**AN ABSTRACT OF THE THESIS OF**

Debbie J. Mustacich for the degree of Doctor of Philosophy in Biochemistry and Biophysics presented on February 2, 1998. Title: The Microtubule System and the Canalicular *mdr2* P-glycoprotein Play a Role in the Intracellular Transport and Biliary Secretion of α-Tocopherol and Phosphatidylcholine in Rats and Mice.

Abstract approved: *Redacted for Privacy*

Donald J. Reed

α-Tocopherol (α-T), a lipid-soluble chain-breaking antioxidant, is important in the protection of biologic membranes and is known to be transported in the circulatory system in association with lipids. However, the mechanism by which α-T is secreted into the bile is not known. Piperonyl butoxide (PIP) is a widely used synthetic methylenedioxyphenyl insecticide synergist. In the first study, bile was collected for 1 hr prior to, and 5 hr following, ip injection of 1g/kg PIP. Within 3 hr of PIP administration, biliary secretion of both α-T and phosphatidylcholine (PC) increased significantly as compared to controls. Hepatic α-T levels of PIP-treated rats decreased to 26% below those of controls at the end of the bile collection period. In a second set of experiments, rats were pretreated with either colchicine or one of two doses of vinblastine; both colchicine and the higher dose of vinblastine prevented the PIP-induced decrease in hepatic α-T and the increase in biliary secretion of both α-T and PC. Next, the P-glycoprotein (Pgp) inhibitor verapamil was utilized to investigate the possible involvement of Pgps in the biliary transport of α-T and PC following treatment with PIP. The cannalicular Pgp encoded by the mdr2 gene has been shown

to transport PC into the bile under basal conditions in mice. When rats were iv injected with verapamil (4 mg/kg) 10 min prior to PIP treatment, verapamil prevented the PIP-induced increases in biliary secretion of α-T and PC and resulted in biliary outputs of α-T that were significantly below controls. Also, we determined that the biliary α-T levels in *mdr2* knockout mice were 25% of those in wildtype mice; furthermore, the α-T and glutathione levels in liver, lung, and kidney tissues of *mdr2* knockout mice differed significantly from those of wildtype mice. To investigate the fate of biliary α-T, $[^{14}C]$α-T was injected into the bile duct cannulae of rats and it was determined that approximately 60% of the radioactivity was reabsorbed within 1 hr. These results indicate that α-T undergoes enterohepatic circulation and that the biliary secretion of both α-T and PC is dependent on a functional microtubule system and *mdr2* Pgp.

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# The Microtubule System and the Canalicular *mdr2* P-glycoprotein Play a Role in the Intracellular Transport and Biliary Secretion of α-Tocopherol and Phosphatidylcholine in Rats and Mice

by

Debbie J. Mustacich

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Debbie J. Mustacich, Author

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## CONTRIBUTION OF AUTHORS

Marda Brown assisted in the collection of samples and performed some of the assays in each of the studies. Jef Shields assisted in the synthesis and purification of $[^{14}C]\alpha$-tocopherol and the collection of samples for the study involving $[^{14}C]\alpha$-tocopherol. Robert Horton assisted in the collection of samples and some of the assays for the PIP/verapamil study. All of the experiments were performed in the laboratory of Dr. Donald J. Reed who also assisted in experimental design and the interpretation of data.

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# DEDICATION

To my husband Jeff, and to Kaeli, my companion of 12 years.

# The Microtubule System and the Canalicular *mdr*2 P-glycoprotein Play a Role in the Intracellular Transport and Biliary Secretion of α-Tocopherol and Phosphatidylcholine in Rats and Mice

## Chapter I.

## Introduction

Debbie J. Mustacich

# Background and Significance

## Historical Perspectives

It has been suggested that the liver functions as a storage site for vitamin E, the major lipid-soluble antioxidant *in vivo*. As discussed below, vitamin E is important in protecting lipids from lipid peroxidation, a process that leads to membrane damage in a number of pathologic conditions and following exposure to certain chemicals. Bjorneboe *et al.* (1986) estimated that 29% of the total body vitamin E content is located in the liver. Therefore, significant decreases in hepatic vitamin E that do not lead to the transport of this important antioxidant into the circulatory system and/or to other tissues, may be detrimental to organs other than the liver, as well as the general health of the animal/human. Previous work in the laboratory of Dr. Donald J. Reed, as well as that reported by other research groups, has shown that hepatic vitamin E is depleted to 70-75% of controls following treatment of rats with a variety of compounds, including methyl ethyl ketone peroxide (MEKP) (Warren and Reed, 1991), cadmium (Shukla and Chandra, 1989), and ethanol (Bjorneboe *et al.*, 1987). Even when multiple acute doses were given in each of these studies, the degree of hepatic vitamin E depletion did not exceed 70% of controls. The results of these studies suggests that approximately 30% of the hepatic vitamin E is available for utilization by the liver and/or secretion from the liver in response to chemical insult. There are two possible pathways for the secretion of vitamin E from the liver, the

plasma and the bile; although the pathway for secretion of vitamin E into the plasma has been investigated extensively, the biliary pathway has received very little attention. In fact, the mechanism by which biliary secretion of vitamin E occurs following chemical insult, and the fate of this biliary vitamin E, has not been previously investigated.

There are a number of human conditions involving vitamin E deficiency in which the enterohepatic circulation is impaired, i.e., chronic childhood cholestasis and cystic fibrosis. A better understanding of the mechanism(s) by which vitamin E is transported within the liver and secreted into both the plasma and the bile, as well as the percentage of this secreted vitamin E that is available for distribution to other tissues, is important to developing future treatment regimes for conditions which may lead to vitamin E deficiency.

## Piperonyl Butoxide

Piperonyl butoxide (PIP) is a synthetic methylenedioxyphenyl compound that has been shown to inhibit cytochrome P450 enzymes (reviewed in Haley, 1978). PIP, first synthesized in 1947 by Herman Wachs, is used as a synergist with pyrethroid insecticides and is frequently used in toxicology studies, at a dose of 1g/kg body wt, to investigate the role of cytochrome P450 in the *in vitro* and *in vivo* metabolism of xenobiotics (Hodgson and Philpot, 1974; Haley, 1978). Although its effect on P450–dependent metabolism has been extensively investigated, its effect(s) on the

liver's protective antioxidant systems has only been investigated with respect to hepatic glutathione status. PIP (1 g/kg body wt) does not have any significant effect on hepatic glutathione status in Sprague-Dawley rats (James and Harbison, 1982; Warren and Reed, unpublished results); however, this same dose of PIP results in the loss of approximately 24% of hepatic vitamin E in rats, as compared to controls, within 1 hour of ip injection (Warren and Reed, unpublished results). This rapid depletion of hepatic vitamin E was not accompanied by an increase in plasma vitamin E levels. The work in this thesis is based on an ongoing interest by our laboratory to determine the mechanism(s) by which hepatic vitamin E levels are decreased following chemical insult *in vivo* and the fate of this vitamin E.

## Role of Vitamin E in Antioxidant Protection

Since the discovery of vitamin E in 1922 by Evans and Bishop, it has been determined that there are eight naturally occurring forms of vitamin E including the four tocopherols, α-, β-, δ-, and γ-tocopherol. All four tocopherols have the same isoprenoid side chain and methyl groups at chiral positions 2, 4', and 8'; however, the four tocopherol compounds differ in the number and position of the methyl groups on their chromanol ring (reviewed in Machlin, 1991). The remaining four naturally occurring forms of vitamin E, α-, β-, δ-, and γ-tocotrienol, also differ from each other in the number and position of the methyl groups on their chromanol ring; however, unlike the tocopherols, these four forms of vitamin E have an unsaturated side chain

containing three double bonds (Machlin, 1991). RRR-α-tocopherol (α-T), the naturally occurring isomeric form of α–tocopherol, is the most biologically active of the naturally occurring forms of vitamin E and is the major lipid soluble antioxidant found *in vivo* (Figure I.1).

Figure I.1. Structure of α-Tocopherol.

The primary source of vitamin E in western-style diets is vegetable oils. The percentage of total vitamin E present in the form of α-T is highest in olive (94%), canola (83%), safflower (83%), and sunflower oils (89%); however, safflower and sunflower oils have the highest amounts of α-T when expressed as mg/g oil (Sheppard *et al.*, 1993). In contrast, only a small percentage of the vitamin E found in corn and soybean oil occurs as α-T (15% and 7%, respectively). The majority of the vitamin E in these two oils is in the form of γ-tocopherol (γ-T) (Sheppard *et al.*, 1993). Almonds, filberts, and sunflower seeds are also good sources of α-T .

Vitamin E deficiency is seldom seen in healthy adult humans and it is difficult to render animals deficient in vitamin E. However, symptoms of vitamin E deficiency such as ataxia and areflexia are seen in adults with certain pathologic conditions, i.e., abetalipoproteinemia, cystic fibrosis, familial isolated vitamin E deficiency, and cholestatic liver disease (Traber *et al.*, 1986; Sokol *et al.*, 1985). In addition, children with chronic cholestasis develop an ataxic neurologic syndrome that has been associated with vitamin E deficiency (Sokol *et al.*, 1983).

α-T has several biologic functions including: (i) the prevention of toxicant- and carcinogen-induced oxidative damage through its ability to trap reactive oxyradicals (reviewed in Liebler, 1993), (ii) the stabilization of membranes (Urano and Matsuo, 1987; Urano *et al.*, 1990), (iii) the regulation of enzymes, i.e., protein kinase C (Azzi *et al.*, 1995), and (iiii) the protection of biologic membranes from lipid peroxidation through its chain-breaking antioxidant capabilities (Tappel, 1962; Niki, 1987; Liebler, 1993) .

Lipid peroxidation, a process by which membranes are damaged in a number of pathologic conditions and following exposure to certain chemicals, occurs in three steps: initiation, propagation, and degradation (Figure I.2). α-T blocks lipid peroxidation reactions at the propagation step; whereby it reacts primarily with lipid

R-CH 2-CH=CH-CH 2-CH=CH-R (PUFA)

(1) INITIATION

•OH

$H_2O$

R-CH 2-CH=CH-CH-CH=CH-R

Molecular Rearrangement

R-CH-CH=CH-CH =CH-CH 2-R

$O_2$

R-CH-CH=CH -CH=CH-CH 2-R

O

O•

Ascorbate •

α-T

Ascorbate

α-T•

PUFA

(2) PROPAGATION

lipid radical

chain reaction

R-CH-CH=CH -CH=CH-CH 2-R

O

O

H

(3) DEGRADATION

$Fe^{2+}$

$Fe^{3+}$

ETHANE

PENTANE

O=CH-CH 2-HC=O

(malondialdehyde)

R-CH-CH=CH -CH=CH-CH 2-R

O•

α-T

Ascorbate •

α-T•

Ascorbate

R-CH-CH=CH -CH=CH-CH 2-R

O

H

Figure I.2. Protective interactions between α-T and ascorbate against lipid peroxidation. The three degradation products, ethane, pentane, and malondialdehyde, may be measured as an indication of *in vivo* lipid peroxidation.

peroxyl radicals ($LOO^\bullet$) to form lipid hydroperoxides (LOOH) and the tocopheroxyl radical ($\alpha$-$T^\bullet$). The $\alpha$-$T^\bullet$ is an unusually stable phenoxyl radical (reviewed in Liebler, 1993). Figure I.2 shows a general scheme for lipid peroxidation of polyunsaturated fatty acids (PUFAs) and the points at which $\alpha$-T reacts with $LOO^\bullet$, as well as lipid alkoxyl radicals ($LO^\bullet$), to form the $\alpha$-$T^\bullet$, LOOH and LOH, respectively. These interactions result in the inhibition of the propagation step of lipid peroxidation. Also indicated in figure I.2 is the proposed function of ascorbate to regenerate and/or "spare" $\alpha$-T. In this context the term "spare" refers to the preferential oxidation of ascorbate instead of $\alpha$-T (reviewed in Keaney and Frei, 1994).

## Role of Vitamin C in Vitamin E Antioxidant Protection

Ascorbate (Vitamin C) is a water-soluble vitamin that is an essential nutrient in humans, monkeys, and guinea pigs. It facilitates both collagen formation and iron absorption, as well as functioning as an antioxidant by scavenging oxygen radicals such as superoxide and hydroxyl radical. It has been suggested by *in vitro* studies that ascorbate and $\alpha$-T act synergistically in the inhibition of lipid peroxidation (reviewed in Reed, 1992; Niki, 1991; Liebler, 1993). In addition, numerous *in vitro* studies have demonstrated that ascorbate is able to regenerate and/or spare $\alpha$-T (Figure I.2) (Scarpa, *et al.*, 1984; Liebler, *et al.*, 1986; Miki *et al.*, 1989; Chan *et al.*, 1991). Furthermore, several *in vitro* models have demonstrated that a threshold level of $\alpha$-T is needed to inhibit lipid peroxidation in membranes and that ascorbate is able to lower

this α-T threshold level (Liebler, *et al*., 1986; Pascoe and Reed, 1987; Tirmenstein and Reed, 1989; Murphy and Kehrer, 1989; Leedle and Aust, 1990). However, such an interaction has not been conclusively demonstrated to occur *in vivo*.

The recent development of the ODS rat that, as primates and guinea pigs, lacks L-gulonolactone oxidase enzyme activity may provide a good model for the determination of a synergistic relationship between α-T and ascorbate *in vivo*. One of the advantages of utilizing ODS rats in *in vivo* studies is that, unlike guinea pigs, these rats show very low individual differences for both α-T and ascorbate concentrations in tissues, plasma, and red blood cells (Igarashi *et al.*, 1991). These rats were recently utilized by Cadenas, *et al.* (1996) to demonstrate that increasing the dietary intake of ascorbate leads to increased plasma α-T levels under basal conditions, suggesting an *in vivo* synergistic between ascorbate and α-T. Studies under various conditions of ascorbate and α-T supplementation in which the metabolites of α-T are both qualitatively and quantitatively determined *in vivo*, are needed to further elucidate the synergistic behavior between these two antioxidants and distinguish between the regeneration of vitamin E and "sparing" of vitamin E *in vivo*.

Role of Glutathione in Vitamin E Antioxidant Protection

In addition to the studies demonstrating an interaction between α-T and ascorbate, a number of *in vitro* studies have shown that glutathione also decreases the threshold levels of α-T needed to prevent lipid peroxidation (Tirmenstein and Reed, 1989). Glutathione is the most abundant low-molecular-weight nonprotein thiol found in cells and the glutathione redox cycle is the major defense system against reactive oxygen species produced by exposure to exogenous compounds, as well as the normal oxidative products of cellular metabolism. Although the interaction between ascorbate and α-T appears to be predominantly a chemical reaction, the results of several studies have suggested that the interaction between glutathione and α-T requires enzymic participation (Tirmenstein and Reed, 1989; Murphy and Kehrer, 1989; Leedle and Aust, 1990). Furthermore, Ursini *et al.* (1982) have suggested that the enzyme phospholipid hydroperoxide glutathione peroxidase participates in the regeneration of α-T by glutathione.

In support of the hypothesis that both ascorbate and glutathione regenerate and/or "spare" α-T *in vivo*, it has been demonstrated in homogenates of human platelets that an oxidized form of α-T is regenerated to the reduced form by the addition of either ascorbate or glutathione. However, in agreement with the previous work cited above, glutathione was unable to regenerate α-T in a similar system under enzyme denaturing conditions (Chan *et al.*, 1991). Unfortunately, the oxidized form of α-T was not conclusively identified in this study. Further studies are needed to

determine if the *in vivo* oxidative fate of α-T is affected by either GSH-dependent or ascorbate-dependent protective systems and the mechanism(s) by which these interactions occur *in vivo*.

