Biochemical Pharmacology, Vol. 34, No. 21, pp. 3857–3865, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

HEPATIC SUBCELLULAR DISTRIBUTION OF MANGANESE IN MANGANESE AND MANGANESE–BILIRUBIN INDUCED CHOLESTASIS

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(*Received* 7 *December* 1984; *accepted* 22 *March* 1985)

Abstract—Administration of non-cholestatic doses of manganese (Mn^{2+}) followed by injection of bilirubin (BR) results in a severe reduction in rat bile flow. Male Sprague–Dawley rats were given various doses of Mn^{2+} (2, 4.5, 8, and 18 mg/kg, i.v.) and killed 0.25, 1, 3, or 5 hr later. $^{54}Mn^{2+}$ was used to evaluate Mn^{2+} content (μg/g protein) in different liver fractions: homogenate, mitochondria, microsomes, cytoplasm, nuclei-membrane fraction and liver cell plasma membrane fractions, one containing bile canalicular complexes (LCPM-BCM), the other containing sinusoidal membranes (LCPM-PM). In LCPM-BCM and LCPM-PM, two time-related patterns of Mn^{2+} content were observed. With non-cholestatic doses (2, 4.5, and 8 mg/kg), Mn^{2+} content decreased with time and rarely exceeded 50 μg/g protein. With 18 mg/kg (a cholestatic dose), Mn^{2+} content increased with time and reached values over 100 μg/g protein (3–5 hr), reflecting possible modification in membrane structure. BR caused a marked increase in Mn^{2+} content (at a dose of 4.5 mg Mn^{2+}/kg) in LCPM-BCM (240%), approaching values seen with 18 mg Mn^{2+}/kg, whereas in LCPM-PM it was less striking (50%). These and other results obtained with various treatments (cholestatic and non-cholestatic) suggest than Mn^{2+} concentration in bile canalicular membranes is a critical factor in both forms of cholestasis, and that BR can facilitate Mn^{2+} incorporation in the bile canalicular membrane.

In the rat, manganese administration in large doses induces intrahepatic cholestasis and mild focal necrosis [1, 2]. If a bilirubin infusion follows the administration of manganese, the resulting necrosis and diminution of bile flow are both more severe [3, 4]; the cytological effect persists, while the cholestasis is reversible. Although the ultrastructural changes observed resemble some of those seen in human cholestasis [5], the necrotic feature restricts the utility of this model for studying chemically induced cholestasis.

In the manganese–bilirubin (Mn^{2+}-BR) model characterized in our laboratory, a non-cholestatic (and non-necrotic) dose of manganese followed by bilirubin results in a severe, but reversible diminution in bile flow, but necrosis is absent [6, 7]. This response is dose dependent for bilirubin but not for manganese, and a critical time interval exists during which the bilirubin must be administered [6, 8]. Cytological changes are synchronous with the onset and recovery of cholestasis [7]. The dose of manganese influences the critical time interval after manganese administration during which one can inject bilirubin and elicit a cholestatic response [6]. Sulfobromophthalein (BSP) can afford protection against Mn^{2+}-BR cholestasis [9, 10]. The magnitude of the protection appears to be related to the amount of BSP remaining unexcreted at the time the bilirubin is injected [6].

de Lamirande and Plaa [6] proposed that an intermediate step is involved between manganese administration and the subsequent interaction with bilirubin to eventually lead to cholestasis. Since metals are known to affect membrane function, they investigated the possibility that the canalicular membrane is the site of action [11]. No correlation was found between the cholestatic treatment (Mn^{2+}-BR) and the various enzymic activities measured in membrane fractions. However, the isolated bile canalicular-enriched membranes (LCPM-BCM) were yellow in color, and an important shift in the recovery of proteins (canalicular to sinusoidal) occurred. This stimulated further research, and led to the following hypothetical sequence: Mn^{2+} → canalicular membrane alteration → BR incorporation → cholestasis. Further evidence of physical alteration of the bile canalicular membrane was noted: Mn^{2+} alone produces important increases in LCPM-BCM cholesterol and phospholipid contents; bilirubin enters or binds to the LCPM-BCM in the combined Mn^{2+}-BR treatment [12].

