Biochemical Pharmacology, Vol. 30, No. 16, pp. 2305–2312, 1981.
Printed in Great Britain.

0006-2952/81/162305-08 $02.00/0

HEPATOCELLULAR MEMBRANE ALTERATION AS A POSSIBLE CAUSE OF MANGANESE–BILIRUBIN-INDUCED CHOLESTASIS

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(*Received* 1 *August* 1980; *accepted* 29 *January* 1981)

Abstract—Canalicular membrane alteration was investigated as a possible mechanism for the cholestasis induced by a manganese–bilirubin (Mn–BR) combination. Hepatic bile canalicular (BCM)- and sinusoidal (PM)-enriched membrane fractions were isolated under both cholestatic (Mn–BR or bile duct ligation) and noncholestatic (manganese or bilirubin alone; manganese + sulfobromophthalein + bilirubin) conditions. The major effect of Mn–BR interaction was an important shift in the recovery of proteins in isolated liver plasma membrane fractions; the shift was from the canalicular to the sinusoidal fraction. Enzymic activities (5'-nucleotidase, leucylaminopeptidase and ATPase) of both BCM and PM fractions were easily depressed even if bile flow was normal and therefore do not appear to be good indicators of altered bile secretory function. Canalicular membranes isolated from animals in Mn–BR cholestasis were yellow and probably incorporated bilirubin. Phalloidin pretreatment afforded progressive and reversible protection against Mn–BR-induced cholestasis.

Manganese-induced intrahepatic cholestasis can cause both morphologic and functional alterations in the livers of rats [1, 2]. This model can be useful for studying chemically induced intrahepatic cholestasis since, with the exception of necrosis caused by large doses of manganese, the ultrastructural changes observed resemble some of those seen in human cholestasis [3].

If manganese and bilirubin injections are combined, the resulting diminution in bile flow is more severe [4, 5], and a bilirubin dose–response relationship is observed [6–8]. The combination of normally noncholestatic doses of manganese and bilirubin produces a dramatic but reversible fall in bile flow in rats, and this response is dose dependent for bilirubin but not for manganese [7, 9]. The dose of manganese, however, does influence the period of time during which manganese–bilirubin (Mn–BR) cholestasis can be elicited.

Protection against Mn–BR cholestasis is afforded by sulfobromophthalein (BSP) [10, 11]. The magnitude of the protection seems related to the amount of BSP remaining unexcreted at the time the bilirubin is injected [7]. Furthermore, the relationship between the minimal protective dose of BSP and the dose of bilirubin was found to be linear and independent of manganese load [7]. BSP appears to act on the bilirubin component of Mn–BR cholestasis rather than on manganese.

It has been proposed that an intermediate step is involved between manganese administration and the subsequent interaction with bilirubin to eventually lead to cholestasis [7]. This intermediate step could be the formation of a reactive chemical complex, the inhibition of an enzyme, a temporary alteration of the canalicular membrane or its pericanalicular microfilament network, etc. These last two possibilities are attractive since metals are known to affect membrane function. Furthermore, the characteristics of the manganese action, especially the "all-or-none" response, are consistent with these two possibilities.

In some models of cholestasis, alterations of bile canalicular membranes (BCM) have been observed. The cholestasis induced by chloropromazine [12, 13], lithocholic or taurolithocholic acid [14], cytochalasin B or norethandrolone [15] is characterized by depression of enzymic activities, especially ATPases, and/or changes in the cholesterol : phospholipid ratio of the membrane lipid layer, and/or diminution of the recovery of canalicular membranes.

The first aim of the present study was to expand these observations to the Mn–BR model and to obtain support for the hypothesis that membrane alteration could be an important event in the development of cholestasis. The second part of our study concerns the possible interaction between manganese and the pericanalicular filamentous network. Recent findings suggest that these microfilaments play a role in bile secretory mechanisms [16–21].

Phalloidin induces a marked thickening of the pericanalicular filamentous network in hepatocytes, accompanied by a decrease in bile flow [16, 17]. Recently, it was shown that phalloidin also alters the protein composition of isolated plasma membranes [22]. Modifications of the Mn–BR-induced cholestasis after a phalloidin pretreatment would give

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indirect evidence that manganese interacts with microfilaments or membranes.

