved are similar enough to homeotherms. Therefore, this discussion of PL metabolism will deal primarily with research on homeotherms, where the most extensive work

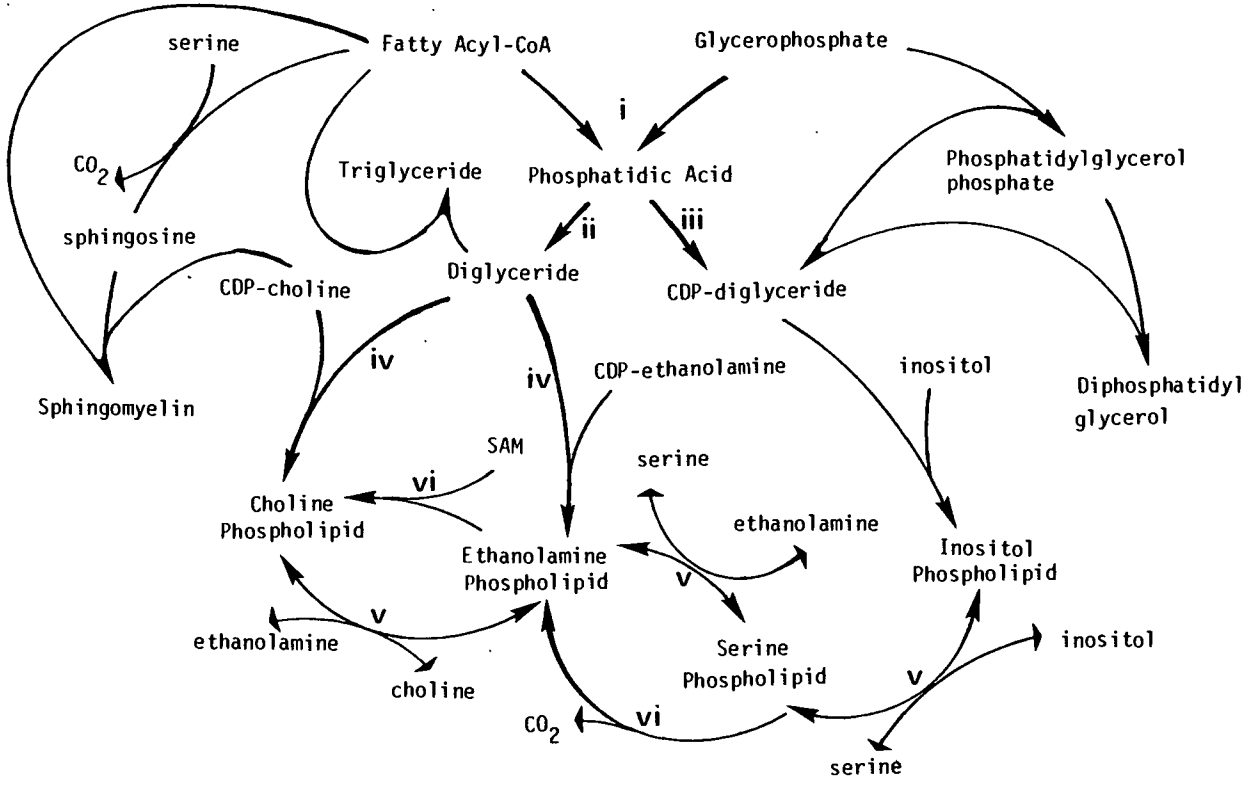

Figure 1. Interrelated pathways of phospholipid biosynthesis.

has been done and the pathways elucidated.

As can be seen in Figure 1, the biosynthetic pathways of various phospholipids are so closely interrelated that a perturbation in one pathway nearly always has repercussions on others. The metabolism of membrane PLs encompasses a large number of bond formations and cleavages that can occur with no single obligatory sequence. The major phospholipids that will be represented in this review are: 1) choline phospholipid (CP), the principal phosphate-containing lipid and a major constituent of cell membranes in most animals, 2) ethanolamine phospholipid (EP), another of the most abundant lipids, and believed to be involved in the formation of hexagonal structures (micelles) within the membrane bilayer, 3) serine phospholipid (SP) only synthesized by base exchange reactions in animals, and 4) phosphatidic acid (PA), the key intermediate in triglyceride and phospholipid synthesis, with a high turnover rate, as determined by labeling with ^{32}P.

Although phosphatidic acid (PA) can arise within the cell via several pathways (see reaction i, Fig. 1), its major source in most cells is through acylation of glycero-3-phosphate. These acylation

reactions exhibit fairly pronounced specificities towards certain fatty acid species (16) and may be important in regulating the fatty acid pattern of PA and, ultimately, its lipid products (17). It has been shown that there is a preference for saturated fatty acids at the one position of PA and other PLs (18). However, the 2-acyl ester of PA and other common PLs is usually a cis-unsaturated moiety (19). This fatty acid at the 2 position is primarily responsible for the degree of expansion, flexibility, and overall fluidity of lipid components within the membranes. More highly unsaturated fatty acids, such as arachidonic acid, do not seem to be used for the synthesis of PA itself but are readily exchanged into some of its products, e.g. ethanolamine and choline phospholipids. Properties of the enzymes involved in PA synthesis have been extensively reviewed by Bremer et al (20) and by Lands and Crawford (6). Utilization of PA involves one of two principal reactions: 1) its dephosphorylation to diglyceride (reaction ii, Fig. 1), which is then utilized for the formation of three major lipid products, EP, CP, or triglyceride (TG), or 2) activation to CDP-diglyceride, which supplies the phosphatidyl moiety of inositol phospholipids (reaction iii). This important branchpoint offers the

opportunity for controlling both the amounts and the fatty acid make up of the phosphatidic acid shunted into the diglyceride vs. the CDP-diglyceride.

Furthermore, two distinct types of physiologically important regulations may be instigated upon utilization of the diglyceride pool (reaction iv, Fig. 1). The bulk of the liver cell membrane phospholipids are formed de novo by this scheme, coupling diglycerides with the activated CDP-bases of ethanolamine and choline. There may be control of diglyceride flux into several lipids, as well as enzyme specificity for particular molecular species of diglyceride. In other words, which diglyceride goes to which CDP-base may be determined by the diglyceride's fatty acid composition. However, the results of some research have suggested that rates of phospholipid synthesis are not strongly affected by quantities of diglyceride available (21). It is more likely that the rate of diglyceride utilization can be regulated by varying the supply of CDP-bases, the cosubstrates for CP and EP synthesis.

Not much more is known regarding the selectivity of diglyceride conversion to phospholipids, although there is some evidence for a preferential use

of hexaenoic diglycerides in the synthesis of rat liver ethanolamine phospholipids (22). In vitro studies have been performed to directly test enzyme specificity, incubating the enzymes with select diglyceride species, and certain differences in preference are apparent (23, 24). To avoid problems of differential substrate solubility which plagued interpretations of other experiments, Kanoh and Ohno (25) generated endogenous diglycerides by reversal of the phosphotransferase reactions. In all cases, the specificity of both ethanolamine- and cholinephosphotransferase towards the diglycerides differed markedly. A few investigators believe that acyl groups present in newly formed EP and CP may be derived from diglycerides generated by reversal of the cholinephosphotransferase reaction, which is enhanced by incubating microsomes with CMP (26). Since all CP species are degraded without detectable preference, the fatty acid pattern established in this PL may significantly influence the diglyceride pool (27). Despite numerous studies (28), the role of these reactions in tailoring the fatty acid composition of membrane phospholipids remains uncertain.

We now have sufficient information on metabolic reactions of membrane phospholipids to recognize that

the various parts of the molecule may be altered without requiring total synthesis of a new phospholipid. A number of the major phospholipids, including CP, EP, SP, and IP, can exchange their polar head group, or base, for another that is present free in the cell (29, 30). Earlier studies have shown these "base exchange" reactions (reaction v , Fig. 1) to be stimulated by Ca^{2+} (31) and inhibited by fatty acids (32). It appears that base exchange may be the sole mechanism for synthesis of serine phospholipids (SP) in animal tissues, since attempts to detect SP formation via the CDP-diglyceride, as demonstrated in bacteria, have been unsuccessful (33). Research has indicated that these base exchange reactions, between free base and phospholipid, may function in transporting bases into the cell or as a mechanism of quickly altering membrane phospholipid distribution in response to different physiological states (29). Although polar head group exchange is important in SP formation, there is no consensus on the quantitative contribution of this reaction to membrane metabolism (34). Kanfer (35) recently presented a very extensive review on the roles of base exchange reactions in PL synthesis.

Such interconversions as the decarboxylation of SP to form EP and the subsequent transmethylation of EP

to CP, via S-adenosylmethionine (SAM), have been well-documented (reaction vi, Fig. 1) (36). Van Golde *et al* (37) reported that the only site in the liver cell where the decarboxylation reaction occurs is in the mitochondria, and the enzyme, phosphatidylserine decarboxylase, is specifically associated with the inner mitochondrial membrane. The stepwise conversion of EP to CP by methyl transfer from SAM takes place in the microsomes (38) and is possibly amplified in the liver of choline-deficient animals (39). Additionally, the hexaenoic species of EP appears to be the preferred substrate, *in vivo*, for these methylation reactions (40). The consequence or significance of this pathway is not yet fully understood, although it has already been demonstrated to be more active in liver than in other organs, such as brain, where it is apparently nonexistent. This may be linked to the role of the liver as a major producer of CPs, not only for cell membranes, but also for transport to plasma and bile. These interconversions may constitute the primary "*de novo*" sources of ethanolamine and choline for many organisms. However, Thompson (7) suggests that it seems unlikely that they play a major role in regulation of membrane lipid composition.

A discussion of phospholipid metabolism would

not be complete without considering the degradative reactions involved as they are continually being found more important in overall cell function. It has been hypothesized that the principal regulatory mechanism for membrane renewal may be in negative control of phospholipid degradation, rather than the positive control of synthetic pathways. Dawson (41) recently suggested that the specific nature of a membrane is partly directed by the degradative enzymes responsible for its turnover. Properties of the degradative enzymes have been considered in some detail by Lands and Crawford (6). Consequently, their mode of action will be only briefly outlined here.

Enzymes are known which are capable of hydrolyzing all the ester and amide linkages of phospholipids, although the most widely distributed lipolytic enzymes are those catalyzing the removal of long-chain fatty acids. The latter can be categorized as either the phospholipase A_1 or A_2 type, hydrolyzing the acyl moiety at the 1 or 2 position of the phospholipid glycerol backbone, respectively. Studies have shown that the unsaturated acyl group at position 2 turns over more rapidly than any other portions of the PL molecule (42). Phospholipases A_1 and A_2 appear

to be primarily utilized for the deacylation-reacylation reactions essential for the constant retailoring of membrane phospholipids. Comprehensive studies by Sundler and his colleagues (43, 44) have demonstrated that base exchange and methylation reactions predominantly involve phospholipids containing unsaturated fatty acids. Consequently, the more common PL acyl moieties, stearic and arachidonic acids, are incorporated largely through these deacylation-reacylation reactions. Furthermore, there are an increasing number of instances where phospholipases appear to be activated during defined physiological events in the cell, e.g. the cleavage of inositol phospholipids in response to cell stimulation (45, 46).

Functions of Membrane Phospholipids in the Cell

Adequate data now exist which confirm the observation that tissues with identical functions also have similar lipid compositions (47). Conversely, tissues within the same organism that have different functions are distinctly different in their lipid content (48). It has also been demonstrated that, even within a particular cell, where individual metabolic

functions are distributed among various organelles, lipid compositions differ from one functional membrane to another (49). The most widespread explanations offered to account for membrane lipid specificity are: 1) the apparent need for an environment of proper fluidity and surface tension for optimal enzyme function (50) or 2) the absolute requirement of some enzymes for a certain lipid in order to maintain functional activity (51, 52).

