Table 1. Comparison of the apparent V_{max} and Michaelis–Menten constants for MAO in the enzymatic deamination of dopamine and serotonin labeled in the α-position with deuterium*

	Dopamine		Serotonin	
	V_{max}	K_m	V_{max}	K_m
N n-deuterated	7.14 ± 0.81	4.3 ± 0.1	6.06 ± 0.50	1.46 ± 0.09
$\alpha\alpha$-d_2-labeled	4.43 ± 0.24†	6.1 ± 0.70‡	2.47 ± 0.12†	2.23 ± 0.03†

* V_{max} [maximum velocities, nmoles · (mg protein)$^{-1}$ · min^{-1}] and K_m [Michaelis–Menten constant, 1×10^{-4} M] values were obtained by Lineweaver–Burk double-reciprocal plots. Experimental data were based on least squares analysis assuming equal variance for the velocities.

† Significantly different from the control, $P < 0.001$.

‡ Significantly different from the control, $P < 0.05$.

Table 2. Deuterium isotope effects on the enzymatic deamination of $\alpha\alpha$-d_2-labeled dopamine and serotonin

Substrate	V_H/V_D	$(V/K_m)_H/(V/K_m)_D$
Dopamine	1.61	2.28
Serotonin	2.45	3.75

group; then it was isolated and purified as its creatinine salt. 5-Hydroxytryptamine-$\alpha\alpha\beta\beta$-d_4 was prepared by refluxing 5-benzyloxyindole-3-acetonitrile, in ethanol-OD, D_2O and NaCN, reduction with lithium aluminum deuteride and hydrogenation over palladium on charcoal.

The extent of deuteration was assessed mass-spectrometrically utilising the dansyl derivatives of the unsubstituted and deuterated amines with an A.E.I. MS-902S high resolution mass spectrometer [1]. It was found that the extent of deuteration was quite high in all cases (> 95%).

The enzymatic deamination of dopamine and serotinin and their deuterated analogues was determined. As can be seen from Fig. 1, the initial rates of oxidative deamination of the $\alpha\alpha$-d_2-dopamine and $\alpha\alpha$-d_2-5-HT were much reduced in comparison with those of the non-deuterated amines by the rat liver mitochondrial MAO. A slight enhancement of MAO activity with respect to $\beta\beta$-d_2-dopamine and a reduction with respect to $\alpha\alpha\beta\beta$-d_4-5-HT, as was the case for the trace amines [1], were also observed.

The kinetic parameters K_m and V_{max} for DA and 5-HT as derived from Lineweaver–Burk plots are summarized in Table 1. A decrease in V_{max} and an increase of K_m were observed with respect to the oxidation of both $\alpha\alpha$-d_2-dopamine and $\alpha\alpha$-d_2-serotonin. The isotope effects of the $\alpha\alpha$-d_2 substitution, expressed as V_H/V_D and $(V/K_m)_H/(V/K_m)_D$ are shown in Table 2. The magnitude of these deuterium isotope effects, as compared with our previous observation of the trace amines [1], was in the order: serotonin > p-tyramine > m-tyramine > β-phenylethylamine > dopamine. The aromatic structures of these amines appear to have affected the transition state and varied the isotope effect.

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Liver cell plasma membrane lipids in manganese–bilirubin-induced intrahepatic cholestasis

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In the rat, administration of manganese followed by injection of bilirubin results in a severe reduction in bile flow; this cholestasis is reversible, varies according to the dose of bilirubin administered, and can be prevented by the administration of sulfobromophthalein [1–6]. Its pathogenesis is not yet known. In a previous study, de Lamirande *et al.* [7] observed that alteration of the enzymic activity of liver cell plasma membranes did not appear to be the cause of cholestasis. However, they reported that a marked shift in the recovery of protein in isolated liver plasma

membrane fractions, from the canalicular to the sinusoidal fraction, occurred during manganese–bilirubin cholestasis. Furthermore, the isolated bile canalicular membranes were yellow in color, suggesting that binding or incorporation of bilirubin to the membrane had occurred. As the bile canalicular membrane protein recovery was lower in manganese–bilirubin cholestasis, despite the possible binding or incorporation of bilirubin in the membrane, this could suggest major changes in the membrane lipids. Therefore, the objective of the present study was to determine the alteration in liver cell plasma membrane lipids and bilirubin binding in manganese–bilirubin-induced cholestasis.