As stated above, in addition to its suggested *in vivo* interaction with α-T to prevent lipid peroxidation, glutathione participates in the glutathione redox cycle. The glutathione redox cycle has been shown to play a major role in protecting cellular thiols and maintaining them in their reduced state (reviewed in Reed, 1990). As discussed below, and in chapters II and III, the integrity of the microtubule system is essential to the intrahepatic transport and subsequent biliary secretion of both phospholipids and α-T (Groen *et al.,* 1995; Gregory *et al.*, 1978; Barnwell *et al.*, 1984; Mustacich, *et al.*, 1996). The major protein in the microtubules is tubulin which contains several sulfhydryl groups that must be maintained in the reduced state in order for the microtubule system to remain functional. Oxidation of any one of these sulfhydryl groups results in the loss of microtubule function. Decreased cellular levels of reduced glutathione and/or α-T have been shown to correlate with decreased levels of protein thiols and impaired microtubule function in hepatocytes following chemical insult (Di Monte *et al.*, 1984; Pascoe *et al.*, 1987). Therefore, it is important to determine both glutathione and α-T status during studies of biliary secretion such as those described in chapters II and III.

As discussed above and in chapter II, it has been previously determined by investigators in our laboratory and other laboratories that piperonyl butoxide, the xenobiotic used in the studies described in chapter II, does not affect glutathione status in Sprague-Dawley rats (James and Harbison, 1982; Warren and Reed, unpublished results). Consequently, the determination of glutathione status was not included in the investigations presented in chapter II.

Mechanisms of α-T Transport

All forms of vitamin E found in the diet are absorbed by the intestine in the form of micelles (Figure I.3) (reviewed in Traber, 1994). Bile acids are essential for the formation of the mixed micelles that deliver both dietary lipids and lipid soluble vitamins to the intestinal lumen for absorption (Sokol *et al.*, 1983). Figure I.3 shows that following absorption by the enterocytes, absorptive cells lining the intestinal lumen, vitamin E is incorporated into chylomicrons that then enter the circulation through the lymphatic system. Once in the plasma, chylomicrons are converted to chylomicron remnants which are taken up by the liver where the lipids are repackaged into very low density lipoprotein (VLDL) and secreted back into the plasma. Studies using deuterated tocopherols have found that VLDL is preferentially enriched with RRR-α-T prior to secretion into the plasma (reviewed in Traber, 1994; Traber *et al.*, 1992; Traber and Kayden, 1989); therefore it is the liver, not the intestine, which

discriminates between α-T and the other forms of vitamin E for distribution of this important antioxidant to the tissues.

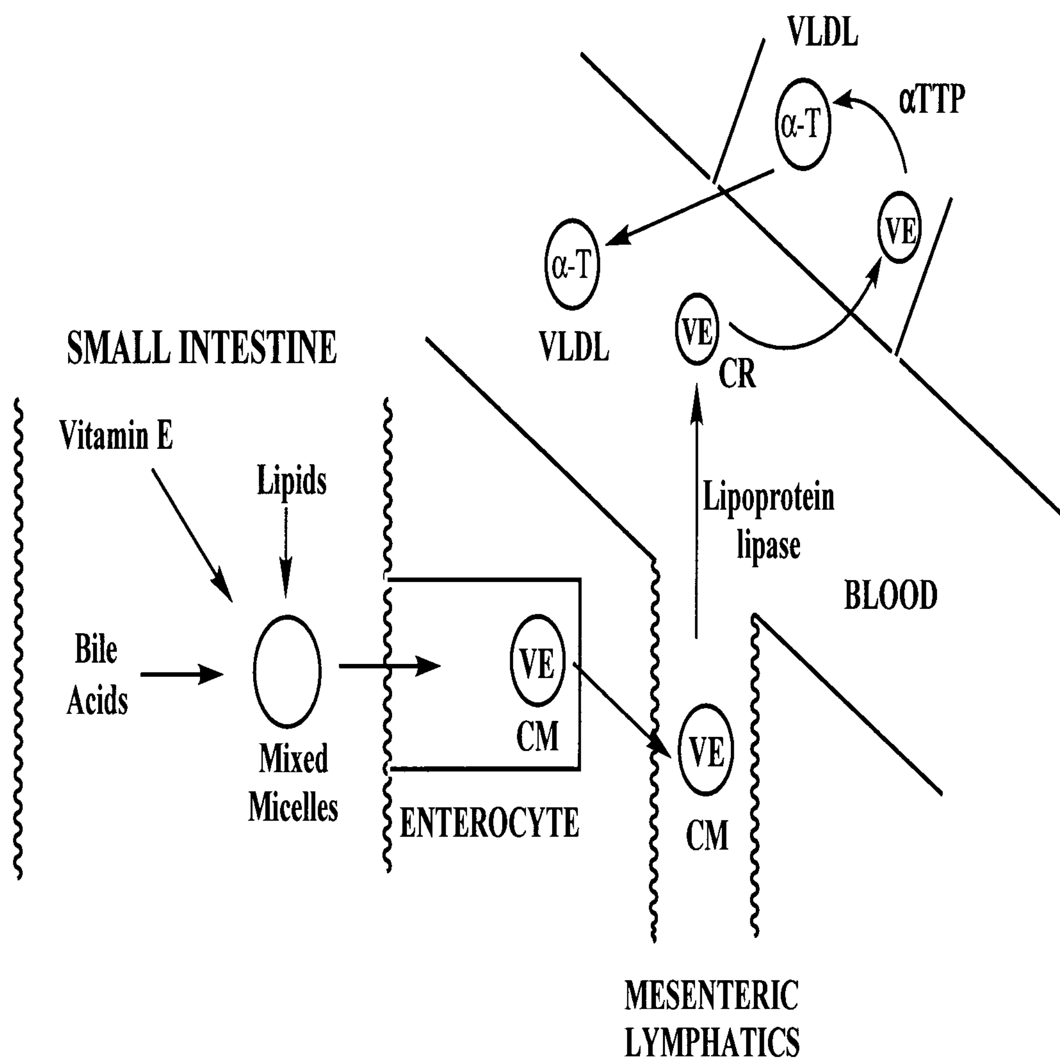


Figure I.3 Absorption and transport of dietary vitamin E. Abbreviations: CM, chylomicrons; VE, vitamin E; CR, chylomicron remnant; αTTP, α–Tocopherol transport protein; VLDL, very low density lipoprotein.

The preferential incorporation of α-T into the VLDL has been attributed to the presence of a hepatic α-T binding and transfer protein (αTTP) (reviewed in Traber, 1994); it is the specificity of this protein that is responsible for the predominance of the α-T form of vitamin E in animal tissues. Unless given daily supplements of α-T (800–1200 IU), humans having a genetic defect in this protein are unable to maintain normal plasma and tissue levels of α-T resulting in the development of neurologic abnormalities that are characteristic of vitamin E deficiency (reviewed in Traber, 1994). The gene for a human α-T binding and transfer protein (hαTTP) has recently been isolated and cloned from human liver tissue (Arita *et al.*, 1995). The corresponding protein has been determined to have 94% homology at the amino acid level with the rat α-T transfer protein (rαTTP) and 37% homology with the yeast SEC14 protein which is known to have phosphatidylinositol/phosphatidylcholine transfer activity (Arita *et al.*, 1995). The 32-kDa rαTTP has been shown to transfer α-T between membranes (Sato *et al.*, 1993; Yoshida, *et al.*, 1992; Mowri *et al.*, 1981).

After entering the blood, VLDL containing α-T is converted to LDL which exchanges α-T with HDL; HDL is the most efficient lipoprotein donor of α-T to tissues (Kostner *et al.*, 1995). These same researchers have recently demonstrated the acceleration of the transfer of α-T between lipoproteins and between HDL and cells by the human plasma phospholipid transfer protein (PLTP) (Kostner *et al.*, 1995). The ability of the PLTP to bind and transfer both phospholipids and α-T, as well as the homology of the hαTTP with phospholipid transfer proteins, and the results

presented in chapters II and III, support our hypothesis that the transport of these two substances is closely related *in vivo*.

Since the liver may function as a major storage site for α-T, it is important to understand the mechanism(s) by which α-T is secreted from the liver into both the plasma and the bile, as well as the fate of α-T once it is secreted from the liver into these two body fluids. The mechanism by which α-T is secreted into the plasma and transported to other tissues has been the focus of numerous α-T transport studies, while biliary secretion has received very little attention. The specific aims of this thesis work were to (i) investigate the mechanism(s) involved in the biliary secretion of α-T, (ii) determine if a relationship exists between the biliary secretion of α-T and the secretion of lipids into the bile, and (iii) determine the fate of α-T secreted into the bile.

## Mechanisms of Phosphatidylcholine Transport

Phosphatidylcholine (PC) constitutes approximately 90% of the phospholipids in the bile of rats and humans; however, the mechanism(s) by which biliary lipids are transported within the hepatocytes and secreted into the bile is still uncertain (Rigotti *et al.*, 1994). Inhibition of biliary PC secretion by microtubule blocking agents has been demonstrated by several groups of researchers (Gregory *et al.*, 1978; Barnwell *et al.*, 1984; Dubin *et al.*, 1980); while still other researchers debate the importance of the microtubule system in the intrahepatic transport and basal secretion of biliary

lipids (Verkade *et al.*, 1995). Hayakawa *et al.* (1990) have demonstrated two possible pathways for intrahepatic transport and biliary secretion of PC; one which is and one which is not inhibited by the microtubule blocking agent, colchicine.

Recent investigations into a physiologic role for the protein encoded by one of the mouse multidrug resistance genes, mdr2, suggest that this p-glycoprotein (Pgp) may be involved in the secretion of PC into the bile (Smit *et al.*, 1993; Ruetz and Gros, 1994). Smit *et al.* (1993) showed that PC was undetectable in the bile of knockout mice which were homozygous for a disruption in the mdr2 gene (–/–). Biliary PC levels in mice heterozygous for the mdr2 disruption (+/–) were approximately half those of mice which were homozygous for the intact mdr2 gene (+/+). Ruetz and Gros (1994) demonstrated that the *mdr2* Pgp encoded by the mouse mdr2 gene functioned as a PC translocase/flippase which did not translocate phosphatidylethanolamine (PE). This PC translocase activity was dependent on the presence of ATP and $Mg^{2+}$ and was inhibited by both vanadate and the Pgp modulator verapamil, but not colchicine and vinblastine (Ruetz and Gros, 1994; Oude Elferink *et al.*, 1997). Colchicine and vinblastine are substrates for other murine canalicular Pgps (*mdr1a* and *mdr1b*). In addition, it has been shown that expression of the *mdr2* protein in secretory vesicles (SV) from yeast results in the enhancement of PC translocation and that this translocation is further enhanced by loading the SVs with the primary bile salt taurocholate (Ruetz and Gros, 1995).

Although the *mdr2* Pgp does not confer multidrug resistance, it is encoded by a member of the multidrug resistance family of genes. This family of genes is a member of a superfamily of transport proteins known as the ATP-binding cassette (ABC) transporter proteins which transport a variety of compounds across membranes and share common structural characteristics (reviewed in Higgins 1992). There are two members of the mdr gene family found in human tissues (MDR1 and MDR3) and three members in rodents (*mdr1a*, *mdr1b*, and *mdr2*). Murine *mdr2* and human MDR3 share 91% identity at the amino acid level and are both found predominantly in the liver where their expression is restricted to the canalicular membrane of hepatocytes (Buschman *et al.*, 1992; van der Valk *et al.*, 1990). This location supports the suggestion that these two proteins have a function in biliary secretion.

Further evidence for the involvement of the *mdr2* Pgp in biliary secretion is the recently demonstrated induction of MDR2 gene expression in cynomolgus monkeys during cholestasis (Schrenk *et al.*, 1993). In addition, the simultaneous stimulation of biliary PC output with an increase in steady-state levels of mdr2 mRNA in mice treated with fibrates has been demonstrated by Chianale *et al.* (1996). It has been proposed that *mdr2* translocase activity may create the asymmetric distribution of PC seen in the outer leaflet of the canalicular membrane leading to PC-rich buds which are pinched off in the presence of canalicular bile acids (Verkade *et al.*, 1995; Ruetz and Gros, 1994).

The results reported in chapter II indicate that following chemical insult the hepatic transport and/or biliary secretion of both PC and α-T requires an intact microtubule system. These results are the first to demonstrate that a relationship exists between the biliary secretion of PC and that of α-T. Therefore, since the mdr2 Pgp has been shown to be responsible for the biliary secretion of PC, and proteins encoded by the mdr genes primarily transport lipid soluble, planar molecules which would be expected to intercalate between phospholipid molecules in the membrane bilayer, and since α-T is known to intercalate into membranes in this way, studies to investigate the possible involvement of the *mdr2* Pgp in the biliary secretion of α-T were undertaken and are described in Chapter III.

Enterohepatic Circulation

Although biliary PC is known to be reabsorbed by the intestine and undergo enterohepatic circulation, the fate of biliary α-T has not been previously determined. The study of the mechanisms of α-T intrahepatic transport, biliary secretion, and enterohepatic circulation, as well as the relationship of these three parameters to phospholipid transport, is very important to our understanding of vitamin E status and homeostasis. It is also important to determine the extent to which biliary α-T is reabsorbed and whether this is a pathway for the redistribution of hepatic α-T to other tissues which may be impaired under certain pathologic conditions. As stated above, basal transport of α-T into the bile has received very little attention and the

mechanism by which biliary output of α-T occurs following chemical insult has not been previously investigated.

## Working Hypothesis and Key Questions

The working hypothesis for the studies presented in chapters II and III is that (1) α-tocopherol, the most predominant form of vitamin E in animal tissues, is secreted into the bile at levels significantly above those of controls following treatment of rats with piperonyl butoxide, (2) biliary α-tocopherol is reabsorbed by the intestine and, thus, the secretion of hepatic α-tocopherol into the bile is a mechanism for enterohepatic distribution of α-tocopherol to other tissues, (3) a relationship exists between the biliary secretion of α-tocopherol and that of lipids, and (4) microtubules and/or the canalicular *mdr2* P-glycoprotein play(s) a role in the biliary secretion of α-tocopherol.

Part I of the working hypothesis is addressed in chapter II by treating rats with 1 g/kg PIP and then collecting bile over a period of 5 hours in both controls and PIP-treated rats to determine if PIP treatment affects biliary output of α-T as compared to controls. Experiments utilizing microtubule-blocking agents are also discussed in chapter II. The purpose of the latter experiments was to determine if the microtubule system is involved in the biliary secretion of α-T and PC under basal conditions and/or following PIP treatment; thereby addressing part 4 of the working hypothesis.

In chapter III we asked the question: Does the canalicular mdr2 Pgp play a role in the biliary secretion of α-T under basal conditions and/or following treatment of rats with PIP? In addition, the possible role of the mdr2 Pgp in basal biliary secretion

of α-T was investigated utilizing wildtype, mdr2 knockout, and mdr1a knockout mice. Furthermore, one final question was asked in chapter III: Is biliary α-T reabsorbed by the intestine such that the secretion of hepatic α-T into the bile is a possible mechanism for enterohepatic distribution of α-T to other tissues? To answer this question [$^{14}$C]α-T was injected into the bile duct cannulae of rats and [$^{14}$C] radioactivity was determined in a number of tissues over a period of 8 hours. Part 3 of the working hypothesis is addressed by including the measurement of biliary PC secretion in all of the experiments described in both chapters II and III. One consistent finding in all of the studies described in this thesis is that the hepatic transport and biliary secretion of α-T and PC appears to be closely related *in vivo*.

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# Chapter II

## Colchicine and Vinblastine Prevent the Piperonyl Butoxide-Induced Increase in Biliary Output of α-Tocopherol

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## Abstract

α-Tocopherol, a lipid soluble chain-breaking antioxidant, is important in the protection of biologic membranes and is transported in the body in association with lipids. It has been estimated that the liver contains up to 29% of the α-tocopherol stores of the body. Piperonyl butoxide (PIP) is a widely used synthetic methylenedioxyphenyl insecticide synergist. Bile was collected for 1 hour prior to, and 5 hours following, ip injection of 1g/kg PIP. Within three hours of PIP administration, biliary α-tocopherol output increased to 132% of pretreatment measurements. Hepatic α-tocopherol levels of PIP treated rats decreased to 26% below those of controls at the end of the bile collection period. Biliary bile acid and phospholipid output also increased significantly following PIP treatment. In a second set of experiments rats were pretreated with either colchicine or one of two doses of vinblastine; both colchicine and the higher dose of vinblastine prevented the PIP-induced decrease in hepatic α-tocopherol and the increase in biliary output of α-tocopherol, bile acids, and phospholipids. These results suggest that (i) PIP-induced biliary secretion of α-tocopherol is associated with altered biliary phospholipid output (ii) a significant portion of hepatic α-tocopherol may function as a highly mobilizable source of antioxidant for possible rapid enterohepatic distribution and (iii) microtubules may be involved in this process.