A number of investigators have demonstrated the contribution of different lipid types to the membrane's physical properties (53, 54). Observations of the response of membrane lipid composition to environmental perturbation (e.g. temperature changes, various ions) have indicated that phospholipids change primarily to regulate membrane fluidity (55) and short-term compositional changes in lipid polar moieties may be able to compensate for extracellular ion fluctuations (56). Since phospholipids interact with one another in a variety of modes, relatively small changes in the lipid composition of the membrane could significantly alter its physical properties (57). Changes in phospholipid and/or fatty acid components have been accomplished by dietary means and then followed by assays for specific cell functions to determine their

effects (58). However, cholesterol has been shown to exert a moderating effect which reduces the influence of interacting phospholipids on membrane properties (59).

A variety of foreign substances have been implicated in alterations of liver phospholipid metabolism and composition. Cell poisons, such as carbon tetrachloride (CCl_4), may operate by modifying lipid composition, as they affect the structure and function of the ER early in development of liver cell injury. Recent studies have demonstrated the selective modification of serine phospholipids by CCl_4 treatment in hepatocyte ER (60). It has also been shown that other substances which induce lipid peroxidation in membranes, such as paraquat, may inhibit phospholipid synthesis and secretion (61). Induction of hepatic drug-metabolizing enzymes by many types of lipophilic compounds has long been associated with a characteristic proliferation of the endoplasmic reticulum (62). Whether such lipophilic substances cause this effect by stimulating synthesis or inhibiting catabolism of the constituent phospholipids is still a mystery. However, recent studies have presented evidence in favor of both mechanisms,

depending on the inducing agent employed (62, 63). A selective increase in CPs by stimulation of sequential methylation of EPs has been specifically associated with phenobarbital induction (64). Furthermore, a number of recent reports have demonstrated that certain dietary lipids (65) and choline (66) are necessary for induction of the MFO system and concomitant accumulation of smooth ER in hepatocytes.

Phospholipid accumulation and changes in membrane composition have also been shown to occur during chemical carcinogenesis (67). It has been established that there is a specificity governing synthesis and positioning of lipids in individual tissue membranes. Consequently, investigators have hypothesized that an alteration in this specificity may lead to a total change in the cell and, eventually, loss of growth control, leading to tumor formation. In cell culture, mouse 3T3 cells normally show alterations in PL turnover upon reaching confluence, due to contact inhibition (68). The rate of ^{32}P incorporation into CP doubled while EP and SP labeling decreased, although the rate of incorporation into total PL remains unchanged. Early studies showed only a decrease in sphingomyelin with viral transformation (69). However, more recent work has demonstrated significant

alterations in PL polar head group turnover in SV-40 transformed cells (70).

Alterations in PL metabolism also appear to be associated with a variety of natural physiological events in the cell; such as the phenomenon of the "phospholipid effect" (71) in which PLs are believed to take part in the transmission of nerve impulses, or the activation of IP turnover as the initial step in leukocyte transformation by phytohemagglutinin (72). Other normal cellular events accompanied by changes in phospholipid metabolism include: phagocytosis and the "release" response (73), platelet aggregation and release (74), thyroid stimulation (75), and membrane fusion (76.)

De Novo Biosynthesis of Liver Microsomal Choline Phospholipids

Choline phospholipid (CP) is by far the major phospholipid in mammalian liver cell membranes, comprising approximately 50-60% of the total PL in rat hepatocyte endoplasmic reticulum (67). Its primary function in the cell appears to be structural. However, species of CP have been implicated in a

variety of physiological roles; from being the major component of pulmonary surfactant (77) to its function in the acclimation of fish to different environmental temperatures (78). Choline phospholipids can be synthesized in the liver via several pathways, e.g. base exchange with other PLs (30), sequential methylation of ethanolamine phospholipids (EP), which accounts for only 20% of liver CPs (79), and acylation of lyso-CP (80) (see Figure 2). However, the major CP biosynthetic pathway, the primary source of CP in all cells, involves the de novo synthesis of CP via phosphorylation of the free base, choline to form phosphocholine, activation of this product, to CDP-choline and subsequent transfer to diacylglycerol, derived from PA. This important pathway, as elucidated primarily by Kennedy and co-workers (81), consists of three steps catalyzed by three separate enzymes; choline kinase (E.C. 2.7.1.32), CTP:cholinephosphate cytidylyltransferase (E.C. 2.7.7.15), and CDP-choline: 1,2-diacylglycerol cholinephosphotransferase (E.C. 2.7.8.2).

It is a general biochemical principle that the first committed step in a pathway is under metabolic control and thus, is rate-limiting. Although choline kinase catalyzes this committed reaction, under most

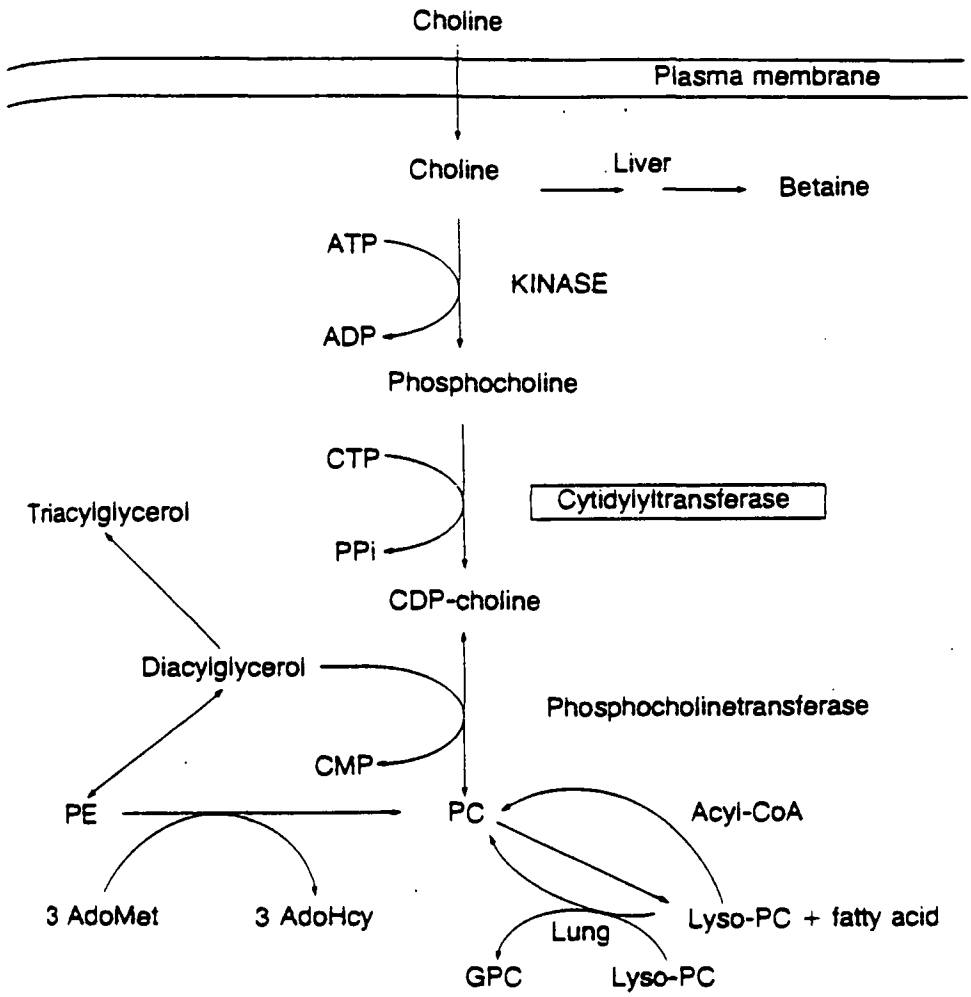

Figure 2. Major metabolic pathways for the biosynthesis of choline phospholipids.

circumstances the enzyme appears to be neither rate-limiting nor regulatory for CP biosynthesis. For example, the relative pool sizes of choline (substrate) to phosphorylcholine (product), measured in liver (82), indicated a rapid conversion of choline to phosphorylcholine with the rate-limiting step following the kinase reaction. Moreover, in most experiments, alterations in the rate of CP production have not been accompanied by corresponding changes in choline kinase activity (83). There is no evidence that the choline kinase reaction is not the first committed step in CP biosynthesis, as phosphorylcholine has not been shown to be metabolized to any compounds other than CDP-choline. In fact, even the degradation of phosphorylcholine to choline by a phosphatase does not appear to occur *in vivo* (84). This paradox might be explained if choline transport and phosphorylation could be shown to be closely linked processes in the cell, as has been suggested for *E. caudatum* (85). Although it was first reported that rat and mouse liver choline kinase were microsomal (86), investigators now agree that this enzyme is found in the 105,000xg supernatant fraction in a variety of mammalian tissues (87, 88). Ishidate *et al* (89, 90) have recently demonstrated the induction of rat liver choline kinase

activity by polycyclic aromatic hydrocarbons (PAHs) and suggested that this induction is involved in the sequence of events leading to the induction of hepatic drug metabolism.

A number of different approaches have provided strong evidence that the regulated step in the production of CP is the reaction catalyzed by CTP:phosphocholine cytidylyltransferase. An early indication for the rate-limiting role of this enzyme arose from the studies of Sundler et al (82). These authors demonstrated that the phosphocholine pool in rat liver was 150 times higher than the CDP-choline pool. More definitive evidence for the rate-limiting role of this reaction has been obtained from pulse-chase experiments in cultured HeLa cells infected with poliovirus (91). Results of these studies have shown that the increased rate of CP biosynthesis, observed in poliovirus-infected cells is due solely to an acceleration of the cytidylyltransferase-catalyzed reaction.

The properties of the cytidylyltransferase purified from rat liver are consistent with its regulatory function as it is distributed in both cytosolic and microsomal fractions of the liver

homogenate (92). Since the amount recovered in each fraction is affected by the homogenization and centrifugation procedures used (93), the enzyme is believed to be weakly associated with the endoplasmic reticulum. Furthermore, the cytidylyltransferase exists in the cytosol in two forms of different molecular size; the L-form (low molecular weight), stimulated by liposomes made from rat liver lipids, and the H-form (high molecular weight), generated from the aggregation of multiple copies of the L-form incubated with liposomes or following storage of the cytosol at 4 C. This aggregation appears to be due to the presence of diacylglycerol in the cytosol (94). Antibodies raised against the cytosolic L-form cross-reacted with both the H-form and the microsomal cytidylyltransferase, suggesting that these are all the same enzyme in different forms and subcellular locations. It was initially demonstrated that various lipids, especially lysophosphatidylethanolamine, dramatically increased the activity of the purified L-form, while choline phospholipids inhibited activity as much as 80% (95). Pelech et al (96, 97) have presented data implicating cytidylyltransferase as an enzyme that is also susceptible to regulation by a phosphorylation-dephosphorylation cycle. More

recently, this same group demonstrated that fatty acids actually promote translocation of the cytidylyltransferase to the endoplasmic reticulum (98). This change was accompanied by aggregation of the enzyme to the high molecular weight species, a concomitant acceleration of the cytidylyltransferase reaction and, ultimately, stimulation of CP synthesis. The cytidylyltransferase also appears to be regulated by changes in the concentrations of activators or inhibitors rather than by changes in the enzyme concentration itself. This has been well documented in choline-deficient rats, where the activity of cytidylyltransferase decreased to 60% of control levels, while the amount of immunochemically reacting enzyme did not change (93).

Experiments on CP production in the lung (99) also implicate the cytidylyltransferase as a regulatory enzyme. Studies with partially purified cytidylyltransferase from rat lung show that the enzyme from fetal lung has low activity which is increased 3-fold within 12 hours of birth (100). This is accompanied by a stimulation of the synthesis of dipalmitoyl-CPs-the major active component of pulmonary surfactant required to maintain the structural integrity of the alveoli. The activation of this

enzyme in the lung by phosphatidylglycerol is associated with aggregation to high molecular weight polymers-the major form of the enzyme in the lungs of adults (101). The latest results confirmed that the active form of cytidylyltransferase in lung cells is the membrane-bound enzyme since the increased synthesis of CP following birth appears to be related to elevated levels of cytidylyltransferase in the microsomal fraction (102).