METHODS

Animals. Male Sprague–Dawley rats (Canadian Breeding Farm Ltd., St. Constant, Québec), weighing 280–330 g for the membrane studies or 175–225 g for the phalloidin studies, were maintained on Charles River Rat Chow and water *ad lib.* The animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and catheters were placed in a femoral vein (PE-50 tubing), and, for the phalloidin studies, in the common bile duct (PE-10 tubing). We also performed experiments in which the common bile duct of rats was ligated for 60 min. Anesthesia was maintained throughout the treatments and/or the bile secretion studies. Body temperature was monitored via a rectal probe (Yellow Springs Instruments Co. Thermoregulator) and maintained at 37° by means of an infrared lamp to eliminate temperature-dependent changes in bile flow [23]. Injection of a small volume of 0.9% NaCl preceded each treatment to wash the femoral vein cannula and to prevent contact between the various substances. In the phalloidin studies, this substance was injected for 1, 2 or 3 days; surgery was performed 24 hr after the last phalloidin injection and the treatments were then administered; bile was collected over 15- or 30-min periods and measured volumetrically.

Injectable materials. Monohydrated manganese sulfate ($MnSO_4 \cdot H_2O$) was dissolved in 0.9% NaCl and injected intravenously at a dose of 15 or 20 mg/kg (4.5 and 6.0 mg Mn/kg respectively) within 3 min. Freshly prepared aqueous solutions containing 0.52 g NaCl, 0.52 g Na_2CO_3, and 0.50 g bilirubin (Sigma Chemical Co. St. Louis, MO) per 100 ml were injected intravenously (0.5 ml/100 g) over a 2-min period. A concentrated BSP solution (5 g/100 ml, Dade Laboratories, Miami, FL) was diluted and injected intravenously (15 mg/kg) in less than 1 min. Phalloidin was dissolved in saline and injected i.p. ($500\ \mu g \cdot kg^{-1} \cdot day^{-1}$) for 1, 2 or 3 days.

Treatments and membrane isolation. Mn–BR-induced cholestasis can be schematized in the following manner [7, 9]:

$$\text{Mn} \xrightarrow{15\,\text{min}} \text{BR} \xrightarrow{60\,\text{min}} \text{cholestasis.}$$

BSP must be injected 10 min before bilirubin to prevent cholestasis [7, 9]. Taking this scheme into consideration, membranes were isolated in nine different situations: (1) without treatment (control); (2) 15 min after manganese injection (Mn 15 min); (3) 75 min after manganese injection (Mn 75 min); (4) 60 min after bilirubin injection (BR 60 min); (5) 10 min after BSP injection (BSP 10 min); (6) 60 min after manganese and bilirubin injections (Mn–BR); (7) 60 min after manganese, BSP, and bilirubin injections (Mn–BSP–BR); (8) 60 min after a bile duct ligation (BDL 60 min); and (9) 60 min after a bile duct ligation combined with a bilirubin injection (BR–BDL 60 min). The last two experiments were performed to compare effects of Mn–BR-induced cholestasis and of extra-hepatic cholestasis with or without bilirubin load.

Membranes were isolated according to the method of Yousef and Murray [24], with some modifications. At designated times, the liver was perfused via the portal vein with ice-cold physiological saline, buffered (pH 7.4) with 1 mM $NaHCO_3$ and 0.5 mM $CaCl_2$, by means of a reservoir elevated to a height of approximately 60 cm. The liver was removed, rinsed, blotted on filter paper, and passed through a plastic tissue press (Hickey Plastic Ltd., Montréal, Canada). The resulting pulp is 90 per cent enriched with parenchymal cells, and amost all connective tissue stays in the press [25]. The liver puree was weighed and 1 mM $NaHCO_3$–0.5 mM $CaCl_2$ solution was added to a total volume in milliliters equal to three times the liver puree weight in grams. Homogenization was performed using a Polytron homogenizer at speed 5 for 3 sec. The homogenate was mixed with $NaHCO_3$–$CaCl_2$ buffer to a total volume of 10 ml/g of liver puree. The homogenate was then distributed among tubes and centrifuged at 500 *g* for 5 min and at 1000 *g* for 10 min (2000 and 2800 rpm, respectively, SS-34 rotor, Sorvall RC2-B centrifuge). The supernatant fractions were removed and used for isolation of microsomes. The pellets were resuspended in the same volume of $NaHCO_3$–$CaCl_2$ buffer and recentrifuged at 1000 *g* for 10 min. The resulting pellets were combined and $NaHCO_3$–$CaCl_2$ solution was added to a total volume, in milliliters, equal to 60–80 per cent of the initial weight of the liver puree in grams. A sucrose solution (d: 1.26) was added in sufficient quantity to give a final density of 1.22 (approximately 5.5 times the volume of the original pellet–buffer suspension). After thorough mixing, 15-ml aliquots of the membrane suspension (d: 1.22) were put into ultracentrifuge tubes over-layered with 7 ml of sucrose (d: 1.18) and then with 5 ml of sucrose (d: 1.16). After a 60-min centrifugation at 66,000 *g* (27,000 rpm, Beckman 30 fixed angle rotor, Beckman L3-40 centrifuge), membrane layers were observed at the upper 1.18 to 1.16 interface (bile canalicular fraction, BCM) and at the lower 1.22 to 1.18 interface (sinusoidal membranes fraction, PM). Both fractions were harvested separately, using a 5-ml glass syringe equipped with a long needle (No. 14) cut over the bevel. The canalicular fraction was diluted with 4–5 vol. of $NaHCO_3$–$CaCl_2$ buffer and centrifuged at 3000 *g* for 15 min. The pellets were pooled, suspended in 30 ml of $NaHCO_3$-$CaCl_2$ buffer, and centrifuged for 10 min at 9000 *g* (8500 rpm). The final pellet (BCM was then suspended in 2–4 ml of buffer according to the collected quantity. The sinusoidal fraction (1.22 to 1.18 interface) required further purification. The weight (W) and volume (V) of the aspirated layer containing the sinusoidal fraction were noted and sucrose (d: 1.26) was added to give a final density of 1.22. The volume of sucrose ($V_{1.26}$) was calculated using the formula:

$$V_{1.26} = \frac{\text{d: } 1.22 - (W/V)}{0.04} V.$$

After thorough mixing, all manipulations, the sucrose gradient, ultracentrifugation, harvesting and washings were performed again. The purity of the BCM and PM fractions was verified by electron microscopy.

The protein content of the membrane suspensions was determined by the method of Lowry *et al.* [26]

as modified by Peterson [27], using crystalline bovine serum albumin as the standard. For all enzymic activities measured we first assured ourselves that the conditions used for the measurement exhibited a tissue concentration-dependent and a time-dependent linear reaction. All incubations were performed at 37° using a Dubnoff metabolic shaker. Activities were measured for all fractions, at two different concentrations, and each sample was paired with another in which the substrate was omitted (substrate blank); finally, the amount of substrate hydrolyzed when incubated was also measured omitting the tissue (tissue blank). The following enzymic activities were measured: glucose-6-phosphatase (G-6-Pase) [28], 5′-nucleotidase [29], leucyl-β-naphthylamidase (LAP) [30], (1-hr incubation), total and Na^+, K^+-ATPases [24], (10-min incubation). Inorganic phosphorous (P_i) was determined by the method of Fiske and Subbarow [31].

Statistics. For studies concerning bile flow (phalloidin studies), statistically significant differences between control values and treatment values were determined by Student's *t*-test (two-tail). For studies concerning membranes, data were submitted to an analysis of variance and treatment means were subsequently tested using the Student–Newman–Keuls procedure [32]. The 0.05 level of probability ($P < 0.05$) was used as the criterion of significance.

RESULTS

Membranes. Tables 1, 2 and 3 show protein recovery and enzymic activities of both BCM and PM fractions isolated after various treatments. G-6-Pase activity of BCM and PM was similar to that observed by Samuels and Carey [12]; electron microscopy did not indicate microsomal or mitochondrial contamination in our preparations. 5′-Nucleotidase and LAP activities of the PM fraction were higher than those reported by Yousef and Murray [24] and could be explained by the presence of a small amount of canalicular membrane in the PM fraction. Although total ATPase activities were comparable to those previously reported, the values obtained for Na^+,K^+-ATPase or Mg^{2+}-ATPase accord with those reported by some, but not all, investigators [24, 33–35]. This variation is understandable in view of the different conditions of isolation of membranes and analytical procedures employed in different laboratories. Isolation and analytical procedures were similar for all of our membrane studies, and the intragroup variability was low. Thus, comparisons can be made between the various experimental groups.

Table 1 shows that, after a short cessation of bile flow (Mn–BR, BDL 60 min, or BR–BDL 60 min), PM and total (PM + BCM) membrane protein recoveries were increased. Mn–BR-induced intrahepatic cholestasis was, however, the sole treatment that affected the PM/BCM ratio; the amount of protein in the BCM fraction was markedly reduced whereas that in the PM fraction was significantly increased. On the other hand, extrahepatic cholestasis (BDL 60 min, or BR–BDL 60 min) improved both BCM and PM recoveries, and the PM/BCM ratio was not statistically different from control values. It is noteworthy that the changes observed after Mn–BR treatment were no longer present in the Mn–BSP–BR group.