# Introduction

Piperonyl butoxide (PIP) is a synthetic methylenedioxyphenyl compound used as a synergist with pyrethroid and carbamate insecticides (Hodgson and Philpot, 1974; Fishbein and Falk, 1969; Haley, 1978). It is used in both commercial and household insecticide preparations and in over-the-counter head lice treatments (Hodgson and Philpot, 1974; Fishbein and Falk, 1969; Vettorazzi, 1977; Sause and Galizia, 1991). PIP is used as a food additive in Japan (Tanaka *et al.*, 1994). PIP's synergistic action is attributed to its ability to inhibit cytochrome P–450 enzymes (Hodgson and Philpot, 1974; Fishbein and Falk, 1969; Haley, 1978; Franklin, 1972). PIP exhibits mixed inhibition kinetics in liver microsomal preparations in the presence of ethylmorphine. Inhibition of P–450 enzymes occurs within 1 hour of a single administration of PIP and is followed by induction of these same enzymes after approximately 24 hours (Haley, 1978; Franklin, 1972; 1976; Fujitani *et al.*, 1993; Goldstein *et al.,* 1973). As a known inhibitor of cytochrome P–450 dependent metabolism, PIP is frequently used in toxicology studies to investigate the role of cytochrome P–450 in the in vitro and in vivo metabolism of numerous xenobiotics. For the purpose of such experiments PIP (1 g/kg) is typically used as a pretreatment 30–60 minutes prior to administration of the xenobiotic of interest. Although the effect of PIP on P–450 dependent metabolism has been extensively studied, its effect(s) on the liver's cellular protective mechanisms during these toxicology studies

is unclear. It was of interest to our laboratory to determine if PIP disturbed the status of hepatic α-tocopherol.

RRR-α-tocopherol (α-tocopherol) is the most biologically active of the eight naturally occurring forms of vitamin E and is the major lipid soluble antioxidant in vivo. As a chain-breaking antioxidant it is important in the protection of biologic membranes from lipid peroxidation. α-Tocopherol prevents carcinogen- and toxicant-induced oxidative damage through its ability to trap reactive oxyradicals. It has been estimated that up to 29% of the total body α-tocopherol content is located in the liver of rats (Bjorneboe *et al.*, 1986). α-Tocopherol is transported in the body in association with lipids and has been found to be secreted into the bile (Kayden and Traber, 1993; Traber, 1994). The mechanism by which this hepatic secretion occurs, including any involvement of the microtubule system, is not known. Bile acids and fatty acids have been shown to be necessary for the movement of α-tocopherol within the intestinal lumen and for uptake by intestinal cells, but it is not known if the three substances are secreted into the bile in conjunction with each other (Traber *et al.*, 1990). Lack of the appropriate intestinal concentrations of bile acids and lipids, as is seen in children with cholestasis and cystic fibrosis, can lead to α-tocopherol deficiency (Traber *et al*, 1990; Sokol, 1993). Decreased levels of α-tocopherol diminish the ability of cells to protect themselves from oxidative damage and in humans a deficiency of α-tocopherol can result in neurologic abnormalities (Kayden and Traber, 1993).

Previous work in our laboratory determined that treatment of rats with 1 g/kg PIP resulted in a loss of approximately 24% of their hepatic α-tocopherol after 1 hour as compared to controls receiving only vehicle (Warren, unpublished results); however plasma α-tocopherol levels did not increase significantly during this time period. The purpose of the present study was two-fold: 1) to determine if PIP has an effect on biliary α-tocopherol, bile acid, cholesterol or phospholipid output and 2) to determine if vinblastine and colchicine, two agents which have been shown to block the transport of both proteins and phospholipids into the bile (Godfrey *et al.,* 1982; Dubin *et al.*, 1980), block the transport of α-tocopherol into the bile. Since α-tocopherol is generally associated with lipids, both within cells and during its transport through the body, we chose to measure biliary levels of cholesterol, bile acids and phospholipids. These substances are the major lipid constituents of the bile. The level of PIP treatment used in this study (1g/kg body wt) is commonly used to inhibit cytochrome P–450 metabolism in toxicology studies, as discussed above, and is not expected to cause hepatic damage.

# Materials and Methods

## Chemicals

α-Tocopherol and δ-tocopherol, as well as tocopherol-stripped corn oil, were purchased from Kodak Chemicals (Rochester,NY). Piperonyl butoxide (PIP) was purchased from Aldrich. Colchicine and vinblastine were purchased from Sigma. All other chemicals used were reagent grade or better.

## Animals

Male Sprague-Dawley rats (265–300 g), obtained from Simonsen Labs Inc., were given Purina rat chow containing 60 ppm of tocopherol (specified by the supplier) and water *ad libitum* (unless otherwise specified). Animals were housed in plastic cages (two per cage) with hardwood chips for bedding and kept on a 12 hr light/dark schedule.

## In Vivo Treatments

All animals were starved for 12–14 hours prior to bile collection. Since commercially available corn oil contains tocopherols, tocopherol-stripped corn oil was used as a vehicle for PIP in all experiments. In the first set of experiments, pairs of rats received either PIP (1g/kg) or vehicle following an initial hour (0 hr) of bile

collection, as described below. In the second set of experiments, pairs of rats were pretreated by i.p. injection with either colchicine (2.5 mg/kg body wt) or vinblastine (3.3 mg/kg or 13.3 mg/kg body wt) 2 hr prior to receiving either PIP or vehicle. In this second set of experiments the 0 hr of bile collection occurred 1 hour after pretreatment and 1hour prior to PIP treatment. Both colchicine and vinblastine were dissolved in saline (0.9% NaCl).

Preparation of animals for bile collection was essentially as described by Marchand and Reed (1989). Briefly, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital, the bile duct was cannulated with PE–10 tubing, and saline (0.9% NaCl) was infused through a jugular vein cannula at a rate of 1 ml/hr to keep the rats hydrated. Rats were placed on a heating pad to maintain body temperature which was monitored with a rectal thermometer. Anesthesia was maintained by intraperitoneal injection of sodium pentobarbital as needed.

Bile was collected by positioning the free end of the cannula tubing above a cryogenic vial in a Dewar bowl; both of which were filled with liquid nitrogen and protected from light. Bile was collected from all rats for 1 hour prior to PIP treatment. At the end of 1 hour, the PIP treated rats were intraperitoneally injected with a solution of 1g/kg PIP and vehicle, while all other rats were injected with vehicle only. Bile was then collected at 1 hour intervals over a 5 hour period. Frozen samples were immediately transferred to a large Dewar flask of liquid nitrogen. Samples were either analyzed at the end of the collection period or stored at –80°C.

At the end of the collection period, while still under anesthesia, the thoracic and abdominal cavities of the rats were opened and blood was obtained via heart puncture using a vacutainer tube containing EDTA. The blood was centrifuged at 4°C and the plasma was stored at –80°C. Livers were thoroughly perfused with saline (0.9% NaCl warmed to 37°C) and placed in liquid nitrogen filled cryogenic vials. The frozen tissue was then ground in liquid nitrogen and 100 mg samples were stored at –80°C until analyzed.

Biochemical Assays

α-Tocopherol content of plasma, liver, and bile was determined by reverse-phase HPLC with a Spectra-Physics P200 pump and FL2000 fluorescence detector (excitation=284, emission=326). α-Tocopherol and lipid were extracted from samples by the method of Burton *et al.* (1985). δ–Tocopherol was added as an internal standard prior to extraction. The n-heptane layer was blown to dryness with a nitrogen stream, reconstituted in methanol, and injected onto a Custom LC 5 μm ODS $C_{18}$ column with an isocratic mobile phase consisting of 80% methanol, 15% 2-propanol and 5% water at a flow rate of 1 ml/min. The retention times for δ-tocopherol and α-tocopherol were 10.5 and 13.7 min, respectively,

Lipid phosphorous was determined by the method of Chalvardjian and Rudnicki (1970). A 10 μl sample of the reconstituted lipid extract, described above, was blown

to dryness with a stream of nitrogen and placed in a drying oven. After addition of 50 µl 70% perchloric acid, samples were heated in a Van Water and Rogers heating block for 2 hr at 160°C. After cooling, 0.4 ml of water, 2.0 ml ammonium molybdate malachite green reagent and 0.08 ml 1.5% Tween 20 were added and levels were determined at 660 nm with a Kontron 810 spectrophotometer.

Cholesterol and bile acids were measured enzymatically with in vitro diagnostic kits from Sigma diagnostics. Abnormal and normal controls and standards for both of these assays were obtained from Sigma.

Statistical Analysis

For the purpose of analysis, biliary responses for hours 1 through 5 were converted to percent of initial value (to control for animal-to-animal variation) and a separate analysis of variance (ANOVA) was conducted at each time point using SAS for Windows version 6.11. To examine pretreatment effects at 0 hour (Table II.2) and for non-PIP rats (Figures II.1-II.3) data were analyzed by ANOVA followed by three pair-wise t-tests (each pretreatment vs control). Data for phospholipid at the zero hour were log transformed for analysis. To examine PIP effects (Table II.1, Figures II.1-II.3), data were analyzed by ANOVA with pairs as nested blocks followed by four pairwise t-tests (PIP vs non-PIP within each pretreatment and the control). Unless otherwise noted, p-values for pair-wise comparisons were conservatively

adjusted by the Bonferroni method (adjusted $p < 0.05$ implies that unadjusted $p < 0.05 / k$ where for pretreatment effects $k=3$ and for PIP effects $k=4$).

# Results

## Effect of PIP on α-Tocopherol

Liver α-tocopherol content was determined for each rat at the end of the 6 hour bile collection period and is expressed as nmol α-tocopherol/g liver. Livers for all rats weighed 11.3 ± 0.72 g (mean ± SE) with no significant difference between treatment groups. Hepatic α-tocopherol levels in PIP-treated rats were significantly decreased to 74% ± 2% of control values (Table II.1). Rats which have not been pretreated with either vinblastine or colchicine and were injected with only vehicle at the end of the 0 hr are referred to as controls. Pretreatment of rats with the low dose of vinblastine (3.3 mg/kg body wt) followed by 1g/kg PIP resulted in a similar significant decrease (74 ± 5%) in hepatic α-tocopherol level as compared to pretreatment with vinblastine alone. PIP treatment following pretreatment with either the high dose of vinblastine (13.3 mg/kg body wt) or colchicine (2.5 mg/kg body wt) resulted in a smaller decrease in hepatic α-tocopherol levels (82 ± 1% and 85 ± 4%, respectively). Plasma concentrations of α-tocopherol, measured at the end of the 6 hour bile collection period, were not significantly different between treatment groups (Table II.1, expressed as nmol/ml plasma).

Bile data were calculated as biliary output (conc. X bile volume). Bile volumes for each individual rat did not change significantly over the 6 hr collection period; also, bile volumes for all groups did not significantly differ from each other at any time

point (data not shown). Zero hr measurements of biliary α-tocopherol and bile acid output were not significantly different within each treatment group (Table II.2). In contrast, biliary phospholipid output was significantly decreased in all pretreated rats compared to controls (Table II.2). This is in agreement with work done by other researchers utilizing rats in which a continuous bile acid infusion was not provided (Gregory *et al.*, 1978). Since we were interested in examining the difference in biliary output over time for each treatment group, we treated the 0 hr measurement for each rat as 100% and have presented the biliary output of α-tocopherol, phospholipid, and bile acids as %0 hr (Figures II.1-II.3).

Table II.1. Liver and Plasma Levels of α-T 5 hr after Treatment of Rats with Piperonyl Butoxide, both with and without Pretreatment.

| | **liver** (nmol/g) | % of respective control | **plasma** (nmol/ml) |
|---|---|---|---|
| Control | 18.2 ± 1.6 | | 4.22 ± 0.61 |
| PIP | 13.6 ± 1.5[a] | 74 ± 2[a] | 3.58 ± 0.48 |
| Colchicine (2.5mg) | 23.1 ± 1.2 | | 3.91 ± 0.11 |
| Col (2.5mg) + PIP | 19.6 ± 0.8[b] | 85 ± 4[b] | 3.66 ± 0.08 |
| Vinblastine (3.3mg) | 22.0 ± 2.1 | | 4.09 ± 0.35 |
| Vin (3.3mg) + PIP | 16.3 ± 2.1[b] | 70 ± 5[b] | 4.07 ± 0.80 |
| Vinblastine (13.3mg) | 21.6 ± 0.3 | | 5.30 ± 1.24 |
| Vin (13.3mg) +PIP | 17.3 ± 0.2 | 82 ± 1[b] | 4.23 ± 0.18 |

*Note*: values are means ± 1SE.
a: significantly different from control, $p< 0.05$
b: pretreatment + PIP results which are significantly different from the respective pretreated only group, $p< 0.05$.

During the 5 hour bile collection period following injection of vehicle, biliary α-tocopherol output in control rats gradually declined to 63% of 0 hr output; a similar decline was seen in all pretreatment only groups such that there was no significant difference in α-tocopherol at any time point (Figure II.1). Three hours following PIP treatment, α-tocopherol output was elevated to 58.3 ± 9.7% above the control %0 hr output (Figure II.1). By 4 hours after PIP treatment, α-tocopherol %0 hr output returned to near control rates. Pretreatment with vinblastine (high dose) or colchicine (2.5mg/kg body wt) completely prevented the observed increase in α-tocopherol

output. In contrast, pretreatment with the low dose of vinblastine did not prevent the PIP-induced increase in α-tocopherol output at 3 hours and, in fact, resulted in a continued significant increase in α-tocopherol output (>50% above controls) which was sustained through 4 hrs after PIP treatment (Figure II.1).

Effect of PIP on Phospholipids

Since α-tocopherol is fat soluble and generally associated with lipids we also determined if phospholipid output into the bile was being affected by treatment with PIP. Bile acids, cholesterol, and phospholipids are the major lipid constituents of bile, with phosphatidylcholine constituting 80-95% of the total biliary phospholipid (reviewed in Coleman and Rahman, 1992; Coleman, 1987).

Biliary phospholipid output for the 0 hr collection period was significantly decreased in all pretreated groups as compared to controls (Table II.2). In Figure II.2, both colchicine + PIP and the higher dose of vinblastine (13.3 mg/kg body wt.) + PIP resulted in phospholipid %0 hr outputs which were not significantly different from those of their respective pretreatment only groups. On the other hand, the low dose of vinblastine did not prevent the PIP-induced increase in %0 hr output. During the fifth hour following PIP treatment the variation in phospholipid output among rats in all pretreated groups was substantial and no significant differences were seen. This

same figure shows that PIP only treatment resulted in a significant increase above controls during hours 1-4.