There is only limited information available regarding manganese distribution in the hepatocyte. Maynard and Cotzias [13] and Klaassen [14] observed high affinity of the mitochondrial fraction for manganese, but it also distributes in other subcellular fractions. However, membrane preparations were not isolated in those studies. This information is of interest since membrane modification seems to be an important feature of this experimental model of cholestasis.

The present study focuses on the manganese component of the cholestatic Mn^{2+}-BR combination. In

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an attempt to localize the site of action, subcellular distribution of this metal in the rat liver was investigated, using various doses of manganese, ranging from non-cholestatic (2, 4.5, and 8 mg/kg) to cholestatic and necrotic (18 mg/kg) [1, 6–8]. The distribution was also studied when bilirubin (various doses) was injected (Mn^{2+}-BR); when protection was afforded by BSP administration (Mn^{2+}-BSP-BR); after recovery from Mn^{2+}-BR cholestasis; when bilirubin was injected after the critical time interval; and when bilirubin was administered before manganese (BR-Mn^{2+}).

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (Charles River Canada Inc., St. Constant, Québec), weighing 250–300 g, were maintained on Charles River Rat Chow and water *ad lib.* The animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and a catheter (PE-50 tubing) was placed in a femoral vein. Anesthesia was maintained throughout the treatments, except for the animals receiving manganese at 8 and 18 mg/kg. The combination of anesthesia and these high doses is frequently lethal; these rats were injected in a caudal vein and were anesthetized 15 min before liver perfusion. 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 fluctuations. Even though manganese excretion is not affected markedly by alteration of body temperature [14], bilirubin, BSP excretion rate and bile flow exhibit temperature dependency [15].

Injectable materials. Monohydrated manganese sulfate ($MnSO_4 \cdot H_2O$) was dissolved in 0.9% NaCl and injected intravenously at a dose of 2, 4.5, 8 or 18 mg Mn^{2+}/kg. A tracer amount of $^{54}MnCl_2$ (carrier free; New England Nuclear, Canada) was added; each animal received 4–8 μCi. The solution was injected over a 2-min period. 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 over a 2-min period, at a dose of 15, 25 or 35 mg/kg. 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. 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.

Treatments. In the first series of experiments, animals received manganese at the doses mentioned above (2–18 mg/kg) and were killed 0.25, 1, 3 or 5 hr later. The liver was then fractionated to evaluate Mn^{2+} content in each subcellular fraction. In a second series of experiments, we studied the distribution of the metal under various conditions. Mn^{2+}-BR induced cholestasis occurs in the following manner [6, 8]: manganese is first administered, followed by bilirubin injection 15 min later; the cholestatic effect reaches its maximal intensity 60 min after BR injection, although the onset occurs sooner; BSP must be injected 10 min before bilirubin to prevent cholestasis [6, 8]. We elected to look at the Mn^{2+} distribution 60 min after Mn^{2+} injection, while the effect is being generated. Taking this scheme into consideration, subcellular fractions were isolated after different experimental situations: (I) Mn^{2+} (4.5 mg/kg) alone (control); (II) Mn^{2+}-BR (15 mg/kg); (III) Mn^{2+}-BR (25 mg/kg); (IV) Mn^{2+}-BR (35 mg/kg); and (V) Mn^{2+}-BSP-BR (25 mg/kg). Finally, three other situations were studied: (VI) BR (25 mg/kg) injected 15 min before Mn^{2+}; (VII) Mn^{2+} followed 15 min later by BR (25 mg/kg) injection, followed by a 285-min period before sacrifice; and (VIII) an interval of 135 min between Mn^{2+} and BR (25 mg/kg) injections. Hence, situations II, III and IV look at the effect of increasing BR dosage on the metal distribution; in situation V, protection against Mn^{2+}-BR cholestasis is afforded by BSP [8]; situation VI looks at a non-cholestatic treatment, where the order of injection has been inverted [14]. In situation VII, the animals are killed when they have recovered from cholestasis [16]. Treatment VIII is a non-cholestatic treatment since BR is injected after the critical time interval [6].