The last reaction in the biosynthesis of CP is catalyzed by a microsomal diacylglycerol cholinephosphotransferase. This enzyme appeared to be an attractive candidate for a role in the regulation of CP production since it acts at the diacylglycerol branchpoint of triacylglycerol and phospholipid synthesis. However, such control appears to be limited to a possible specificity for certain diacylglycerol species (24) since cholinephosphotransferase activity does not seem to influence the rate of CP biosynthesis (103). For example, rats fed an acute dose of ethanol show a marked increase in liver diacylglycerol synthesis (104). However, this excess diacylglycerol does not affect the rate of CP production, as might be expected if cholinephosphotransferase was a regulatory enzyme. The diacylglycerol dependency of

cholinephosphotransferase has been examined using both exogenous (105) and membrane-bound (25) substrates and conflicting reports have been presented. Some investigators failed to demonstrate any selectivity of the enzyme for certain diacylglycerol species (106) while others claim it shows a definite preference for a particular fatty acid composition (105). These authors suggest that this may account, in part, for differences in fatty acid constituents in CP and EP.

More recent research has concentrated on the selectivity of lung cholinephosphotransferase in an effort to determine the significance of the CDP-choline pathway in generating the pulmonary surfactant, dipalmitoyl-CP (107, 108). Again, there were conflicting results. These discrepancies may be due to the inherent complexity of the enzyme assay system in which difficulties arise when trying to deliver amphipathic substrates to a membrane-bound enzyme. Investigation of the cholinephosphotransferase reaction in trout liver microsomes has suggested a role for the CDP-choline pathway in the acclimation of fish to changes in environmental temperature (109). Since fish cell membranes respond to temperature alterations by changes in their PL fatty acid composition, perhaps research on cholinephosphotransferase in this species

will help answer the question of the selectivity of this enzyme for diglycerides in mammalian systems (110).

Regulation of Choline Phospholipid Metabolism

The regulation of the synthesis of choline phospholipid, the principal mammalian phospholipid, is currently being studied under a variety of developmental and physiological conditions (111-113). Investigators have now demonstrated that the apparent coordination of cholesterol and choline phospholipid (CP) synthesis involves regulation at the cytidylyltransferase step (114). Furthermore, the most recent research on control of de novo CP biosynthesis has confirmed the regulatory role of the cytidylyltransferase in mammalian cells. Sleight and Kent (115-117) have shown that CP production is subject to some kind of feedback regulation in both embryonic chick muscle and Chinese hamster ovary (CHO) cell lines. In addition, they found that activation of the regulatory enzyme, cytidylyltransferase, was accompanied by a concomitant shift in its subcellular distribution, from the cytosol to particulate fractions (116). Their work suggested that activation of

cytidylyltransferase required a change in membrane phospholipid composition and this was shown experimentally by phospholipase C treatment of the microsomal PLs. Further research led them to propose that the mechanism of control of CP synthesis involved a monitoring of membrane PL composition. They concluded that regulation is at least partly dependent on information transfer from membranes to the cytidylyltransferase such that, in the presence of CP-deficient membranes, the enzyme is activated and associates with the endoplasmic reticulum (117). Another investigation recently demonstrated that modifying membrane PL composition may trigger CP synthesis via the methylation pathway as well (118).

The significance of transmethylation of ethanolamine phospholipids to form CP in the liver has not yet been established. Recent studies have suggested that this pathway is primarily important for supplying CP during choline deficiency, as long as there is sufficient S-adenosylmethionine (methyl donor) available (39). Most observations have provided evidence that the contributions of the de novo and methylation pathways are reciprocal and compensate for one another in the production of the large quantities of CP required for normal liver functions (119).

Current research has implicated a more important role for EP methylation activity with the discovery of its influence on membrane viscosity. Jaiswal et al (120) have demonstrated that an increase in N-methyltransferase is followed by an increase in membrane fluidity in the microsomes of rat aorta. In addition, they found that induction of rat liver microsomal P-450 by either phenobarbital (PB) or 3-methylcholanthrene (3-MC) was accompanied by an increase in microsomal membrane fluidity (121). Based on this evidence, they have concluded that changes in methyltransferase activity and the resulting alterations in membrane viscosity may contribute to the process of MFO induction.

It is now believed that phenobarbital induction of P-450 is accompanied by a proliferation of the endoplasmic reticulum and, more specifically, an increase in CP primarily via the methylation pathway (122). Moreover, recent research by Ishidate et al (90) has shown that PB and 3-MC have opposite effects on the enzymes of de novo CP synthesis. This indicates a possible relationship between induction of microsomal drug-metabolizing activity and modulation of CP production in animal liver. These investigators have also established that 3-MC causes counteractive effects

on CP biosynthesis, induction of choline kinase activity and inhibition of cholinephosphotransferase activity, both of which may participate in a concomitant increase in the phosphocholine pool size in rat liver (123). Based on previous evidence that phosphocholine may be involved in physiological events in the cell, they suggested that phosphocholine may play an important role in P-450 induction. Phorbol ester tumor promoters (e.g. TPA) have also been shown to influence de novo CP metabolism (124, 125), presumably via activation of the cytidylyltransferase (126). This sensitivity of CP metabolism is of special interest since the response parallels the tumor-promoting activity of the different phorbol derivatives.

The Mixed-Function Oxidase System

It is well established that the liver microsomal cytochrome P-450 mixed-function oxidase (MFO) system is primarily responsible for the oxidative metabolism of a variety of lipophilic substrates. The MFO system is responsible for the metabolism of endogenous (i.e. steroids, cholesterol, prostaglandins) (127-129) and exogenous (i.e. pesticides, pollutants,

drugs) (130-132) compounds. This enzyme complex is associated with an internal membrane system, the endoplasmic reticulum, in many tissues of various animals (133, 134) and plants (135). Cytochrome P-450, coupled with other MFO enzymes, renders many foreign substances more soluble and thus, more readily excretable, by numerous oxidative transformations. In this process, drugs and carcinogens may be detoxified or converted to substances with greater toxicity or biological activity (136).

Strobel et al (137), and Lu et al (138) were among the first to identify three distinct fractions from solubilized liver microsomes that were required for successful reconstitution of a functional MFO system, capable of substrate hydroxylation. These three fractions contained: (a) a soluble form of cytrochrome P-450, identified spectrally as the CO complex; (b) a soluble form of NADPH-cytochrome P-450 reductase, a flavoprotein previously termed NADPH-cytochrome c reductase, and (c) a heat-stable chloroform-soluble factor, subsequently identified as phospholipid. All three components were necessary, in addition to NADPH and molecular oxygen, for the hydroxylation of a variety of substrates, including polycyclic hydrocarbons, fatty acids, and drugs (139).

Cytochrome P-450 and the reductase are involved in electron transfer reactions, accompanied by consumption of molecular oxygen, to yield a hydroxylated product from the lipid-soluble substrate.

The third component of the MFO system, phospholipid, is by no means less important. In fact, it appears there is a specific phospholipid requirement for rapid electron transfer from NADPH to the cytochrome and thus for rapid substrate hydroxylation. The significance of phospholipids to cytochrome P-450 function has been confirmed by studies which demonstrated that rats require not only dietary protein but also specific lipids for normal synthesis of microsomal cytochrome P-450 and hydroxylating enzymes (140). Phosphatidylcholine, the most abundant species of the liver microsomal phospholipids (141), also appeared to be the most active in regenerating P-450 function. However, more recent studies, using reconstituted vesicles, have revealed a preference of cytochrome P-450 for negatively-charged phospholipids, such as phosphatidic acid (142) and serine phospholipids (143), and for "hexagonal (non-lamellar) lipids" such as phosphatidylethanolamine (144). Current studies have demonstrated increased monooxygenase activity in vesicles of defined lipid composition.

This activity was proportional to the amount of negatively charged phospholipids in the vesicle (143).

The lipid environment of the membrane undoubtedly contributes to many of the unique characteristics of the microsomal electron transport system, and to regulation of reactions involved in drug metabolism. Based on research thus far, performed *in vitro* with microsomes or reconstituted systems (145-148), there are several conflicting views of the arrangement of the MFO system in the microsomal membrane. Some describe cytochrome P-450 and the reductase as randomly distributed in a fluid lipid bilayer (145), while others have proposed a semi-rigid or rigid multienzyme complex (146, 147) and still another view suggested various degrees of aggregation, depending on their functional state (148). The model proposed by Stier *et al* (144) suggested an accumulation of ethanolamine phospholipids in the proximity of the cytochrome P-450 system in the form of inverted micelles. These authors also postulated that this non-lamellar phase was necessary for the incorporation and function of the MFO enzymes in the membrane and was associated with changes in their conformation and aggregation. However, more recent evidence (149) has completely discounted Stier's hypothesis, indicating

that all phospholipids are homogeneously distributed in the lamellar phase of the rough microsomal membrane. Saturation transfer-electron paramagnetic resonance (st-EPR) studies by Schwarz et al (147) strongly supported the well-defined rigid model, where 8-12 molecules of P-450 are arranged in a cluster about a central reductase molecule. Tarr et al (150) most recently proposed a detailed model for the secondary structure and membrane topology of phenobarbital-induced liver microsomal cytochrome P-450 (P-450LM2) based on the complete amino acid sequence of the enzyme.

Although studies with reconstituted systems provide much information, their validity as models for the functioning microsomal MFO system has been questioned (151). Some reconstitution studies are non-vesicular, as the relative quantities of lipid in the lipid:protein complexes fall below the critical micelle concentration (CMC). Such results with soluble enzymes in micelles may not accurately reflect their true organization and activity in vivo. Recent investigations have attempted to correct this by employing artificial membranes, where purified P-450 and the reductase are incorporated into phospholipid vesicles or liposomes (152-154). Some available

evidence suggests that the properties of these bilayers differ from non-vesicular reconstituted systems (155) and this may often be the reason for conflicting reports of phospholipid effects on proteins (e.g. vesicles vs. micelles). For example, soluble cytochrome P-450 was demonstrated to be more thermolabile than P-450 incorporated into liposomal membranes (156).

Mechanism of Action of the MFO System

The accompanying diagram (see Figure 3) summarizes our present understanding of the mechanism of action of cytochrome P-450 on lipophilic substrates, as adapted from Coon *et al* (157, 158). The action of the MFO system usually results in formation of metabolites more polar and excretable than the parent compound. The events leading to activation of molecular oxygen and subsequent insertion into the substrate are best visualized as follows. In step 1 the substrate (RH) binds to the oxidized form of P-450. The second step involves the transfer of an electron from NADPH via the reductase to reduce the P-450(Fe^{3+})-RH complex to the ferrous state. This is followed by addition of molecular oxygen and rapid

formation of the oxycytochrome P-450-substrate complex (step 3) which is believed to be in equilibrium with a ferric-superoxide form. The latter form may decay to regenerate the ferric-substrate complex with release of the superoxide anion, ultimately as hydrogen peroxide. H_2O_2 is capable of destroying the heme moiety of P-450 or initiating lipid peroxidation, either of which would be detrimental to MFO activity.

In step 4 a second electron is donated by the reductase, or cytochrome b5, to yield peroxycytochrome P-450, a species at the oxidation level of H_2O_2. If protonated, the peroxide anion could dissociate, with concomitant formation of H_2O_2 and again produce "uncoupling" of the MFO system. Step 5 involves the peroxide anion undergoing protonation and elimination of H_2O, generating the highly reactive oxene P-450. Deuterium isotope studies indicate that the substrate undergoes hydrogen abstraction, yielding a free radical intermediate (step 6) which can then react with the activated O_2 to give a hydroxylated product (ROH) (step 7). Upon removal of the product (step 8), oxidized P-450(Fe^{3+}) is regenerated. For a more detailed explanation of the MFO mechanisms of action there are quite a few excellent reviews available (159-161).