Table II.2. Biliary Output of α-Tocopherol, Phospholipids, and Bile Acids during the 0 hr of Bile Collection.

| | Tocopherol (pmol/hr) | n | Phospholipid (mmol/hr) | n | Bile Acids (mmol/hr) | n |
|---|---|---|---|---|---|---|
| Control | 1137 ± 125 | 8 | 2.7 ± 0.21 | 7 | 9.66 ± 0.63 | 3 |
| Colchicine (2.5mg) | 1169 ± 167 | 3 | 0.5 ± 0.04[a] | 3 | 8.14 ± 0.51 | 3 |
| Vinblastine (3.3mg) | 1065 ± 160 | 3 | 0.4 ± 0.04[a] | 3 | 8.19 ± 0.45 | 3 |
| Vinblastine(13.3mg) | 1198 ± 182 | 3 | 0.4 ± 0.06[a] | 3 | 8.56 ± 0.73 | 3 |

*Note*: values are means ± 1SE.

a: significantly different from control, $p < 0.05$

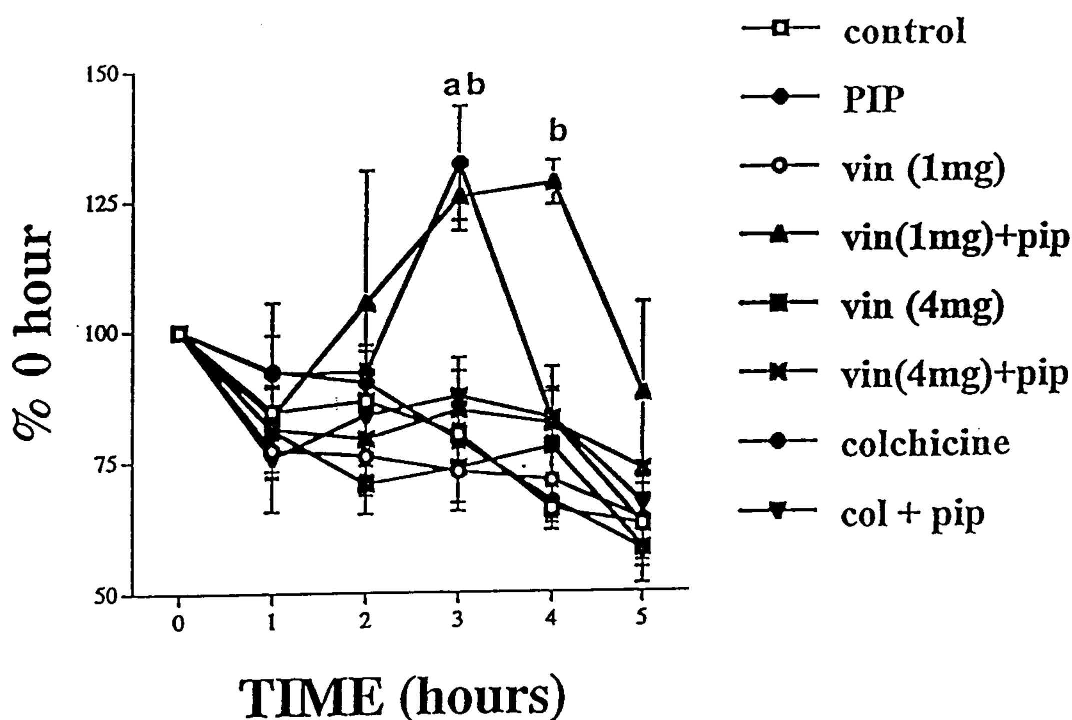


Figure II.1. Effect of colchicine and vinblastine on the PIP-induced increase in biliary α-T output. Colchicine and vinblastine were given 1 hr prior to the start of bile collection and bile was collected for 1 hr prior to PIP treatment. Data are expressed as %0 hr as described in results and are presented as mean ± 1 SE, n=7-8 for controls and PIP only treatment, n=3 for all pretreatment and pretreatment + PIP values. Letters a and b represent points at which PIP treatment resulted in a significant increase, $p < 0.05$ Bonferroni adjusted (adjusted $p < 0.05$ implies that unadjusted $p < 0.05 / k$ where k=3 for PIP effects). a = treatment with PIP alone is significantly different from control. b = treatment with PIP following pretreatment with vinblastine (3.3mg/kg) is significantly different from vinblastine (3.3mg/kg) alone.

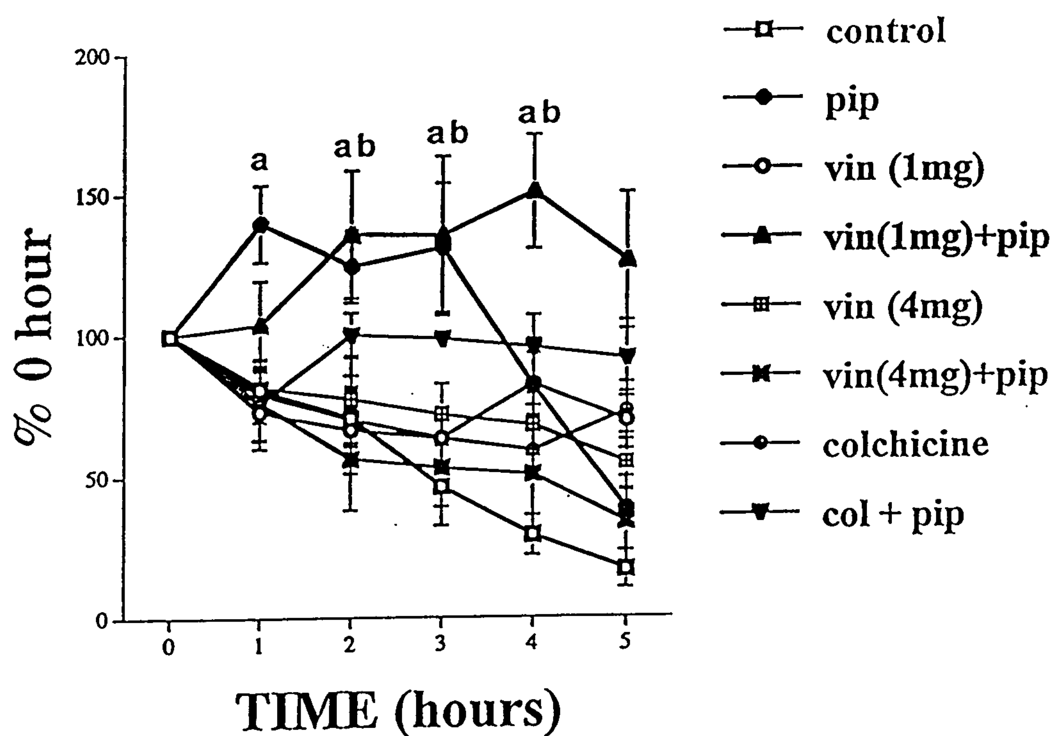


Figure II.2. Effect of colchicine and vinblastine on the PIP-induced increase in biliary output of phospholipid. Conditions are as described in Figure II.1. Letters a and b represent points at which PIP treatment resulted in a significant increase, $p < 0.05$ Bonferroni adjusted (adjusted $p < 0.05$ implies that unadjusted $p <. 0.05 / k$ where $k=3$ for PIP effects). a = treatment with PIP alone is significantly different from control. b = treatment with PIP following pretreatment with vinblastine (3.3mg/kg) is significantly different from vinblastine (3.3mg/kg) alone.

Effect of PIP on Bile Acids and Cholesterol

Bile acid output was assessed in bile for each hour of collection for the last three pairs of control and PIP only treated rats and for all of the pretreated rats. These results are presented in Figure II.3. The mean value for bile acid output for all groups during the 0 hr collection period is shown in Table II.2 and is in the range of values found in the literature (Katagiri *et al.*, 1992, Crawford *et al.*, 1988). Three hours after treatment with PIP, bile acid output had increased 149 ± 39.9% above %0 hr output for controls; however, all three pretreatments were able to block the effect of PIP on bile acid output (Figure II.3). The bile acid concentration in PIP treated bile during the 3rd hour had a mean value of 66 mmol/l and was below the maximum inducible concentrations (80-90 mmol/l) reported by Coleman *et al.* (1988) and Erlinger (1988). The mean value for biliary cholesterol output during the 0 hr collection period was 0.46 ± .05 μmol/hr and is in agreement with values reported by Kuipers *et al.* (1987). PIP had no significant effect on biliary cholesterol output (data not shown); therefore biliary cholesterol output was not measured in the second set of experiments with pretreatments.

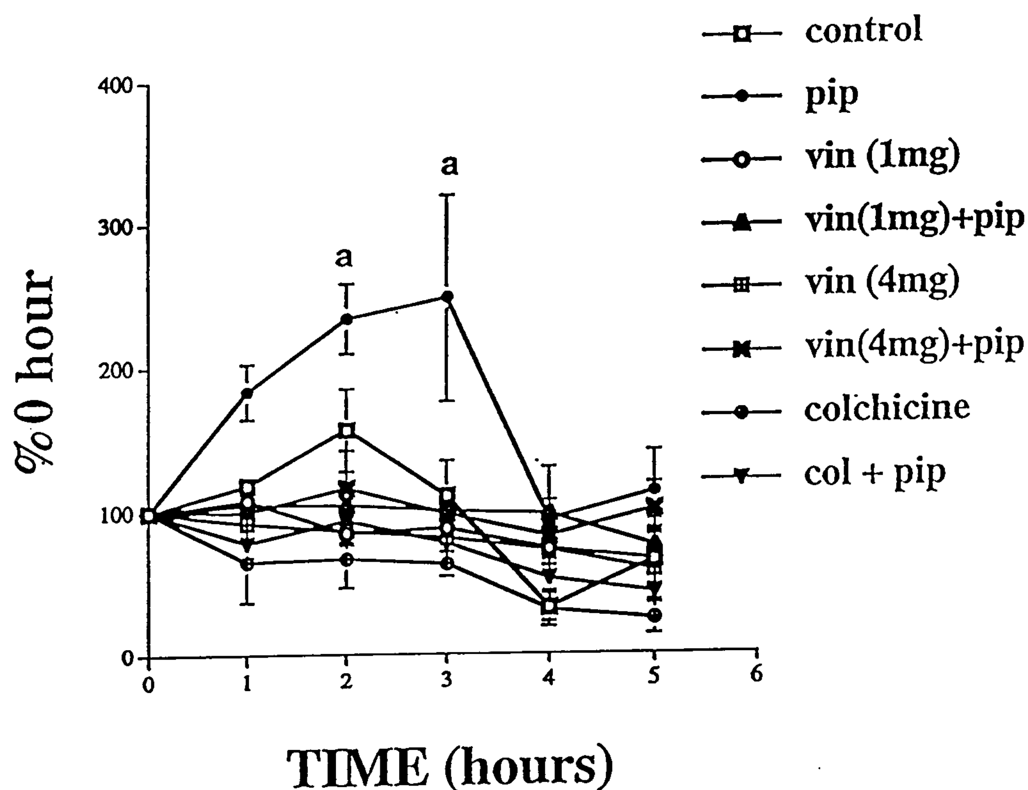


Figure II.3. Effect of colchicine and vinblastine on the PIP-induced increase in biliary output of bile acids. Conditions are as described in Figure II.1. Letter a represents points at which PIP treatment resulted in a significant increase above controls, $p < 0.05$ Bonferroni adjusted (adjusted $p < 0.05$ implies that unadjusted $p <. 0.05 / k$ where $k=3$ for PIP effects).

## Discussion

We have demonstrated that i.p. injection of PIP has a significant and rapid effect on the transport of a mobilizable pool of α-tocopherol into the bile and that this is accompanied by a significant decrease in hepatic α-tocopherol as compared to controls. We have also demonstrated that colchicine and vinblastine prevent this PIP-induced increase in biliary output, suggesting an involvement of microtubules; a dose effect was demonstrated for vinblastine. Treatment of rats with PIP (1g/kg) has previously been shown by our laboratory to cause depletion of hepatic α-tocopherol levels to 76% of controls 1 hour after treatment without a concurrent increase in plasma α-tocopherol levels (Warren *et al.*, unpublished results). In the current study, we investigated the secretion of α-tocopherol into the bile following treatment of rats with PIP and tested the hypothesis that colchicine and vinblastine would prevent the PIP-induced increase in the biliary output of α-tocopherol.

α-Tocopherol is lipid soluble and is transported in bodily fluids in association with lipids (Bjorneboe *et al.*, 1986; Traber, 1994). The major lipids found in the bile are phospholipids, cholesterol, and bile acids. Figures II.1and II.2 show that the biliary output of α-tocopherol and phospholipids, both follow a similar pattern of gradual decline in controls. The progressive depletion of biliary lipids following interruption of enterohepatic circulation by bile duct cannulation has been noted by others (Coleman and Rahman, 1992; Coleman *et al.*, 1988). The pattern seen in Figure

II.3 for the output of bile acids in controls may be due to circadian rhythms (Kuipers *et al.*, 1985). The biliary secretion of α-tocopherol has apparently not been comprehensively studied, either basally or following chemical treatment to the liver. α-Tocopherol has been shown to be secreted by the liver into the plasma in association with very low density lipids under both physiological conditions and when chemically challenged (Traber, 1994; Warren and Reed, 1991).

Our data demonstrate that treatment of rats with PIP results in a significant increase in the biliary output of α-tocopherol above controls during hour 3 (Figure II.1). This increase can be prevented by pretreatment with colchicine (2.5mg/kg body wt.) or vinblastine (13.3mg/kg body wt.), both of which have been shown to disrupt microtubules and prevent the secretion of a variety of substances into the bile including proteins and phospholipids (Dubin *et al.*, 1980; Godfrey *et al.*, 1982; Gregory, *et al.*, 1978; Lowe, *et al.*, 1984; Hayakawa *et al.*, 1989). The low dose of vinblastine, on the other hand, was not expected to be sufficient to interfere with microtubule function (Godfrey *et al.*, 1982; Gregory, *et al.*, 1978). The results presented in Figure II.1 show that not only did the low dose of vinblastine not prevent the PIP-induced increase, but it resulted in the prolongation of elevated biliary α-tocopherol output.

The effect of PIP treatment on %0 hr output of phospholipid is presented in Figure II.2. PIP treatment of rats results in a significant increase in the %0 hr biliary phospholipid output above controls over a 4 hr period. Again, as was seen with α-

tocopherol, pretreatment with colchicine or the high dose of vinblastine completely prevented the effect of PIP. The inability of the low dose of vinblastine to prevent the PIP-induced increase in both phospholipid and α-tocopherol is indicative of a dose effect for vinblastine on the biliary outputs of these two substances.

In addition to their effect on microtubules, vinblastine and colchicine are also known to interact with transporter proteins in the canalicular membrane of hepatocytes (reviewed in Higgins, 1992 and Boyer, *et al*., 1992). Metabolites of PIP have been shown to be excreted very rapidly, within 1 hour, into the bile by Fishbein and Falk, 1969. The observations presented in this study suggest an interesting question. Could the low dose of vinblastine, while not being sufficient to completely interrupt microtubule function, be interfering with the transport of PIP and its metabolites into the bile— thereby prolonging the effect of chemical treatment on the output of α-tocopherol? Possible effects of vinblastine and colchicine on the intracellular transport and/or the movement of PIP and its metabolites across the canalicular membrane have not been reported in the literature.

All three pretreatments prevented the PIP-induced increase in biliary output of bile acids, but did not significantly affect basal output of bile acids into the bile (Figure II.3). This is in agreement with both Crawford and Gollan (1988) and Dubin *et al.* (1980). They demonstrated that although colchicine and vinblastine did not affect basal transport and secretion of bile acids, both drugs were able to impair bile acid secretion into the bile following a bolus injection of bile acid in both the basal and bile

acid-depleted rat. These results, as well as ours, may indicate that bile acid output under certain non-physiological conditions occurs by a colchicine- and vinblastine-sensitive pathway.

Although increases in biliary phospholipid output are usually accompanied by increases in biliary cholesterol output, previous studies have demonstrated that it is possible to elicit differential effects on the biliary secretion of these two lipids and to dissociate cholesterol output from the biliary output of bile acids (Nervi *et al.*, 1984; Stone *et al.*, 1985). Our results indicate that this occurred during our experiments with PIP.

Similar levels of hepatic α-tocopherol depletion have been seen following treatment of rats with methyl ethyl ketone peroxide (Warren and Reed 1991), 1,2-dibromoethane (Warren *et al.*, 1991), cadmium (Schukla and Chandra, 1989) and ethanol (Bjorneboe *et al.*, 1987). Even when multiple acute doses are given, the degree of α-tocopherol depletion remains within the range of 70% of controls (Warren and Reed, 1991, Warren *et al.*, 1991, Bjorneboe *et al.*, 1987). We propose that the results presented in this study further support the following suggestions by Warren *et al.* (1991): i) that there is a discretionary pool of hepatic α-tocopherol which is sensitive to chemical treatment and ii) that this discretionary α-tocopherol pool constitutes approximately 30% of the total α-tocopherol available to the liver. We suggest that the model which we are studying may represent a common transport mechanism for the mobilization and enterohepatic distribution of this antioxidant.

