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Title: Chlordecone Impaired Biliary Excretion: In Vivo and In Vitro Correlates

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The liver is a target organ for the organochlorine insecticide chlordecone (CD) and site of highest CD tissue concentrations. CD-induced hepatobiliary dysfunction is characterized by impaired biliary excretion of organic anions including imipramine polar metabolites (Mehendale, 1977), phenolphthalein glucuronide (PG) (Curtis and Mehendale, 1979) and taurocholate (Curtis and Hoyt, 1984). The focus of this research was to investigate mechanisms of impaired biliary excretion localized to the bile canaliculus. Two modes of CD action were investigated: 1) direct effects on organic anion transport at the bile canaliculus; and/or 2) general membrane perturbation, indirectly affecting anion transport proteins.

Bile canaliculi-enriched fractions (BCEF) were isolated from rat livers in order to characterize effects of CD on this domain of the plasma membranes. CD (0.08 and 0.50 μmol/mg protein) inhibited the initial rate leading to a peak Na^+-stimulated [^{3}H]L-glutamate uptake in BCEF. CD inhibition of the initial or Na^+-gradient driven

phase of [^{3}H]L-glutamate uptake suggested that CD was affecting maintenance of the Na^{+} gradient by the BCEF membrane vesicles.

In vivo PG anion excretion was inhibited as well as in vitro [^{3}H]L-glutamate transport at 24 hr following in vivo CD treatment (60 mg/kg body weight) of rats. Seventy-two hr following CD treatment, rats recovered to control PG excretion levels. PG excretory performance was regained in 72 hr pretreated rats despite an increase in liver CD concentration. Liver CD concentrations in 24 hr pretreated rats were approximately 50% (1.18 nmol CD/mg protein) of the concentrations (1.81-2.75 nmol CD/mg protein) in 72 hr pretreated rats.

At low CD concentrations, there was no evidence of general membrane perturbation in terms of immobilization of the lipid electron spin resonance probe, 16-doxyl stearate, in BCEF. Mobility of 16-doxyl stearate in BCEF was reduced at in vitro CD concentrations of 0.20 μmol/mg protein or greater. CD did reduce hepatobiliary permeability to [^{14}C]mannitol in 24 and 72 hr pretreated rats; perhaps restricting movement through membrane aqueous pores.

CHLORDECONE IMPAIRED BILIARY EXCRETION: *IN VIVO* AND *IN VITRO* CORRELATES

by

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CONTRIBUTION OF AUTHORS

Terry Miller provided assistance with the electron spin resonance techniques in terms of providing the necessary instrumentation and helping with technical design of the experiments.

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INTRODUCTION

The liver serves the important function of excreting many different high molecular weight endogenous and exogenous substances from the body (Smith, 1971). Chlordecone is one of many chlorinated hydrocarbon toxicants including tetrachlorodibenzene-p-dioxin (TCDD), polychlorinated biphenyls, mirex and 3-methylcholanthrene (Klaassen and Watkins, 1984) that are excreted via the bile. During biliary excretion, the liver is exposed to these toxicants with the possibility of resulting liver damage. Primary mechanisms of liver damage have been difficult to define. Chlordecone, for example, causes liver hypertrophy, proliferation of the endoplasmic reticulum and P-450 isoenzyme induction, increased bile secretion and reduced biliary excretion of polar imipramine metabolites and phenolphthalein glucuronide (PG) (Guzelian, 1982).

Biliary excretion of exogenously administered high molecular weight compounds has been used as a diagnostic test for hepatobiliary performance (Smith, 1971). Intrahepatic cholestasis is defined as a reduction in bile secretion or reduced excretion of bile constituents including diagnostic marker compounds occurring within the

hepatocyte. It has been demonstrated classically with estrogen treatment (Forker, 1969) and also with the chlorinated hydrocarbons, TCDD (Eaton and Klaassen, 1979), mirex, and chlordecone (Mehendale et al., 1979; Curtis and Mehendale, 1981). Intrahepatic cholestasis was defined as an endpoint of a common sequence of mechanisms. Recently, intrahepatic cholestasis has been shown to have so many different manifestations that there may be many different mechanisms involved. The purpose of this study was to define the primary mechanisms of the intrahepatic cholestasis induced by chlordecone.

LITERATURE REVIEW

Bile secretion

Solutes passing through the liver from blood to bile may either move transcellularly (absorbed through the basolateral or sinusoidal portion of the hepatocyte membrane and secreted through the canalicular or apical portion) or paracellularly (through tight junctions between adjacent hepatocytes)(Erlinger, 1982). It is generally accepted that bile formation is an osmotic filtration process and not governed by perfusion pressures (Brauer et al., 1954; Sperber, 1959). Movement of some solutes via the transcellular route involves an energy-dependent translocation system. The translocation system in most epithelial-type tissues involves Na^+,K^+-ATPase coupled to a porter protein. Criteria for such a system have been confirmed in the sinusoidal surface of the hepatocyte (Blitzer and Boyer, 1978; Boyer and Reno, 1975; Emmelot et al., 1964; Evans et al., 1976). Paracellular solute movement probably depends on osmotic gradients established by the energy dependent systems of the transcellular route (Boyer, 1980). Even though canalicular secretion has not been clearly defined, bile canalicular transport systems have been characterized as predominantly Na^+-independent but dependent on electrochemical gradients (Meier, 1984; Inoue, 1984) and

in the case of bile acids, might involve vesicular exocytosis (Lamri et al., 1988).

Markers of membrane permeability

Biliary excretion of inert molecules injected into the bloodstream has been used to delineate paracellular from transcellular pathways. [^{14}C]Sucrose and [^{32}P]orthophosphate have been used to study changes in paracellular permeability (Krell et al., 1982; Jaeschke et al., 1987). [^{14}C]Mannitol has been an indicator of transcellular permeability (Alpini et al., 1986). In the past, mannitol was used to estimate bile secretion rates and bile volume contributed by the bile canalicular domain of the plasma membrane. Results from past permeability studies have had to be reinterpreted recently, however, due to the possible contribution of other diffusion routes including transcellular, paracellular, ductular and fluid phase exocytosis (vesicular).

Increased mannitol clearance from blood to bile could indicate not only increased plasma membrane permeability but also increased paracellular diffusion. Increased permeability to sucrose would not usually reflect increased membrane permeability since it is a molecule which has a diameter of 10.4 Å that is too large to diffuse through membrane pores (Alpini et al., 1986). Under certain cholestatic conditions, however, the

paracellular junction is disrupted to permit increased diffusion of sucrose into the bile canalicular space (Layden and Boyer, 1977; Elias et al., 1980; Layden et al., 1978). Recent studies have also demonstrated that sucrose is moved by intracellular vesicles (Lake et al., 1985). There have also been findings of permeability of bile duct epithelial cells to these marker compounds (Smith and Boyer, 1982). Nonetheless, it is possible to discern qualitative differences in biliary permeability between treatments using biliary clearance of inert carbohydrate markers (Tavoloni, 1988). Special techniques have been developed to isolate and quantitate the amount that each route of diffusion contributes to biliary permeability (Alpini et al., 1986; Jaeschke et al., 1987).

Markers of plasma membrane active transport

Hepatic membrane active transport systems have been established for several exogenous markers including the anions; bromosulfophthalein (BSP) (Goresky, 1964; Scharschhmidt et al., 1975), PG (Uesugi et al., 1974), imipramine polar metabolites (Moldowan and Bellward, 1974) and tertiary (Nayak and Schanker, 1969) and quaternary (Schanker and Solomon, 1963; Hirom et al., 1984) ammonium cations. While excretion of these markers has been studied in whole animals, classification of the transport systems has required more simple systems such as perfused

livers, isolated hepatocytes or isolated plasma membranes.

Hepatocytes are polar cells and different regions of the plasma membrane (sinusoidal, lateral and canalicular) are characterized by different lipid and protein compositions (Yousef and Murray, 1978). Therefore, a compound could be transported into hepatocytes by one transport protein and transported out by another. Some compounds are transported by different transporter proteins even within a region of the plasma membrane (Arias, 1986). Secretion across the bile canaliculus is the rate limiting step in the transcellular active transport of many compounds from blood to bile (Paumgartner et al., 1975; Poupon et al., 1976). Characteristics of the canalicular transport proteins could determine how long a compound would reside within hepatocytes as well as how readily a compound would be excreted from the body via the bile.

Characterization of bile canaliculi

Research on characterization of bile canalicular transport systems has only recently been exploited due to inherent sampling difficulties. The contents of intact bile canaliculi can not be sampled directly due to their small size and lack of tubular organization and structure. Therefore, indirect methods of sampling have been employed. These methods have included isolated hepatic

plasma membrane fractions enriched in bile canaliculi.

Various isolation techniques have evolved over the past ten years in an attempt to gain higher purity of the bile canalicular domain. Isolation of this domain of the membrane has been difficult for several reasons. Two confounding factors are: 1) the apical or canalicular domain comprises only 13% of the plasma membrane (Weibel et al., 1969); and 2) the canaliculus is bordered on both sides by tight junctions (Boyer, 1980). The cohesiveness of the tight junctions makes it difficult to shear away other portions of the membrane and leave the canaliculus intact. Contamination of the isolated fraction with other membrane regions seems unavoidable.

Meier et al. (1984) have succeeded in isolating a bile canalicular enriched isolated membrane fraction that retains transport properties and their fraction is the most enriched in bile canalicular marker enzymes of any published. In working with such a pure fraction, they have been able to characterize several different bile canalicular transport systems.

Purity of isolated membrane fractions has been based on the ratio of isolated fraction to liver homogenate marker enzyme activities. Hepatocytes, like other epithelial cells, exhibit polarity of the surface membrane in terms of enzyme activity (Evans, 1980). Histochemical studies have shown several enzymes that are concentrated

at the bile canaliculus including phosphatases, such as ATPases, and aminopeptidases (Wachstein and Meisel, 1957; Essner et al., 1958; Novikoff et al., 1962; Sierakowska et al., 1963). Inoue et al (1983) have provided evidence, using antibodies bound to isolated hepatocytes, that γ-glutamyl transpeptidase is located on the luminal side of the canalicular membrane. Other enzymes that have been localized to the canaliculi are 5'-nucleotidase, oligomycin insensitive Mg^{2+}-ATPase (Curtis and Mehendale, 1979; Kraus-Friedman et al., 1982, Inoue et al., 1983), leucine aminopeptidase (Roman and Hubbard, 1983) and alkaline phosphatase (Blitzer and Boyer, 1978).

Organic anion transport at the bile canaliculus

Transport studies, which are modifications of renal and intestinal vesicle studies have been applied to liver plasma membranes within the past decade (Arias, 1986). The most widely studied transport systems in hepatic plasma membranes have been organic anion transport systems. Organic anion transport systems have been popular subjects due to interest in elucidating the mechanisms of bile secretion for the most prevalent class of endogenous compounds - the bile acids. Average intracellular bile acid concentrations are 0.1 to 0.3 mM and concentrations in bile are approximately 2 mM (Okishio and Nair, 1966). The 10:1 concentration gradient suggests

that bile acids are actively transported out of the cell against this gradient but canalicular mechanisms are still unclear. All of the canalicular anion carriers so far characterized are distinct from sinusoidal carriers and depend on either an established ion gradient or a membrane potential gradient. The transporters that have been localized to the canaliculus are: Na^+-independent, but electrogenic transporters of taurocholate and other physiologic bile acids (Meier et al., 1984), bilirubin, reduced glutathione (Inoue et al., 1983), glutathione disulfide (Akerboom et al., 1984), and a Na^+-dependent transporter of glutamate (Ballatori et al., 1986). Anion exchange mechanisms for bicarbonate which could serve to drive other anions out of the cell have also been identified (Meier et al., 1985).

Putative mechanisms for CD-induced cholestasis

Hepatobiliary dysfunction resulting from CD is characterized by reduced biliary excretion of the organic anions, PG and imipramine polar metabolites (Mehendale, 1977; Curtis and Mehendale, 1979). From these findings, it was postulated that CD may be impairing plasma membrane transporter proteins (Curtis and Mehendale, 1981). There was evidence that other integral membrane proteins, Na^+,K^+-ATPase and Mg^{2+}-ATPase were also inhibited by CD (Curtis and Mehendale, 1981; Curtis, 1988). There was

also evidence that, in the transport of compounds from blood to bile, the transport from the hepatocyte into the bile canalicular space was inhibited by CD (Mehendale, 1977). Therefore, putative mechanisms for CD liver toxicity might involve the bile canaliculus and associated transport proteins (Curtis and Mehendale, 1981).