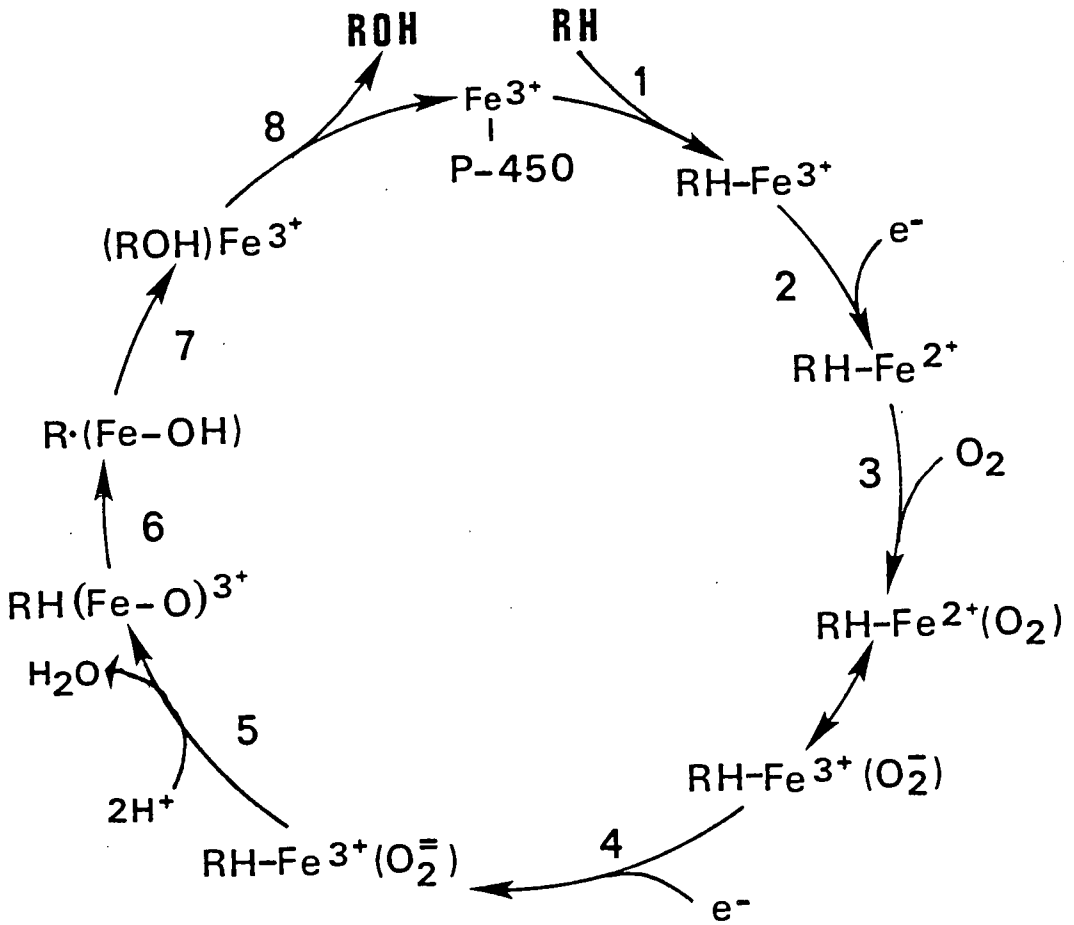

Figure 3. Schematic representation of the mechanism of action of the MFO system.

Ingelman-Sundberg et al (143) have recently presented evidence that certain steps of this cytochrome P-450 reaction scheme may be influenced by membrane phospholipid composition. According to their data, the interaction of NADPH-cytochrome P-450 reductase with cytochrome P-450 (steps 2 and 4) is dependent upon the surface charge of the liposomal membrane, which determines the overall hydroxylation rate in the reconstituted system. Mannering (162) recently classified regulation of the MFO system into three categories: 1) that imposed by inherent properties of components of the system, 2) by endogenous factors such as genes and hormones, or 3) by exogenous factors such as inducing agents.

Overall Effects of Induction of the MFO system on the Cell

Enzyme induction plays an important role in normal development and differentiation, in regulation of key metabolic pathways, in cellular actions of numerous hormones (163), and in the response of an organism to drugs (132). Enzyme induction can be defined as an adaptive increase in the number of

molecules of a specific enzyme as a result of either an increase in its rate of synthesis or a decrease in its rate of degradation.

The phenomenon of induction of liver microsomal P-450-mediated monooxygenase activity by xenobiotics and endogenous compounds has been extensively studied and reviewed over the past twenty years (164, 165) and yet we still do not completely understand molecular events within the cell that trigger the induction process. According to Remmer (166), induction of drug metabolizing enzymes is determined by the following characteristics of the inducing agent: high lipid solubility-enabling the inducer to reach lipophilic membranes of the endoplasmic reticulum, and long duration of pharmacological activity-leading to prolonged association of the substrate with the enzyme. However, as researchers began efforts to correlate observed stimulation of particular microsomal enzymes with administration of specific xenobiotics, they found that induction of the MFO system was clearly more diverse than previously suspected. They discovered that P-450 was not a single component, but a complement of multiple molecular species or isoenzymes, with varying but overlapping substrate specificities (167-169). Apparently, all inducing agents stimulate a

particular "profile" of molecular forms of P-450 (170) and a change in this profile may result in different ratios of toxic, biologically active or inactive metabolites produced from the same substrate (171). Multiple forms of cytochrome P-450 have been identified according to their "induction patterns" produced by administration of various xenobiotics (172). Selective induction of certain cytochromes P-450 by xenobiotics is now used as a tool for generating large numbers of specific forms of P-450 for purification and characterization (173).

Additionally, many of the foreign substances that induce mammalian MFO activity have been classified according to the types of cytochromes P-450 they stimulate (174). Most inducing agents can be classified into two major groups; 1) typified by phenobarbital (PB), which induces several molecular forms of P-450 and monooxygenase activities, directed toward a wide variety of substrates, and 2) represented by 3-methylcholanthrene (3-MC), a polycyclic aromatic hydrocarbon (PAH), that apparently induces 1-2 subspecies of cytochrome P-450, and a more limited group of associated enzyme activities. However, initial division of the induction phenomenon into two classes has now been recognized as an

oversimplification since other types of inducing agents have been discovered (e.g. hexachlorobenzene, Aroclor 1254) that appear to share the properties of both PB and PAH groups (175).

The mechanism by which PB and many other drugs stimulate the synthesis of the hepatic microsomal drug-metabolizing enzymes has long been assumed to be different from the mechanism whereby PAHs produce their inductive effects. Many studies have been conducted to demonstrate how differently these two classes of inducers affect the MFO system and related cell processes (176-178). It has been well-documented that PB induction of the hepatic microsomal enzymes is accompanied by proliferation of the smooth ER (179, 180). This morphological response precedes increases in P-450 and related drug-metabolizing enzyme levels that occur via enhanced synthesis of enzyme protein, DNA-dependent RNA and specific mRNAs- as evidenced by inhibition of induction by select antibiotics (181). PB-induced membrane hypertrophy has been shown to occur via initial increased synthesis (182) and subsequent decreased catabolism (183) of constituent phospholipids. Although there are some theories available (184, 185), the molecular mechanism by which PB produces its inductive effect remains to be

elucidated.

In contrast, much more is known about the mechanism by which "3-MC type" inducers (TCDD, B-naphthoflavone, polycyclic hydrocarbons) exert their effects. Induction by these compounds differs from PB-type induction in that P-450 levels are less elevated and several related drug-metabolizing enzymes (e.g. reductase, N-demethylase) are not at all affected. At the same time, a specific enzyme activity, aryl hydrocarbon (benzo[a]pyrene) hydroxylase (AHH), increases several-fold (186). Additionally, a unique hypochromic shift in the P-450 CO-reduced difference spectra to 447-448 nm has been shown. This induced enzyme has been termed cytochrome P-448 (187). 3-MC induction in mammals is further characterized by absence of any marked membrane proliferation or cell hypertrophy and little or no observed increase in phospholipid synthesis (177).

All of these differences have been explained by the discovery of a specific "cytosolic" receptor for PAHs and all "3-MC type" inducers (e.g. TCDD) in rat and mouse liver (188). The mechanism of PAH induction appears to involve stereospecific, high affinity binding of the hydrocarbon to this "cytosolic" receptor

protein and subsequent translocation from the cytoplasm to the nucleus (189). Once in the nucleus, this inducer-receptor complex actually binds to specific sites in the genome, termed the Ah (aryl hydrocarbon) locus (190), and enhances transcription of select genes that code for AHH activity, cytochrome P-448 and a host of conjugating enzymes involved in phase 2 reactions. A very recent discovery (191) has led to a slight alteration in the above hypothesized mechanism. Studies have now demonstrated that this "cytosolic" receptor is actually only located in the nucleus in vivo. The highly lipophilic PAHs are believed to passively diffuse through the cell and penetrate the nucleus where they then bind to the receptor and subsequently induce transcription of the Ah locus. It is interesting to note that the toxicity of TCDD (a 3-MC type inducer) has recently been shown to be due to induction of genes not related to the Ah locus (192).

If this is the case, the question arises whether any other enzymes are influenced during induction by PAHs, in particular those of phospholipid metabolism, as phospholipids have been shown to specifically influence membrane proteins (193). More recently, Ishidate and coworkers (194) have demonstrated both activation and inhibition of select

enzymes of the de novo synthesis of choline phospholipids (CPs) following administration of 3-MC. Earlier studies have also shown that an increase in phospholipid synthesis in liver of PB-treated rats resulted from a stimulated synthesis of CPs via methylation of ethanolamine phospholipids (EPs) (64). Furthermore, increased activities of S-adenosylmethionine:PE methyltransferase (195), acetyl CoA carboxylase (196) and acyl Co A:sn-glycerol-3-phosphate acyltransferase (197) have been demonstrated in vitro at an early time point following injection with PB. Others have found that elevated levels of microsomal phospholipid were due more to inhibition of degradative enzymes, such as phospholipases, rather than increased synthesis (180).

Most of the previous work on induction of the MFO system has concentrated on the P-450 drug metabolizing enzyme complex, and on separation and purification of the enzymes (198, 199). There is little information available on the effects of these inducing agents on other cellular activities. Since the cytochrome P-450-dependent monooxygenase system is also responsible for the metabolism of endogenous substrates (200), it might be expected that induction would lead to important modifications in metabolism of endogenous

lipids. Kupfer et al (201) have demonstrated the induction of prostaglandin metabolism by P-450 upon administration of either PB or PAHs. Conney et al (202) and others (203, 204) have also shown significant alterations in steroid metabolism as a result of P-450 induction.

A Comparison of the Induction Response in Homeotherms vs. Poikilotherms

Initial studies twenty years ago suggested that fish did not possess mixed-function oxidase activity (205). Subsequent work has not only established the existence of cytochrome P-450-dependent MFO activity in aquatic species (206) but has also demonstrated some rather unique properties. For instance, whereas mammals respond to PB treatment with a proliferation of hepatic endoplasmic reticulum and increases in select P-450 forms, fish appear refractory to such compounds (207, 208). This lack of response is also seen with other PB-type inducers, such as non-copolanar polybrominated biphenyl isomers (209) and DDT (210). On the other hand, fish appear very responsive to induction by the polycyclic aromatic hydrocarbons (PAHs), e.g. 3-methylcholanthrene (3-MC), TCDD and B-naphthoflavone (BNF) that produce significant increases in P-450 levels and Ah-associated activities

(211, 212). It must be established whether fish in general are not responsive to PB-type inducers or whether the negative data produced in past experiments with fish may have been related to experimental variables. Several studies have already demonstrated an influence of age, sex, temperature, and season on monooxygenase activities in fish and their response to several inducers (213, 214). Furthermore, seasonal variation in both xenobiotic and steroid metabolism was recently reported (215).

Many recent studies and reviews have compared the effects of various inducing agents on the liver MFO systems of mammals (e.g. rat) versus poikilotherms (e.g. trout) (216-218). The cytochrome P-450 of both untreated and PAH treated trout (219, 220) share several common characteristics with mammalian P-448, including spectral properties, substrate specificity, and sensitivity to inhibitors in vitro. When specific PAHs are active as inducers, they produce a pattern of induction of MFO activities characteristic of 3-MC induction in mammals, accompanied by a general increase in cytochrome(s) P-450 levels. However, the characteristic hypochromic shift in the Soret λ max to 448 nm has not been observed in most fish studies (221); hence its reclassification as a P_1-450 (190).