Our research indicates that both α-tocopherol and phospholipids are transported into the bile following chemical treatment by a pathway which is sensitive to both colchicine and vinblastine i.e., the microtubule system. The results of this study suggest that although basal biliary output of α-tocopherol does not depend on an intact microtubule system, biliary output following chemical challenge does require a fully functional microtubule system. We further propose that hepatic α-tocopherol is a highly mobilizable source of antioxidant for possible rapid enterohepatic distribution following a toxicant-induced stress.

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# Chapter III

## Biliary Secretion of α–Tocopherol and the Role of the mdr2 P-Glycoprotein in Rats and Mice.

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## Abstract

The mechanism by which α-tocopherol is secreted into the bile is not known; however, we have previously demonstrated that treatment with piperonyl butoxide (PIP, 1g/kg) results in increases in the biliary output of both α-tocopherol and phosphatidylcholine within 3 hours of ip injection in rats and that the biliary output of both substances was prevented by chemicals that disrupt microtubules (*Toxicol. Appl. Pharmacol.* **139**, 411–417 (1996)). The P-glycoprotein encoded by the mdr2 gene has been shown to transport phosphatidylcholine into the bile; therefore, in the current study, we utilized the P-glycoprotein inhibitor verapamil to investigate the possible involvement of P-glycoproteins in the biliary secretion of α-tocopherol. When rats were iv injected with verapamil (4mg/kg) 10 minutes prior to PIP treatment, verapamil prevented the PIP-induced increases in biliary α-tocopherol and phosphatidylcholine output and resulted in biliary α-tocopherol outputs that were significantly less than controls. Also, we determined that the biliary α-tocopherol levels in mdr2 knockout mice were 25% of the biliary levels in wildtype mice; furthermore, mdr2 liver, lung, and kidney levels of α-tocopherol and glutathione differed from those of wildtype. To investigate the fate of biliary α-tocopherol, we injected [$^{14}$C]α-tocopherol into the bile duct cannulae of rats and determined that more than 60% of the radioactivity was reabsorbed within 1 hour. Our results indicate that α-tocopherol undergoes enterohepatic circulation and that the biliary secretion of

$\alpha$–tocopherol, under basal conditions and following chemical treatment, is dependent on the presence of a functioning mdr2 p-glycoprotein in rats and mice.

## Introduction

RRR-α-Tocopherol (α-T) is the most biologically active of the eight naturally occurring forms of vitamin E and is the major lipid soluble antioxidant *in vivo*. α-T prevents toxicant- and carcinogen-induced oxidative damage through its ability to trap reactive oxyradicals and is important in the protection of biologic membranes from lipid peroxidation through its chain-breaking antioxidant capabilities. All eight forms of vitamin E are absorbed from the intestine and transported, along with dietary lipids, to the liver where α-T is preferentially incorporated into very low density lipoproteins (VLDL) and secreted into the plasma; therefore it is the liver, not the intestine, which discriminates between α-T and the other forms of vitamin E for distribution of this important antioxidant to the tissues (2,3). Once it enters the plasma, VLDL is converted to LDL which exchanges α-T with HDL; HDL is the most efficient lipoprotein donor of α-T to tissues. Kostner *et al.* (4) have recently demonstrated the acceleration of the net transfer of α-T between lipoproteins and between HDL and cells by the human plasma phospholipid transfer protein (PLTP) which is known to transfer phospholipids, including phosphatidylcholine, between plasma lipoproteins.

In addition to incorporating α-T into VLDL for distribution to other tissues, the liver also functions as a major storage site for α-T; it has been estimated that 29% of the total body α-T content is located in the liver of rats (5). Work in our laboratory,

as well as that reported by others, has shown that hepatic α-T is depleted to 70–75% of controls following treatment of rats with a variety of compounds, including methyl ethyl ketone peroxide (6), cadmium (7), and ethanol (8). In all of these studies, even when multiple acute doses were given, the degree of hepatic α-T depletion remained in the range of 70–75% of controls. Warren *et al.* (9) found that plasma α-T levels were increased following depletion of hepatic α-T and these researchers have proposed that there is a discretionary pool of hepatic α-T, constituting approximately 30% of the total α-T available to the liver, that is available for distribution to other tissues and is sensitive to chemical insult. Two possible pathways for this distribution are the secretion of α-T into the plasma and secretion into the bile; the latter has received very little attention.

The liver secretes lipids and lipid soluble substances into the bile including α-T and phosphatidylcholine (PC); PC constitutes 90+% of the phospholipids found in the bile of mice, rats, and humans. Although biliary PC is known to be reabsorbed by the intestine and undergo enterohepatic circulation, the fate of biliary α-T has not been previously determined. In addition, it has been shown by several researchers that both microtubules and the hepatic multidrug resistance P-glycoprotein (Pgp), mdr2, play a crucial role in the biliary secretion of PC (10, 11, 12-14); however, the mechanism by which the liver secretes α-T into the bile, including any involvement of transport proteins, is not known. In furtherance of our investigations into the fate of hepatic α-T following chemical insult, we have recently reported that the biliary

secretion of both α-T and PC increases significantly in rats within 3 hours of treatment with the pesticide synergist piperonyl butoxide (PIP) and that this chemically induced biliary secretion of both PC and α-T can be blocked by agents that disrupt the microtubule system (1). These results were the first to indicate that the integrity of the microtubule system is important in the secretion of α-T into the bile following chemical insult. Furthermore, these results, as well as the ability of the PLTP to increase the net transfer of both phospholipid and α-T between membranes, indicate that the transport of these two substances *in vivo* may be closely related. The current study was undertaken to further elucidate (i) the mechanism by which α-T is secreted into the bile following chemical insult, (ii) the apparent *in vivo* relationship between PC and α-T transport, and (iii) the extent to which biliary α-T is reabsorbed by the body.

The multidrug resistance (mdr) gene family is a small family of isozymes consisting of two members in humans (MDR1 and MDR3) and three members in rodents (mdr1a, mdr1b, and mdr2). These genes encode for Pgps that are part of a superfamily of transport proteins which transport a variety of compounds across membranes and share common structural characteristics (15). Murine mdr2 and human MDR3 share 91% identity at the amino acid level and, unlike mdr1a and mdr1b, are found predominantly in the liver where their expression is restricted to the canalicular membranes of hepatocytes (16–18). This location supports the suggestion that the mdr2 and MDR3 Pgps are involved in hepatic secretion into the bile. Recent

investigations into a physiologic role for the mdr2 Pgp suggest that it is involved in the secretion of PC into the bile (10, 11, 19). These investigations have included in vitro studies demonstrating that both the mdr2 and MDR3 Pgps function as PC translocase/flippases that do not transport other phospholipids; this PC translocase activity is dependent on the presence of ATP and $Mg^{2+}$ and is inhibited by the Pgp modulator verapamil (11, 19). In addition, PC is not detected in the bile of knockout mice that are homozygous for a disruption in the mdr2 gene (–/–) and biliary PC levels in mice heterozygous for the mdr2 disruption (+/–) are approximately half of those of mice that are homozygous for the intact mdr2 gene (+/+) (10). The possible role of canalicular Pgps in the biliary secretion of $\alpha$-T has not been previously investigated. Since the mdr2 Pgp has been shown to transport PC, we were interested in investigating if the secretion of $\alpha$-T into the bile, both basally and following chemical treatment with PIP, would be altered by the inhibition and/or deletion of this canalicular Pgp.

In addition to decreased biliary secretion of PC, the mdr2 knockout mouse (–/–) has been shown to have decreased biliary glutathione secretion (10). Previous data from our laboratory, as well as that of other researchers, have demonstrated that disruption of the microtubule system leads to decreased biliary secretion of a variety of substances (1, 12, 13). The major protein in microtubules is tubulin which contains several cysteine residues; certain of these tubulin sulfhydryl groups must be maintained in the reduced form in order for microtubules to execute their numerous

functions, including the intracellular transport of substances to be secreted from a cell (20, 21). Glutathione, a tripeptide that is known to protect cells against oxidative damage from the normal oxidative products of cellular metabolism and detoxify the electrophilic metabolites of numerous xenobiotics, plays a major role in protecting cellular protein thiols and maintaining them in their reduced state (reviewed by Reed, 22). Decreases in reduced glutathione (GSH) and/or α-T levels have been shown to correlate with decreased levels of protein thiols and impaired microtubule function in hepatocytes following chemical insult (22–24). Therefore, since both α-T and GSH are important in maintaining the integrity of the structures involved in biliary secretion, e.g., the sulfhydryl groups of the microtubules and other cytoskeletal proteins, we determined the tissue levels of both α-T and GSH in the mdr2 knockout mouse and compared them to levels found in wildtype and mdr1a knockout mice. Mdr1a knockout mice were included in order to determine if variations in GSH and/or α-T tissue levels occur in both or only one of these two strains in which specific canalicular transport proteins have been deleted.

The purpose of the present study was four-fold: i) to determine if verapamil, an inhibitor of Pgps, affects the PIP-induced biliary output α-T and/or PC, ii) to determine if either of the Pgps found in the canalicular membranes of the liver, mdr1a or mdr2, are required for basal biliary α-T transport, iii) to determine if the absence of either of these two Pgps affects the tissue GSH and/or α-T status of mice, and 4) to determine the extent to which biliary α-T undergoes enterohepatic circulation.

# Materials and Methods

## Chemicals

α-Tocopherol, γ-tocopherol, and δ-tocopherol, as well as tocopherol-stripped corn oil, were purchased from Kodak Chemicals (Rochester,NY). Piperonyl butoxide was purchased from Aldrich. Verapamil was purchased from Sigma. [$^{14}$C]Formaldehyde was purchased from New England Nuclear. [$^{14}$C]α-tocopherol ($^{14}CH_3$- bonded to the 5 position of the chromanol moiety) was synthesized, purified by HPLC, and the radiochemical purity and radiospecific activity were determined as described by Liebler *et al.* (25). The level of radioactivity was determined to be 2.0 μCi/mg [$^{14}$C]α-tocopherol. Soluene 350 was purchased from Packard, Meridan, CT. All other chemicals used were reagent grade or better.

## Animals

Male Sprague-Dawley rats (300–325 g) were purchased from Charles Rivers with bile duct and femoral vein cannulae already in place. Male and female wildtype and mdr2 knockout mice were purchased from Jackson Lab (Bar Harbor, Maine); mdr1a knockout mice were purchased from Taconic (Germantown, NY). Mice were 10–12 weeks of age at the time of sacrifice. All animals were given the appropriate Purina rodent chow containing 60 ppm of α-tocopherol (specified by the supplier)

and water *ad libitum* (unless otherwise specified). Animals were housed in plastic cages (one per cage) with hardwood chips for bedding and kept on a 12 hr light/dark schedule.

In Vivo Treatments

All animals were fasted for 12–14 hours prior to bile collection. Since commercially available corn oil contains tocopherols, tocopherol-stripped corn oil was used as a vehicle for PIP in all experiments. In the first set of experiments, rats were anesthetized with an intraperitoneal (ip) injection of sodium pentobarbital; in addition, saline (0.9% NaCl) was infused through the femoral vein cannula at a rate of 1 ml/hr to keep the rats hydrated for the duration of the experiment. Rats were placed on a heating pad to maintain body temperature which was monitored with a rectal thermometer. Anesthesia was maintained by ip injection of sodium pentobarbital as needed. Bile was collected, as described below, for 50 minutes at which time rats were iv injected with 4mg/kg verapamil ($LD_{50}$ 16 mg/kg) or saline (0.9% NaCl). Ten minutes following verapamil injection rats were ip injected with either PIP (1g/kg) or vehicle; this initial 60 minutes of bile collection is referred to as the 0 hr of bile collection. The dose level of PIP used in these studies is 10% of the $LD_{50}$ for rats.

Bile was collected by positioning the free end of the cannula tubing above a cryogenic vial in a Dewar bowl; both of which were filled with liquid nitrogen and protected from light. Following the initial 0 hr collection period, which ended with the ip injection of either PIP (1g/kg) or vehicle, bile was collected at 1 hr intervals for an additional 5 hr period. Frozen samples, in their cryogenic vials, were immediately transferred to a large Dewar flask of liquid nitrogen at the end of each hour. Samples were either analyzed at the end of the 6 hour collection period or stored at −80°C. All samples were analyzed within 2 days of the experiment date.

At the end of the bile collection period, while still under anesthesia, the thoracic and abdominal cavities of the rats were opened and blood was obtained via heart puncture with a vacutainer tube containing EDTA. The blood was centrifuged at 4°C and the plasma was stored at −80°C. Livers were thoroughly perfused with saline (0.9% NaCl warmed to 37°C) and placed in liquid nitrogen filled cryogenic vials. The frozen tissue was then ground to a powder in liquid nitrogen and 100 mg samples were stored at −80°C until analyzed.

In the second set of experiments, mice were anesthetized with sodium pentobarbital and placed under a 60 W light bulb to maintain body temperature. The abdominal cavity was opened, the bile duct was ligated, and bile was collected from the gallbladder for up to 1 hour and stored in liquid nitrogen. Bile was extracted immediately following the collection period and assayed for phosphatidylcholine, bile acids, and α-T, as described below. The liver, lungs, and one kidney from each mouse

were removed, rinsed with saline, and placed immediately into liquid nitrogen and ground to a powder. The liver and lungs were assayed in duplicate for reduced and oxidized glutathione and α-T on the same day as the experiment; the pulverized kidney was assayed in duplicate for α-T. The remaining kidney from each mouse was removed and immediately homogenized in 10% perchloric acid containing γ-glutamylglutamate as internal standard and assayed in duplicate for reduced and oxidized glutathione by the method of Fariss and Reed (26).

In experiments to determine the reabsorption of biliary α-T, the bile duct cannula tubing of male Sprague-Dawley rats was cut and 0.50 mg [$^{14}$C]α-T (2.0 μCi/mg) was injected into the bile duct cannula. The bile duct cannula of these rats was then reconnected using a short (20 mm) piece of P-20 tubing to reestablish enterohepatic circulation. At each of the following time points, 0.5, 1, 2, 4, and 8 hours, three rats were anesthetized with sodium pentobarbital and bile was collected for 10 minutes. While still under anesthesia, the abdominal and thoracic cavities were opened, blood was collected from the heart as described above, both the intestine and the liver were removed and rinsed with saline, and fecal samples were collected. All tissues, feces, and bile samples were weighed, the tissues were minced, and all samples were solubilized with Soluene 350 as specified by the manufacturer and assayed in duplicate for radioactivity with a Packard Instrument Co., Model 1600TR, scintillation counter. Plasma was separated from blood as described above and duplicate samples of 0.4 ml were assayed for radioactivity.

Biochemical Assays

Tissue, bile, and plasma α-T content was determined by reverse-phase HPLC with a Spectra-Physics P200 pump and FL2000 fluorescence detector (excitation, 284 nm; emission, 326 nm). α-Tocopherol and lipid were extracted from samples by the method of Burton *et al.* (27) and δ-tocopherol was added as an internal standard prior to extraction. The n-heptane layer was blown to dryness with a nitrogen stream, reconstituted in methanol, and injected onto a Custom LC (Houston, TX) 5–μm ODS $C_{18}$ column with an isocratic mobile phase consisting of 80% methanol, 15% 2-propanol, and 5% water at a flow rate of 1 ml/min. The retention times for δ-tocopherol and α-tocopherol were 10.5 and 13.7 min, respectively,

Frozen ground liver and lung tissue was added to 10% PCA containing γ-glutamylglutamate as an internal standard for determination of reduced and oxidized GSH. These samples were assayed by the method of Fariss and Reed (26) using reverse-phase HPLC with a Spectra Physics P200 pump and Applied Biosystems 759A detector (wavelength, 365 nm). Bile acids and phosphatidylcholine were measured enzymatically with *in vitro* diagnostic kits from Sigma diagnostics and Wako Chemicals, respectively.