Another interesting observation concerns the color of the BCM fractions obtained. Canalicular membranes isolated during Mn–BR cholestasis, and only then, were yellow. The color could not be removed by further washing in aqueous medium but was readily extracted by chloroform. We therefore suggest that it was due to bilirubin accumulation in the lipid layer of membranes. In all other situations (BR 60 min, BDL 60 min. Mn–BSP–BR, and even BR–BDL 60 min) the canalicular fraction was almost white. These data support the contention that both the manganese and bilirubin injections, given in the proper sequence, are essential for cholestasis, and they reinforce the hypothesis that a membrane intermediate, induced by manganese, reacts with bilirubin to lead to cholestasis.

Table 2 shows the effect of the various treatments

Table 1. Recovery of protein in canalicular and sinusoidal membrane fractions in control and treated rat livers*

Situation		BCM	PM	PM/BCM
Control	(8)	0.35 ± 0.03	1.18 ± 0.13	3.3 ± 0.4
Mn (15 min)	(5)	0.48 ± 0.05	1.47 ± 0.09	3.2 ± 0.3
Mn (75 min)	(4)	0.39 ± 0.05	1.42 ± 0.10	3.8 ± 0.3
BR (60 min)	(5)	0.38 ± 0.02	1.37 ± 0.15	4.7 ± 0.7
BSP (10 min)	(2)	0.51 ± 0.02	1.67 ± 0.10	3.3 ± 0.3
Mn–BR	(8)	0.23 ± 0.02†	2.83 ± 0.13‡	12.8 ± 1.4†
Mn–BSP–BR	(4)	0.33 ± 0.06	1.54 ± 0.38	5.5 ± 0.5
BDL	(4)	0.47 ± 0.05	2.03 ± 0.09‡	5.3 ± 0.4
BR–BDL (60 min)	(2)	0.46 ± 0.05	2.56 ± 0.05‡	5.5 ± 0.5

* Values are means ± S.E. of the numbers of experiments indicated in parentheses. Treatments are described in Methods. Abbreviations: Mn, manganese; BR, bilirubin; BSP, sulfobromophthalein; and BDL, bile duct ligation. Recoveries are expressed in milligrams of membrane protein per gram of liver puree. The PM/BCM ratio was calculated with data obtained from membranes isolated in the same experiment.

† Significantly different from all other vertical values.

‡ Values Mn–BR, BDL and BR–BDL are significantly higher than the other vertical values.

Table 2. Effects of various treatments on glucose-6-phosphatase, 5′-nucleotidase and leucine aminopeptidase activites of liver cell membrane fractions*

Treatment	BCM fraction			PM fraction		
	Glucose-6-phosphatase	5′-Nucleotidase	Aminopeptidase†	Glucose-6-phosphatase	5′Nucleotidase	Aminopeptidase
Control (8)	1.3 ± 0.2	58.9 ± 5.1	5.23 ± 0.34	2.6 ± 0.3	32.7 ± 4.0	0.99 ± 0.14
Mn (15 min) (5)	1.5 ± 0.2	38.2 ± 4.4‡	4.44 ± 0.31	2.3 ± 0.2	19.3 ± 1.7‡	1.29 ± 0.12
Mn (75 min) (4)	1.1 ± 0.3	54.3 ± 4.7	3.79 ± 0.16	2.1 ± 0.2	21.8 ± 0.9‡	1.19 ± 0.03
BR (60 min) (5)	1.7 ± 0.2	48.0 ± 3.1	4.02 ± 0.10	3.1 ± 0.5	26.0 ± 4.8	0.94 ± 0.21
BSP (10 min) (2)	1.5 ± 0.1	41.1 ± 1.2	3.76 ± 0.19	2.3 ± 0.1	23.1 ± 2.0	1.39 ± 0.07
Mn–BR (8)	1.7 ± 0.1	46.3 ± 3.7	2.39 ± 0.12	3.1 ± 0.2	16.8 ± 2.1‡	1.05 ± 0.07
Mn–BSP–BR (4)	1.3 ± 0.2	40.7 ± 4.8‡	2.83 ± 0.35	1.8 ± 0.3	16.0 ± 1.8‡	0.72 ± 0.09§
BDL (60 min) (4)	0.8 ± 0.1		1.07 ± 0.05	2.7 ± 0.3		1.23 ± 0.03
BR–BDL (60 min) (2)	0.9 ± 0.1	41.6 ± 1.9	1.37 ± 0.10	2.1 ± 0.1	21.6 ± 1.1	1.06 ± 0.06

* Values are means ± S.E. of the number of experiments indicated in parentheses. Treatments are described in Methods. Abbreviations: Mn, manganese; BR, bilirubin; BSP, sulfobromophthalein; and BDL, bile duct ligation. Glucose-6-phosphatase and 5′-nucleotidase activities are expressed in μmoles P_i released per mg protein per hr. Aminopeptidase activity is expressed in μg β-naphthylamine released per mg protein per hr.