Plasma membrane transport processes may be affected by: 1) general lipid bilayer perturbation, 2) direct inhibition of a specific transporter, 3) inhibition of or damage to an ATPase (usually creates energy for the transporter), or 4) damage to components of the cytoskeleton that are responsible for the tone of the canaliculi. The effects of CD on ATPase activity in rat liver plasma membranes have already been investigated (Curtis and Mehendale, 1981; Curtis, 1988). An oligomycin-insensitive Mg^{2+}-ATPase constitutes 75% of the canalicular membrane ATPase activity and it is inhibited by CD treatment at an IC_{50} of 2.5 μmol CD/mg protein. Mechanisms 1 and 2 were investigated during the course of this study.

CHAPTER I: Chlordecone Impairs Na^+-Stimulated [^{3}H]L-Glutamate Transport and Mobility of 16-Doxyl Stearate in Rat Bile Canalicular Enriched Vesicles.

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Abstract

The effect of *in vitro* chlordecone (CD) treatment on hepatic plasma membranes was investigated for possible mechanisms of *in vivo* liver dysfunction. Liver plasma membranes (BCEF) enriched 14- to 19- fold in the bile canalicular markers γ-glutamyl transpeptidase, alkaline phosphatase, and leucine aminopeptidase were isolated from male Sprague-Dawley rats. CD inhibited the bile canalicular-specific active transport of Na+-stimulated [^{3}H]L-glutamate in BCEF vesicles. CD (0.08 and 0.50 μmol/mg protein) reduced both the initial velocity and maximum level of Na+-stimulated [^{3}H]L-glutamate uptake without significantly reducing nonspecific uptake. Nonspecific uptake was [^{3}H]L-glutamate uptake in the absence of an extravesicular to intravesicular Na+ gradient.

In vitro treatment of BCEF with CD (0.2 - 1.0 μmol/mg protein) also reduced the mobility of a 16-doxyl stearate spin label probe in a concentration-dependent manner. No change in mobility was apparent at CD concentrations below 0.2 μmol/mg protein.

These results demonstrated that CD impaired 1) a bile canalicular-specific anion transport system and 2) induced liver plasma membrane perturbation. Na^+-stimulated [^{3}H]L-glutamate uptake was more sensitive to CD than was mobility of the spin label probe.

Introduction

Chlordecone (CD), and other organochlorine toxicants including polychlorinated biphenyls, 2,3,7,8-tetrachlorodibenzo-p-dioxin, and carbon tetrachloride, impair biliary excretion (Klaassen and Watkins, 1984), although probably by different mechanisms. Polychlorinated aromatic compounds and CCl_4 cause hepatocellular necrosis (Poland and Knutson, 1986; Slater, 1982) but CD does not (Guzelian, 1982; Curtis and Hoyt, 1984).

Biliary clearance of marker compounds such as actively transported dyes has been used to assess *in vivo* hepatobiliary performance (Klaassen and Watkins, 1984; Smith, 1971). In rats, CD treatment impaired biliary excretion of organic anions (polar metabolites of imipramine and phenolphthalein glucuronide) despite increased bile flow (Curtis and Menhendale, 1979). The hypothesis that CD interacts with bile canalicular-specific transport processes to impair biliary excretion was suggested by Mehendale (1977) and can be supported by several findings. First, CD decreased biliary excretion of polar metabolites of imipramine from perfused livers without affecting rate of uptake from the perfusate (Menhendale, 1977). Second, in isolated hepatocytes, CD decreased the efflux rate of actively transported ouabain without affecting initial uptake velocity (Eaton and

Klaassen, 1979). Finally, CD inhibited bile canalicular oligomycin-insensitive Mg^{2+}-ATPase with an IC_{50} of 2.5 μmol/mg membrane protein (Curtis, 1988). Oligomycin-insensitive Mg^{2+}-ATPase accounts for at least 70% of total ATPase activity in bile canaliculi and has been suspected of facilitating transport (Curtis, 1988).

From these findings it was not possible to determine if CD interacted with canalicular membrane transport proteins directly or if CD induced nonspecific membrane perturbation, indirectly impairing transport. Several studies have investigated the correlation between nonspecific membrane perturbations and direct inhibition of either transport systems or integral membrane enzymes. Mills et al. (1987) have directly correlated decreased membrane fluidity with increased taurocholate uptake in canalicular vesicles treated *in vitro* with calcium or ethanol. Effects on membrane fluidity directly correlated with Na^+,K^+-ATPase activity but not Mg^{2+}-ATPase in canalicular vesicles from rats treated *in vivo* with several agents which induce hepatobiliary dysfunction (Keeffe et al., 1979). Chlorpromazine decreased bile flow concomitant with reduced Mg^{2+}-ATPase activity and shifts in the protein profile of plasma membranes (Tavoloni et al., 1979).

Sodium-stimulated [^{3}H]L-glutamate transport is located specifically in the bile canalicular domain of the hepatic

plasma membrane (Ballatori et al., 1986a). In vivo, glutamate is recycled by reabsorption into the hepatocyte from canalicular bile (Ballatori et al., 1986b; Ballatori et al., 1988). In the present study, [^{3}H]L-glutamate uptake into isolated plasma membrane vesicles was used to measure the effect of CD on a transport system specific to the bile canaliculus. Nonspecific membrane perturbation was inferred from effects on the mobility of a fatty acid spin label probe.

Methods

Animals

Male Sprague-Dawley rats (Simonsen Breeding Labs, Gilroy, CA) weighing 160-200 g were maintained on Purina rat chow and water ad libitum.

Membrane isolation

Bile canaliculi enriched fractions (BCEF) were isolated according to modifications by Curtis and Mehendale (1981) of a method by Song et al. (1969). Rats were asphyxiated in a CO_2 chamber and livers were excised, weighed, and minced in ice cold 1.15 M KCl. The minced livers were homogenized in 10 mM $NaHCO_3$ and centrifuged at 3500 x g and then at 3200 x g, to yield a mixed-membrane fragment pellet. The pellet was re-suspended and layered on top of a discontinuous sucrose gradient and centrifuged in a Sorvall SV-288 vertical head rotor at 35,000 x g for 1 hour. The layer that had migrated between densities 1.16 and 1.18 was collected with a pipette and washed twice with 25 mls of 10 mM $NaHCO_3$ to yield BCEF.

Membrane vesiculation and treatment

The BCEF pellet from each liver was re-suspended and brought to 9 ml with a buffer which contained 250 mM sucrose, 0.2 mM $CaCl_2$, 50 mM Hepes-Tris (pH 7.5) and 100 mM KCl. For CD-treated membranes, 20-25 μl of 20, 50 or

100 mM CD (Chem Services Inc, Westchester, Pa) (99+% pure, verified by GC-MS, Mass Spectrometry Core Unit, Environmental Health Sciences Center, Oregon State University) in ethanol was added to the BCEF resuspension (0.7 mg protein/ml) while vortexing. BCEF, sucrose buffer and ethanol vehicle or CD in ethanol were vigorously homogenized by 20 strokes in a tight-fitting ground glass homogenizer. The BCEF were treated with an amount of CD 3-10% of the IC_{50} (2.5 μmol/ mg protein) for oligomycin-insensitive Mg^{2+}-ATPase (Curtis, 1988).

The BCEF membranes were pelleted at 27,000 x g for 20 min and resuspended in sucrose buffer (5 mg protein/ml) by forcing through a 22 g needle. Freshly isolated membranes were used in the transport studies. Membranes used in the eletron spin resonance (ESR) studies were stored at -20°C under argon for no more than one week.

Determination of bile canalicular enrichment

Enrichment of the BCEF was assessed by marker enzyme activities. Enrichment was expressed as the ratio of γ-glutamyl transpeptidase (γ-GT), 5'-nucleotidase (5'-N), alkaline phosphatase (AP), and leucine aminopeptidase (LAP) activities of the BCEF to those of crude liver homogenate. Contamination of BCEF by microsomes and golgi was assayed as enzyme enrichment of glucose-6-phosphatase (E.C. 3.1.3.9) (Schwartz and Bodansky, 1961) and

contamination by mitochondria as enrichment of succinate dehydrogenase (EC 1.3.99.1) (Pennington, 1961; Shepherd and Hubscher, 1969). γ-GT (EC 2.3.2.2) activity was determined by a continuous assay from Boehringer Mannheim Diagnostics based on a method by Szasz and Persijn (1974) for glutamate transfer from L-γ-glutamyl-3-carboxy-4-nitroanilide to glycylglycine. 5'-Nucleotidase (EC 3.1.3.5) activity was assayed as total phosphate release minus nonspecific phosphatase according to a Sigma Chemical modification of the method of Dixon and Pardon (1954) and Fiske and Subbarow (1925). AP (EC 3.1.3.1) was assayed by a continuous method by Keefe et al. (1979) and LAP (E.C. 3.4.1.1) activity by a discontinuous method by Goldbarg and Rutenberg (1958). Protein was determined according to the dye-binding method (Bradford, 1958). The enzyme activities of freshly isolated membranes were measured.

Determination of membrane associated CD

Isolated BCEF from each liver were divided in half and treated with CD according to the protocol above with 0.08 μmol CD/mg protein or 0.50 μmol CD/mg protein. Each CD concentration included 0.5 μCi [^{14}C]CD (Sigma Chemical, 5.8 mCi/mmol). The final membrane pellet was re-suspended in 400 μl sucrose buffer, layered on top of 500 μl 41% sucrose (d=1.18) and centrifuged in a microcentrifuge at

15,000 x g for 5 min. The top layer, which contained membranes and membrane-incorporated CD, was separated from the bottom layer and tube surface. The two fractions were suspended in ACS (Amersham) and counted in a Packard-TriCarb liquid scintillation counter.

Glutamate uptake

Transport of [^{3}H]L-glutamate (Dupont, NEN, 69.7 Ci/mmol) into membrane vesicles was measured according to a rapid filtration method by Ballatori et al. (1986a). Briefly, 80 μl of 25°C 1.5 μM [^{3}H]L-glutamate incubation buffer was added to 17-20 μl prewarmed (25°C for approximately 3 min) BCEF vesicle suspension (100 μg protein) and incubated at 25^{o}C. Glutamate incubation buffer contained either 100 mM KCl, to maintain an equal intravesicular and extravesicular K^+ concentration, or 100 mM NaCl to create an extravesicular to intravesicular Na^+ gradient. Uptake was stopped by the addition of 3 ml ice cold stop buffer and rapid filtration through a 0.45 μm Millipore filter (HAWP) on a Hoefer single place filter holder. To ensure consistent results, special attention had to be given to precision pipetting of the viscous sucrose-membrane solutions, rapid dilution with ice cold stop buffer, reproducible and rapid filtration (approximately 1 ml/1.5 sec) and uniform wash times. The filters were dissolved in 10 ml ACS and counted in a

liquid scintillation counter. Dependence of [^{3}H]L-glutamate uptake on medium osmolarity was assayed by incubating BCEF vesicles for 10 min in incubation buffer containing increasing concentrations of sucrose (0.55, 0.75, 0.90, 1.05 osm).

Electron spin resonance (ESR)

16-Doxyl stearate (Syva Corp., Palo Alto, California) in 0.8 μl ethanol [10.5 mM] was added to 0.4 ml of BCEF suspension while vortexing. The molar ratio of spin label probe to lipid was approximately 1:100. The membrane solution was injected into a quartz flat cell and the cell was mounted in a Varian E-4 ESR spectrometer. Mobility of the nitroxide radical was calculated as the ratio of the amplitude of the peak A formed from splitting of peak B (the first peak downfield from the center of the spectrum), to the amplitude of peak B (Butler et al., 1973; Farmer et al. 1985). Each amplitude ratio was the mean of values for at least three different membrane preparations.

Statistical methods

Differences in [^{3}H]L-glutamate uptake among times and treatments was tested by fitting a quadratic curvilinear regression equation to each time course and comparing with an F-test. The Statistical Interactive Programming System

(SIPS, Dept of Statistics, Oregon State University) software was used for the analyses. Other multiple comparisons were made by one-way analysis of variance followed by the Students Newman Keuls test. All glutamate uptake values were expressed as means $\pm$ SE and enzyme activities as means $\pm$ SD. A correlation coefficient (r) was calculated for the relationship between ESR mobility and CD concentration. Differences were significant at $P < 0.05$.