Regardless of how this P-450 is classified, similarities between trout P-450 and BNF induced rat P-448 have been demonstrated using BP metabolite profiles and covalent DNA binding studies (222). Recently, a number of investigations with both fish microsomes and partially purified P-450 (223, 224) have suggested the presence of P-448 in PAH treated fish.

Another interesting difference between mammalian and fish microsomal MFO activity is the temperature optima (225) which is lower, in most cases, for fish than for mammals. This lower optima in fish appears to reflect their poikilothermic nature as certain warm water fish have been found to have higher temperature optima than the cold water trout (226, 227). However, most aquatic species have MFO temperature optima 10-20 C higher, in vitro, than the temperature to which they are acclimated. Thus, fish normally metabolize xenobiotics much more slowly than do mammalian species and this may be why PAH-type MFO induction is more persistent and occurs at lower doses in fish than in mammals (228). Researchers have suggested that the lower temperature optima seen in trout might be due to 1) different fluidity properties of fish and mammalian microsomal membranes that could decrease fish MFO activity at higher temperatures; or

2) possible variations in the structural arrangement of the MFO system within the trout/mammalian microsomal membrane.

Characterization of the properties of fish MFO enzymes has lagged considerably behind the vast number of sophisticated studies in mammals. This is due mainly to the lack of purified fish isozymes. Bend et al (225) were among the first to report solubilization and partial purification of P-450 isozymes from fish hepatic microsomes. More recently, Stegeman and coworkers have successfully purified multiple forms of P-450 from BNF-pretreated scup (229). They also identified AHH activity in this marine fish without BNF treatment (230). In addition, Williams and Buhler (231) have developed a procedure for the simultaneous purification of NADPH P-450 reductase and multiple forms of P-450 from BNF-treated rainbow trout. These authors have demonstrated that the trout hemoprotein was similar to rat P-448 and investigated differences in temperature optima of reconstituted versus microsomal trout enzymes. They have also recently reported the existence of five forms of P-450 purified from BNF-fed trout (232) and determined the qualitative and quantitative changes in these isozymes following treatment with BNF, PCB and PB (233).

At present, Dannan et al (234) have reported the resolution of eight forms of distinct liver microsomal P-450 isozymes from the rat. Similarities between fish P-448 and mammalian P-448 suggest the existence of an Ah locus, with the structural genes that code for synthesis of these P-450 forms and a regulatory gene that codes for the cytosolic receptor that binds Ah inducers. However, presence of an Ah receptor has not, as yet, been demonstrated in fish. For a more thorough treatment of this subject several current reviews are now available pertaining to the induction of MFO activity in aquatic organisms (235-237).

The Significance of Studying Effects of MFO Induction in Poikilotherms

In the last ten years there have been numerous demonstrations of experimental induction of mixed-function oxidases in fish (238-240). Many of the inducers are PAHs, ubiquitous major environmental pollutants that pose a potential hazard not only to human life but to other forms of life as well. Aquatic species may be at risk because they serve as sinks for PAHs from many sources. Moreover, many fish species

have been shown to be very sensitive to induction of AHH activity by PAHs, BNF etc. due to bioaccumulation from water. The basal AHH activity of wild fish shows a great deal of variability (134, 241) and has actually been correlated to the presence of environmental pollutants, presumably acting as inducers of the fish hepatic monooxygenase system (242). It has been proposed that measurements of the induction of various aromatic hydrocarbon monooxygenases (e.g. AHH) might serve as a useful index of exposure to petroleum pollution (243-245). There are both advantages and limitations of using PAH-type enzyme induction as a monitor for pollutants in aquatic ecosystems. However, detailed investigations of the hepatic monooxygenase system in untreated and PAH-treated fish are necessary to determine specific conditions for enzyme induction in aquatic species so they may be used as biochemical monitors of environmental pollution.

Furthermore, characterization of induction of the MFO system in fish may provide useful information with respect to evolutionary and comparative properties of this detoxication mechanism. Many of the factors which influence mammalian MFO activity (sex, age, diet) have already been shown to affect the MFO system of aquatic species (245, 247). Studies on the structural

organization and functional interaction of MFO enzymes and membrane phospholipids in trout microsomes may provide a model for comparative work with mammalian systems. Finally, the rather unique properties of the response of fish to inducers may prove to be useful for the elucidation of molecular mechanisms involved in regulation of induction.

β-Naphthoflavone Induction and Its Effect on Hepatic Phospholipid Metabolism in Rainbow Trout (*Salmo gairdneri*)

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ABSTRACT

B-Naphthoflavone (BNF) induction of microsomal cytochrome P-450 and its effects on various aspects of liver phospholipid (PL) metabolism were examined in rainbow trout 24 and 96 hours after intraperitoneal injection. Spectrophotometrically-measured microsomal cytochrome P-450 content from BNF-induced trout liver showed no significant elevation 24 hours after injection, but increased substantially at 96 hours to approximately double the P-450 content in control liver. This corresponds to results from computer-analyzed laser densitometry scans of LDS-polyacrylamide gels of 96-hour BNF-treated liver microsomal proteins, where a tentatively-identified cytochrome P-448 (P-450LM4) increased 90% relative to controls.

BNF-induced alterations in microsomal phospholipid (PL) composition of trout liver occurred before and after the actual observed increase in cytochrome P-450 contents. A substantial elevation in liver microsomal PL/mg protein (34%) was noted at 24 hours after BNF injection, with a significant accumulation of choline, ethanolamine and inositol PLs. This was accompanied by a slight reduction in serine

PLs when compared to 24-hour controls. At 96 hours much of the initial increase of individual PLs had subsided, although total PL content remained higher in BNF-treated microsomes. This was apparently due to a sustained elevation of ethanolamine PLs, which remained at the same level at 96 hours as at 24 hours. The most significant observation at 96 hours was the substantial depletion of serine PLs (33%). The observed alterations in PL composition and possible metabolic pathways responsible are discussed in relation to previous findings.

The fatty acid compositions of the two major microsomal lipid constituents, choline and ethanolamine PLs, were not significantly different from controls at either 24 or 96 hours after a single injection of BNF. In addition, the enzyme activities of de novo hepatic choline phospholipid synthesis were not significantly altered at 24 hours. Following 96 hours of exposure to BNF, some differences in enzyme activity were noted; choline kinase and cytidylyltransferase activities were reduced, while cholinephosphotransferase activity was substantially increased. In light of the current information available on how induction affects liver microsomal PL metabolism in mammals, these results suggest that trout hepatic enzymes may not be able to

respond with the same degree of complexity as mammalian systems to inducers of monooxygenase activity.

INTRODUCTION

The induction of drug metabolizing enzymes by xenobiotics is a complex biochemical process manifested not only by increases in cytochrome P-450 and related activities (171, 172) but also by alterations in endoplasmic reticulum (ER) membranes (179, 180). This induction process is further complicated by the fact that different substances influence the MFO system and cellular functions in different ways. One group of inducers, the phenobarbital (PB) type, induces cytochrome P-450 (P-450LM2), enhances the metabolism of a variety of compounds and causes ER membrane hypertrophy. Another group, of which 3-methylcholanthrene (3-MC) is a prototype, induces cytochrome P-448 (P_1-450, P-450LM4) and stimulates the metabolism of relatively few substances without any noticeable increase in ER membrane components. On the other hand, polychlorinated biphenyls (PCBs) appear to share the properties of both groups. They have been shown to induce a P-448 cytochrome form, as observed with 3-MC, while eliciting a more general enzyme induction and ER proliferation similar to PB-type effects.

Most research on xenobiotic induction of the

mixed-function oxidase (MFO) system has concentrated solely on the effects of inducers on the cytochrome P-450 monooxygenase complex, and on separation and purification of the component enzymes (198, 199). Consequently, little work has been done to examine how inducing agents influence other cellular processes, such as phospholipid (PL) metabolism. Nevertheless, the lipid environment of the membrane undoubtedly contributes to many of the unique characteristics of this microsomal electron transport system and to regulation of the reactions involved in drug metabolism. The most striking evidence for the significance of PLs in the MFO complex was a demonstration that the reconstituted system of solubilized components cannot function without PL (137, 138). Recent studies have emphasized the importance of PLs and their constituent fatty acids as integral components of the MFO system by demonstrating their effects on cytochrome P-450, cytochrome b_5 and NADPH cytochrome P-450 reductase (248, 249). The essential role of PL is also indicated by the fact that exposure of liver microsomes to organic solvents (250) or phospholipases (251) significantly reduces drug metabolizing activity.

The regulation of choline phospholipid (CP)

synthesis, the principal mammalian liver microsomal PL, is currently being investigated under numerous physiological and developmental conditions (39, 112, 115). Alterations in mammalian CP metabolism have been associated with drug induction (90), tumor promotion (125), viral transformation (70), and lipid peroxidation (61). The production of CP in liver may occur via several pathways, e.g. sequential methylation of ethanolamine phospholipid (EP) (79), acylation of lyso-CP, and base exchange with other PLs (30). However, the major biosynthetic pathway involves the de novo synthesis of CP via phosphorylation of the free base, choline, to form phosphorylcholine, activation of this product to CDP-choline and subsequent transfer to diacylglycerol to yield CP. Of the three enzymes in this pathway, a number of reports have shown that CTP:phosphocholine cytidylyltransferase catalyzes the key reaction that regulates CP synthesis (82, 91). Sleight and Kent (115-117) most recently confirmed the regulatory role of cytidylyltransferase in mammalian cells.

The differences between mammalian and fish microsomal MFO activity have been well-documented (217, 218). For instance, whereas mammals respond to PB-type inducers with a proliferation of hepatic ER and

increases in select forms of P-450 isozymes, fish appear refractory to these compounds (207). Fish have also been shown to be partially refractive to PCBs and related "mixed-type" inducers in that they may not show increases in all related enzyme activities or P-450 content, as seen in mammalian species exposed to these compounds (211). On the other hand, fish are very sensitive to induction by polycyclic aromatic hydrocarbons (PAHs), responding with increased levels of P-448 and aryl hydrocarbon (Ah) associated enzyme activities (252). Furthermore, certain chemicals that have low toxicity in mammalian systems have been shown to be highly toxic to rainbow trout (253). The extent of hepatic MFO metabolism of these compounds has been thought to play a major role in their toxicity.

Fish possess a lower temperature optima for microsomal MFO activity (225), and have been shown to metabolize xenobiotics more slowly than mammalian species (228). In addition, PAH-type MFO induction is more persistent and occurs at lower doses in fish than in mammals. However, it has been proposed that measurements of fish monooxygenase activities induced by PAHs in their natural habitat might serve as a useful biochemical monitor of environmental pollution for man (243, 244). The differences between mammals and

fish, cited above, establishes the importance of efforts to thoroughly characterize the fish MFO system and cellular events related to induction before fish can be relied on as indicators of aquatic pollution for all species.

The present study was undertaken to further characterize the overall consequences of induction by PAHs in rainbow trout by examining the effects of B-naphthoflavone (BNF) on various aspects of PL metabolism.

MATERIALS AND METHODS

Chemicals

[Me-^{14}C]choline chloride (50.5 Ci/mol), [Me-^{14}C]phosphorylcholine (55.0 Ci/mol), and [Me-^{14}C]cytidine diphosphocholine (42.35 Ci/mol) were obtained from New England Nuclear (Boston, MA). Unlabeled choline chloride, phosphorylcholine, and cytidine diphosphocholine were from Sigma Chemical Co. (St. Louis, MO). 1-acyl-2-oleoyl-sn-glycerol was purchased from Nu Chek Prep (Elysian, MN). All reagents used in LDS-PAGE were obtained from Bio-Rad Laboratories. All other chemicals used were reagent grade.

Animals

Fish used in this study were one-year old Mt. Shasta strain rainbow trout (Salmo gairdneri) spawned and raised at the Oregon State University Fish Toxicology and Nutrition Laboratory and fed a semi-purified control diet (254). B-Naphthoflavone (Sigma), suspended in corn oil, was injected intraperitoneally (IP) into trout starved overnight at a dose of 100 mg/kg. Control trout were injected with

an equivalent dose of carrier alone. Control and treated trout were sacrificed by a blow to the head, one group at 24 hours and another at 96 hours. Animals were fasted 24 hours before being killed.