Statistical Analysis

For the purpose of analysis, biliary responses in rats for hours 1 through 5 were converted to percentage of initial value (to control for animal-to-animal variation) and a separate analysis of variance (ANOVA), followed by pairwise *t* tests comparing each treatment group with controls and with each other, was conducted at each time point with SAS for Windows version 6.11 (Figures 1 and 2). To examine PIP and verapamil effects on α-T levels in liver and plasma, data were analyzed by ANOVA followed by pairwise *t* tests comparing each treatment group with controls and with each other (Table III.1). For mouse experiments, an ANOVA (with LSD protection) was conducted followed by pairwise *t* tests comparing each of the three strains with the other two.

# Results

## Effect of Verapamil on α-Tocopherol and Phosphatidylcholine in Rats

Hepatic α-T content was determined for each rat at the end of the bile collection period and is expressed as nmol α-T/g (fresh wt) liver. There were no significant differences between treatment groups for liver weights; mean ± SE for all rats was 11.5 ± 0.83 g. Rats injected with only vehicle are referred to as controls. Treatment of rats with 1 g/kg PIP resulted in hepatic α-T levels that were significantly decreased to 74 ± 2% of controls while pretreatment with verapamil (4 mg/kg body wt) prior to PIP resulted in hepatic α-T levels that were not significantly different from controls (Table III.1). Plasma α-T concentrations were also determined at the end of the bile collection period and are expressed as nmol α-T/ml plasma (Table III.1). There were no significant differences when each of the three treatment groups were compared to control plasma concentrations.

Bile data for rats were calculated as biliary output (concentration × bile volume) per hour. Bile outputs for all treatment groups during the 0 hr of bile collection were 5.27 ± 0.91 nmol α-T/hr. Bile volumes for individual rats did not significantly change over the 6 hours of bile collection and bile volumes were not significantly different between treatment groups (data not shown). Since we were interested in examining the difference in biliary output over time for each treatment group, we treated the 0 hr

measurement for each rat as 100% and have presented the biliary output of α-T and PC as percentage 0 hr (Figs. III.1 and III.2).

TABLE III.1 α-Tocopherol Levels in the Liver and Plasma of Rats 5 hours after Treatment with Piperonyl Butoxide, both with and without Verapamil Pretreatment.

| Treatment Goup | Liver (nmol/g) | Plasma (nmol/ml) |
|---|---|---|
| Control | $30.50 \pm 2.66$ | $7.04 \pm 0.80$ |
| PIP | $22.66 \pm 1.01^{*}$ | $5.72 \pm 0.62$ |
| Verapamil | $40.10 \pm 5.29^{\dagger}$ | $9.19 \pm 0.91^{\dagger}$ |
| Verapamil + PIP | $32.50 \pm 1.79^{\dagger}$ | $9.21 \pm 1.24^{\dagger}$ |

*Note*: values are means ± 1 SE.
* Significantly different from control; $p < 0.05$.
† Significantly different from PIP treatment group; $p < 0.05$.

During the 5 hours of bile collection following injection of vehicle, the biliary output of α-T in control rats gradually declined to 63.0 ± 5.9% of their 0 hr output; however, the percentage 0 hr biliary output of rats treated with PIP increased to 58.3 ± 9.7% above the percentage 0 hr output of control rats during the third hour following PIP treatment and then returned to near control rates during the fourth and fifth hours (Fig. III.1). In Figure III.1 we show that injection of verapamil (4 mg/kg) 10 minutes prior to PIP treatment (Ver + PIP treatment group) not only prevented the PIP-induced increase in biliary output of α-T during hour 3, but resulted in biliary α-T outputs that were significantly lower than those of PIP-treated rats at hours 2, 3,

and 4 ($p < 0.05$). Furthermore, verapamil + PIP outputs were significantly decreased below control biliary α-T outputs at hours 2 and 3 ($p < 0.05$). Biliary α-T output from rats injected with verapamil (4 mg/kg), followed by vehicle, were also significantly decreased below control biliary outputs at hours 2 and 3 (Fig. III.1).

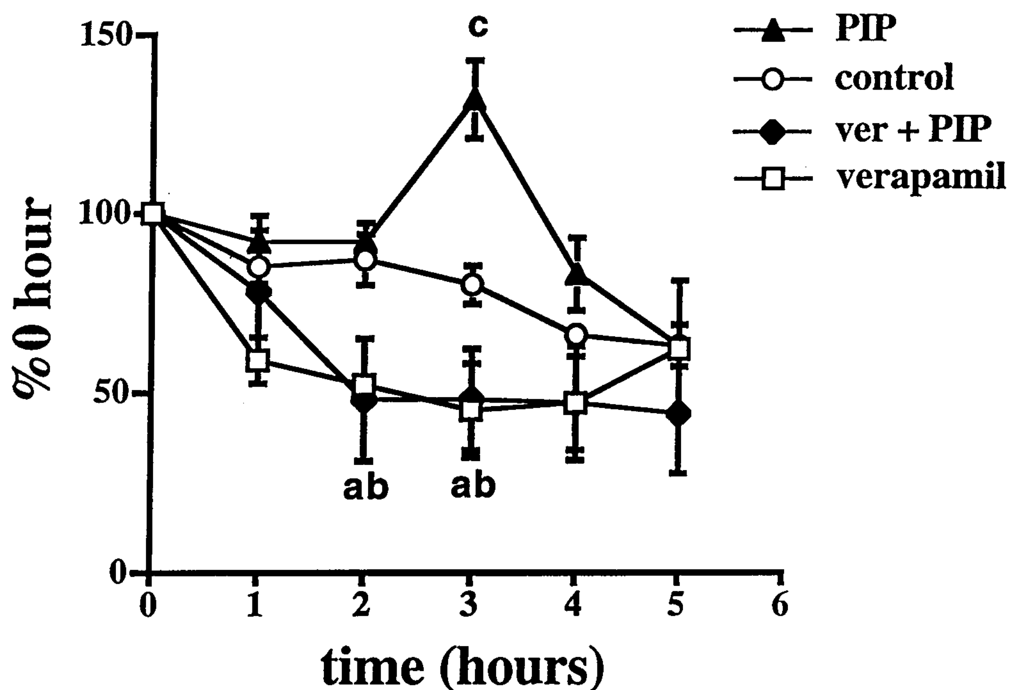


Figure III.1. Effect of verapamil pretreatment on the PIP-induced increase in biliary α-T output in rats. Verapamil was given 50 minutes after the start of bile collection and PIP was given 10 minutes later at the end of the first hour of bile collection. Bile collection continued for an additional 5 hours and data are expressed as % 0 hr α-T, as described in the methods, and are presented as mean ± 1 SE, n = 7–8 for controls and PIP only treatment, n = 3 for verapamil and verapamil + PIP values. Letters a, b, and c represent points at which treatment(s) resulted in a significant difference in biliary α-T output as compared to controls, $p < 0.05$. Letter c = treatment with PIP alone is significantly increased above controls. Letter b = treatment with verapamil + PIP is significantly decreased below controls and letter a = treatment with verapamil alone is significantly decreased below controls.

PC constitutes >90% of the biliary phospholipids in rodents and man (28). Since α-T is fat soluble and generally associated with lipids during its transport within the lymph and blood, and because the mdr2 Pgp has been shown by Smit *et al.* (10) to transport PC into the bile, we also determined if biliary output of PC was affected by the Pgp inhibitor verapamil. PIP treatment resulted in biliary PC outputs that were significantly increased as compared to control PC outputs during hours 1–4 (Fig. III.2). As was seen with biliary α-T output, pretreatment with verapamil prevented the PIP-induced increase at all time points.

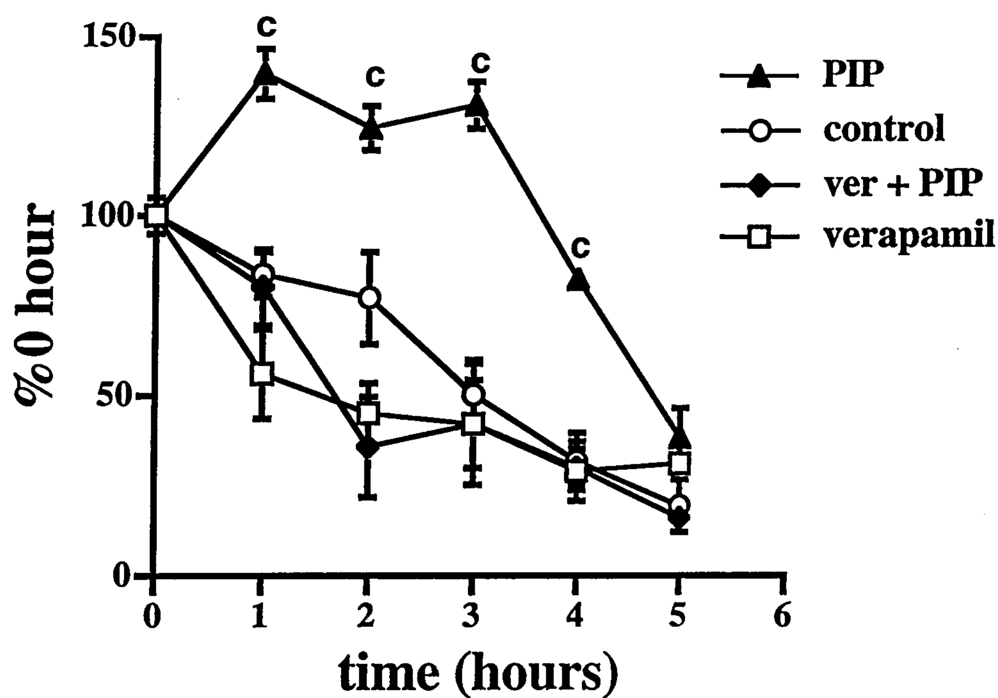


Figure III.2. Effect of verapamil pretreatment on the PIP-induced increase in biliary phosphatidylcholine (PC) output in rats. Conditions are as described in the legend to figure III.1. Data are expressed as % 0 hr PC, as described in the methods, and are presented as mean ± 1 SE, n = 7-8 for controls and PIP only treatment, n = 3 for verapamil and verapamil + PIP values. Letter c = time points at which treatment with PIP alone is significantly increased above controls.

Knockout Mice Studies

Verapamil inhibits substrate transport by both the mdr1a and mdr2 Pgps (11, 29–30); these two Pgps are the most abundant Pgps found in the hepatic canalicular membranes of rodents and man. In order to further elucidate the role of one or both of these Pgps in the secretion of α-T into the bile, we determined the biliary output of α-T, as well as α-T and GSH levels in the liver, lungs, and kidneys, in wildtype mice, mdr1a knockout mice, and mdr2 knockout mice.

α-T Levels in Wildtype and Mdr Knockout Mice

Bile was collected from mice as described in the methods and is expressed as nmols α-T/ ml bile. A striking finding of this study was that biliary α-T levels for the mdr2 knockout mice (0.315 ± 0.09 nmol α-T/ml) were only 25% of the levels found in both the wildtype and mdr1a knockout mice, 1.27 ± 0.03 and 1.22 ± 0.01 nmol α-T/ml, respectively (Fig. III.3). In addition, Figure III.3 shows that α-T levels in the kidneys, lungs, and livers of both strains of knockout mice were significantly elevated above wildtype tissue levels. In contrast, there were no significant differences between the elevated levels of α-T in tissues of mdr1a knockout mice and those of the mdr2 knockout mice; also, with respect to plasma α-T levels, there were no significant differences among the three strains (Fig. III.3).

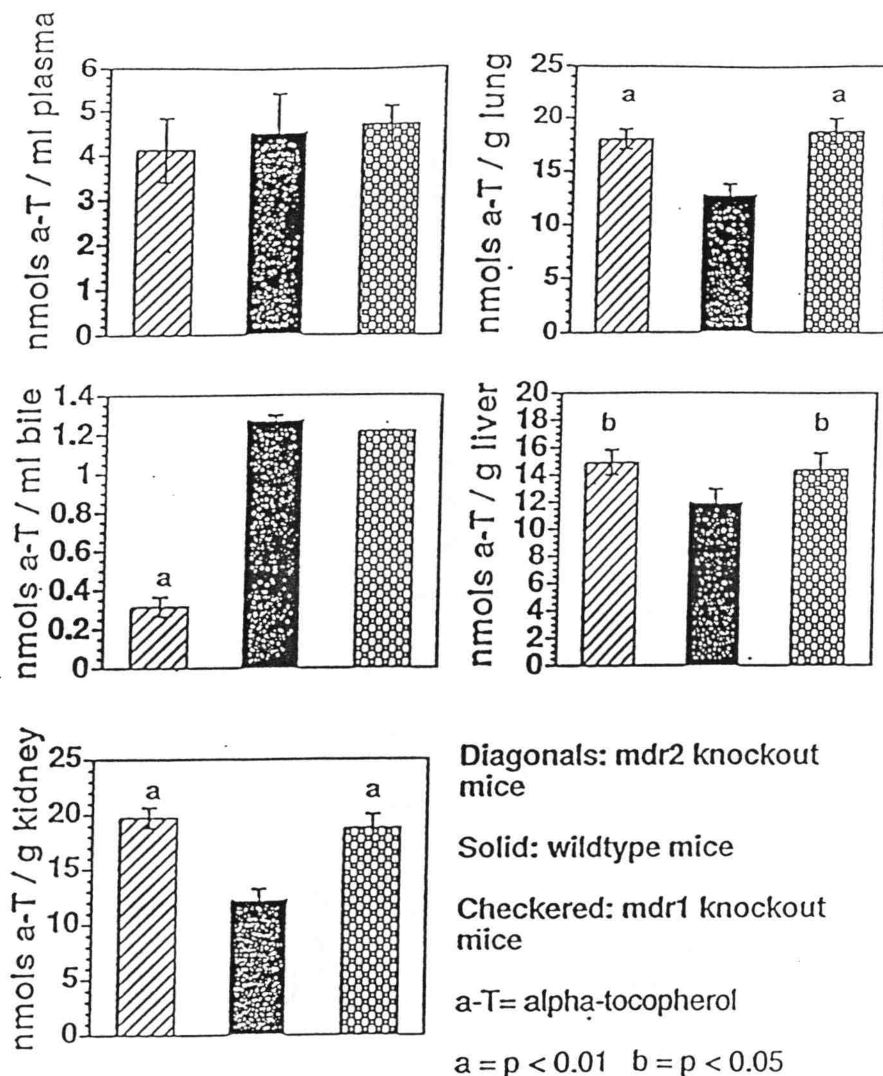


Figure III.3. Plasma, bile, kidney, lung, and liver levels of α-T in mdr2 knockout mice (hatched bars), wildtype mice (solid bars), and mdr1a knockout mice (checkered bars) 10–12 weeks of age. Samples were collected between 10 and 11 AM and either assayed the same day or stored at –80°C for analysis within 48 hr, as described in the methods. Data are expressed as nmol/g of tissue for liver, lung, and kidney, and as nmol/ml of plasma or bile. All data are presented as mean ± 1 SE, n = 6–11. The letter a represents data points that are significantly different from wildtype, $p < 0.01$ and the letter b represents data points that are significantly different from wildtype, $p < 0.05$.

Biliary Bile Acid and PC Levels in Wildtype and Mdr Knockout Mice

Bile acids and PC are two of the major lipids found in the bile and have been shown to be necessary for the optimal movement and absorption of α-T within the intestinal lumen. Since our results from the PIP/verapamil experiments demonstrated that the biliary secretion of both PC and α-T was affected by treatment with the Pgp inhibitor verapamil, we were interested in determining the biliary levels of PC and bile acids in wildtype and Pgp knockout mice. The affect of PIP on bile acid secretion in rats was determined in our previous study (1). Biliary PC and bile acid levels for the three strains of mice are expressed as millimolar and are shown in Table III.2. As was seen with biliary α-T levels, biliary PC levels were significantly decreased in mdr2 knockout mice ($0.7 \pm 0.40$ mM PC) as compared to controls ($8.7 \pm 0.74$ mM) while there was no significant difference between wildtype and mdr1a biliary PC levels. The biliary bile acid levels did not differ significantly between the three strains of mice (Table III.2).