Materials and methods

The animals and treatments used were identical to those previously reported by de Lamirande *et al.* [7]. To induce cholestasis, manganese (Mn) (4.5 mg/kg, i.v.) was given to male Sprague–Dawley rats 15 min before bilirubin (BR) (25 mg/kg, i.v.) injection; the cholestasis was observable 60 min after BR administration. When sulfobromophthalein (BSP) was administered (15 mg/kg, i.v.) to prevent the Mn–BR cholestasis, it was given 10 min before the BR. The liver cell plasma membranes were isolated into two fractions, one rich in the canalicular surface (BCM) and the other rich in the sinusoidal and lateral surfaces (PM), by the methods previously described by Yousef and Murray [8]. The membranes were isolated from nine different groups of rats: (1) control, (2) 15 min after Mn injection (Mn-15), (3) 75 min after Mn injection (Mn-75), (4) 60 min after BR injection (BR-60), (5) 10 min after BSP injection (BSP-10), (6) 60 min after Mn plus BR injection (Mn–BR), (7) 60 min after Mn, BSP plus BR injection (Mn–BSP–BR), (8) 60 min after bile duct ligation (BDL), and (9) 60 min after bile duct ligation plus bilirubin injection (BR–BDL). The last two groups were used to compare the effects of Mn–BR cholestasis and those of extrahepatic cholestasis, with or without BR. Bile flow, liver cell plasma membrane enzyme activities, and proteins were analyzed and reported elsewhere [7]. Cholesterol and phospholipid were extracted from a known amount of membrane into a mixture of chloroform–methanol (2:1). Cholesterol was measured by gas–liquid chromatography using 5-β cholanic acid as an internal standard [9]. Phospholipids were measured by the colorimetric method of Bartlett [10]. BR was measured in the lipid extract by a modification of the procedure of Lathe and Ruthven [11]. The data were subjected to an analysis of variance, followed by the Student–Newman–Keuls procedure, to detect statistically significant ($P < 0.05$) differences between means [12].

Results

Table 1 shows the cholesterol and phospholipid contents of the BCM fraction. There was a significant and similar increase in BCM cholesterol (about 2-fold) in: Mn-15, BR-60, BDL and BR–BDL. Therefore, it appears that Mn and BR injections alone are similar to BDL in their effect on membrane cholesterol. However, the Mn–BR treatment increased membrane cholesterol by at least four times over the control level. It is noteworthy that BSP injection blocked this response to Mn–BR treatment (Mn–BSP–BR < Mn–BR; $P < 0.05$).

The effect on phospholipid content differed in that phospholipids increased significantly in: Mn-15, Mn-75, Mn–BR and Mn–BSP–BR membranes. However, BR injection alone (BR-60) did not significantly influence the phospholipid content.

Bilirubin was detectable only in the BCM fractions obtained from the Mn–BR group; the mean content was 238 ± 11 ng/mg protein. It is noteworthy that BSP treatment (Mn–BSP–BR) prevented this effect. Furthermore, the injection of BR during cholestasis (BR–BDL) did not cause BR to enter into BCM.

Table 2 summarizes the composition of the PM fraction. None of the treatments resulted in an increase of cholesterol or phospholipids. Nearly all of the treatments resulted in a significant reduction in both cholesterol and phospholipid content. Bilirubin was only detectable in the PM fractions from the Mn–BR group; the mean content was 39 ± 2 ng/mg protein.

Discussion

One hypothesis concerning the pathogenesis of chemically induced intrahepatic cholestasis is that the molecular organization of the bile canalicular membrane is altered, which renders the membrane impermeable to water and results in a reduction of bile flow [13]. This hypothesis is supported by analysis of BCM fractions following lithocholic acid [14], chlorpromazine [15] and ethinyl estradiol [16] treatments in which cholesterol content was increased, and cytochalasin B or norethandrolone [17] treatments, where cholesterol contents were reduced. The data reported in the present study show that bile duct ligation induced changes in BCM similar to those observed with lithocholic acid [14], since the cholesterol/phospholipid ratio was increased from 0.27 to about 1.0. In all these models, bile flow is reduced, significantly, and the main alteration is a modification in membrane cholesterol. Both an increase and a decrease of cholesterol can be associated with a decrease in membrane fluidity [18], and recently cholesterol has been shown to influence the surface tension

Table 1. Cholesterol and phospholipid contents of bile canalicular membranes from control and treated rat livers*