Results

Enzyme characterization of the BCEF

BCEF enzyme activities (Table I.1) demonstrated 14 to 19-fold enrichment in plasma membrane associated enzymes (5'-N) including enzymes specifically localized to the bile canaliculus (AP, γ-GT, and LAP). Membrane G-6-P or SD activity was not enriched which indicated minor contamination with microsomes and mitochondria.

Membrane associated CD

After the BCEF pellet was treated with CD, vigorously homogenized, and pelleted, approximately 50% of the CD (for all CD concentrations) and 50% of the BCEF protein was recovered. At 0.08 μmol CD/mg protein, 56 $\pm$ 11% CD was recovered and at 0.41 μmol/mg, 47 $\pm$ 12% CD was recovered. At both CD concentrations, 59 $\pm$ 5% of the BCEF protein was recovered. Therefore, the concentration of CD (μmol/mg protein) in the treated mixed membranes was approximately equal to the concentration in the final BCEF.

Glutamate uptake into BCEF membrane vesicles

BCEF vesicles demonstrated characteristics of active [^{3}H]L-glutamate (glutamate) transport. BCEF vesicles were osmotically reactive as verified by increased extravesicular osmolarity experiments. When the

intravesicular space was reduced by increasing incubation buffer osmolarity, glutamate uptake was decreased proportionally. Extrapolation of glutamate uptake to infinite extravesicular osmolarity (zero intravesicular space) indicated that only 10 to 20% of glutamate uptake could be accounted for by membrane binding.

[^{3}H]L-Glutamate uptake into BCEF vesicles demonstrated biphasic kinetics including a rapid initial phase 0-5 min, followed by a slower phase (5-15 min) (Fig. I.1A). In untreated BCEF, during the rapid initial phase in the presence of extravesicular Na^+ (Na_e) and intravesicular K^+ (K_i), maximum accumulation was 1.35 pmol [^{3}H]L-glutamate/mg protein at 5 min. When extravesicular [K^+] (K_e) was equal to intravesicular [K^+] (K_i), the initial rate (0-5 min) of [^{3}H]L-glutamate uptake was decreased. During the latter phase (5-15 min), the Na^+ gradient (Na_e/K_i) uptake time course was parallel to, or the same rate as the isoosmolar K^+ (K_e/K_i) time course. Maximum accumulation at 5 min in the presence of a Na^+ gradient (Na_e/K_i) was approximately 1.4 times [^{3}H]L-glutamate uptake in isoosmolar K^+ (K_e/K_i).

In vesicles treated with 0.08 μmol CD/ mg protein (Fig. I.1B), the rapid initial phase (Na_e/K_i) was shortened from 0-5 min to 0-3 min and the rate appeared more closely parallel to the rate of uptake in the absence of extravesicular Na^+ (K_e/K_i). With 0.50 μmol CD/ mg

protein (Fig. I.1C), the rapid initial phase disappeared and the rate of uptake (Na_e/K_i) was equal to the rate of uptake (K_e/K_i) for the entire time course. The initial rate of uptake (Na_e/K_i) in BCEF treated with 0.50 μmol CD/ mg protein was significantly decreased from control BCEF and BCEF treated with 0.08 μmol CD/ mg protein. Despite the appearance of a decreased initial rate in isoosmolar K^+ (K_e/K_i) between control BCEF and CD-treated BCEF as well, an F-test did not detect any significant difference.

Na^+-stimulated uptake was dissected from total glutamate uptake by subtracting uptake in the absence of Na^+ (K_e/K_i) from uptake in the presence of extravesicular Na^+ (Na_e/K_i) (Fig. I.2). Peak Na^+-stimulated uptake occurred at 5 min in control BCEF vesicles. Peak Na^+-stimulated uptake after the rapid initial phase appeared to occur earlier in the time course and was decreased in CD-treated BCEF. The peak disappeared with <u>in vitro</u> CD (0.50 μmol/mg protein). Testing among all times, an F-test demonstrated a significant difference between uptake in BCEF treated with 0.50 μmol CD/mg protein and controls only. One-way analysis of variance followed by the Student Newman Keuls test however, indicated 0.08 μmol CD/mg significantly reduced [^{3}H]L-glutamate uptake at 5 min. The Na^+-stimulated uptake at 5 min in membranes treated 0.50 μmol CD/mg protein was approximately 25% of the control peak uptake. Equilibrium values (after 10 min)

for Na^+-stimulated uptake were approximately 0.2 pmol [^{3}H] L-glutamate/mg protein in the control membranes and membranes treated with 0.08 μmol CD/mg protein and nearly zero pmol [^{3}H]L-glutamate/mg protein in membranes treated with 0.50 μmol CD/mg protein.

Electron spin resonance (ESR) studies

A typical ESR spectrum of 16-doxyl stearate spin label incorporated in control BCEF is shown in Figure I.3A. This spectrum is typical of the pattern for 16-doxyl stearate in a fluid lipid model membrane (Jost et al., 1973). The ESR signal was stable in the membrane for at least one hour (data not shown).

Two new components, one low field and one high field, appeared in the spectra of the spin label incorporated into membranes treated <u>in vitro</u> with CD (see vertical lines at the edges of the spectrum in Figure I.3B). Appearance of these extrema due to splitting of the outermost peaks in the spectrum, is characteristic of restricted mobility of the spin label probe (Jost et al., 1973). Immobilization of the probe was quantitated by comparing the ratio of the heights of peak A, representing the more highly immobilized spin label, to peak B, representing the less immobilized spin label. The peak height ratio varied directly with CD concentration in the membranes, however, peak splitting could not be detected

below 0.2 μmol CD/mg protein (Fig. I.4). CD had no direct effect on the spin label in buffer without membranes (data not shown).

Discussion

In vitro CD treatment of BCEF caused nonspecific membrane perturbation, affecting mobility of a lipid spin label probe and impaired bile canalicular-specific Na^+-stimulated [^{3}H]L-glutamate transport. CD also inhibited Na^+-stimulated [^{3}H]taurocholate transport in BCEF (Rochelle et al., 1986), a transport process which may have been in either basolateral or canalicular membranes.

Although the membrane fractions we isolated were, by marker enzyme analyses, not as enriched in canaliculi as another reported preparation (Meier et al., 1984), the endpoint we have analyzed, Na^+-stimulated [^{3}H]L-glutamate transport, has been specifically localized to the canalicular domain of hepatic plasma membranes (Ballatori et al, 1986a). Therefore, Na^+-stimulated [^{3}H]L-glutamate transport in our membrane vesicles was most likely a measure of canalicular transport and not transport in other plasma membrane domains.

Canalicular glutamate active transport is driven by a Na^+ gradient, oriented in an extravesicular (extracellular) to intravesicular (intracellular) direction (Ballatori et al., 1986). BCEF vesicles

demonstrated rapid glutamate uptake. The rapid initial phase was within the first five minutes of incubation (Fig. I.2) due to transient maintenance of the Na^+-gradient by the vesicles (Murer amd Kinne, 1977; Hubbell and McConnell, 1971). At five minutes, peak Na^+-stimulated active transport (Na_e/K_i-K_e/K_i) accounted for 28% of total accumulation (Na_e/K_i). The temporal shift in peak Na^+-stimulated uptake (or decrease in longevity of the rapid initial phase) in CD-treated BCEF may indicate an effect of CD on maintenance of the Na+ gradient by the vesicles. Lower absolute glutamate uptake (pmol/mg protein) in BCEF as compared to the preparations of Ballatori et al. (1986a) could be due to either the lower glutamate concentration used here (1.5 μM as compared to 2.5 μM), and/or lower canalicular enrichment.

Initial rapid glutamate uptake (0-5 min) (Na_e/Ki) in CD-treated BCEF (0.50 μmol/mg protein) was significantly reduced from the initial uptake rate in control BCEF (Fig I.1). Despite the appearance of decreased initial uptake velocity by CD in the absence of Na^+ (K_e/K_i), there was no significant difference between CD-treated or control BCEF. Consequently, lower total [^{3}H]L-glutamate accumulation and decreased uptake velocity induced by CD during the initial uptake phase was probably due to inhibition of the active Na^+-stimulated process. CD (0.50 μmol/ mg protein) also decreased equilibrium Na^+-stimulated [^{3}H]L-glutamate

uptake (5-15 min) (Fig. I.2). Reduced Na^+-stimulated [^{3}H] L-glutamate uptake during the rapid initial phase as well as at equilibrium confirms that the active Na^+ gradient driven transport was sensitive to CD over the entire time course.

Effects on the ESR spectrum of 16-doxyl stearate indicated that CD caused a nonspecific membrane perturbation. When incorporated into membranes, 16-doxyl stearate orients parallel to acyl chains in the lipid bilayer. The nitroxide free radical locates near the center of the lipid bilayer and away from surface polar head groups (Hubbell and McConnell, 1971). Thus, immobilization of the 16-doxyl stearate spin label incorporated into the lipid bilayer (Fig. I.4) would suggest that CD affects the environment of the nitroxide moiety located on the membrane interior. Even though spin label mobility decreased with increasing CD, there was no measurable change in mobility at concentrations below 0.2 μmol CD/mg protein. Therefore, at 0.08 μmol CD/ mg protein, a concentration that affected [^{3}H]L-glutamate transport, there was no detectable effect in the ESR spectra. These results suggest spin label mobility in BCEF was less sensitive to CD than Na^+-stimulated [^{3}H]L-glutamate transport or that the ESR measurements were a less sensitive technique.

Results from this study demonstrate CD inhibits the

canalicular [^{3}H]L-glutamate anion transport system at concentrations of 0.08 μmol/mg protein and also induces nonspecific membrane perturbation at concentrations of 0.2 μmol/mg protein or greater. These findings provide biochemical evidence for CD inhibition of a bile canalicular transport system and a canalicular site of action.

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Table I.1. Marker Enzyme Activities in BCEF

Plasma Membrane Enzyme[a] (μmol product/ mg protein/hr)	Homogenate	BCEF	Ratio[b]
5'N (Pi)	0.79(0.32)[c] (n=16)	14.79(4.84) (n=14)	18.86(8.03)
γ-GT (5-amino-2-nitro-benzoate)	0.11(0.03) (n=6)	1.40(0.53) (n=6)	14.00(4.60)
AP (p-nitrophenol)	0.12(0.04) (n=9)	2.22(0.53) (n=8)	18.85(7.57)
LAP (β-naphthylamine)	0.55(0.05) (n=4)	8.44(1.69) (n=4)	15.32(3.20)
Contaminant Enzymes			
G-6-P (P_i)	0.21(0.03) (n=4)	0.33(0.12) (n=4)	1.55(0.39)
SD (foramazan)	734(170) (n=5)	735(160) (n=6)	1.06(0.13)

[a] 5'N = 5' nucleotidase
γ-GT = γ-glutamyl transpeptidase
AP = alkaline phosphatase
LAP = leucine amino peptidase
G-6-P = Glucose-6 phosphatase
SD = Succinate dehydrogenase

[b] ratio = BCEF/Homogenate

[c] Results are means(SE)