†BDL < Mn–BR < Mn (15 min), Mn (75 min) < Control
BDL + BR < Mn–BSP–BR < BSP (10 min), BR (60 min)
‡ Significantly different from control.
§ Significantly different from BSP (10 min), Mn (15 min), Mn (75 min) and BDL (60 min).

on G-6-Pase, 5′-nucleotidase and LAP activities of liver cell membrane fractions. G-6-Pase activity did not change significantly from one experiment to another; therefore, the increase of PM protein recovery during bile flow obstruction cannot be explained by increased microsomal contamination. 5′-Nucleotidase activity was more easily depressed in the PM than in the BCM fraction; all treatments that included manganese injection exhibited that effect. No correlation between depressed 5′-nucleotidase activity and presence of cholestasis was observed since values obtained during protection (Mn–BSP–BR) were low. In the BCM fraction, LAP activity was depressed by all treatments, the lowest activity being found with bile duct ligation (with or without BR injection). Other treatments can be

Table 3. Effects of various treatments on the ATPase activities of liver cell membrane fractions*

Treatment	BCM fraction			PM fraction		
	Total ATPase	Na^+,K^+-ATPase	Mg^{2+}-ATPase	Total ATPase	Na^+,K^+-ATPase	Mg^{2+}-ATPase
Control (8)	78.2 ± 4.3†	47.5 ± 1.2†	30.4 ± 4.0‡	40.7 ± 4.1§	17.6 ± 2.1‖	23.1 ± 0.8§
Mn (15 min) (5)	42.6 ± 6.9	24.9 ± 4.2	17.6 ± 2.8	27.9 ± 5.5	13.5 ± 2.3¶	14.5 ± 3.5
Mn (75 min) (4)	66.4 ± 3.7§·**	30.9 ± 1.8††	35.0 ± 3.0‡·¶	32.1 ± 1.1	11.5 ± 0.4	20.5 ± 1.2‡
BR (60 min) (5)	51.2 ± 2.7††	24.0 ± 1.7	27.0 ± 1.7	32.0 ± 0.2	11.6 ± 0.7	20.4 ± 0.8
BSP (10 min) (2)	61.3 ± 0.2††	29.0 ± 0.5	32.3 ± 0.7‡	36.7 ± 6.5	21.4 ± 0.3‖	15.3 ± 6.0‡
Mn–BR (8)	35.4 ± 2.8	18.7 ± 1.5	16.8 ± 1.6	26.6 ± 2.0	9.9 ± 0.7	16.6 ± 1.5
Mn–BSP–BR (4)	45.2 ± 2.7	22.9 ± 0.9	21.9 ± 3.2	22.5 ± 0.6	7.9 ± 1.1††	14.6 ± 1.7
BR–BDL (2)	54.8 ± 0.9††	22.1 ± 0.2	32.8 ± 0.7‡	39.9 ± 0.5¶	9.8 ± 0.4	30.1 ± 1.0†

* Values are means ± S.E. of the number of experiments indicated in parentheses. Treatments are described in Methods. Abbreviations: Mn, manganese; BR, bilirubin; BSP, sulfobromophthalein; and BDL, bile duct ligation. Activities are expressed in μmoles P_i released per mg protein per hr. Mg^{2+}-ATPase was calculated by subtraction (total ATPase minus Na^+,K^+-ATPase).

† Significantly different from all other groups.
‡ Significantly different from Mn–BR and Mn (15 min).
§ Significantly different from Mn–BSP–BR, Mn (15 min) and Mn–BR.
‖ Control = BSP (10 min) > all other groups.
¶ Significantly different from Mn–BSP–BR.
** Significantly different from BR (60 min).
†† Significantly different from Mn–BR.

divided into two groups: cholestasis (Mn–BR) or protection (Mn–BSP–BR) and manganese or BSP or bilirubin administered singly. Activities obtained in BCM and PM fractions during Mn–BR cholestasis do not permit us to conclude that increased contamination of the PM fraction by BCM was responsible for the changes in recoveries for these fractions. Furthermore, as with 5′-nucleotidase, LAP activity was not related to the presence of cholestasis.