Treatment		Cholesterol	Phospholipid	Mean cholesterol/phospholipid ratio
Control	(8)	229 ± 11	843 ± 33	0.27
Mn-15	(5)	549 ± 28†	3268 ± 167†	0.17
Mn-75	(4)	341 ± 21	3497 ± 164†	0.10
BR-60	(5)	554 ± 25†	842 ± 42	0.66
BSP-10	(2)	305 ± 28	632 ± 38	0.48
Mn–BR	(8)	1015 ± 44†	2334 ± 95†	0.43
Mn–BSP–BR	(4)	358 ± 20	2701 ± 147†	0.13
BDL	(4)	510 ± 31†	591 ± 36	0.86
BR–BDL	(2)	600 ± 66†	540 ± 56	1.11

* Treatments are described in Materials and Methods. Values are means ± S.E. and are expressed as nmoles/mg of membrane protein. (N) = number of rats.

† Significantly different from control ($P < 0.05$).

Table 2. Cholesterol and phospholipid contents of sinusoidal and lateral membranes from control and treated rat livers*

Treatment		Cholesterol	Phospholipid	Mean cholesterol/phospholipid ratio
Control	(8)	187 ± 11	246 ± 12	0.76
Mn-15	(5)	169 ± 9	241 ± 17	0.70
Mn-75	(4)	118 ± 8†	204 ± 13†	0.58
BR-60	(5)	64 ± 5†	137 ± 11†	0.47
BSP-10	(2)	75 ± 8†	175 ± 27†	0.43
Mn–BR	(8)	71 ± 6†	106 ± 6†	0.67
Mn–BSP–BR	(4)	61 ± 4†	74 ± 4†	0.82
BDL	(4)	174 ± 13	201 ± 12†	0.87
BR–BDL	(2)	164 ± 12†	197 ± 16†	0.83

* Treatments are described in Materials and Methods. Values are means ± S.E. and are expressed as nmoles/mg of membrane protein. (N) = numbers of rats.
† Significantly different from control ($P < 0.05$).

of the membrane [19]. Therefore, membrane cholesterol levels may be viewed as modulators of both membrane fluidity and membrane permeability [17, 18].

The data reported in the present study show that manganese and bilirubin, when given alone, can result in increased BCM cholesterol content; when given in combination the increase appeared to be additive (Mn–BR), but was absent in the presence of BSP (Mn–BSP–BR). However, one must recall that manganese or bilirubin given alone do not depress bile flow, whereas the combination (Mn–BR) reduces bile flow [2, 4]. This suggests that the cholesterol increase in itself may not be the pathogenic factor in this cholestatic model.

A striking observation was the remarkable increase in BCM phospholipid content present following treatment with manganese, either alone (Mn-15, Mn-75) or in combination with bilirubin (Mn–BR). However, phospholipids were elevated in the presence of BSP (Mn–BSP–BR), a treatment that abolishes Mn–BR cholestasis [2, 4]. This suggests that the phospholipid increase also may not lead to cholestasis.

The bilirubin data may help to reconcile the situation. Bilirubin was found in the BCM fraction only after Mn–BR treatment. When BSP was given with the combination (Mn–BSP–BR), bilirubin was absent in BCM. Previous work [2] has shown that cholestasis is dose dependent on bilirubin and that BSP protects against the cholestasis by acting on the bilirubin component, rather than the manganese component, of the Mn–BR cholestatic model. Furthermore, bilirubin has been shown to affect lipid secretion in bile [20]. Therefore, it is possible that bilirubin incorporation into the BCM may modify the configuration and/or the interaction between the membrane lipids, causing a decrease in membrane fluidity. The data do not explain how bilirubin binds to the membrane. One could speculate that the increase in phospholipids caused by manganese facilitates bilirubin entry. When the bilirubin contents of the BCM fraction were compared to the log phospholipid content, an excellent correlation was obtained ($y = -1500 + 500 \log x$; $r = 0.96$). BSP protection may be due to its competition with bilirubin incorporation. Although the mechanisms are unknown, it appears reasonable to suggest that manganese causes a disruption in the lipid bilayer, allowing bilirubin incorporation into the membrane and, consequently, a change in the composition of the lipid bilayer. In this context, the initial increase in phospholipids caused by manganese would be more important than the alteration in cholesterol.

The changes in the sinusoidal surface (PM fraction) are less dramatic. No pattern consistent with cholestasis is discernible from the data for cholesterol and phospholipid contents. This seems to indicate that the manganese–bilirubin effect is specific for the secretion step involving the canalicular membrane.