Tissue preparation

Livers from freshly killed trout were perfused with ice-cold saline, homogenized in 4 volumes of 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA (pH 7.4) buffer and centrifuged at 2,000xg for 10 min. and 10,000xg for 20 min. to obtain the postmitochondrial fraction. Microsomal and supernatant fractions were obtained by centrifugation of the postmitochondrial fraction at 105,000xg for 90 min. Microsomes were washed in 0.15 M Tris-HCl (pH 8.0) buffer and recentrifuged at 105,000xg for 90 min. The original supernatant and washed microsomal fractions were then prepared according to their use.

Polyacrylamide gel electrophoresis

One-dimensional (1-D) 7.5-15% sucrose stabilized linear gradient LDS-PAGE gels with dimensions of 14 x 32 x 1.5 cm, and a 5% stacking gel, were prepared as previously described (255). BNF-treated and control trout liver microsomal and

supernatant samples were solubilized in LDS sample buffer as previously described (256). Molecular weight standards (256) (Bio-Rad Laboratories, Richmond, CA), also solubilized in sample buffer, were run in an adjacent lane. Following electrophoresis, the gels were protein-specific stained with 0.2% Coomassie Blue R 250 in 45% methanol, 10% acetic acid overnight, and destained with 45% methanol, 10% acetic acid. Coomassie blue stained gels were scanned with a Zeineh soft laser scanning densitometer (Biomed Instruments, Inc., Fullerton, CA) interfaced with an Apple IIe computer. Relative quantities of protein were determined by integrating areas under the scan using a software package (Model ER1P3 Biomed Instruments, Inc., Fullerton, CA). Relative protein mass levels in the P-448 band were expressed as % of total densitometric scan area from the Coomassie blue stained gels.

Cytochrome P-450 content was determined by methods described previously (257). Cytochromes P-450 (P-450LM2) and P-448 (P-450LM4) were localized on 1-D gradient gels using purified trout liver microsomal P-450 and P-448 which were kindly provided by David E. Williams and Donald R. Buhler.

Phospholipid Analysis

Microsomal lipids were extracted with chloroform/methanol 1:2 by the method of Bligh and Dyer (258), and stored in benzene at -80 C. Separation of phospholipids was accomplished by two-dimensional TLC on silica gel H impregnated with 7.5% magnesium acetate (259) developed in $CHCl_3$/methanol/concentrated NH_4OH/H_2O (65:24:4:1, by vol) in the first dimension, dried under N_2 at room temperature for 20 min, and developed in the second direction with $CHCl_3$/acetone/methanol/acetic acid/H_2O (30:40:10:7:5, by vol). Phospholipids were visualized with iodine vapor and quantified by phosphorous determination as described by Bartlett (260). Fatty acid composition of choline and ethanolamine phospholipids was determined according to the methods of Selivonchick and Roots (261) using a Varian Series 1200 gas chromatograph with a Hewlett-Packard (3380A) integrator. All protein determinations were made according to the procedure of Lowry et al (262) using crystalline bovine serum albumin as the standard.

Enzyme Assays

Choline Kinase (ATP:choline phosphotransferase, E.C. 2.7.1.32) activity was determined by a

modification of the method of Weinhold and Rethy (87) where the incubation mixture contained 0.1 M Tris-HCl (pH 8.0), 15 mM $MgCl_2$, 15 mM ATP, 0.1 mM (Me-^{14}C) choline (4 Ci/mol) and 80-120 ug of supernatant protein in a final volume of 0.5 ml. This supernatant fraction was diluted to a concentration of approximately 2.0 mg/ml with homogenizing buffer, frozen in liquid nitrogen and stored at -80 C. The reaction was initiated by addition of enzyme, incubated for 30 min at 12 C and stopped by placing the tube into a boiling water bath for 2 min. Blanks were run by stopping the reaction immediately after addition of the enzyme. A 100-ul fraction of the reaction mixture was spotted on a Silica gel 60 TLC plate (E. Merck, 5 x 20 x 0.25 cm) which was developed in methanol/0.6% NaCl/NH_4OH (50:50:5, by vol). Bands (0.5 cm) were scraped into vials to which 0.5 ml of water was added followed by 5 ml of Aquasol II (New England Nuclear, Boston, MA) and counting of radioactivity in a Beckman scintillation counter.

Cytidylyltransferase (CTP:cholinephosphate cytidylyltransferase, EC 2.7.7.15) activity was assayed according to Natarajan et al (263) using both the microsomal and supernatnant fractions in separate

incubation mixtures to determine which contained the most activity due to our isolation procedure. The standard incubation mixture contained 15 mM Tris-succinate (pH 7.5) buffer, 9 mM magnesium acetate, 2 mM CTP, 0.12 mM phospho(Me-^{14}C) choline (8 Ci/mol) and either microsomal or supernatant fraction (100-150 ug protein), in a final volume of 0.5 ml. The microsomal pellet was resuspended in homogenizing buffer to a concentration of 1-2 mg/ml, frozen in liquid nitrogen and stored at -80 C. Blanks were run same as above. The assay was started by addition of enzyme, incubated at 12 C for 30 min and stopped by placing the tube into a boiling water bath for 2 min. Quantitation of the product (CDP-choline) was determined in the same manner as for choline kinase.

Cholinephosphotransferase (CDP-choline:1, 2-diacylglycerol cholinephosphotransferase, EC 2.7.8.2) activity was measured essentially as described by Natarajan *et al* (263) at 12 C in a total volume of 0.5 ml. The reaction mixture contained 175 mM Tris-HCl (pH 8.5), 30 mM $MgCl_2$, 0.2 mM EGTA, 200 ug BSA, 100 uM 1-acyl-2-oleoyl-*sn*-glycerol (dissolved in 5 ul of 100% ethanol), 100 uM CDP (Me-^{14}C) choline (4 Ci/mol) and 50-100 ug microsomal protein prepared as noted above.

The incubation was terminated after 30 min by addition of 0.3 ml of 2% $HClO_4$. Phospholipids were extracted by the method of Bligh and Dyer (258) as modified by Coleman and Bell (105). It was determined by TLC in $CHCl_3$/methanol/H_2O (65:25:4, by vol) that greater than 95% of the extracted labeled material was choline phospholipid. A sample of the final $CHCl_3$ phase was pipetted into a scintillation vial, evaporated under N_2, scintillation fluid was added and samples were counted as previously mentioned.

RESULTS AND DISCUSSION

P-448-type induction by B-naphthoflavone (BNF)

Figure 4 shows a comparison of cytochrome P-450 content in liver microsomes from control and BNF-treated rainbow trout 24 and 96 hours after IP injection. It is readily apparent that BNF induction of the MFO system is a time-dependent process such that, in trout, an exposure time greater than 24 hours is necessary for a significant increase in P-450 levels. Williams and Buhler (231, 232) previously established that BNF preferentially induces liver microsomal cytochrome P-448 (P_1-450, P-450LM4) in rainbow trout. Liver samples were taken 24 hours after a single dose of BNF to look for possible transient effects of induction and examine for early cellular events (e.g. ER proliferation) in the induction process which might be otherwise overlooked at a later time point. The 96-hour time point was chosen because it was previously established that PAHs increase P-450 activities to a plateau between 4 and 7 days after injection (218).

Induction of a cytochrome P-450 isozyme thought to be P-448 was confirmed by separation of the proteins via one-dimensional gradient polyacrylamide gel

electrophoresis (PAGE). The upper half of Figure 5 consists of two representative lanes from an LDS-PAGE gel containing 96-hour samples of liver microsomal protein from BNF-treated (upper lane) and control (lower lane) trout. The increased protein level at 57,000 Mr was tentatively identified as cytochrome P-448 using purified trout liver microsomal cytochromes P-448 and P-450. The purified P-448 comigrated with the 57,000 Mr protein, as indicated by the arrows. The lower half of Figure 5 illustrates the results from computer-analyzed laser densitometry scans of the above gel. These scans have been expanded to emphasize the immediate region of P-448. The shaded area represents the cytochrome P-448 "peak" which was integrated to determine the relative percent increase in P-448 that occurred upon induction with BNF. A 90% increase in P-448 levels was observed 96 hours after a single IP injection of BNF. This corresponds with spectral results presented in Figure 4, where BNF-induced cytochrome P-450 levels were shown to be approximately double the control levels at 96 hours. No other significant alterations in protein levels were identified on the gels.

Effects of BNF on microsomal phospholipid composition of trout liver

Few findings have been reported concerning alterations in phospholipid (PL) metabolism and/or composition due to administration of PAH-type inducers, such as BNF. Furthermore, little or no research on phospholipids has been linked with studies of MFO induction in poikilotherms. The effects of BNF administration on liver microsomal PL composition in rainbow trout 24 and 96 hours following IP injection are presented in Table 1. Differences in PL alterations before and after the actual observed increase in cytochrome P-448 are evident. As was previously mentioned, 24-h samples were examined to detect early events in the induction process that may occur before cytochrome P-450 levels have increased. It was predicted that this early time point may very likely involve changes in PL metabolism as this has already been established from time course studies of PB induction in mammals (177).

Ishidate et al (62, 177) have extensively investigated the effects ot the three known groups of cytochrome P-450 inducers on liver PL metabolism in mammals. These authors confirmed previous results from

other studies when they demonstrated that PB-induced ER proliferation was due to stimulation of PL synthesis during the early induction process, followed by a decreased turnover rate of these newly-synthesized microsomal PLs later. However, further research indicated that ER membrane hypertrophy following administration of PCBs was not due to enhanced PL production but solely to inhibited catabolism of membrane PLs and possibly depressed lipoprotein secretion. Unlike the effects in PB or PCB-treated rats, no significant increase in microsomal PL or protein content was observed in 3-methylcholanthrene (3-MC)-treated rats, suggesting no apparent proliferation of ER membranes. As a result of the different cellular manifestations observed with each type of inducer, these authors have suggested that there exists a possible relationship between induction of the drug-metabolizing enzymes and regulation of PL metabolism.

Unlike Ishidate's results with 3-MC induction in rat liver, we observed a significant (34%) increase in liver microsomal PL content/mg protein (Table 1), accompanied by a less pronounced increase in protein levels (mg protein/ g liver), only 24 hours after BNF injection of trout. Although comparisons of these

results with experiments in mammalian systems will be made, it must be kept in mind that species differences may be the major reason for the different responses to induction. For example, poikilotherms are known to metabolize xenobiotics more slowly than mammals which makes them more sensitive to several PAHs. At the same time these animals appear refractory to PB, a well-known inducer of mammalian MFO activity. In this study there was no apparent change in the total PL/mg lipid, suggesting triglyceride levels also increased with induction. In addition, a 30% increase in total mg lipid/mg protein was observed. This may not represent the dramatic proliferation observed with PB induction in mammals but it establishes that there is some increase in the amount of ER membrane in the cell, which would seem necessary to accommodate the elevated microsomal protein levels.

The most significant alterations in individual PLs occurred at 24 h following BNF injection, with a pronounced accumulation of choline phospholipids (CP), ethanolamine phospholipids (EP) and inositol phospholipids (IP), accompanied by a slight reduction (16%) in serine phospholipids (SP) when compared to 24-h controls (Table 1). Since phospholipids interact with one another in a variety of modes, relatively

small changes in the lipid composition of the membrane could significantly alter its physical properties (57). At 96 h after a single injection of BNF, much of the initial elevation in individual PLs has subsided, reflected in the reduction of total PL content/mg protein as compared to 24 h BNF-treated trout. However, total PL levels are still higher in BNF-treated trout when compared to controls, apparently due to a sustained increase in EP, the only PL that remains at the same level at 96 h as at 24 h. Both CP and IP have returned to control levels accompanied by a substantial depletion of SP (33%). The large reduction in SP coupled with a sustained elevation of EP might imply an increase in decarboxylation of SP to form EP upon induction. This decarboxylation reaction is restricted to the mitochondria which may suggest an effect of MFO induction on mitochondrial enzyme systems. However, since SP did not decrease drastically at 24 h, it is not likely that stimulation of this pathway would provide the necessary increase in EP observed. Thus, other possibilities must be considered. Some investigations have indicated that polar head group exchange between various membrane PLs and free bases may be a mechanism for quickly altering membrane PL distribution in response to different

physiological states (29). For example, changes in PL head group turnover have been noted in SV40-transformed fibroblasts (70). In addition, it appears that base exchange is the sole mechanism for synthesis of SPs in animal tissues (33). Since the most pronounced fluctuations in PL content occurred just 24 h after BNF injection, an alteration in the various base exchange reactions may be the reason for the initial increase in CP, EP and IP and the later depletion of SP.