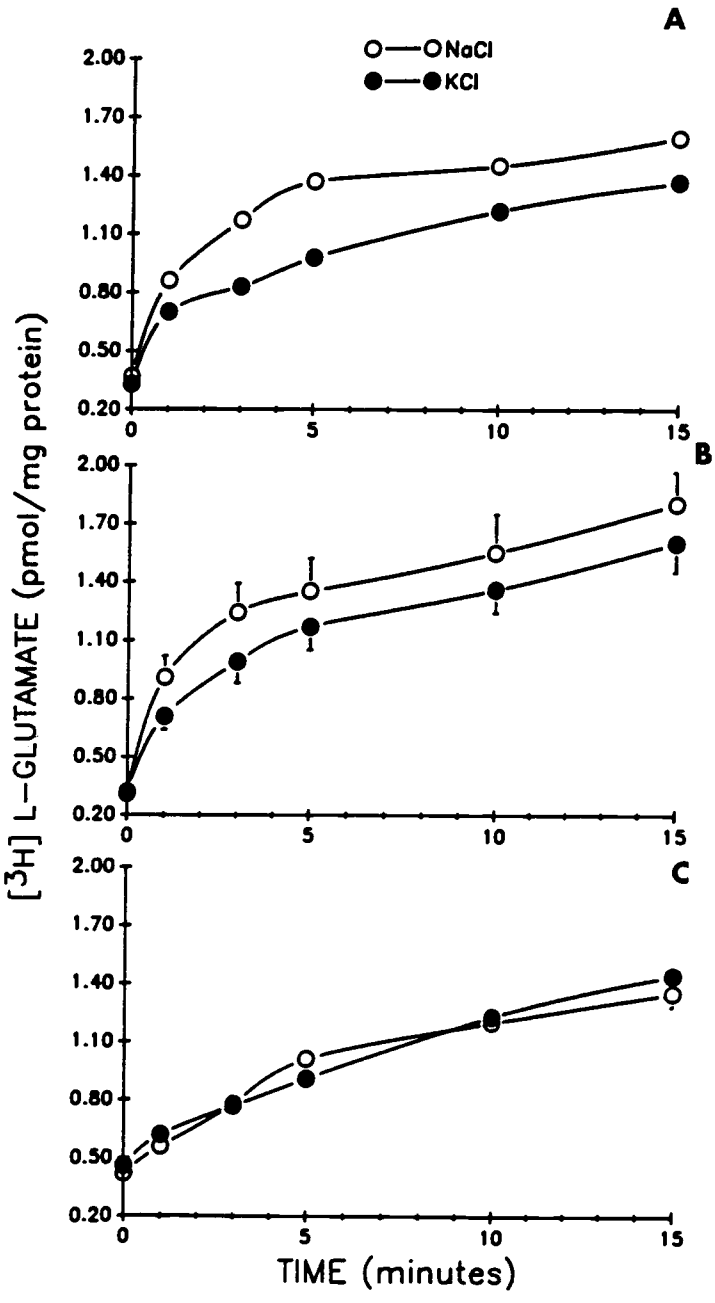

Figure I.1: [^{3}H] L-glutamate uptake into rat liver BCEF vesicles A. untreated, B. treated with 0.08 μmol CD/mg protein, C. treated with 0.50 μmol CD/mg protein. Freshly isolated BCEF were resuspended in 250 mM sucrose, 0.2 mM $CaCl_2$, 100 mM KCl and 50 mM Hepes (pH 7.5, Tris). Uptake of 1.5 μM [^{3}H]L-glutamate was measured at 22.5°C in 195 mM sucrose, 0.2 mM $CaCl_2$, 20 mM TMA-gluconate, 5 mM $MgCl_2$, 50 mM Hepes (pH 7.5, Tris) and either 100 mM NaCl O or 100 mM KCl ● [14]. Values are expressed as means ± SE for three or four experiments. The symbols include SE ≤ 0.07.

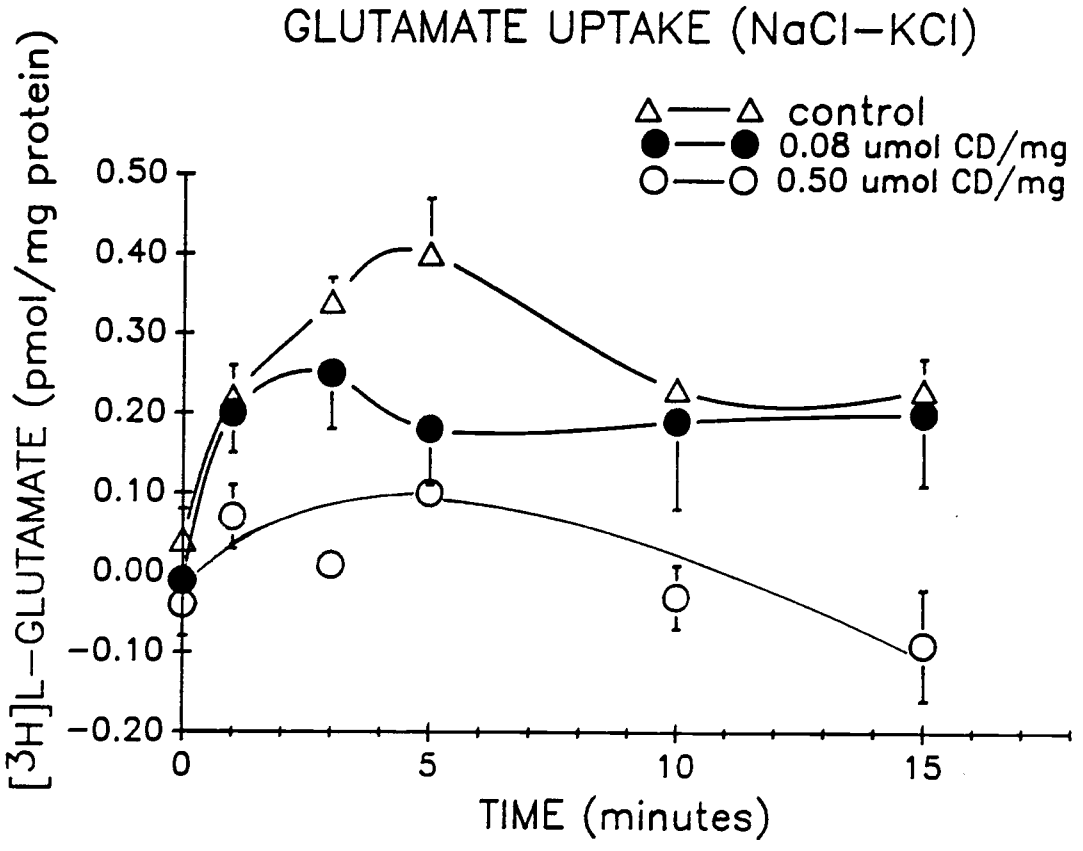

Figure I.2: Na^+-stimulated [^{3}H] L-glutamate uptake into BCEF vesicles. The values were derived by subtracting [^{3}H]L-glutamate uptake in equal osmolar K^+ (K_e/K_i) from total uptake in the presence of extravesicular Na^+ (Na_e/K_i) for BCEF treated with ethanol vehicle Δ , 0.08 μmol CD/mg protein ● , or 0.50 μmol CD/ mg protein O . Values are expressed as means ± SE.

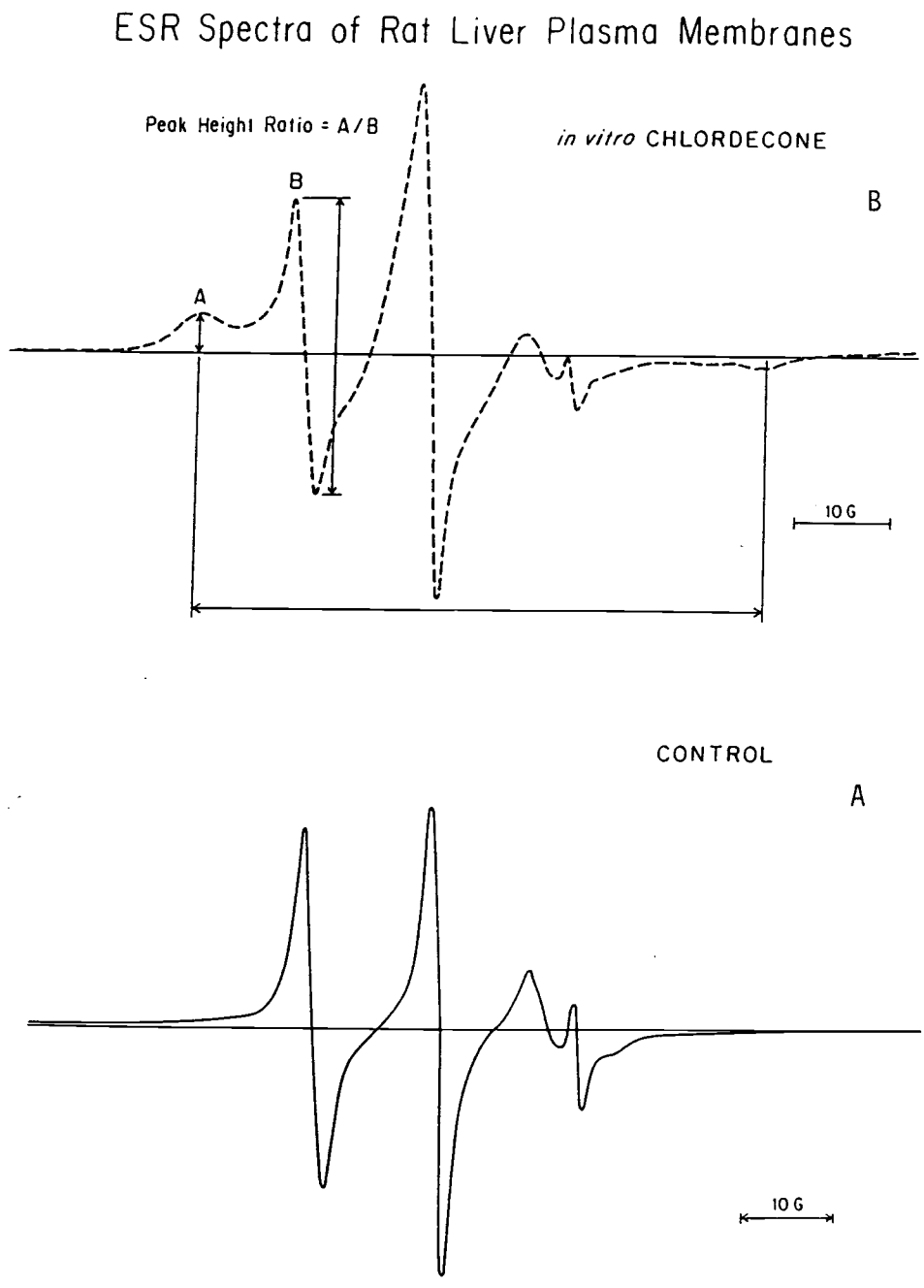

Figure I.3: ESR spectra of 16-doxyl stearic acid spin label probe in BCEF suspensions (5 mg protein/ml). A.) Spectrum of untreated BCEF, B.) spectrum of BCEF treated in vitro with CD (1.0 μmol/mg protein). Spectrometer settings were: field set 3348 G, microwave power 10 mW, modulation amplitude 0.82 G, microwave frequency 9.415 gigaHz, scan range 100 G. The filter time constant varied from 0.3 to 1 sec with scan speeds of 8 to 16 min, depending on the gain. Gain varied from 2 x 10^5 to 5 x 10^5. All spectra were recorded at room temperature.

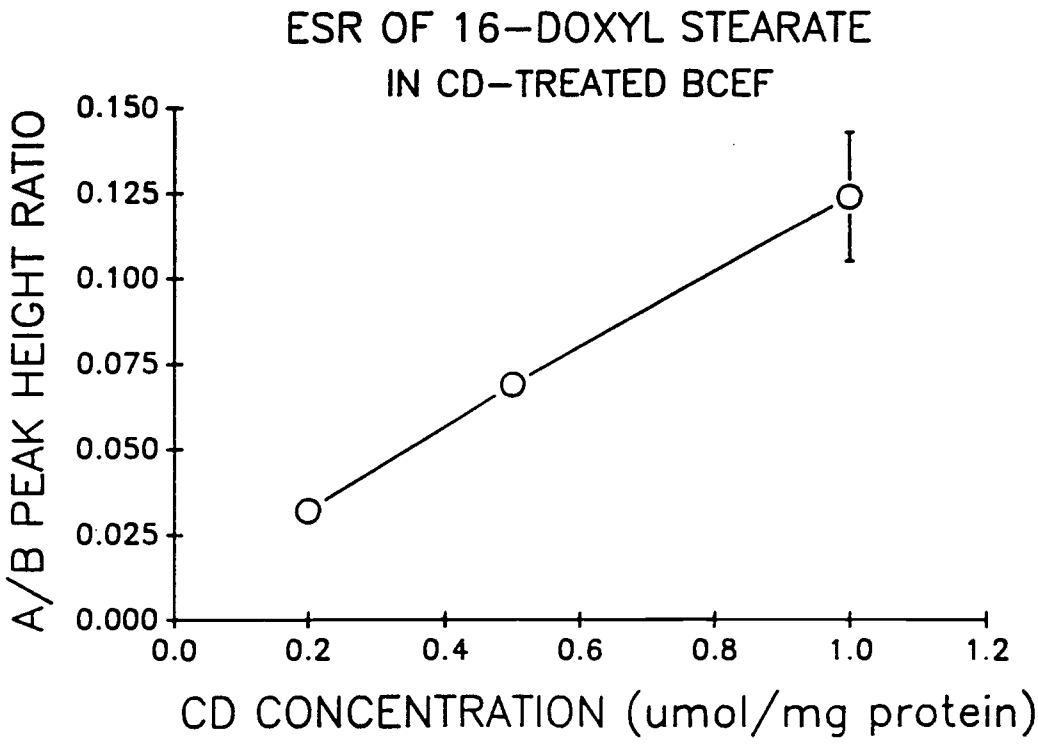

Figure I.4: CD concentration dependence of 16-doxyl stearate immobilization. There was a direct correlation between immobilization of 16-doxyl stearate (peak height ratio A/B) and increasing concentrations of CD in BCEF (r=1.0).