Isolation of membrane fractions. Membranes (LCPM-BCM: liver cell plasma membrane containing bile canalicular membranes; LCPM-PM: liver cell plasma membrane containing sinusoidal membranes) were isolated according to the method of Yousef and Murray [17], as modified in our laboratory [11]. At designated times, the liver was perfused via the portal vein with ice-cold physiological saline (15 ml) 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% enriched with parenchymal cells, and almost all connective tissue stays in the press [18]. 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 7 for 2 sec. 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 saved for isolation of microsomes, mitochondria and cytoplasm (see below). 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% of the initial weight of the liver puree in grams. An aliquot of this suspension (nuclei-membrane fraction) was saved. A sucrose solution (d: 1.26) was added in sufficient quantity to give a final density of 1.22 (5.5 times the volume of the original pellet-buffer suspension). After thorough mixing, aliquots (15 ml) of the membrane suspension (d: 1.22) were put into ultracentrifuge tubes over-layered with 7 ml 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 (LCPM-BCM) and at the lower 1.22 to 1.18 interface

(LCPM-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 7000 *g* (7500 rpm) for 20 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 was then suspended in 2 ml of buffer. The sinusoidal fraction (1.22–1.18 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:

$$V_{1.26}\frac{1.22-(W/V)}{0.04}V$$

After thorough mixing, all manipulations, the sucrose gradient, ultracentrifugation, harvesting and washings were performed again.

Mitochondrial fraction isolation. The supernatant fraction saved from the first centrifugation (1000 *g*) was centrifuged at 12,000 *g* (10,000 rpm) for 10 min. The pellets were resuspended in 30 ml of the $NaHCO_3$–$CaCl_2$ buffer and recentrifuged in the same conditions. The final pellet was suspended in 15 ml of the buffer.

Isolation of microsomal and cytoplasmic fractions. The microsomal fraction was isolated according to the method of Cinti *et al.* [19]. To the supernatant fraction obtained from the post-mitochondrial centrifugation was added $CaCl_2$ to a final concentration of 10 mM. The supernatant fraction was centrifuged at 27,000 *g* (15,000 rpm) for 15 min; the supernatant obtained from this centrifugation contains the cell soluble fraction. The pellets were gently washed with 5 ml of Tris (0.02 M)–KCl (0.15 M), pH 7.4, buffer, combined and suspended in 15 ml of the same buffer, and recentrifuged at 27,000 *g* for 15 min. The pellets were washed again with 5 ml of the buffer, combined, and suspended in 15 ml of the Tris–KCl buffer.

$^{54}Mn^{2+}$ quantification. The concentration of $^{54}Mn^{2+}$ in the various samples was determined in a Packard Auto-Gamma spectrometer.

Protein content. The concentration of protein in each subcellular fraction was determined by the method of Lowry *et al.* [20], as modified by Peterson [21], using crystalline bovine serum albumin as the standard.

Characterization of membrane fractions. To evaluate the purity and comparability of our LCPM-BCM and LCPM-PM fractions, the following enzymic activities were measured: glucose-6-phosphatase [22], 5′-nucelotidase [23], and leucyl-β-naphthylamidase (LAP) [24]. All incubations were performed in a Dubnoff metabolic shaker. Inorganic phosphorus (P_i) was determined by the method of Fiske and Subbarow [25]. The purity of the membrane fractions was verified by electron microscopy.

Statistics. Data were submitted to an analysis of variance. When multiple comparisons were required, treatment means were subsequently tested using the Student–Newman–Keuls procedure [26]. The 0.05 level of probability ($P < 0.05$) was used as the criterion of significance.