TABLE III.2. Biliary Phosphatidylcholine (PC) and Bile Acid Levels for Wildtype, Mdr2 Knockout, and Mdr1a Knockout Mice.

| Mouse Genotype | PC (mM) | Bile Acid (mM) |
|---|---|---|
| Wildtype | 8.7 ± 0.74 | 14.2 ± 0.38 |
| mdr2 | 0.7 ± 0.40* | 12.2 ± 0.84 |
| mdr1a | 9.2 ± 0.60 | 15.4 ± 0.39 |

*Note*. Values are means ± 1 SE, n = 5–7.
* Significantly different from control; $p < 0.01$.

GSH Levels in Wildtype and Mdr Knockout Mice

Because of the possible association of antioxidant status in the various organs, reduced (GSH) and oxidized (GSSG) glutathione levels were determined in the lungs, liver, and kidneys of wildtype, mdr1a knockout, and mdr2 knockout mice at 10–12 weeks of age with total glutathione tissue levels and GSH tissue levels expressed as μmol GSH/g tissue. In Figure III.4, lung GSH levels, 1.36 ± 0.11 μmol/g tissue, were significantly decreased in mdr2 mice compared to wildtype lung GSH levels, 1.66 ± 0.09 μmol/g tissue. The total glutathione (GSH + GSSG) measured in the lungs of mdr2 knockout mice and wildtype mice did not significantly differ, but the percentage of total glutathione present as GSSG in the lungs of mdr2 knockout mice, 22.02 ± 2.94%, was significantly higher than that determined for wildtype lung tissue, 13.6 ± 1.7% (Table III.3). In contrast, both the total glutathione (2.12 ± 0.18 μmol/g tissue) and the reduced glutathione levels (1.97 ± 0.14 μmol/g tissue) in the kidneys of mdr2

mice were significantly decreased from wildtype levels (2.76 ± 0.19 and 2.65 ± 0.19, respectively), whereas the percentage of total glutathione present as GSSG in the kidneys of mdr2 knockout mice was not significantly different from wildtype levels (Fig. III.4 and Table III.3). There were no significant differences in either of the two knockout strains for any of the three glutathione parameters measured on their liver tissue as compared to wildtype livers.

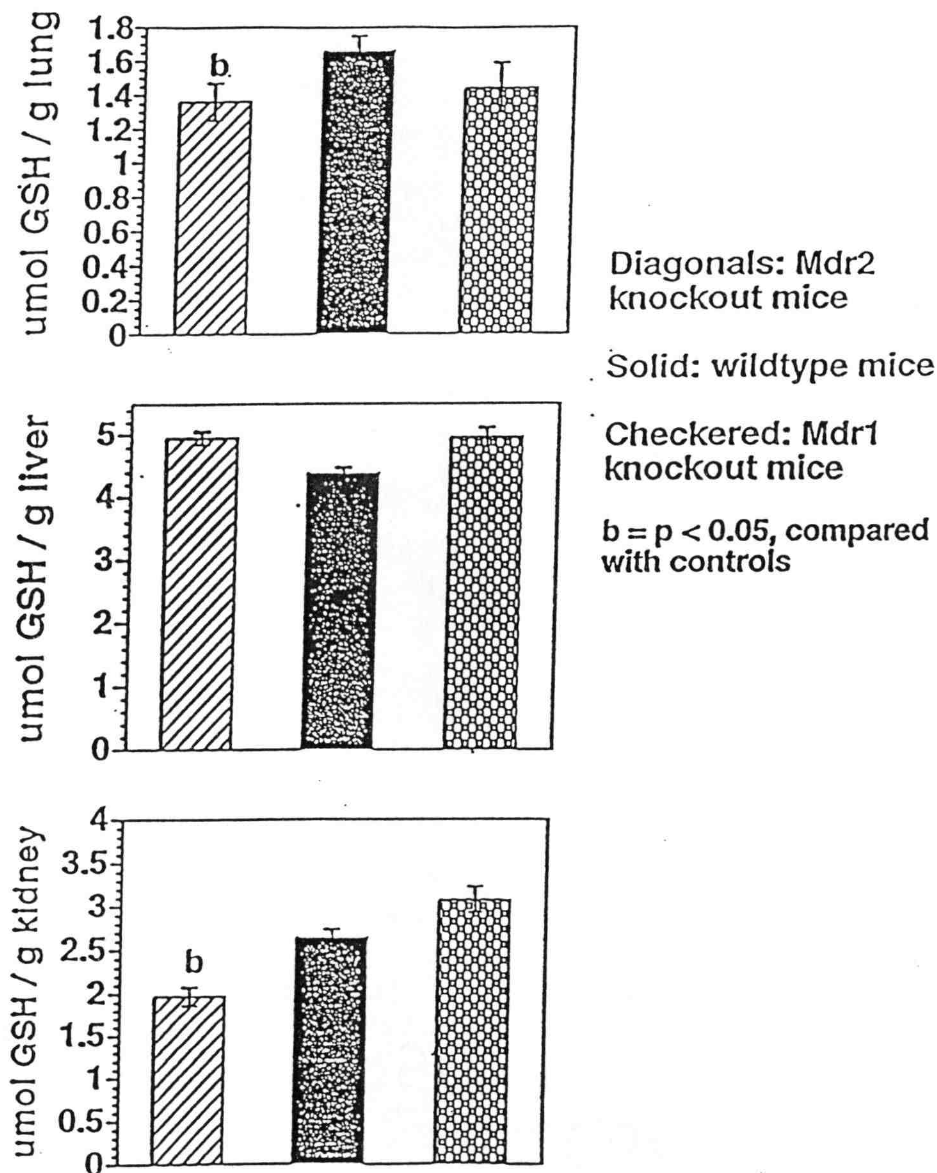


Figure III.4. Reduced glutathione (GSH) levels in the lung, liver, and kidneys of mdr2 knockout mice (hatched bars), wildtype mice (solid bars), and mdr1a knockout mice (checkered bars) and10–12 weeks of age. Conditions are as described in the legend to Figure 3. Data are expressed as μmol/g of tissue. All data are presented as means ± 1 SE, n = 6–11. The letter b represents data points that are significantly different from wildtype, $p < 0.05$.

Table III.3. Liver, Lung, and Kidney Glutathione Levels (μmol GSH/g tissue)for Wildtype, Mdr2 Knockout, and Mdr1a Knockout Mice.

| Tissue | Total GSH (GSH + GSSG) | | | % Total Glutathione present as GSSG | | |
|---|---|---|---|---|---|---|
| | wildtype | mdr2 | mdr1a | wildtype | mdr2 | mdr1a |
| liver | 4.87 ± 0.2 | 5.27 ± 0.3 | 5.19 ± 0.20 | 9.6 ± 1.6% | 11.8 ± 1.4% | 4.7 ± 1.5% |
| lung | 1.90 ± 0.2 | 1.94 ± 0.19 | 1.73 ± 0.12 | 13.6 ± 1.7% | 22.0 ± 2.9% | 16.7 ± 4.7% |
| kidney | 2.76 ± 0.2 | 2.12 ± 0.18* | 3.30 ± 0.18 | 5.4 ± 0.8% | 9.2 ± 2.1% | 8.9 ± 1.7% |

*Note*. Values are means ± 1 SE, n = 6–10.
* Significantly different from control; $p < 0.05$.

Reabsorption of biliary α-T

Since our results indicate that biliary α-T output can be modulated by both chemical exposure and the presence of the mdr2 Pgp, we investigated the fate of biliary α-T, with respect to enterohepatic circulation, by injecting $[^{14}C]$α-T into the bile of rats via bile duct cannulae. $^{14}C$ radioactivity data are presented in Table III.4 and expressed as percentage of $[^{14}C]$α-T dose present in the intestine, blood, liver, bile, and feces at time points during the first 8 hours after injection. Our results show that 26.5 ± 1.2% of the administered $^{14}C$ radioactivity was absorbed by the intestine within the first 30 minutes with peak intestinal absorption occurring 1 hour after the dose was given ( Table III.4). Plasma $^{14}C$ radioactivity reached a plateau at hour 1 with 2.06 ± 0.08% of the $^{14}C$ radioactivity present in the plasma at that time; plasma

$^{14}C$ radioactivity did not significantly differ for any of the subsequent time points when compared to hour 1 or each other. Within 1 hour of injection of [$^{14}C$]α-T into the bile duct cannulae of rats, 3.65 ± 0.55% of the $^{14}C$ radioactivity had reached the liver. Hepatic $^{14}C$ radioactivity decreased slightly at the 4 hour time point, but this decrease was not statistically significant when compared to hours 1 and 2. By 8 hours following injection of [$^{14}C$]α-T, hepatic $^{14}C$ radioactivity had significantly increased as compared to hours 1, 2, and 4 (Table III.4). Further research is needed to elucidate the mechanism of this increase, its chemical identity, and to determine its possible significance in the time course for α-T distribution to body tissues and/or the enterohepatic circulation of α-T. Recovery of $^{14}C$ radioactivity from the feces steadily increased to 7.15 ± 0.45% over the 8 hour period.

TABLE III.4. Percentage of $^{14}C$ Present in Whole Organ or Tissue Collected at Time Points Over an 8 Hour Period Following Injection of 0.50 mg [14C]α-T into the Biliary Cannulae of Rats.

| | 30 min | 1 hour | 2 hour | 4 hour | 8 |
|---|---|---|---|---|---|
| Sm. intestine | 26.5 ± 1.20 | 63.0 ± 2.00 | 20.1 ± 3.10 | 7.30 ± 0.91 | 5.37 ± 1.53 |
| plasma* | 0.56 ± 0.15 | 2.06 ± 0.08 | 2.16 ± 0.15 | 1.00 ± 0.19 | 1.46 ± 0.25 |
| liver | 1.57 ± 0.52 | 3.65 ± 0.55 | 3.15 ± 0.65 | 2.30 ± 0.32 | 5.65 ± 0.65 |
| bile‡ | 0.20 ± 0.02 | 0.15 ± 0.02 | 0.56 ± 0.10 | 0.95 ± 0.05 | 0.41 ± 0.01 |
| feces | 1.83 ± 0.17 | 1.70 ± 0.20 | 2.90 ± 0.20 | 3.70 ± 0.22 | 7.15 ± 0.45 |

*Note*. Values are means ± 1 SE, n = 3.
*Total value calculated on the assumption that the plasma volume of a rat is 3.28% of its body weight (41).
‡This value was expressed as percentage of dose per ml of bile since no estimate of total mass was made.

## Discussion

In this study, we have demonstrated that treatment of bile-duct cannulated rats with PIP (1 g/kg) results in a rapid and significant increase in the secretion of both α-T and PC into the bile and that verapamil, a Pgp inhibitor, prevents the PIP-induced biliary secretion of both of these compounds. In addition, we have shown that mice lacking a functional mdr2 Pgp have biliary α-T levels that are 25% of those in wildtype mice, whereas the biliary α-T levels of mice lacking the mdr1a Pgp do not significantly differ from wildtype levels.

α-T is transported in bodily fluids in association with lipids and has been shown to be secreted into the plasma by the liver under both physiological conditions and following chemical challenge (2, 6). As discussed in the introduction, it has been suggested that a discretionary pool of hepatic α-T which is sensitive to chemical insult is available for distribution to other tissues (9). One possible mechanism for this distribution is via an enterohepatic pathway in which hepatic α-T would be secreted into the bile, reabsorbed by the intestines, and then taken up by the blood for distribution to the body tissues. Although the hepatic biliary secretion of α-T has not been comprehensively studied, either basally or following chemical treatment, treatment of bile-duct-cannulated rats with microtubule blocking agents prior to ip injection of PIP (1 g/kg) has previously been shown by our laboratory to prevent the PIP-induced increase in biliary secretion of both α-T and PC (1). We have suggested

that these previous results indicate that a relationship exists between the biliary output of α-T and that of PC. Recently, it has been shown that the mdr2 Pgp is responsible for the presence of PC in the bile of mice (10). Therefore, in the current study, we further investigated the mechanism by which α-T is secreted into the bile by testing the hypothesis that the mdr2 Pgp plays a role in the biliary secretion of α-T under basal conditions and following chemical treatment.

Our data demonstrate that following treatment of rats with PIP the total biliary output of α-T over a 5 hour period increases by 48 ± 4% compared to controls; however, pretreatment of rats with verapamil (4 mg/kg body wt) prevents this PIP-induced increase and results in total biliary α-T outputs that are decreased to 60% of controls (Fig. III.1). Furthermore, treatment of rats with verapamil followed by vehicle also resulted in total biliary α-T outputs that were significantly decreased below controls (Fig. III.1). As was seen with α-T, PIP treatment resulted in a significant increase in the biliary output of PC that was prevented by pretreatment with verapamil (4 mg/kg body wt). Verapamil pretreatment resulted in biliary PC outputs that were equal to or less than those of controls (Fig. III.2). The ability of verapamil to block the PIP-induced increase in biliary secretion of both α-T and PC, and to decrease the biliary secretion of α-T and PC in control rats, further supports our suggestion that the *in vivo* transport of these two substances is closely related.

In Table III.1 we show that PIP treatment resulted in hepatic α-T levels that are 74 ± 2% of controls. In contrast, treatment with verapamil prior to PIP resulted in

hepatic α-T levels that were not significantly different than controls. This level of depletion (26 ± 2%) is within the range of hepatic α-T depletion noted by other researchers following chemical treatment of rats (6–9) and further supports the suggestion by Warren *et al.* (9) that a discretionary pool of hepatic α-T exists which is sensitive to chemical treatment and constitutes approximately 30% of the total α-T available to the liver. The amount of α-T secreted into the bile during the 6 hours of bile collection following PIP treatment was 37 ± 3% of that lost by the livers of PIP-treated rats.

Since we have shown that chemical treatment can significantly alter the biliary output of α-T, as may certain disease states, e.g., cholestasis and cystic fibrosis, we were interested in determining if biliary α-T is reabsorbed by the body and undergoes enterohepatic circulation. Several α-T absorption studies have studied oral administration of α-T (31, 32), but the reabsorption of biliary α-T has not been previously reported. In our reabsorption study we injected [$^{14}$C]α-T into the bile duct cannula of rats and determined the level of $^{14}$C radioactivity in the plasma, bile, and organs involved in enterohepatic circulation. The percentage dose of $^{14}$C radioactivity reported in Table III.4 for plasma and tissues, at the indicated time points, are similar to those reported by Krishnamurthy and Bieri (31); these researchers administered an oral dose of [$^{14}$C]α-T that was the same as the dose used in our study (0.50 mg [$^{14}$C]α-T). In addition, these researchers identified 99+% of the $^{14}$C radioactivity isolated in the tissues and plasma of rats, during the first 24 hours

following administration, as unchanged α-T. Since we measured $^{14}C$ radioactivity during the first 8 hours following administration of [$^{14}C$]α-T, the previous work by Krishnamurthy and Bieri indicates that the percentage dose levels reported in Table III.4 represent [$^{14}C$]α-T. The percentage dose of $^{14}C$ radioactivity reported in Table III.4 for plasma and organs of the enterohepatic circulation indicate that α-T secreted into the bile is reabsorbed by the body and, like PC, undergoes enterohepatic circulation. As stated earlier, α-T functions to protect lipids from peroxidation; therefore, in addition to being reabsorbed and distributed to tissues in the body, the presence of α-T in the bile may function to prevent the peroxidation of phospholipids which has been shown to be involved in the formation of cholesterol gallstones in the bile (33).