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CHAPTER II: Evidence for a Bile Canalicular Site of Action in Chlordecone Impaired Organic Anion Transport

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Abstract

This study evaluated the hypothesis that organic anion transport systems at the bile canaliculus are sites of action in chlordecone(CD)-induced hepatobiliary dysfunction. CD-induced hepatobiliary dysfunction is characterized by impaired excretion of organic anions (Curtis and Mehendale, 1979). Rats were gavaged (0 or 60 mg CD/kg in corn oil) and biliary excretion of phenophthalein glucuronide (PG) was determined 24 and 72 hr following CD treatment in bile-fistulated, intact preparations. Biliary excretion of PG, a marker for hepatocellular active transport, was impaired in 24 hr but not 72 hr CD pretreated rats. Biliary excretion of a 10 umol PG/kg bolus was reduced by approximately 25% in 24 hour pretreated compared to control rats. Seventy-two hr pretreated rats recovered to control PG excretion levels, despite a higher liver CD concentration than in 24 hr CD pretreated rats. Biliary clearance of [^{14}C]mannitol, a marker for plasma membrane permeability, was inhibited in both 24 and 72 hr pretreated rats.

Na^+-stimulated [^{3}H]L-glutamate transport was inhibited in bile canaliculi enriched fractions (BCEF) from 24 hr pretreated rats. Marker enzyme enrichments and electron microscopic examination of BCEF indicated that inhbition of [^{3}H]L-glutamate transport was not due to CD-induced differences in isolation of the BCEF fractions.

There was no evidence of general lipid bilayer perturbations as determined by electron spin resonance mobility of 16-doxyl stearate in BCEF. These results suggest that CD directly impairs bile canalicular active anion transport system(s).

Introduction

Liver dysfunction caused by the chlorinated pesticide, chlordecone (CD), is characterized by impaired biliary excretion of organic anions without evidence of cholestasis or hepatocellular necrosis. Excretion of actively transported organic anions such as polar imipramine metabolites, phenolphthalein glucuronide, and taurocholic acid is inhibited in bile-fistulated, intact rat preparations (Curtis and Mehendale, 1979: Curtis and Hoyt, 1984). This hepatobiliary toxicity may be due to the high concentrations of CD which persist in the liver. Hepatic concentrations 72 hr following CD exposure in rats are over 200% of other tissues except adrenal (Egle et al., 1978). Impaired biliary excretion occurs after an apparent threshold dose of 10 mg/kg body weight or a liver concentration greater than 0.052 μg/g (0.106 nmol/g).

The site of CD toxicity was proposed by Mehendale (1977) to occur somewhere in the passage of compounds from hepatocytes into the bile canalicular space. Curtis and Mehendale (1981) proposed the bile canalicular domain of the plasma membrane as a site of CD action. Transport across the bile canaliculus is the rate limiting step in the transport of many substances from blood to bile (Paumgartner et al., 1974: Poupon et al., 1976). Therefore, impedence of transport at the bile canaliculs could account for reduced biliary excretion. Rochelle et

al. (Chapt. I) have presented *in vitro* evidence for active transport at the bile canaliculus as a site of action for CD.

CD could impair biliary excretion by directly inhibiting membrane transport proteins or by affecting membrane structure, indirectly inhibiting integral membrane proteins such as enzymes and transport proteins. The Na^+-stimulated [^{3}H]L-glutamate transport system which is specifically localized to the bile canaliculus (Ballatori et al., 1986) is inhibited by CD *in vitro* (Chapt. I). *In vivo* dietary CD (162 mg total CD/kg body weight over a 15 day period) inhibits biliary excretion of the organic anion, phenolphthalein glucuronide(PG) (Curtis and Mehendale, 1981). Curtis and Hoyt (1984) presented evidence that *in vivo* dietary CD over 15 days inhibits excretion of the bile acid anion, taurocholate. In the present study, we examined the effects of peak liver concentrations of a smaller CD dose administered as a bolus. We were interested in the more temporally immediate effects of CD in order to investigate primary mechanisms. Liver concentrations peak in 2 to 3 days after a CD bolus (Egle et al, 1978).

The present study relates biochemical properties of bile canaliculi enriched fractions (BCEF) from CD pretreated rats to *in vivo* hepatobiliary dysfunction. Effects of *in vivo* CD pretreatment (60 mg/kg) on *in vitro*

[^{3}H]L-glutamate transport and *in vivo* transport of PG were investigated. Bile canalicular enzyme activities in untreated and CD pretreated rats were assayed to compare CD effects on other integral membrane proteins and/or changes in BCEF characteristics. Effects on membrane proteins were compared to general membrane effects. Biliary clearance of [^{14}C]mannitol was used as an *in vivo* indicator of general plasma membrane permeability. Mannitol passively diffuses transcellulary from the blood to the bile (Tavoloni, 1984: Alpini et al., 1986) and has been used as an indicator of biliary tree permeability (Hewitt et al., 1985: Davidson and Fujimoto, 1987). Effects of *in vivo* CD pretreatment on the biliary excretion of [^{14}C]mannitol were compared to reduced mobility of a lipid electron spin resonance probe; a measure of lipid bilayer perturbation.

In order to relate *in vitro* effects to *in vivo* effects it was necessary to estimate the amount of CD reaching the bile canaliculus. The amount of CD reaching the bile canaliculus was estimated by measuring the amount of CD remaining in the isolated BCEF and comparing this to the amount of CD in other subcellular fractions and in the whole liver.

Methods

Animals

Male Sprague Dawley rats from Simonsen Breeding Laboratories (Gilroy, CA) were maintained on Purina rat chow and water ad libitum. Rats weighing 300 to 365 grams were used for intact animal preparations and rats weighing 160 to 200 grams were used for BCEF isolation. A bolus of CD (60 mg/kg body weight) in corn oil vehicle (64 mg/ml) or corn oil alone was administered by gavage. Rats were gavaged 24 or 72 hr before surgery and 24 hr before BCEF isolation.

BCEF Isolation and Treatment

The BCEF from each excised liver was isolated on a sucrose density gradient, vesiculated and treated with in vitro CD by methods described in Chapter I.

Determination of liver and subcellular CD concentration

Rats were dosed according to their body weight with 0.15 to 0.19 ml of the CD solution (64 mg/ml) in corn oil which included 1 μCi [^{14}C]CD (Sigma Chemical, 5.8 mCi/mmol). The BCEF was isolated as previously described but duplicate 0.7 ml samples of homogenate and subsequent membrane fractions were taken after each centrifugation. The final BCEF pellet was also collected. A 15 μl subsample was taken from each 0.7 ml sample for protein

determination. The remaining sample was treated for tissue digestion (Carpenter and Curtis, 1989).

In vivo biliary clearance studies

The bile duct, femoral artery and vein were cannulated in pentobarbitol-anesthetized rats (Curtis and Hoyt, 1984). Renal pedicles were ligated to prevent renal clearance of mannitol and PG. Bile collection was initiated and a bolus of 0.2 ml (55 mCi/mmol) of [^{14}C]mannitol (0.5 μCi/ml) in normal saline was administered via the venous cannula. Fifteen min after [^{14}C]mannitol adminstration, PG (10 μmol/kg body weight) in normal saline (1 μmol/ml), was administered as a bolus via the venous cannula. Bile was collected for 1 hr after mannitol administration in 15 min samples. The total volume of bile in each sample was recorded. Two 0.3-0.4 ml of blood were collected in heparinized tubes via the arterial cannula. Blood samples were drawn at 7.5 and 30 min after [^{14}C]mannitol administration. Livers were excised and weighed after bile collection.

Biliary clearance sample treatment

Heparinized blood was centrifuged for 15 minutes at 2000 rpm to separate plasma. Plasma and bile samples (50 μl) were added to 10 ml of Scintiverse scintillation cocktail and counted in a Packard TriCarb liquid

scintillation counter to obtain [^{14}C]mannitol concentrations. Bile-to-plasma [^{14}C]mannitol concentration ratios were calculated and [^{14}C]mannitol clearance was calculated as the mean bile-to-plasma ratio multiplied by the mean bile flow for each animal.

Bile samples were also analysed for PG concentration by a spectrophotometric method (Gustafson and Benet, 1974). The PG in each 35 ul bile sample was hydrolyzed with 8 N HCl at 100°C for 1 hr. The pH was adjusted to 10.4 and absorbances were recorded at 550 nm. The percent PG dose excreted was calculated from the concentration in each sample and the volume of bile secreted during that period.

Enzyme assays

Alkaline phosphatase, γ-glutamyl transpeptidase, leucine aminopeptidase, and 5'-nucleotidase were assayed as previously described (Chapt. I).

Electron spin resonance (ESR)

The mobility of a 16-doxyl stearate lipid spin resonance probe (Syva Corp, Palo Alto, California) in BCEF was determined as described in Chapter I.

Glutamate uptake

Transport of [^{3}H]L-glutamate (Dupont, NEN, 69.7

Ci/mmol) into BCEF vesicles was measured according to a rapid filtration method of Ballatori et al (1986) as described in Chapter I. BCEF vesicles (100 μg protein) were incubated in 1.5 μM [^{3}H]L-glutamate buffer with either 100 mM KCl (to maintain an equal intravesicular to extravescicular K+ concentration) or 100 mM NaCl (to create an extravesicular to intravesicular Na+ gradient).

Statistical methods

Differences among times and treatments with respect to bile flow and PG excretion were compared with a repeated measures analysis of variance (BMDP Statistical Software, Inc., Los Angeles, California) followed by a Newman-Keuls multiple comparison test for pairwise differences among times by treatments. Differences in [^{3}H]L-glutamate uptake among times and treatments was tested by fitting a quadratic regression equation piecewise to the initial phase (0 to 5 min) and latter phase (5 to 15 min) of each time course and comparing with an F-test. The Statistical Interactive Programming System (SIPS; Dept. of Statistics, Oregon State University) was used for the analysis. Treatment differences with respect to [^{14}C]mannitol clearance were compared with one-way analysis of variance. All values were expressed as means $\pm$ SE. A correlation coefficient (r) was calculated for the relationship between ESR mobility and CD concentration. Differences

were significant at $P(f) < 0.05$.

Results

CD Concentration in BCEF Isolated from *in vivo* treated rats

CD-treated livers when compared to control had less rounded edges and a dark red color, apparently due to blood that was not easily washed from the livers.

Twenty-four and 72 hr following *in vivo* [^{14}C]CD treatment (0.118-0.145 μCi [^{14}C]/mg CD and 60 mg/kg body wt), 6.5 (1.2 nmol/mg protein) and 13 (3.0 nmol/mg protein) percent of the [^{14}C]CD dose was recovered in the liver homogenate, respectively (Table II.1). Figure II.1 follows the percent of liver CD in the membrane pellet during BCEF isolation. Amounts of [^{14}C]CD (μCi and % homogenate total) in the membrane pellet during the liver fractionation presented in Figure II.1 were from 72 hr pretreated rats. Even though liver homogenates from rats pretreated for 24 hr contained a smaller fraction of the total [^{14}C]CD dose, the percent of homogenate CD recovered at each step was approximately equal to the percent recovered in the 72 hr pretreated rats until the last homogenization-vesiculation step. After the last homogenization, the mean final [^{14}C]CD concentrations in BCEF from rats pretreated for 24 and 72 hr were approximately 2.4 to 3.0 nmol/mg protein. Consequently,

even though the percent homogenate CD recovered in the 24 and 72 hr pretreated BCEF did not appear to be different, the ratio of BCEF/whole homogenate [^{14}C]CD concentration was clearly different between the two pretreatments (2.0 in 24 hr and 1.1 in 72 hr pretreated livers).