It is also probable that augmentation of CP, EP and IP in trout microsomal membranes could occur via stimulation of de novo synthesis pathways. A number of reports have shown that tumor-promoting phorbol esters may stimulate de novo CP production (126). However, the pronounced increase in CP after 24 h followed by a return to control levels after 96 h may be due to short-term stimulation of the transmethylation of EP. Many investigators have demonstrated elevated levels of CP via stimulation of the methylation pathway in the liver of PB-induced mammals (64). Recent studies have also suggested the significance of this pathway for supplying CP during choline deficiency (39). Current research has implicated a more important role for EP methylation activity with the discovery of its influence on membrane viscosity (120). Sastry et al

(121) have recently presented evidence to suggest that changes in methyltransferase activity and the resulting alterations in membrane fluidity may contribute to the process of MFO induction. On the other hand, the accumulation of PLs in the membrane may just as well be due to inhibition of their catabolism, as was found to be the sole cause of elevated PL content with PCB induction.

The initial accumulation of IP at 24 h followed by a return to control levels is particularly interesting in light of our current knowledge of the role of IPs in signal transmission for a variety of neurotransmitters, hormones and growth factors (45, 46) and the most recent discovery of IP as a potential target for oncogenes in eliciting malignant transformation of cells (264).

Effects of BNF on fatty acid composition of choline and ethanolamine phospholipids

Davison and Wills (265) reported that with PB induction the proportion of linoleic acid in CP and EP increased to 120% of the control whereas oleic, arachidonic, and docosahexaenoic acid composition

decreased. However, the results of the present study showed no significant differences in acyl group composition of CP or EP between BNF-treated and control trout at either 24 or 96 h after treatment (see Table 2). These data support the contention that the mechanism of BNF induction is different from that of PB induction. It has been shown that alterations in constituent acyl groups of PLs may influence membrane fluidity that may then affect integral membrane proteins such as P-450 (266, 267). In addition, it is sometimes possible to predict a pathway used to synthesize a particular PL by analyzing its fatty acid composition as some enzymes have been shown to preferentially metabolize PLs containing specific acyl moieties (17, 18). Thus, any drastic changes in a particular metabolic pathway may be detected by a deviation in the PL's fatty acid composition.

Effects of BNF on the enzymes of de novo choline phospholipid synthesis

As shown in Figure 6, a single injection of BNF did not significantly alter any of the three enzyme activities after 24 h when compared to controls. Following 96 h of exposure to BNF, some differences in

enzyme activities can be noted; choline kinase activity is slightly decreased (10%), cytidylyltransferase is significantly reduced (21%), while cholinephosphotransferase activity shows a substantial increase of 25% over control values.

The metabolism of PLs, especially CP, has been shown, in vivo, to be dramatically influenced by such inducers as PB, 3-MC and PCBs (177). Ishidate et al (90) recently investigated the mechanism of interaction between these various inducers and the enzymes of de novo CP synthesis in rat liver. These authors suggested that the observed opposite effects of PB and 3-MC on these enzymes indicate a possible relationship between modulation of CP synthesis and induction of microsomal drug-metabolizing enzymes in rat liver. They also discovered that 3-MC and PCBs cause counteractive effects on de novo CP synthesis in rat liver; 1) stimulation of choline kinase and 2) simultaneous inhibition of cytidylyltransferase and cholinephosphotransferase, leading to an accumulation of intermediates, diacylglycerol and phosphocholine. Results of experiments by Holub et al (109) indicated that the CDP-choline pathway is operative in the formation of CP in the liver of rainbow trout, and may play a role in the acclimation of fish to environmental

temperature.

The results of Ishidate's work and the transient increase in CP we observed led us to examine the de novo synthesis pathway with the expectation that an adaptive change of the enzyme levels due to BNF induction might be the cause of the increased CP content. Ishidate et al (194, 123) recently demonstrated the similarities between the induction patterns of both choline kinase activity and P-448 due to 3-MC, suggesting that the increased choline kinase activity may be involved in the mechanism of 3-MC-mediated induction of the rat hepatic microsomal MFO system. However, we did not observe increased choline kinase activity upon induction with BNF in trout liver (Figure 6, A). This may be entirely due to interspecies variation and, thus, further studies of this apparent absence of induced kinase activity in trout may help elucidate the mechanism of MFO induction by PAHs in both species.

The activity of the key regulatory enzyme, cytidylyltransferase, was significantly reduced after 96 h (Figure 6, B) when compared to control enzyme in trout hepatic microsomes. We also found that the supernatant cytidylyltransferase activity was not

affected (data not shown) by BNF induction even when the microsomal activity was significantly reduced. This would suggest a separate control mechanism for the soluble and membrane-bound cytidylyltransferase activities in liver CP synthesis. Ishidate et al (123) concluded from their studies that the cholinephosphotransferase step could be a site of 3-MC inhibition of rat liver CP synthesis whereas our data suggests that the only site of regulation in trout liver is the reaction catalyzed by cytidylyltransferase.

The last reaction in CP formation is catalyzed by cholinephosphotransferase. It has been demonstrated that this enzyme's activity does not regulate the rate of CP synthesis (103), although it does act at a branchpoint of PL metabolism and has shown specificity for certain diacylglycerol species (24). In the present study, BNF significantly increased the activity of cholinephosphotransferase in trout liver microsomes at 96 h following injection (Figure 6, C). The implications of this increased activity accompanying the reduced elevation of CP at this time point are unclear. Nevertheless, it seems possible that the observed alterations in ER membrane PL composition may have an activating effect on this enzyme in vitro that

would not be evident *in vivo*. This may be due to inhibition of the regulatory cytidylyltransferase which limits the pool of CDP-choline available for CP formation. Schneider *et al* (268) have conclusively shown that the activity of membrane enzymes may be sensitive to the ratio of PL to protein. Furthermore, recent investigations have established that regulation of CP production involves monitoring membrane PL composition such that modifying the constituent PLs in the membrane can apparently stimulate both *de novo* and methylation pathways of CP synthesis (117, 118).

The lack of consistency between effects of BNF on CP levels and the enzymes of *de novo* CP synthesis suggests that it may be beneficial to investigate alternative pathways of both CP anabolism and catabolism. Previous studies have demonstrated an increase in the N-methyltransferases upon PB induction, which is presumed to be responsible for the observed increase in microsomal CP content (195). At the same time, others have shown a PCB-induced increase in CP and other PLs to occur via decreased catabolism and turnover of membrane PLs (183). Dawson (41) recently suggested that the specific nature of a membrane is partly directed by the degradative enzymes responsible for its turnover. The question to be addressed in

future experiments is what mechanism of CP accumulation would most likely be activated during induction by PAHs to permit the sudden intital elevation of CP and subsequent return to control levels. The data from the present study suggest that, in comparison to Ishidate's work in rat liver, trout hepatic enzymes may not be capable of responding with the same degree of complexity to inducers of monooxygenase activity. Elucidation of possible reasons for the different responses of trout liver PL metabolism to PAH induction may lead to a better understanding of the development of the inductive response in all species.

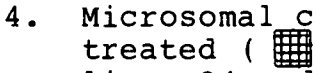

Figure 4. Microsomal cytochrome P-450 content of BNF-treated (▦) and control (▢) trout liver 24 and 96 hours following IP injection. Bars represent means ± S.D. of duplicate measurements from 3 sample groups of 4-5 pooled livers.

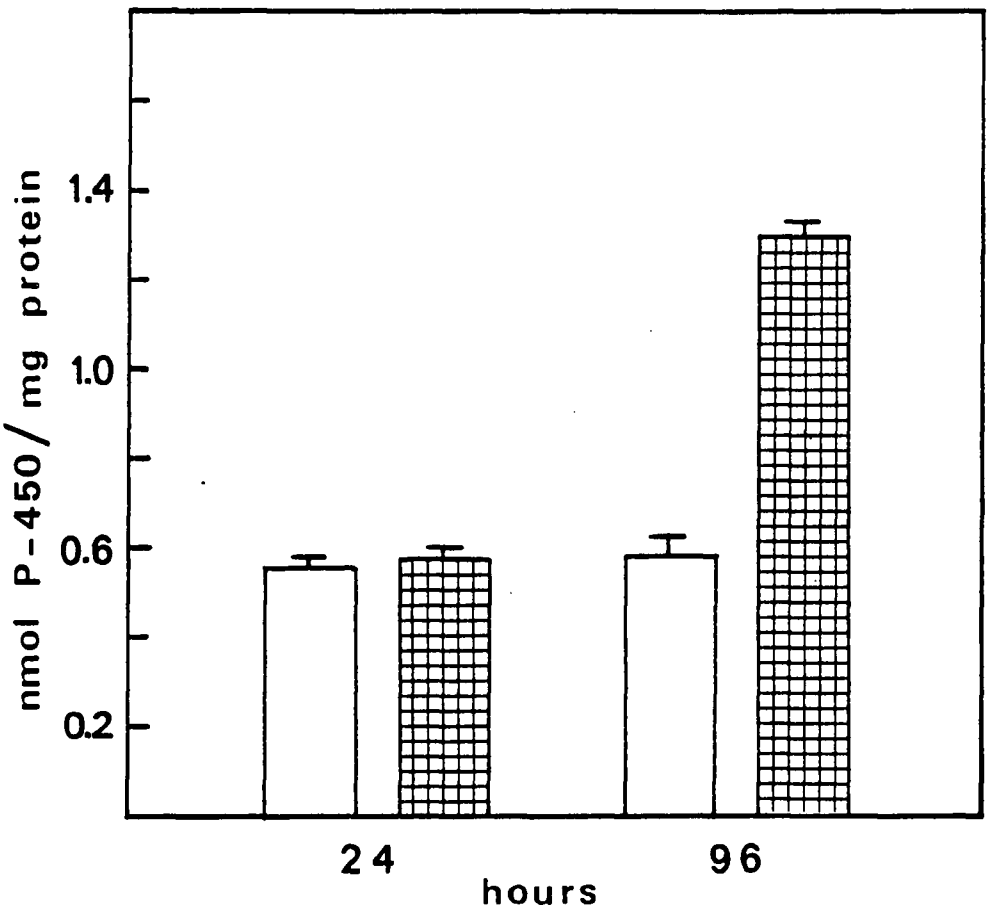

Figure 4

Figure 5. Gradient LDS-PAGE of BNF-treated and control 96 hour trout liver microsomes accompanied by computer-analyzed laser densitometry scans of cytochrome P-448 region.
Two representative lanes from a one-dimensional gradient LDS-PAGE of control (lower lane) and BNF-treated (upper lane) trout liver microsomal proteins at 96 hours after injection. The elevated 57Kd Mr (relative molecular weight) protein comigrated with purified trout microsomal cytochrome P-448. Molecular weight (Mr x 10^{-3}) markers are given above the gel. Lower half of figure shows computer-analyzed laser densitometry scans of the above gel expanded in the immediate region of P-448- Panel A corresponds to the BNF-treated lane and Panel B represents the control lane. Shaded area corresponds to zone of P-448 comigration.