Table 1. Enzyme activities of liver cell plasma membrane fractions*

Liver fraction	Control			Mn^{2+}-BR		
	Glucose-6-phosphatase (8)	5′-Nucleotidase (8)	Aminopeptidase (4)	Glucose-6-phosphatase (2)	5′-Nucleotidase (6)	Aminopeptidase (6)
Homogenate	3.6 ± 1.0	2.3 ± 0.6	0.59 ± 0.04	2.9 ± 0.6	3.4 ± 0.7	0.45 ± 0.02
LCPM-BCM	1.5 ± 0.2	65.6 ± 5.8	5.25 ± 0.80	1.3 ± 0.1	59.9 ± 4.0	4.29 ± 0.12
LCPM-PM	3.0 ± 0.2	32.3 ± 5.8	2.04 ± 0.20	3.5 ± 0.1	31.4 ± 3.3	1.63 ± 0.26

* Values are means ± S.E., calculated from (N) animals. Mn^{2+}-BR treatment is described in Methods. Glucose-6-phosphatase and 5′-nucleotidase 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.

RESULTS

Characterization of membrane fractions. Table 1 shows enzyme activities found in the homogenate and in both LCPM-BCM and LCPM-PM fractions isolated from control animals. Enzyme activities listed fall within the wide range reported by other investigators for aminopeptidase (0.96 to 9.40 μg β-naphthylamine/mg protein/hr), 5′-nucleotidase (45.7 to 92.2 μmoles P_i/mg protein/hr), and glucose-6-phosphatase (0.2 to 1.8 μmoles P_i/mg protein/hr) of LCPM-BCM isolated by similar techniques [27–31]. Our LCPM-PM fraction showed higher enzymic activities for aminopeptidase and 5′-nucleotidase than those reported by Yousef and Murray [17], which suggests some contamination by canalicular membranes. Glucose-6-phosphatase activity was somewhat elevated and may reflect microsomal contamination. Electron microscopic examination of control membrane preparations was in agreement with the enzyme profile. The LCPM-BCM fraction revealed intact bile canaliculi and tight junctions; no microsomal nor mitochondrial contamination was noted in that fraction. The LCPM-PM fraction showed mainly vesicles and membrane sheets; tight junctions and endoplasmic reticulum were seen occasionally.

Activities of aminopeptidase, glucose-6-phosphatase and 5′-nucleotidase were also measured in fractions isolated from Mn^{2+}-BR-treated animals (Table 1). Values obtained for the treated animals did not differ significantly from control values, thus permitting comparisons between these groups.

Affinity of the subcellular fractions for manganese. Figure 1 shows manganese content (μg Mn^{2+}/g protein) found in each subcellular fraction at various times after manganese administration (8 mg/kg). Mitochondrial, nuceli-membrane and cytoplasmic fractions showed the highest relative affinity for manganese (similar to that of the homogenate), followed by the microsomal fraction and finally by the LCPM-PM and LCPM-BCM fractions. Klaassen [14] found the highest manganese concentration in the 600 *g* (nuclei-membrane) and in the 10,000 *g* (mitochondrial) fractions, following 10 mg Mn^{2+}/kg, but the manganese content in their cytoplasmic fraction was much lower. This difference could be explained by the added manipulations required in our study for isolation of the membrane fractions. Some redistribution of the metal may have occurred, leading to a higher cytoplasmic content. The same relative order of affinity was observed with the other manganese doses (2, 4.5 and 18 mg/kg) employed (data not shown).

Influence of manganese dose and time on the manganese content in subcellular fractions. Figure 2 illustrates the variation of manganese content (μg/g protein) with time and dose for the total homogenate. Similar curves were obtained for microsomal, mitochondrial, cytoplasmic and nuclei-membrane fractions (data not shown). Maximal manganese content in these fractions was reached within the first hour and, by 5 hr, levels were reduced markedly to about 15–25% of the 15-min value, except for the 18 mg/kg dose for which elimination of the metal was slower (at 5 hr, >50% of the 15-min value).