We propose that the biliary and hepatic results of our PIP/verapamil study, as well as the results discussed above with [$^{14}C$]α-T, further support our suggestion that the in vivo transport of PC and α-T are closely related (1). The results from several other studies also support this suggestion, including: (i) the ability of the PLTP to accelerate the transfer of both phospholipids and α-T between membranes (4), (ii) the 37% homology that the hepatic α-T binding and transfer protein (hαTTP) protein shares with the yeast SEC14 protein that is known to have phosphatidylinositol/phosphatidylcholine transfer activity (34), and (iii) our previous work demonstrating that compounds that disrupt microtubules block the transport of both PC and α-T into the bile (1). In addition, our results with verapamil indicate that

a Pgp is involved in the biliary secretion of both PC and α–T; thereby supporting and extending the work of other researchers that have shown that the mdr2 and MDR3 Pgps are involved in the biliary secretion of PC (10, 19, 35). However, verapamil is known to prevent substrate transport by both the mdr1a and mdr2 Pgps (11, 29), both of which are found in the canalicular membranes of rats (11, 35–37). Consequently, based on the results of the above experiments, it was not possible to conclusively determine which of these Pgps, if not both, was involved in the *in vivo* biliary secretion of α-T. In order to further elucidate the mechanism by which α-T is secreted into the bile, we determined the biliary α-T levels of both mdr2 and mdr1a knockout mice and compared their biliary α-T levels with those of wildtype mice.

A significant finding of this study was that the biliary α-T levels of mdr2 knockout mice were approximately 25% of both wildtype and mdr1a levels, while the biliary α-T levels in mdr1a knockout mice were not significantly different from wildtype levels. Similar to results reported by Smit *et al.* (10), we found that biliary PC levels in mdr2 knockout mice were $< 10\%$ of wildtype biliary PC levels. Our biliary PC results in mdr2 knockout mice, and our verapamil data in rats, support the work by other researchers indicating that the mdr2 Pgp functions as a PC translocase/flippase and is required for the transport of PC into the bile (10, 11, 29, 38). The affect of verapamil on the biliary output of PC and α-T, under both basal conditions and following chemical treatment, as well as the biliary α-T levels of mdr2 knockout mice, has not been previously reported. Therefore, our results are the first

to indicate that (i) the inhibition or deletion of the mdr2 Pgp leads to decreases in the transport of α-T into the bile under basal conditions and (ii) pretreatment with the Pgp inhibitor verapamil blocks the chemically induced increases in the biliary secretion of both PC and α-T following treatment with PIP.

As discussed in the introduction, both GSH and α-T are important in maintaining the protein thiol status of the cell and thus the integrity of the microtubules which contain numerous sulfhydryl groups that must be maintained in the reduced state in order for the microtubules to be fully functional (22–24). Since we have previously demonstrated that microtubules are involved in the transport of both α-T and PC (1), the hepatic glutathione and α-T levels of both the mdr1a and mdr2 knockout mice were determined and compared with wildtype levels. Antioxidant status has not been previously reported for these two knockout strains; therefore, because of the possible association of antioxidant status in the various organs, we chose to include the lungs and kidneys in our glutathione and α-T tissue determinations. These organs were chosen because the kidneys have a high rate of turnover for GSH ($t_{1/2}$= 1–5 hours), similar to that of the liver, and both the lungs and kidneys have high rates of turnover for α-T ($t_{1/2}$= 7–11 days) which are similar to the α-T turnover rate in the liver ($t_{1/2}$= 9 days) (39, 40).

The increase in both total glutathione and GSSG, along with the decreased level of GSH in the lungs of mdr2 knockout mice may be indicative of the presence of an oxidative stress in this organ. On the other hand, both total glutathione and GSH were

decreased below wildtype levels in the kidneys of mdr2 knockout mice without a concurrent increase in GSSG levels. In addition, our results indicate that both the mdr1a and mdr2 knockout strains have increased α-T levels in their livers, lungs, and kidneys as compared to wildtype mice. Additional research is needed to determine the mechanism(s) by which the absence of the mdr2 Pgp differentially affects lung and kidney glutathione status, as well as, the mechanism(s) by which the absence of either of these two Pgps results in increased α-T levels in liver, kidney, and lung tissues compared to wildtype levels.

In conclusion, the results of this study further support the suggestion by Mustacich *et al.* (1) that (i) hepatic α-T is a highly mobilizable source of antioxidant that is available for distribution to other tissues via an enterohepatic pathway and (ii) a close relationship exists between the biliary secretion of α-T and that of PC. In addition, we propose that the mdr2 Pgp has a role in this relationship. Furthermore, we suggest that the model we are studying may represent a common mechanism for the rapid mobilization and enterohepatic distribution of α-T and that alterations in the enterohepatic distribution pathway, such as is seen in certain disease states, may lead to a compromised ability to respond to conditions of oxidative stress and/or chemical toxicants.

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# Chapter IV

# Discussion

Debbie J. Mustacich

## Summary

The studies described in this thesis provide valuable data with respect to *in vivo* transport and homeostasis of $\alpha$-T during basal conditions and following chemical insult. The data presented here are the first to indicate that both microtubules and the mdr2 Pgp play a role in the biliary secretion of $\alpha$-T. During this thesis research study, four key questions were addressed: (1) Is $\alpha$-T secreted into the bile at levels significantly above those of controls following treatment of rats with PIP? (2) Is biliary $\alpha$-T reabsorbed by the intestine such that the secretion of hepatic $\alpha$-T into the bile is a possible mechanism for enterohepatic distribution of $\alpha$-T to other tissues? (3) Does a relationship exist between the biliary secretion of $\alpha$-T and that of lipids? and (4) Does the microtubule system and/or the canalicular mdr2 Pgp play a role in the biliary secretion of $\alpha$-T?

Since cholesterol and PC are two of the most abundant lipids secreted into the bile, we investigated the possibility of a relationship existing between the biliary secretion of $\alpha$-T and the biliary secretion of either or both of these two lipids following treatment with PIP. The results of the studies described in chapter II demonstrate that ip injection of bile-duct-cannulated rats with PIP (1 g/kg) results in a rapid and significant increase in the secretion of $\alpha$-T and PC, but not cholesterol, into the bile and that this increased biliary secretion is accompanied by a significant decrease in hepatic $\alpha$-T as compared to controls. A similar decrease in hepatic $\alpha$-T

levels has been observed following treatment of rats with several other drugs, i.e, MEKP and ethanol, suggesting that approximately 30% of the total α-T available to the liver is available for distribution to other tissues and is sensitive to chemical insult.

The PIP-induced increase in biliary secretion of both α-Tand PC, as well as the decrease in hepatic α-T levels, was prevented by pretreatment with either colchicine or vinblastine in a dose-dependent manner. However, following pretreatment with colchicine and vinbastine the biliary outputs of α-T and PC were not significantly different from controls indicating that basal secretion had not been affected by the inhibition of microtubule function. It is possible that the hepatic phosphatidylcholine transfer protein (PC-TP), as well as other transfer proteins, i.e., αTTP, are involved in the basal transport of PC and α-T to the canalicular membrane and that this transport pathway is not inhibited by microtubule blocking drugs.

The results presented in chapter II support the suggestion by Groen *et al.* (1995) that a vesicular pathway exists for the supply of PC to the bile under high flux conditions, while a non-vesicular pathway supplies PC under basal conditions. Furthermore, the data presented in chapter II suggests that biliary PC and α-T are supplied by similar pathways under both basal conditions and following a chemical insult. These results are the first to indicate that the biliary secretion of α-T and PC are closely related *in vivo* and that the microtubule system is involved in the transport of α-T into the bile, thus supporting parts three and four of the working hypothesis presented in the introduction.

In chapter III, the studies of the biliary secretion of α-T and PC in mdr1a and mdr2 knockout mice demonstrate that a functional mdr2 Pgp, but not a functional mdr1a Pgp, is necessary for the basal biliary secretion of both α-T and PC. In addition, treatment of rats with verapamil, an inhibitor of both mdr1a and mdr2 Pgp transport (Oude Elferink *et al.*, 1997), not only blocked the PIP-induced increase in biliary secretion of α-T and PC, but resulted in biliary outputs of both α-T and PC that were below those of controls. The results of the knockout mice studies suggest that it is the ability of verapamil to inhibit mdr2 Pgp transport, rather than transport by mdr1a, that results in the decreased biliary secretion of α-T and PC in the PIP/verapamil rat studies. Both the murine mdr2 Pgp and the human MDR3 Pgp have been shown to transport PC across membranes (Ruetz and Gros, 1994; Smit *et al.*, 1993; Smith *et al.*, 1994), but this is the first study to investigate biliary α-T secretion in relationship to the presence of a functional mdr2 Pgp.

The data presented in chapter III demonstrates that a functional mdr2 Pgp is necessary for both the basal and PIP-induced secretion of α-T and PC, thereby supporting part four of the working hypothesis. Also, the results obtained in these studies with both rats and mice further support part three of the working hypothesis by providing additional evidence that suggests a close relationship between the *in vivo* transport of α-T and that of PC.

The studies of tissue α-T and glutathione status in the *mdr*2 knockout mice indicate that a deficiency of this Pgp results in alterations in both α-T and glutathione

homeostasis in both the lungs and kidneys. As discussed in chapter III, the increased level of GSSG in the lungs of mdr2 knockout mice may be indicative of the presence of an oxidative stress in this organ. Further research is needed to determine the mechanism(s) by which the absence of the *mdr2* Pgp alters both $\alpha$-T and glutathione status in the tissues of these mice; particularly in light of a recent pilot study indicating that patients with a subtype of progressive familial intrahepatic cholestasis (PFIC) lack the human homologue of the murine mdr2 Pgp, the MDR3 Pgp (Deleuze *et al.*, 1996). Patients with this subtype of PFIC present with similar biochemical and histological pathologies as those seen in mdr2 knockout mice and die prior to adolescence; therefore, studies investigating the antioxidant homeostasis in the mdr2 knockout mice may be of assistance in developing future treatment regimes for patients with this condition.

In order to investigate part 2 of the working hypothesis, $[^{14}C]\alpha$-T was injected into the bile duct cannulae of rats and the fate of this biliary $\alpha$-T was determined and is presented in chapter III. The results of the $[^{14}C]\alpha$-T studies in chapter III demonstrate that more than 50% of the $\alpha$-T secreted into the bile is reabsorbed by the intestine and that this reabsorbed $\alpha$-T enters the circulatory system and is taken up by the liver. This is the first study to investigate the fate of biliary $\alpha$-T and determine that $\alpha$-T, as PC, undergoes enterohepatic circulation. These results indicate that $\alpha$-T secreted into the bile is not merely excreted from the body, but is available for reabsorption and distribution to tissues.

In addition to the above-mentioned pathologic condition, (PFIC), observations reported in this thesis study provide information that should be considered during research and treatment of patients involving drugs that increase the biliary secretion of PC, i.e., hypolipidaemic drugs such as fibrates. Fibrates and other peroxisome proliferators have recently been shown to induce mdr2 gene expression in conjunction with increased biliary PC secretion in mice (Chianale *et al.*, 1996; Miranda *et al.*, 1997). These drugs are used to treat patients with higher than normal concentrations of plasma lipids. Patients with these high plasma lipid levels, particularly if the lipids are in the form of low density lipoproteins, have an increased risk for atherosclerosis. α-T has been shown to inhibit the oxidation of low density lipoproteins, a process that has been proposed to be one of the initial steps in the development of atherosclerosis (reviewed in Keaney and Frei, 1994). Therefore, a sustained increase in the biliary secretion of α-T, even with 50+% reabsorption of α-T by the intestine, may result in a decrease in the plasma α-T/lipid ratio resulting in an increased risk of atherosclerosis in these patients. Hence, considering the results presented in this thesis, it may be prudent to determine if treatment with hypolipidaemic drugs adversely affects the plasma α-T/lipid ratio and/or the oxidizability of LDL.

Finally, recent investigations into the etiology of cholesterol gallstone formation indicates that peroxidation of biliary PC, as well as insufficient quantities of biliary PC, may lead to the formation of cholesterol crystals in the gallbladder (Eder *et al.*, 1996). Furthermore, a pilot study of antioxidant intake in patients with cholesterol

gallstones suggests that low daily intakes of $\alpha$-T plays a role in human gallstone disease (Worthington *et al.*, 1997). It is interesting to note that crystals were found in the gallbladders of all mdr2 knockout mice, but were not observed in either wildtype or mdr1a knockout mice (Mustacich and Reed, unpublished results). Unfortunately, the composition of these crystals has not been determined. Based on the cholesterol gallstone studies cited above and the findings presented in this thesis, it is interesting to speculate that one function of the close association between $\alpha$-T and PC during transport *in vivo* is the protection of PC from lipid peroxidation that may be a part of the etiology of a number of pathologic conditions, including atherosclerosis and cholesterol gallstone formation.

## Conclusions

The work in this thesis was based on an ongoing interest by our laboratory to determine the mechanism(s) by which hepatic vitamin E levels are decreased following chemical insult *in vivo* and the fate of this vitamin E. Numerous disease states are known to involve oxidative stress which may be exacerbated by exposure to toxic chemicals. In addition, pathologic conditions, i.e., cholestasis and cystic fibrosis, alter the ability of the body to distribute compounds by an enterohepatic pathway and may lead to a compromised ability to respond to conditions of oxidative stress and/or chemical toxicants. Therefore, the study of the mechanisms of $\alpha$-T intrahepatic transport, biliary secretion, and enterohepatic circulation, as well as the relationship of these three parameters to phospholipid transport, is very important to our understanding of the relationship between oxidative stress and the *in vivo* function of $\alpha$-T and to the development of treatment regimes for patients with relevant conditions.

The results of the studies described in Chapters II and III demonstrate that the biliary secretion of both $\alpha$-T and PC are inducible following PIP treatment and that colchicine and vinblastine prevent the PIP-induced increase in the biliary secretion of $\alpha$-T and PC, thus suggesting an involvement of the microtubules in the intrahepatic transport and/or biliary secretion of these two compounds. In addition, the results of the experiments in chapter III with verapamil, as well as the studies of the biliary

secretion of α-T and PC in mdr2 knockout mice, demonstrate that a functional mdr2 Pgp is necessary for both the basal and PIP-induced secretion of both α-T and PC. Finally, the [$^{14}$C]α-T study showed that biliary α-T, as PC, undergoes enterohepatic circulation, thereby making biliary α-T available for distribution to body tissues .

The findings presented here, along with the ability of the PLTP to bind and transfer both phospholipids and α-T and the homology of the hαTTP with phospholipid transfer proteins, suggest that the transport of α-T and PC is closely related *in vivo.* Further elucidation of the relationship between α-T and PC transport is important to our understanding of the *in vivo* function of α-T and to the development of treatment regimes for patients with relevant pathologic conditions.

## Future Studies

In an extension of the work presented here with PIP, future studies are planned to further elucidate the mechanism(s) by which biliary secretion of α-T occurs under normal and oxidative stress conditions. One aim of these studies, is to determine if increased biliary output of α-T and PC is a mechanism common to a variety of chemical insults. Included in these studies will be (i) chemicals that challenge the liver via the formation of oxyradicals and lipid peroxidation, (ii) chemicals that challenge hepatic antioxidant status without lipid peroxidation, and (iii) fibrates and other peroxisome proliferators.

In addition, a study has been designed to determine if the profile of hepatic, plasma, and biliary α-T metabolites varies quantitatively and/or qualitatively following treatment by chemicals from the three categories listed above. The specific aims of this set of studies will be to (i) identify and quantitate the α-T metabolites in the liver, plasma, and bile following a variety of chemical insults, and (ii) determine if the plasma α-T metabolite profile may serve as a biomarker for specific antioxidant reactions and/or the extent of hepatic oxidative stress that may be occurring due to pathologic conditions and/or the exposure of the patient to a chemical insult.

Furthermore, the results presented in this thesis do not distinguish between a direct or an indirect mechanism for the biliary secretion of α-T with respect to the

mdr2 Pgp. Further studies are needed to determine if this Pgp binds and transports α-T or if the increase in biliary α-T secretion seen in the experiments presented here was the result of a secondary effect due to the increased biliary secretion of PC by this Pgp. Also, additional studies are needed to determine if bile acids play a role in the biliary secretion of α-T.

A question that requires further study, but is not directly related to the work presented in this thesis is whether the *in vivo* oxidative fate of α-T, under basal and oxidative stress conditions, is affected by either GSH-dependent or ascorbate-dependent protective systems and the mechanism(s) by which these interactions occur *in vivo*.

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