From these data we can speculate on the shift in the protein recovery of the BCM observed in the Mn–BR group and reported previously [7]. Since the BCM fraction of the Mn–BR group contains more lipids per unit of protein, rearrangement of membrane protein may occur in a fashion similar to that seen with lithocholic acid [21]. Membrane proteins might translocate in the lateral part of the membrane to allow the incorporation of the excess lipid.

In conclusion, the data suggest that manganese administration increases both the cholesterol and phospholipid content of the canalicular membrane. We propose that this allows the incorporation of bilirubin into the lipid bilayer, possibly altering the cholesterol–phospholipid interaction [22, 23], thus decreasing canalicular membrane fluidity and permeability; this may result in the observed cholestasis.

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Aldrin epoxidation kinetics in small samples of human liver

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In vitro studies of the activity of hepatic microsomal mono-oxygenases in man have been limited by the quantity of tissue that is conventially removed during diagnostic liver biopsy (rarely more than 60 mg wet wt is surplus to histological requirements) and by the sensitivity of methods for measuring the products of the enzyme reactions. Benz[*a*]pyrene hydroxylation, ethylmorphine demethylation and the kinetics of amylobarbitone hydroxylation have been measured in human liver biopsies [1–3]. Mono-oxygenases, including antipyrine hydroxylation [4], phenacetin-*O*-de-ethylase and 7-ethoxycoumarin de-ethylase [5], have also been investigated in tissue wedges obtained at laparotomy.

Aldrin epoxidation activity has been shown to be readily measured in needle biopsies of rat liver. The major metabolite, dieldrin, is detectable with a high degree of sensitivity by electron capture gas chromatography [6]. Wolff has demonstrated in the rat that aldrin epoxidation is readily inducible wtth phenobarbitone but not with 3-methylcholanthrene [6]. After purification of cytochrome P-450 forms, activity is limited to the phenobarbitone-inducible form of cytochrome P-450 [7]. These observations suggest that aldrin epoxidation might be a useful probe for investigating mono-oxygenase activity in small samples of human liver.

Materials and methods

Human liver, surplus to diagnostic requirements, was obtained at either diagnostic needle biopsy (six patients), or from a wedge biopsy removed during laparotomy (two patients). In all instances, liver biopsy was performed for suspected hepatic disease: in the six patients undergoing needle biopsy, no hepatic pathology was found and the liver was histologically normal. The two wedge biopsies were obtained from non-malignant tissue of patients undergoing partial hepatectomy for primary liver cell cancer.

Liver was either frozen immediately in liquid nitrogen and stored at −80°, or placed into ice-cold phosphate buffer (0.25 M potassium phosphate containing 0.15 M potassium chloride and 1.0 M EDTA, pH 7.25). The microsomal pellet was prepared as described by Boobis *et al.* [1] and resuspended in 0.25 M phosphate buffer containing 30% v/v glycerol. Enzyme activity was usually assayed immediately, although storage of microsomes at −80° for up to 2 months did not alter activity. Aldrin epoxidation was measured by a micro scale modification of the method described by Wolff *et al.* [6]. Incubations were for 20 min at 37° in a final vol. of 0.1 ml containing 0.005 M phosphate buffer (pH 7.5), 0.5 mM NADPH, microsomal protein (20–40 μg) and aldrin added in 1 μl methanol. Dieldrin was extracted into hexane and measured by electron capture gas chromatography. The lower limit of detection of the method was 0.5 pmoles dieldrin formed/mg microsomal protein/min. Protein was measured by a modification of the method of Lowry *et al.* [8]. Inhibitors (metyrapone and α-napthaflavone) were added to the incubation medium in 1 μl ethanol before addition of the substrate. Control incubations contained 1 μl ethanol.

Results and discussion

Aldrin epoxidation activities measured in microsomes prepared from 100-mg portions of wedge biopsy tissue were similar to those prepared from 10-mg portions of the same tissue [108.3 ± 7.3 (n = 4) and 104.9 ± 7.0 (n = 4) pmoles dieldrin/mg/min, respectively]. Aldrin epoxidation was linear with time to 30 min, and proportional to microsomal protein between 0.1 and 0.5 mg/ml at a substrate concn of 100 μM. Protein concns of less than 0.1 mg/ml resulted in variable activity measurements. Optimum activity was measured at pH 7.6, but between pH 7.4 and pH 7.8 activity was greater than 90% of the maximum. Activity was reduced by 97% when NADPH was omitted from the