In contrast, LCPM-BCM and LCPM-PM fractions exhibited a totally different behaviour from that of the homogenate and all other fractions. Figure 3 shows how manganese content (μg/g protein) varied with time and dose in the LCPM-BCM fraction. The three lower doses (2, 4.5 and 8 mg/kg) yielded values ranging from 10 to 48 μg Mn^{2+}/g protein, decreasing with time, while the highest dose (18 mg/kg) produced a disproportionate rise of manganese content, over 100 μg/g protein after 1 hr and increasing with time to reach 140 μg/g protein after 5 hr. Thus, manganese accumulates markedly in the LCPM-BCM fraction when the 18 mg/kg dose (cholestatic) is administered, whereas it is at a low level when non-cholestatic doses are used.

Figure 4 illustrates the results obtained for the

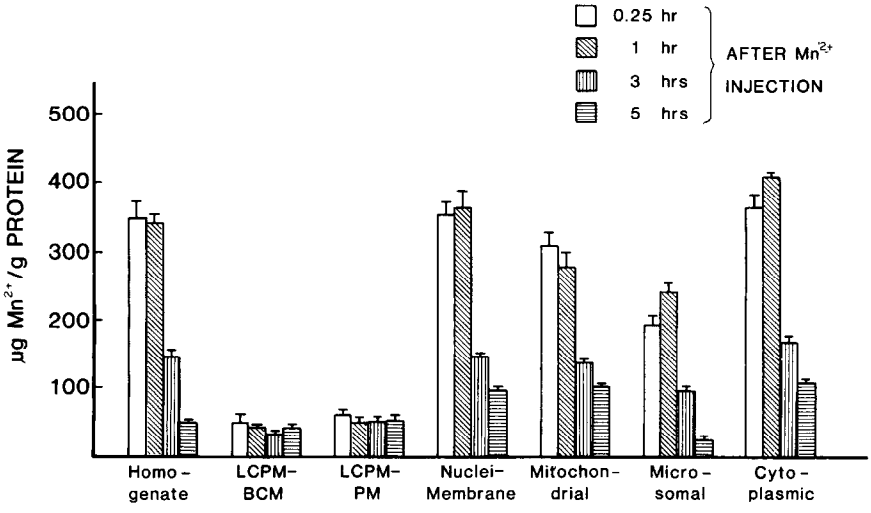

Fig. 1. Manganese content (μg Mn^{2+}/g protein) of the subcellular fractions at various times following administration of manganese (8 mg/kg). Values are expressed as the mean ± S.E.; four rats were used in each group.

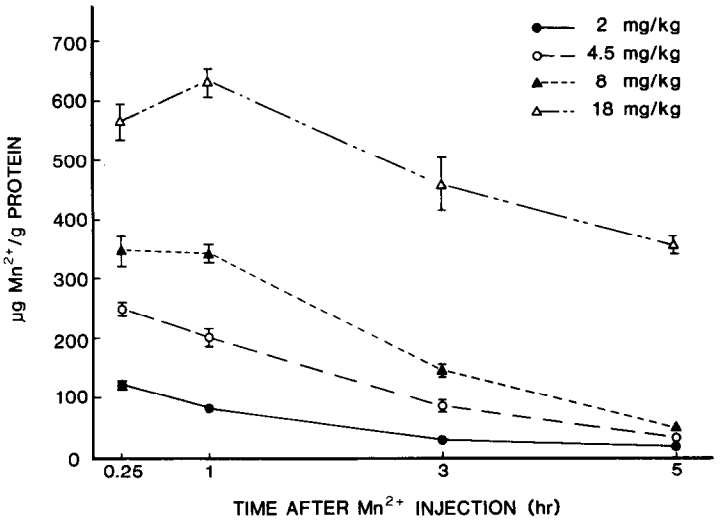

Fig. 2. Variation in manganese content of the homogenate with time and dose. Values are means ± S.E. determined in four rats.