Table 3 shows the effect of various treatments on the activities of the ATPase of liver cell membrane fractions. Mg^{2+}-ATPase activity was calculated as the difference between total ATPase and Na^+,K^+-ATPase. ATPase activities were quite variable and could exist at low levels even if bile flow was normal (Mn 15 min, Mn–BSP–BR). We must also note that the manganese effect was rapidly reversible; in both BCM and PM fractions 15 min after manganese injection Na^+,K^+- and Mg^{2+}-ATPases were depressed but, 60 min later (Mn 75 min), Mg^{2+}-ATPase activity was restored. Values for Na^+,K^+-ATPase in BCM were higher than those reported by some investigators [24, 33]. This can be explained by the fact that our preparations contained bile canaliculi with relatively long lateral membranes. Poupon and Evans [36] have shown that the highest activity of Na^+,K^+-ATPase is predominantly localized in a membrane subfraction containing large numbers of intercellular junctions.

These experiments on liver cell membranes showed that the most important change was not related to the diminution in the specific activity of enzymes but to a profound change in the distribution of protein between BCM and PM fractions. Biliary obstruction caused an increase of total (BCM + PM) recovery, but Mn–BR treatment was the only one to affect separation of these fractions.

Phalloidin. We first noted that phalloidin ($500\ \mu g \cdot kg^{-1} \cdot day^{-1}$) itself induced a small but significant decrease in bile flow (10.3, 11.3 and 16.2 per cent after 1, 2 or 3 days respectively). This decrease was not as marked as that reported (19 and 34 per cent after 1 and 3 days) by Dubin *et al.* [17]; these authors, however, expressed bile flow in terms of liver weight rather than body weight.

Figure 1A shows that, after 3 days of pretreatment, bile flow was not affected by bilirubin injection but was temporarily decreased by manganese (4.5 mg/kg) injection. Mn–BR cholestasis was less severe in phalloidin-pretreated rats and the protective effect seemed related to the duration of the pretreatment (Fig. 1A, Table 4). We thereafter increased the manganese load to 6.0 mg/kg and observed a 52.3 ± 13.6 per cent decrease of bile flow 60 min after bilirubin injection (Fig. 1B). This result is important since normally there is no manganese dose–response relationship in Mn–BR cholestasis [7, 9]. We concluded that the protection afforded by phalloidin pretreatment occurred on the manganese component of the interaction and that thickening of the microfilament network could be related to the protective effect.

DISCUSSION

It is logical to suppose that intrahepatic cholestasis is associated with enzymic and structural alterations at the liver cell membrane level. Enzymic activities, however, appear to be a poor index of bile secretory function since few correlations can be established between these activities and the presence of cholestasis. In almost all models of cholestasis, partial enzymic inhibition has been reported in the canalicular membrane fraction, but the pattern of this inhibition is quite variable, depending on both the enzyme (5′-nucleotidase, Mg^{2+}- and Na^+,K^+-