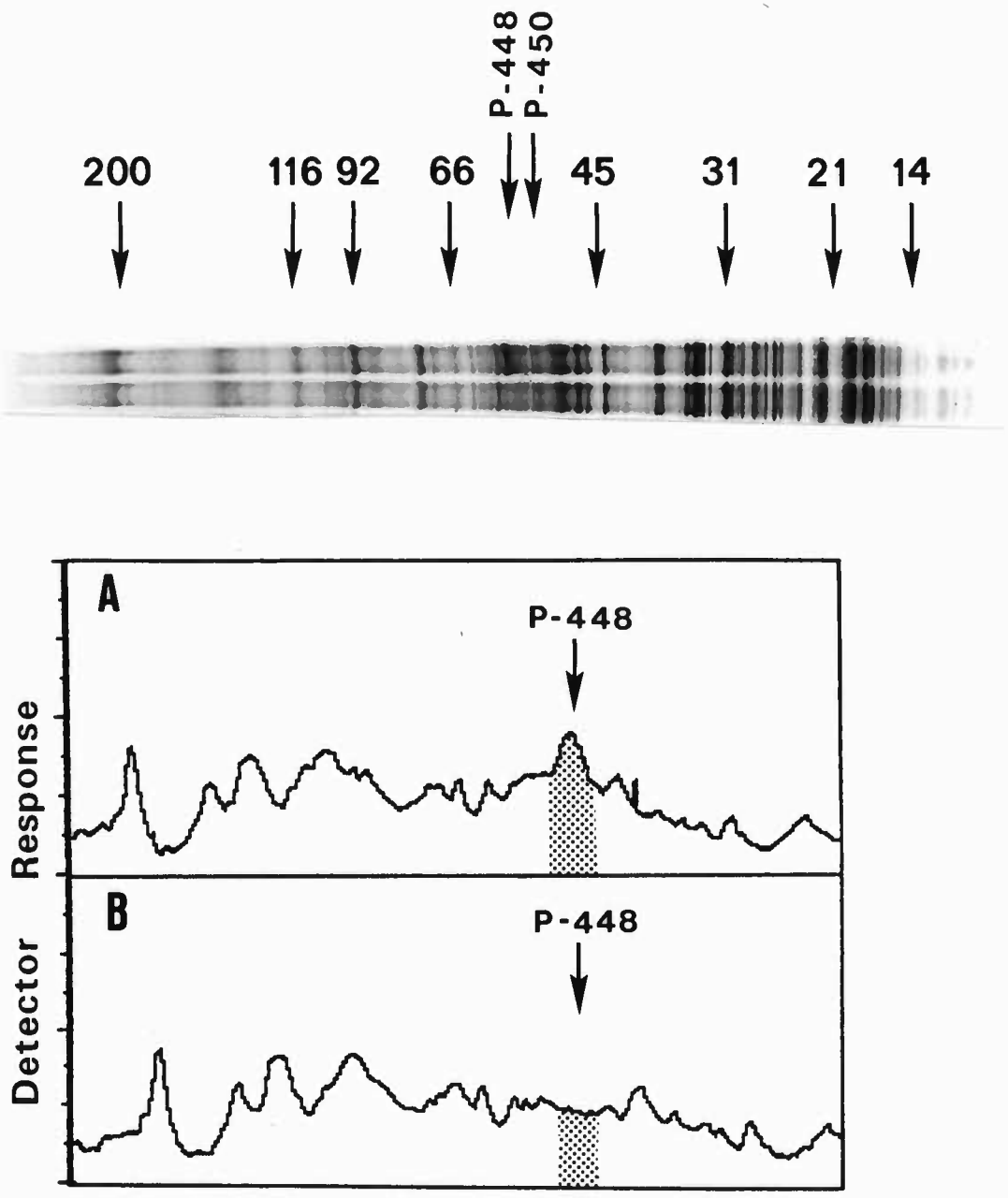
P-448
P-450
200
116
92
66
45
31
21
14
A
P-448
Response
B
P-448
Detector

Figure 5

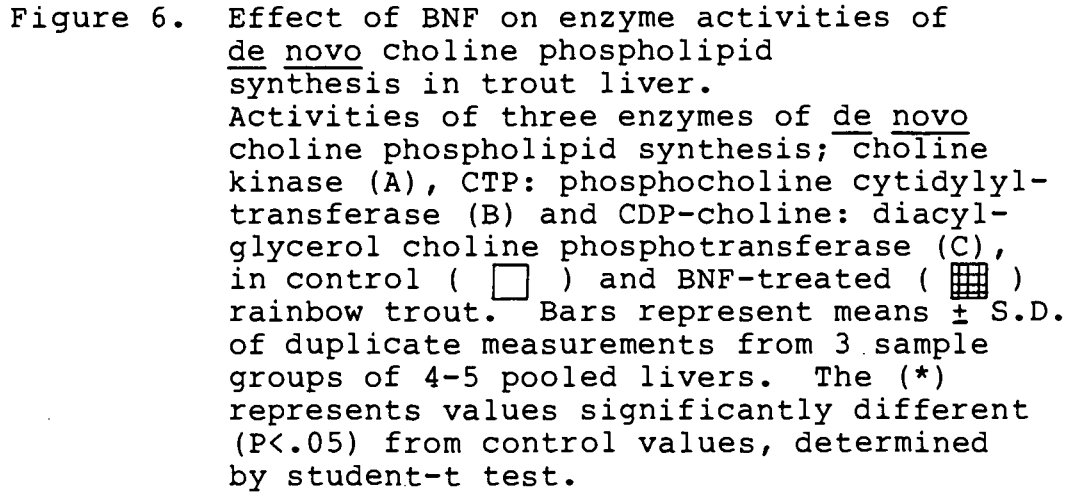

Figure 6. Effect of BNF on enzyme activities of de novo choline phospholipid synthesis in trout liver. Activities of three enzymes of de novo choline phospholipid synthesis; choline kinase (A), CTP: phosphocholine cytidylyl-transferase (B) and CDP-choline: diacyl-glycerol choline phosphotransferase (C), in control (□) and BNF-treated (▦) rainbow trout. Bars represent means ± S.D. of duplicate measurements from 3 sample groups of 4-5 pooled livers. The (*) represents values significantly different ($P<.05$) from control values, determined by student-t test.

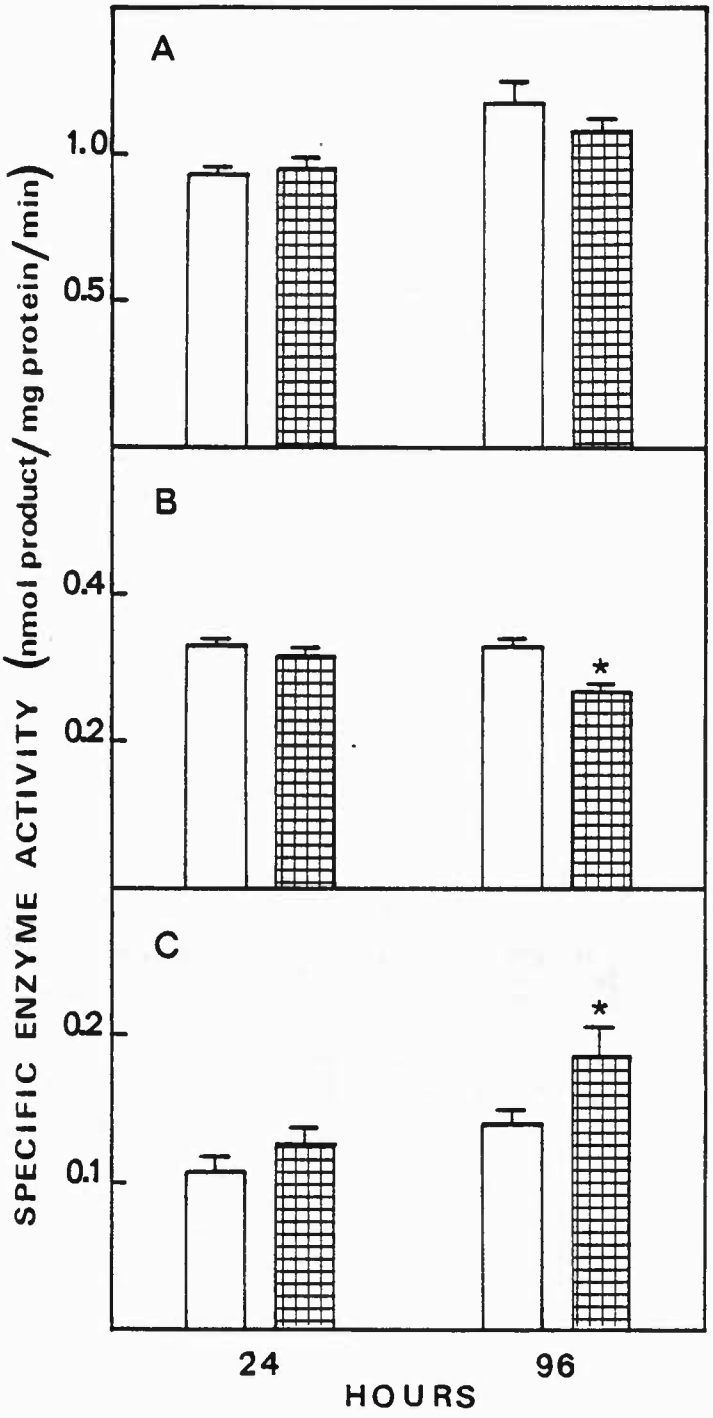
A
B
C
1.0
0.5
0.4
0.2
0.2
0.1
*
*
SPECIFIC ENZYME ACTIVITY (nmol product/mg protein/min)
24
96
HOURS

Figure 6

TABLE 1

MICROSOMAL PHOSPHOLIPID COMPOSITION OF CONTROL VS. BNF-TREATED TROUT LIVER

	nmol phospholipid/mg protein[a]			
	24 hours		96 hours	
	Control	BNF	Control	BNF
TOTAL PHOSPHOLIPID	364 ± 11	489 ± 8[b]	384 ± 9	448 ± 20[b]
CHOLINE PHOSPHOLIPID	258 ± 13	328 ± 10[b]	249 ± 14	274 ± 14
EHTANOLAMINE PHOSPHOLIPID	70 ± 7	87 ± 7[b]	70 ± 5	87 ± 3[b]
SERINE PHOSPHOLIPID	14 ± 2	12 ± 0.6	15 ± 1	10 ± 0.6[b]
INOSITOL PHOSPHOLIPID	27 ± 2	34 ± 3[b]	27 ± 2	27 ± 1
SPHINGOMYELIN	16 ± 1	18 ± 1[b]	17 ± 0.6	14 ± 2
PHOSPHATIDIC ACID	tr	tr	tr	tr

[a] Values represent means ± S.D. for 3 sample groups consisting of 4-5 fish each.

[b] Significantly different ($p<.05$) from control values according to student-t test.

TABLE 2

COMPOSITION OF CONSTITUENT FATTY ACIDS OF LIVER MICROSOMAL CHOLINE AND ETHANOLAMINE PHOSPHOLIPIDS FROM FISH INDUCED WITH B-NAPHTHOFLAVONE [a]

	Choline phospholipids				Ethanolamine phospholipids			
	24 hours		96 hours		24 hours		96 hours	
Acyl group	Control	BNF	Control	BNF	Control	BNF	Control	BNF
14:0	1.8	1.6	1.2	1.2	tr	tr	tr	tr
16:0	19.9	21.4	19.1	19.2	8.7	7.9	10.8	7.8
16:1	4.2	3.9	3.9	4.1	1.4	1.4	1.6	1.3
18:0	6.5	7.1	6.2	6.8	6.9	6.7	7.4	6.5
18:1	12.8	11.9	13.5	14.4	20.6	20.8	21.9	22.8
18:2	0.7	0.9	1.0	1.5	1.0	1.2	1.4	1.8
18:3	2.5	2.1	2.2	2.1	6.2	5.8	5.5	5.6
20:4	3.1	2.8	3.5	3.2	1.4	1.3	1.1	1.3
20:5	5.6	5.6	6.7	5.1	4.7	5.2	5.4	5.3
22:5	1.4	1.5	1.5	1.3	1.2	1.3	1.4	1.2
22:6	38.5	38.9	38.4	38.3	44.3	44.7	40.3	43.1

Trace amounts of 20:1, 20:2, 20:3, 22:4, and 22:5 were observed.

a Composition is expressed as percent of total fatty acids.

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