LCPM-PM fraction. Dose–time relationships similar to those described for the LCPM-BCM fraction were observed for this fraction. Again, the high dose (18 mg/kg) produced accumulation of the metal, while with lower doses values were low and decreased with time. Globally, administration of manganese at a high dose (18 mg/kg) resulted in an accumulation of manganese in both fractions of the hepatocellular membrane.

Influence of sulfobromophthalein and bilirubin on manganese content in subcellular fractions. Table 2 shows manganese content values in subcellular fractions following various treatments: (1) Mn^{2+}: administration of manganese alone; (2) Mn^{2+}-BR: a cholestatic treatment; and (3) Mn^{2+}-BSP-BR: where protection from the Mn^{2+}-BR cholestasis is afforded by BSP. No significant treatment-related changes were noted in the cell fractions, except for the LCPM-BCM fraction. Administration of bilirubin 15 min after manganese produced a significant increase (3-fold; $P < 0.05$) in LCPM-BCM manganese content. This interaction is quite striking since Mn^{2+} content in the LCPM-BCM fraction with a low dose of Mn^{2+} (4.5 mg/kg) in the presence of bilirubin

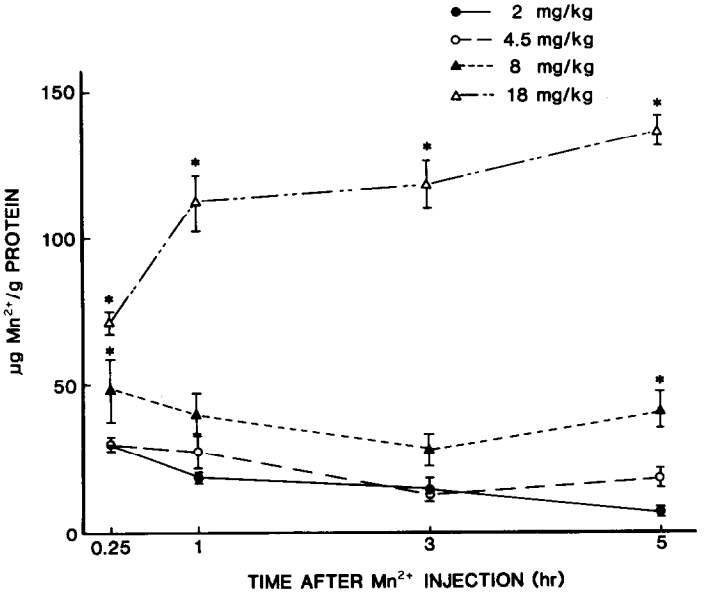

Fig. 3. Variation in manganese content of the liver cell plasma membrane fraction containing bile canalicular complexes (LCPM-BCM) with time and dose. Values are means ± S.E. determined in four rats. An asterisk (*) indicates significantly different ($P < 0.05$) from the mean of all values of lower manganese content, at the same post-injection time.