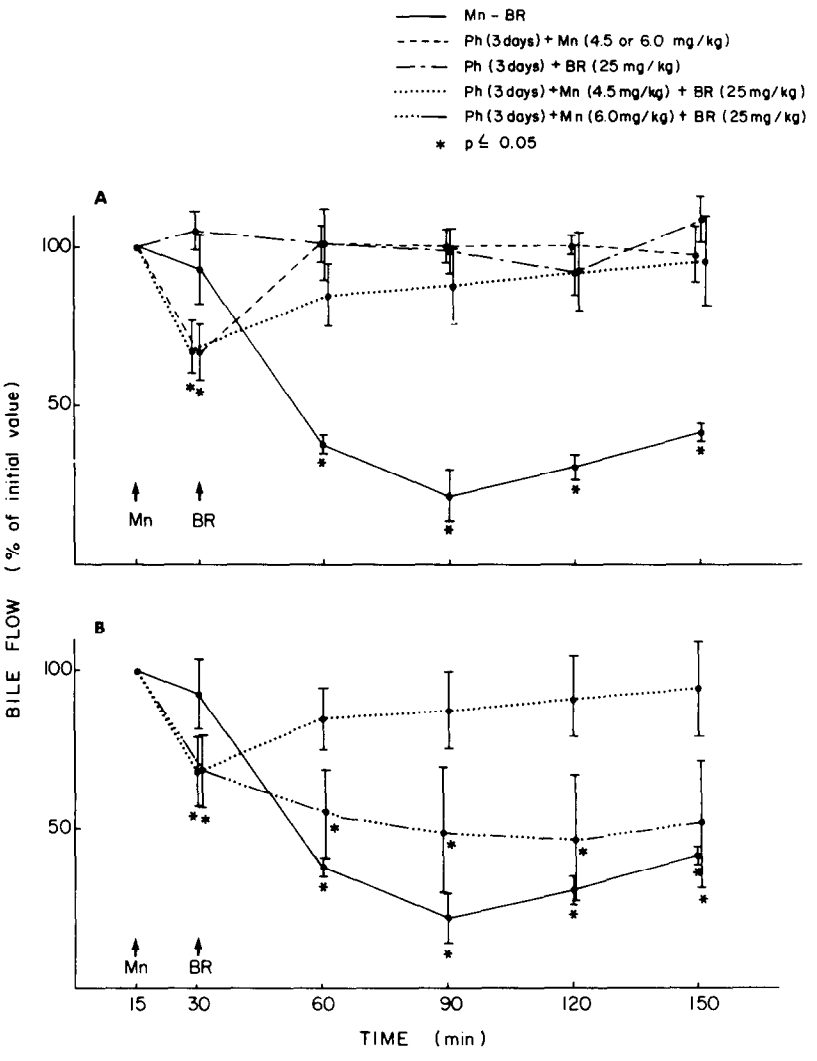

Fig. 1 Effect of phalloidin pretreatment on manganese–bilirubin-induced cholestasis. Manganese (4.5 or 6.0 mg/kg) was injected 15 min before bilirubin (25 mg/kg). Panel A depicts the control values (Ph + Mn or Ph + BR) as well as the protective effect of phalloidin when the lower dose of manganese (4.5 mg/kg) was combined with the bilirubin. In Panel B the effects of phalloidin on both doses of manganese (4.5 or 6.0 mg/kg), when combined with bilirubin, are compared. Bile flow is expressed as a percentage of initial bile flow. Values are means ± S.E. for four to eight rats. Phalloidin was injected i.p. ($500\ \mu g \cdot kg^{-1} \cdot day^{-1}$) for 3 days. The Mn–BR bile flow curve obtained without pretreatment is given for comparison. Key: Mn, manganese; BR, bilirubin; and Ph, phalloidin. Asterisks indicate $P \leq 0.05$ compared to initial value.

ATPase, LAP) and the cholestatic agent (norethandrolone, cytochalasm B, lithocholic acid, chlorpromazine) studied [13–15].

The manganese–bilirubin model can be useful for studying the presence or absence of correlations between enzymic activities and cholestasis since the model involves two components (manganese and bilirubin) that are not cholestatic when given alone but result in cholestasis when given together. Furthermore, the cholestatic response of the combination can be prevented if a third substance, BSP, is added to the combination. Thus, one can study the enzymic responses to the individual components, to the combination, and during the presence or absence of cholestasis. 5′-Nucleotidase, LAP and the ATPases were frequently depressed even when bile flow was normal (example: BR 60 min, Mn 15 min, Mn–BSP–BR). Furthermore, when Mn–BR cholestasis was prevented by the coadministration of BSP, the depressed enzymic activities did not always return to normal. Thus there was a lack of correlation between enzymic activities and cholestasis.

When manganese alone was administered, 5′-nucleotidase and Mg^{2+}-ATPase were partly inhibited 15 min after injection (Mn 15 min), but they were almost restored 60 min later (Mn 75 min). With this dose of manganese (4.5 mg/kg), bilirubin must be injected 15 min after manganese to elicit a maximum cholastatic response; if bilirubin injection is delayed 60 min, bile flow is not affected by the treatment

Table 4. Effect of phalloidin pretreatment on the decrease of bile flow observed after manganese–bilirubin combination*

Days of pretreatment	Decrease of bile flow 60 min after bilirubin injection (% of initial bile flow)
0	78.6 ± 3.3
1	52.3 ± 13.6
2	18.2 ± 8.5
3	11.3 ± 8.4

* Each value is the mean ± S.E. for four to eight rats. Phalloidin was injected (i.p.), 500 $\mu g \cdot kg^{-1} \cdot day^{-1}$. Statistical differences ($P \leq 0.05$) can be expressed: 0 day > 1 day > 2 days = 3 days. Manganese (4.5 mg/kg) was injected 15 min before bilirubin (25 mg/kg).

[7, 9]. Thus, a parallelism seems to exist between the moment at which manganese, by itself, produces maximum effects on these enzymic activities and the moment at which bilirubin should be injected for the induction of cholestasis.