Bile flow in cannulated rats

Bile flow (μl/min/g liver) was constant during the first 45 min of bile collection but was significantly reduced during the last 15 min. There was also no significant difference in bile flow among control, 24 hr and 72 hr pretreated rats (data not shown).

Cumulative bile flow (ml/kg body weight), during the four sampling periods, was slightly higher in 72 hr pretreated rats than in control or 24 hr pretreated rats (Fig. II.2). Statistical tests for significant differences between 72 hr pretreated and the other treatments were inappropriate due to unequal variances. Cummulative bile flow in control rats was marginally but significantly higher than in 24 hr pretreated rats in the last three 15 minute periods.

PG excretion

PG was excreted rapidly after i.v. administration and excretion rate (nmol/min/g) decreased with time. There were no significant differences in excretion rates among

treatments (data not shown). Based on analysis of variance, there were significant differences in percent PG-dose excreted over time among all treatments with percent PG dose excreted by the 24 hr CD pretreated rats (22% at 15 min to 43% at 45 min) lower than that of the control or 72 hr pretreated animals (Fig. II.3). The percent of the PG dose excreted by 72 hr pretreated and control rats was approximately equal; 30% at 15 min to 55% at 45 min.

[^{14}C]Mannitol excretion

[^{14}C]Mannitol was cleared rapidily into the bile after i.v. administration. The bile-to-plasma ratios were approximately 1.0 for control rats while those for CD pretreated rats were significantly reduced to approximately 0.8 (Fig II.4). Consequently, the [^{14}C]mannitol concentration in the bile of CD pretreated rats was lower than in the plasma. [^{14}C]Mannitol clearance, which was calculated from this ratio multiplied by the bile flow (μl/min/kg body wt), was also significantly reduced in CD pretreated rats when compared to controls (Fig. II.4).

BCEF characterization

BCEF from CD-treated livers moved to a slightly higher

level in the density gradient, indicating that they were less dense than the control BCEF. No difference in density was noted between 72 and 24 hr pretreated BCEF. CD-pretreated BCEF was more likely to form clumps and less likely to evenly homogenize than control BCEF.

In untreated rats, plasma membrane associated enzymes (5'-nucleotidase) including enzymes specifically localized to the bile canaliculus (alkaline phosphatase, γ-glutamyl transpeptidase and leucine amino peptidase) were enriched 11- to 25-fold in BCEF activity as compared to liver homogenate activity. There were no differences in enzyme activites between 24 hr pretreated rats and untreated rats (Table II.2). Enzyme activities from 72 hr pretreated rats were not studied.

Electron spin resonance

There was no evidence in the ESR spectrum of BCEF from 24 hr CD pretreated rats for an effect on 16-doxyl stearate mobility. Immobilization of the spin label increased, however, with additional <u>in vitro</u> concentrations of CD (0.4 to 1.0 μmol/mg protein) in both untreated and 24 hr pretreated BCEF (Fig. II.5). There was no significant difference between the extent of immobilization with greater CD concentration in BCEF from control and 24 hr pretreated rats.

[3H]L-Glutamate Uptake

BCEF vesicles from control and 24 hr CD pretreated rats demonstrated characteristics of active [^{3}H]L-glutamate transport. [^{3}H]L-Glutamate uptake into BCEF was characterized by biphasic kinetics including a rapid initial phase (0-5 min), followed by a slower phase (5-15 min) (Fig II.6). Sodium-stimulated [^{3}H]L-glutamate uptake has been characterized as active transport specifically localized to the bile canalicular domain of the plasma membrane (Ballatori et al., 1986). Sodium-stimulated uptake was dissected from total glutamate uptake by subtracting uptake in isoosmolar K^+, extravesicular and intravesicular (K_e/K_i), from total uptake in the presence of extravesicular Na^+ (Na_e/K_i). At all time points there was greater [^{3}H]L-glutamate accumulation in the presence of extravesicular Na^+ (Na_e/K_i) than in the presence of isoomolar K^+ (Ke/K_i). The initial rate (0-5 min) was more rapid in the presence of an extravesicular to intravesicular Na^+ gradient (Na_e/K_i) than in isoosmolar K^+ (K_e/K_i). Maximum accumulation occured in 3 to 5 min. During the late phase (5-15 min), the Na^+ gradient (Na_e/K_i) uptake rate was parallel to, or the same rate as the time course with isoomolar K^+ (K_e/K_i). Whereas [^{3}H]L-glutamate uptake in control BCEF vesicles reached a peak at 3 to 5 min and then declined, [^{3}H]L-glutamate uptake in BCEF from 24 hr pretreated rats rose more slowly to a

steady plateau (Fig. II.6).

Discussion

Most other studies of CD-induced hepatobiliary dysfunction have involved CD dietary administration for 10 to 15 days (Curtis and Mehendale,1979; Curtis and Mehendale, 1981; Curtis and Hoyt, 1984). While this regimen is useful for simulating environmental exposure, there are difficulties in quantifying and replicating doses. In the present study, we wanted to estimate absorbed doses accurately and maintain dosing consistency among test animals. In order to extrapolate CD effects in isolated BCEF to effects in cannulated rats, it was necessary to closely monitor the amount of CD administered, the amount that reached the liver and the amount incorporated in the BCEF.

In the present study, a dose of 60 mg CD/kg body wt by gavage resulted in liver concentrations of 0.085 mg/g and 0.188 mg/g in 24 and 72 hours after treatment, respectively. These concentrations were comparable to the concentrations achieved by feeding rats 100 ppm CD in the diet for 10 days (0.090 mg/g liver; Mehendale, 1981) and slightly higher than those attained by feeding 10 ppm CD for 15 days (0.052 mg/g; Curtis and Mehendale, 1981). Our CD concentrations in rat liver were also lower than those attained by feeding rats 100 ppm for 15 days (0.400 mg/g;

Curtis and Mehendale, 1981).

We found CD effects on hepatobiliary performance involved a time factor in addition to a concentration factor. PG excretion was significantly reduced 24 hr after in vivo CD and recovered to control levels 72 hr after in vivo CD. Mehendale (1981) also observed hepatobiliary system recovery with time. In his study, rats recovered to control PG excretion levels 15 days following a 35 day exposure to CD. There was a general correlation of decreased PG excretion with increased liver concentration but slight increases in CD concentration did not always lead to a proportional reduction in PG excretion. Also there was no correlation between bile flow and PG excretion.

In the present study, PG excretion returned to control levels at 72 hr despite an increase in liver CD concentration. The fact that the same concentration of CD (approximately 2.4 nmol/mg protein) remained in BCEF from both 24 and 72 hr pretreated rats suggested that liver distribution of CD into BCEF was changing with time. This finding is consistent with the hypothesis of Carpenter and Curtis (1989) that the liver compensates 3 days after CD pretreatment by adjusting CD processing and transport. Compensation by the liver could involve changes in membrane composition similar to those resulting from treatment with ethanol and other anesthetics (Roth, 1979;

Pang et al., 1980). The compositional change could include membrane proteins that directly affect anion transport, or lipids that indirectly affect anion transport proteins.

Desaiah et al. (1977) and Curtis and Mehendale (1981) presented evidence that CD is lost to supernatant fractions during BCEF isolation. Curtis and Mehendale (1981) hypothesized that it was extracted from the membranes into the resuspension buffers due to its aqueous solubility (0.4% at 100°C) and low lipophilicity relative to other organochlorine compounds (oil/water partition coefficient=40). In the present study, approximately equal amounts of CD and protein were lost into the supernatant with each pellet resuspension. The only exception was in 24 hr pretreated BCEF during the last homogenization-vesiculation step when about twice as much CD/protein was recovered in the BCEF (0.64% homogenate CD/0.31% homogenate protein). These results suggest that 1) CD was closely associated with liver protein and 2) CD may have been associated with a different protein fraction in the 24 hr pretreated BCEF than in the 72 hr pretreated BCEF.

Our time course data for disposition of [^{14}C]CD (Table II.1) and hepatobiliary dysfunction (Fig. II.3) do not suggest that gene expression is involved in CD toxicity at the plasma membrane organic anion transport

site. Biliary excretion of organic anions was impaired 24 hr after CD treatment and at lower liver concentrations than were achieved at 72 hr. Conversely, secondary events linked to receptor signals would be expected to be more highly expressed with time after exposure. These findings differ from the time course associated with the toxic mechanisms of the prototype organochlorine TCDD which are receptor-mediated responses. Our data are consistent with a more direct action of CD at the bile canaliculus.

Other evidence for a direct effect of CD was found from the effects of CD on the transcellular movement of [^{14}C]mannitol. Biliary clearance and bile-to-plasma ratios of [^{14}C]mannitol were significantly reduced from controls in 24 hr and 72 hr CD pretreated rats. This is similar to other findings which proposed reduced biliary permeability with CD treatment (50 mg/kg and 10 mg/kg/day for four days) based upon retrograde administered mannitol (Davidson and Fujimoto, 1987; Hewitt et al., 1986).

In this study, reduced excretion of PG served as a sensitive indicator of CD-induced hepatobiliary dysfunction (Curtis and Mehendale, 1979; Curtis and Mehendale, 1981) and was also the basis for our dosing and testing schedule for the biochemical endpoints. Since there was a significant effect on PG excretion 24 hr following 60 mg CD/kg, we used this regimen to analyse the *in vitro* endpoints.

In vivo 24 hr CD pretreatment (60 mg/kg) reduced peak Na^+-stimulated [^{3}H]L-glutamate uptake into BCEF vesicles although the effect was not statistically significant. The rapid intial phase (0-5 min) led to a peak of Na+-stimulated [^{3}H]L-glutamate uptake in control BCEF that was characteristic of ion gradient driven active transport (Murer and Hopfer, 1974). This initial phase was due to transient maintenance of the extravesicular to intravesicular Na^+-gradient by the BCEF vesicles. In BCEF from 24 hr pretreated rats, the peak was replaced by a slow approach to a plateau. This time course indicates that CD may have affected BCEF maintenance of the Na^+ gradient across the BCEF vesicle. In our previous work, *in vitro* CD (0.08 and 0.50 μmol CD/mg BCEF protein) reduced initial rate as well as the peak of Na^+-stimulated uptake. CD concentrations (2.4 - 3.1 nmol/mg BCEF protein) in the present study were approximately 3% of the lowest concentration used in our *in vitro* study.

Changes in ESR mobility of 16-doxyl stearate in BCEF was not as sensitive to CD as [^{3}H]L-glutamate uptake. We found that CD concentrations greater than 0.20 μmol/mg protein were required to alter the ESR spectra (Chapt. I). Therefore, it was not surprising that BCEF from 24 hr pretreated rats yielded no detectable effects on mobility of the 16-doxyl stearate spin label probe at CD concentrations below 0.20 μmol/mg protein. Adding *in*

vitro CD to BCEF from CD pretreated rats or to BCEF from control rats immobilized 16-doxyl stearate to the same degree, suggesting that there were no adjustments in plasma membrane fluidity in 24 hr pretreated rats.

From these results, we conclude that reduced biliary excretion of the PG anion and impaired transport of the [^{3}H]L-glutamate anion by the BCEF were sensitive and acute indicators of CD-induced hepatobiliary dysfunction. There was no evidence of general lipid bilayer perturbation in in vivo pretreated plasma membranes although there was evidence from reduced biliary mannitol clearance that plasma membrane aqueous pores were restricted by CD. Compared to 24 hr pretreated rats, BCEF from 72 hr pretreated rats contained less CD despite higher liver concentrations. Recovery to control biliary PG excretion in 72 hr pretreated rats may have been a result of redistribution of CD within the liver.

Table II.1: [^{14}C]CD equivalents in liver homogenates and BCEF from rats pretreated (60 mg/kg body wt).