Our most striking finding, however, was not related to the depression of the specific activity of enzymes but to a profound shift in the recovery of protein in isolated liver plasma membrane fractions. The shift was from the canalicular (BCM) to the sinusoidal (PM) fraction. Decreases in BCM recovery have previously been reported after the administration of a few cholestatic substances: cytochalasin B, norethandrolone, and lithocholic acid (decreases of 28, 34 and 25 per cent respectively) [14, 15]. Tavolini *et al.* [13] also noted, in isolated rat livers perfused with chlorpromazine, that the recovery of BCM was lessened by 46 per cent but that of PM was increased by 213 per cent, total recovery (BCM + PM) being slightly increased. With the Mn–BR model we observed that a short cessation of bile flow (Mn–BR, BDL 60 min, or BR–BDL 60 min) caused an increase in total (BCM + PM) protein recovery (240, 172 and 217 per cent respectively). Mn–BR intrahepatic cholestasis was, however, the only treatment that affected the PM/BCM ratio; BCM recovery decreased (34 per cent), whereas PM recovery increased (240 per cent). Neither enzymic activities nor electron microscopy indicated a possible contamination of PM fraction by canalicular membranes. Therefore, the observed change in the PM/BCM ratio may have been due to an important structural alteration of the canalicular membrane leading to an increase in density. This structural modification would not have been the consequence of the decreased bile flow since bile duct ligation did not induce such a change. It rather appears to have been an event preceding, or simultaneous to, the intrahepatic cholestasis and, therefore, it could possibly be its cause.

The hypothesis of a structural modification of canalicular membranes is supported by the fact that the BCM fraction obtained after Mn–BR treatment was yellow in color; this color was readily extracted by chloroform but not by an aqueous medium. We therefore suppose it was due to bilirubin, solubilized or attached, in some way, to membrane lipids. Furthermore, since the yellow color was observed only after the manganese treatment preceded the bilirubin load, it appears that manganese must first induce a modification of these membranes to permit bilirubin incorporation. We hypothesize therefore that a manganese-induced intermediate is formed at the canalicular membrane level, probably in the lipid layer; this modification would possibly permit bilirubin incorporation and finally lead to cholestasis. Bilirubin–lipid association in BCM appears reasonable. This kind of association has already been observed in a macromolecular aggregate, isolated from human gall bladder and hepatic bile, and is constituted of bile pigments, cholesterol and phospholipids [37]. It is possible that a manganese-induced alteration permits binding of bilirubin to membrane lipids; the total Mn–lipids–BR interaction would hinder normal bile secretory function. Modification of membrane lipid layers has been observed in other models of cholestasis [14, 15]. We have not yet analyzed membrane lipids of our BCM and PM fractions, but we can expect changes after the Mn–BR treatment since the BCM fraction is not well separated from the PM fraction and probably contains bilirubin. Manganese might induce a slight change in BCM lipids and thus facilitate bilirubin incorporation.

The phalloidin experiments, although preliminary and limited in scope, provide additional support to the view that manganese acts on hepatocellular membranes. Mn–BR cholestasis was less severe in phalloidin-pretreated rats; the protective effect seemed related to the duration of the pretreatment and was partially reversed by increasing the manganese load from 4.5 to 6.0 mg/kg. The mechanism of this protection is as yet unknown, but we can suppose that: (1) it is mediated by the microfilament network since the protective effect increases in a parallel manner to the phalloidin-induced thickening of this network [17]; (2) it occurs on the manganese component of the Mn–BR interaction since metals are known to act on these microfilaments and since the protective effect could be reversed by an increase in manganese dose; and (3) it could also affect the bilirubin component of the Mn–BR interaction, since with the isolated rat liver preparation clearance of bilirubin is blocked when phalloidin is added to the perfusate [38]. It seems unlikely, however, that decreased bilirubin uptake would be the dominant factor in the protective effect observed in our experiments.

In conclusion, these studies show that the activities of 5′-nucleotidase, LAP, and ATPase, of both the BCM and the PM fractions, can be easily depressed even if bile flow is normal and therefore are not good indicators of altered bile secretory function. The major effect of Mn–BR interaction, possibly leading to cholestasis, was a modification of BCM; there was an important shift in the recovery of proteins in isolated liver plasma membrane fractions; the shift was from the canalicular to the sinusoidal fraction. Furthermore, the BCM fraction was yellow and may have contained bilirubin. We suppose that manganese induces a slight modification of the membrane lipid layer that permits bilirubin incorporation and subsequent cholestasis. The progressive and reversi-

ble protection afforded by phalloidin pretreatment is also indicative of a manganese–membrane interaction.

Acknowledgements—This work was supported by a grant and a studentship from the Medical Research Council of Canada. The expert technical assistance of Miss Thérèse Vaillancourt is gratefully acknowledged.

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