	Pretreatment	
	24 hr	72 hr
CD/liver (μmol/g)	0.174(0.00)*	0.382(0.09)
(mg/g)	0.085(0.00)	0.188(0.04)
CD/homogenate protein		
(nmol/mg)	1.18(0.01)	2.75(0.94)
(μg/mg)	0.58(0.01)	1.35(0.46)
Percent of CD dose	6.5(0.0)	13.0(4.0)
CD/BCEF protein		
(nmol/mg)	2.43(0.00)	3.08(0.60)
(μg/mg)	1.19(0.00)	1.52(0.30)
% Homogenate CD in BCEF	0.64(0.00)	0.40(0.19)
% Homogenate protein in BCEF	0.31(0.00)	0.33(0.12)

*Results are means (SE) for 2 to 3 individual preparations.

Table II.2: Marker enzyme activities in BCEF from control versus in vivo (60 mg CD/kg body wt) pretreated rats.

Plasma Membrane Enzymes[a] (μmol product/ mg protein/hr)	Homogenate	BCEF	Ratio[b]
5'N (P_i)			
Control	0.65(0.24)[c]	14.41(2.63)	23.40(4.36)
Treated	0.39(0.10)	9.70(2.47)	25.40(6.67)
γ-GT (5-amino-2-nitro-benzoate)			
Control	0.08(0.02)	1.07(0.55)	12.43(4.05)
Treated	0.11(0.07)	1.02(0.25)	10.83(4.05)
AP (p-nitrophenol)			
Control	0.12(0.03)	3.30(1.77)	25.67(8.81)
Treated	0.16(0.03)	3.38(1.09)	21.50(4.96)
LAP (β-naphthylamine)			
Control	0.66(0.17)	6.89(3.17)	11.30(6.33)
Treated	0.87(0.02)	4.74(0.42)	5.43(0.55)

[a] 5'N = 5' nucleotidase
γ-GT = γ-glutamyl transpeptidase
AP = alkaline phosphatase
LAP = leucine amino peptidase

[b] ratio = BCEF/Homogenate

[c] Results are means (SE) for 3 individual preparations

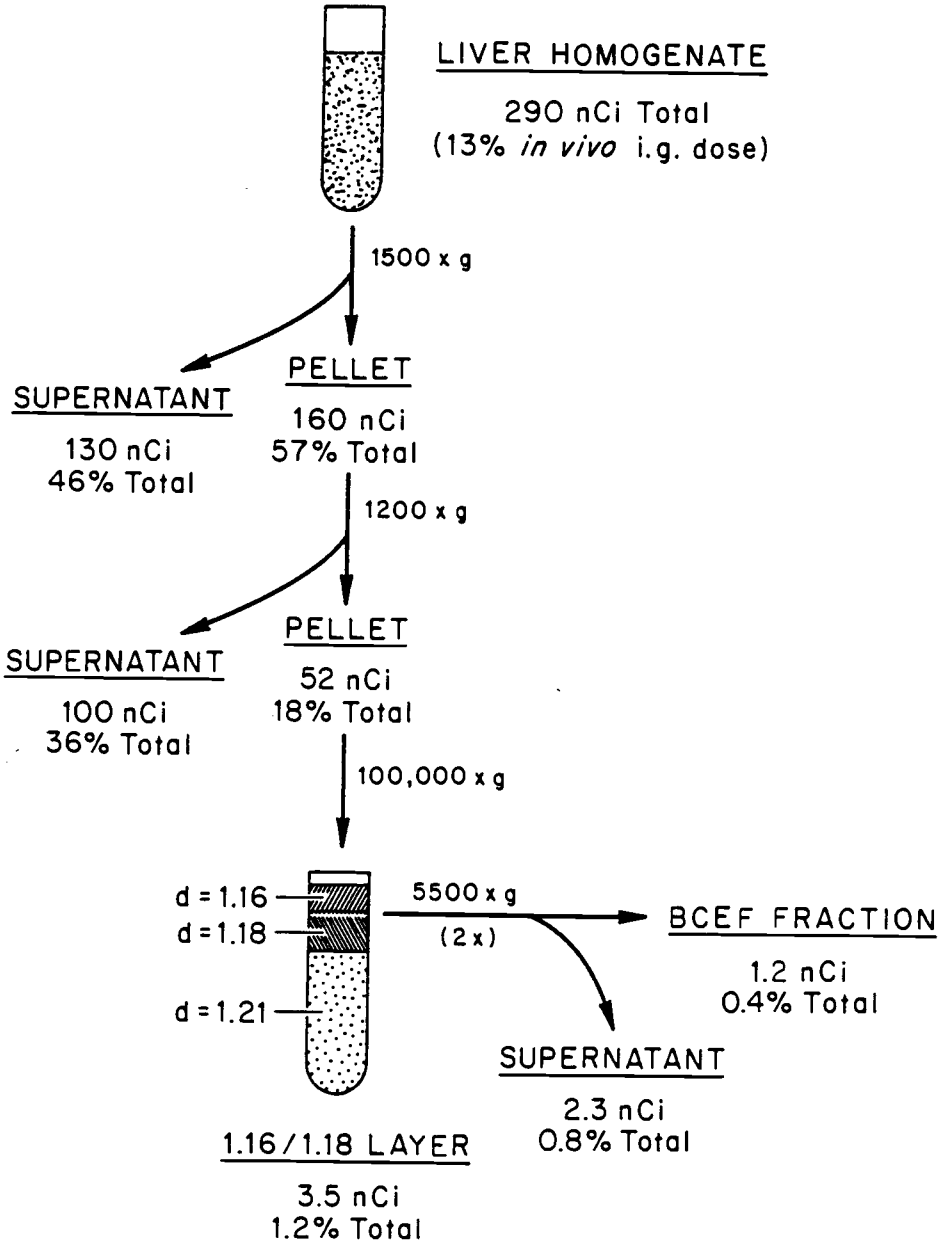

Figure II.1 [^{14}C]Chlordecone recovery during BCEF isolation from livers of 72 hr pretreated (60 mg CD/kg body weight) rats. Each arrow depicts one resuspension and centrifugation step. Values are expressed as mean [^{14}C] equivalents (nCi)) and mean percent of total liver homogenate [^{14}C] for three individual BCEF preparations.

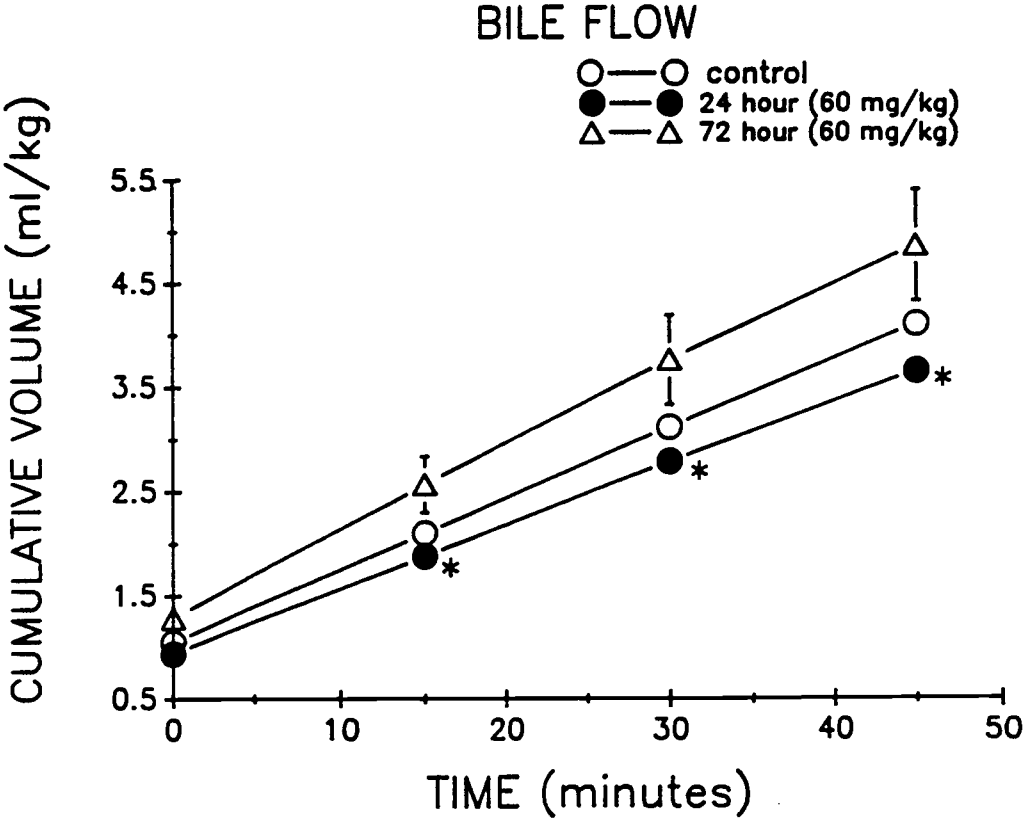

Figure II.2: Cumulative volume (ml/kg body weight) of bile collected over 45 min in control O , 24 hr CD pretreated ●, and 72 hr Δ(60 mg CD/kg body wt) pretreated rats. Values are expressed as means ± SE for 4 to 8 rats.

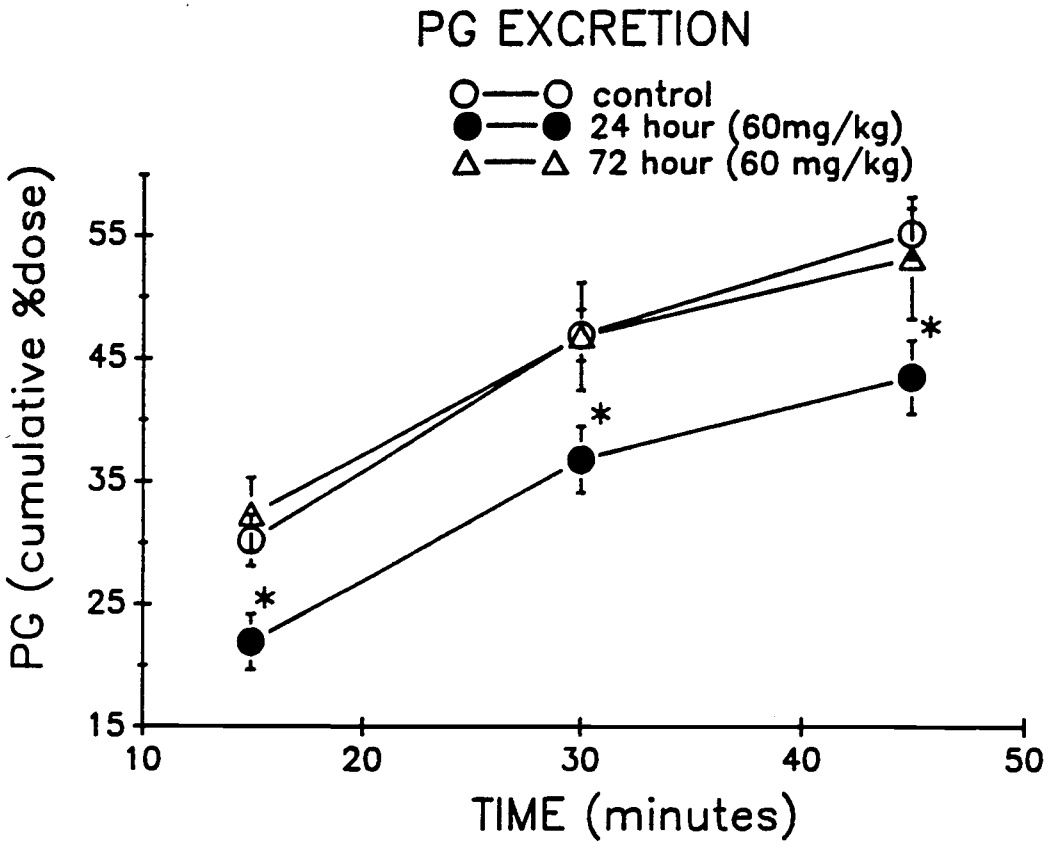

Figure II.3: Cumulative percent dose of a phenolphthalein glucuronide (PG) bolus (10 umol/kg body weight) excreted versus time in control O , 24 hr ● , and 72 hr Δ (60 mg CD/kg body wt) pretreated rats. Values are expressed as means ± SE for 4 to 8 rats.

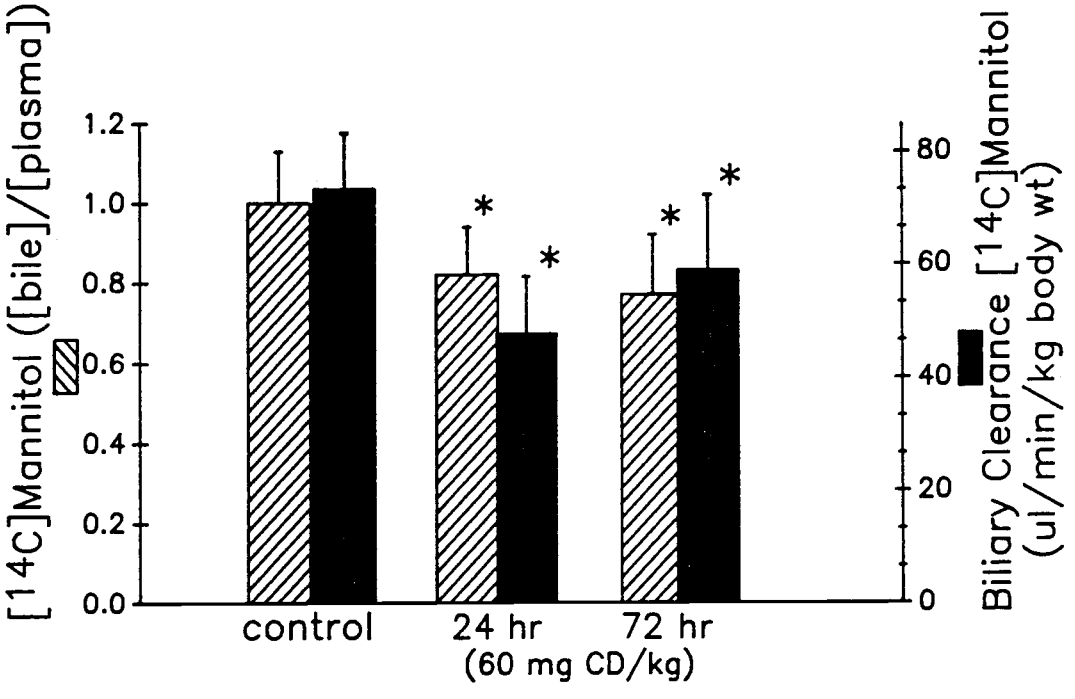

Figure II.4: Biliary excretion of [^{14}C]mannitol in control, 24 hr and 72 hr pretreated (60 mg CD/kg body wt) rats.

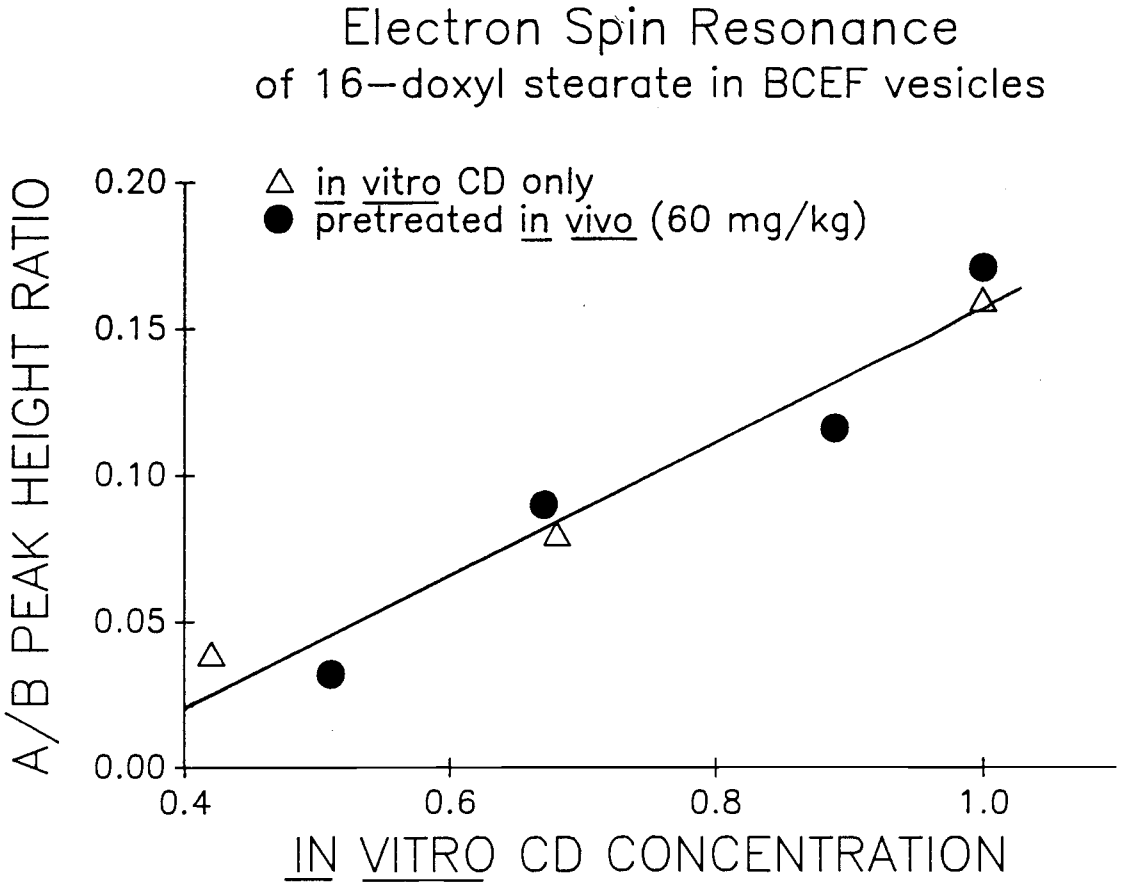

Figure II.5: Mobility of the lipid spin label 16-doxyl stearate in CD in vitro treated BCEF isolated from 24 hr CD pretreated ● and control △ rats. There was a direct correlation between immobilization of 16-doxyl stearate (peak height ratio, A/B) and increasing concentrations of in vitro CD in BCEF for both pretreated and control rats. Each point represents 2 individual BCEF preparations combined. For 24 hr pretreated BCEF: A/B=0.26(CD in vitro concentration) - 0.09, (r=0.97). For control BCEF: A/B=0.21(CD in vitro concentration) - 0.05, (r=0.99)

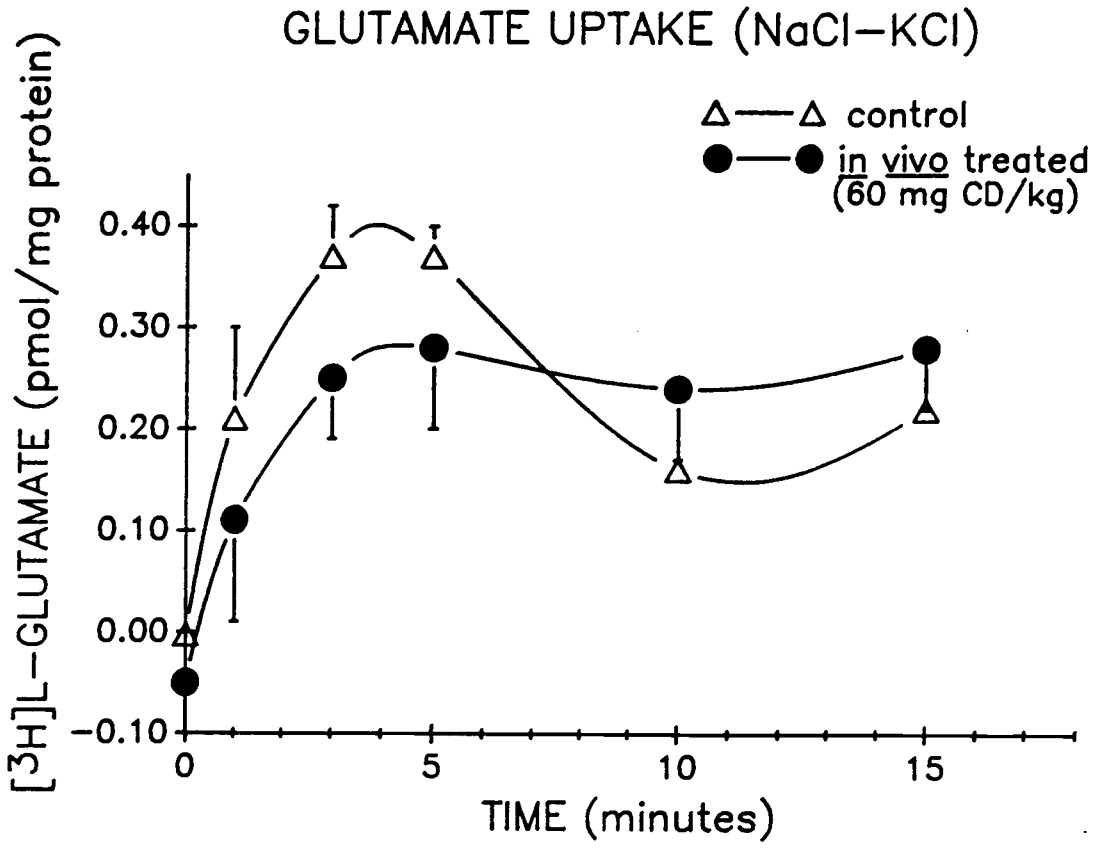

Figure II.6: Na^+-stimulated [^{3}H]L-glutamate uptake into BCEF vesicles. The values were derived by subtracting [^{3}H]L-glutamate uptake in equal osmolar K^+ (K_e/K_i) from total uptake in the presence of an extravesicular to intravesicular Na^+ gradient (Na_e/K_i). BCEF were isolated from 24 hr CD pretreated ● , or control Δ rats. Values are expressed as means ± SE for 3 individual BCEF preparations.

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SUMMARY AND CONCLUSIONS

The objective of this research was to determine the mechanisms for chlordecone-impaired biliary excretion of organic anions. Impaired biliary excretion of organic anion is the primary hepato-toxic manifestation of CD exposure in rats. The key working hypothesis was that CD inhibited anion transport proteins integral to the bile canaliculus. The two modes of action investigated in this study were 1) direct inhibition of anion transport proteins and 2) perturbation of the lipid-protein membrane environment, indirectly affecting anion transport.

In order to study bile canalicular specific effects, plasma membranes enriched in bile canaliculi (BCEF) were isolated from whole liver homogenates. BCEF were suspended in 550 mOsm sucrose buffer to yield sealed plasma membrane vesicles with anion transport systems intact. Isolated biochemical characteristics of membranes were related to biliary excretion in intact rat preparations (24 and 72 hr after 60 mg CD/kg body weight).

CD inhibited transport of organic anions in BCEF as well as in intact rats 24 hr after CD treatment. The bile canaliculus as a primary site of action fit these criteria:

1) bile canalicular-specific organic anion transport was inhibited at very low CD concentrations (2.4 nmoles/mg

protein).

2) CD was found closely associated with the bile canalicular fraction and directly correlated with protein recovery.
3) There was a direct correlation between increased inhibition with increased concentrations of CD.

Lipid bilayer perturbation was monitored in terms of the mobility of an electron spin resonance probe. There was no evidence of lipid environment changes with low concentrations of CD. Reduced [^{14}C]mannitol biliary clearance, however, indicated reduced hepatobiliary membrane permeability. Lipid bilayer effects, therefore, were not indicated as primary mechanisms of action in this study although restricted movement through membrane aqueous pores could be associated with primary effects on transport proteins. Nonetheless, at higher concentrations (0.2 μmol/mg protein), CD produced a direct physical effect on the BCEF lipid bilayer.

There was evidence of secondary events associated with CD treatment suggested by, but not fully explored in this study. These secondary events involve recovery of hepatobiliary performance within three days after CD treatment. Liver and BCEF CD concentrations remained elevated three days following CD treatment but the CD was associated with another subcellular fraction. The

supernatant subcellular fractions were not identified, but the membrane isolation behaved quantitatively (in terms of recovered CD) different between 24 and 72 hr pretreated livers during BCEF isolation.

This research provides evidence for bile canalicular anion transport proteins as sites for primary mechanisms of CD toxicity. It does not suggest that the PG-anion transporter is the same one as the BCEF [^{3}H]L-glutamate transporter, nor does it demonstrate that both transporters are located in the same region of the plasma membrane. It does prove that impaired transport of specific anions is the most acutely sensitive hepatobiliary effect so far determined.

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