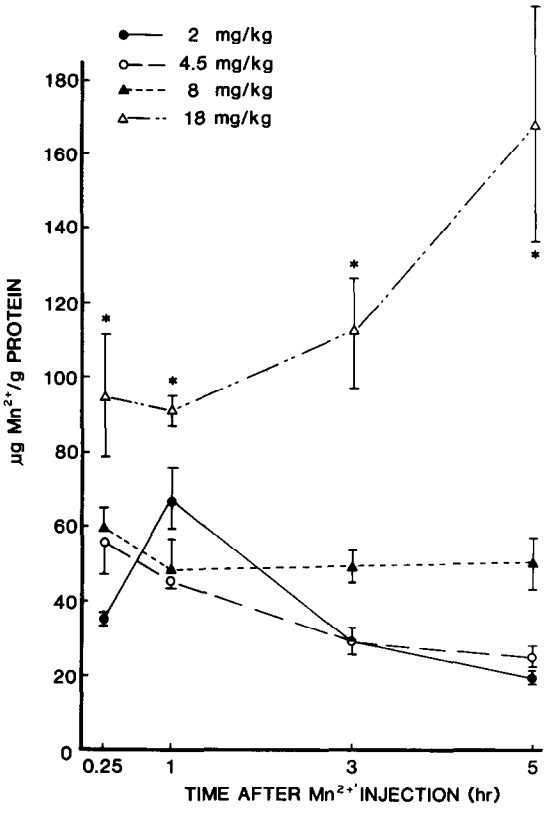

Fig. 4. Variation in manganese content of the liver cell plasma membrane fraction containing sinusoidal membranes (LCPM-PM) with time and dose. Values are means ± S.E. determined in four rats. An asterisk (*) indicates significantly different ($P < 0.05$) from the mean of all values of lower manganese content, at the same post-injection time.

attained levels that were comparable to those observed with a cholestatic dose of 18 mg Mn^{2+}/kg (Fig. 2). When BSP injection was inserted between manganese and bilirubin administrations (Mn^{2+}-BSP-BR), the increase in LCPM-BCM manganese content was partially blocked (36% lower than corresponding Mn^{2+}-BR value; $P < 0.05$). Thus, BSP, which provides protection against Mn^{2+}-BR cholestasis, reduces also the bilirubin enhancement of Mn^{2+} passage into LCPM-BCM.

The relationship between cholestasis and LCPM-BCM content was further investigated in other experiments. In these experiments, we varied the treatment schedules (agents, doses, treatment times and time of sacrifice) so that we would have both cholestatic and non-cholestatic situations. The treatment protocols are depicted in Fig. 5.

Table 3 shows manganese content values in LCPM-BCM and LCPM-PM fractions when the treatments depicted in Fig. 5 were administered. We had shown [6] that, when bilirubin is administered more than 120 min after manganese (4.5 mg/kg), cholestasis does not occur. Under these conditions (treatment VIII), we observed a low manganese content in the LCPM-BCM fraction, similar to the one observed with manganese alone and well under ($P < 0.05$) the values associated with the Mn^{2+}-BR cholestatic treatments III and IV. Thus, when bilirubin is injected in a time sequence that does not lead to cholestasis, the manganese content in the LCPM-BCM fraction remains at a low level.

One interesting feature of this model is the reversibility of the cholestasis. Four hours after injection of manganese and bilirubin (treatment VII), cholestasis is no longer present [16]; manganese content values are accordingly low ($P < 0.05$ versus III and IV), again similar to the one observed when the small dose of manganese was administered alone. Klaassen

Table 2. Manganese content of the subcellular fractions*

	Manganese (µg/g protein)		
	Treatment		
Fraction	Mn^{2+}	Mn^{2+}-BR	Mn^{2+}-BSP-BR
Homogenate	200 ± 16	195 ± 15	200 ± 14
LCPM-BCM	28 ± 6†	93 ± 5†	59 ± 3†
LCPM-PM	45 ± 1	64 ± 7	57 ± 7
Nuclei-membrane	187 ± 12	205 ± 19	181 ± 6
Mitochondria	156 ± 10	160 ± 7	178 ± 11
Microsomes	109 ± 6	115 ± 6	118 ± 7
Cytoplasm	196 ± 12	208 ± 17	218 ± 15
Cholestasis	Absent	Severe	Mild

* Values are expressed as the mean ± S.E. determined in four experiments. Mn^{2+}: Rats received manganese (4.5 mg/kg) and were killed 60 min later. Mn^{2+}-BR: Rats received bilirubin (25 mg/kg) 15 min after manganese, and were killed 45 min after bilirubin injection. Mn^{2+}-BSP-BR: Rats received BSP (15 mg/kg) 5 min after manganese and were given bilirubin 10 min later; they were killed 45 min after bilirubin injection.

† Significantly different ($P < 0.05$) from all other values listed horizontally.

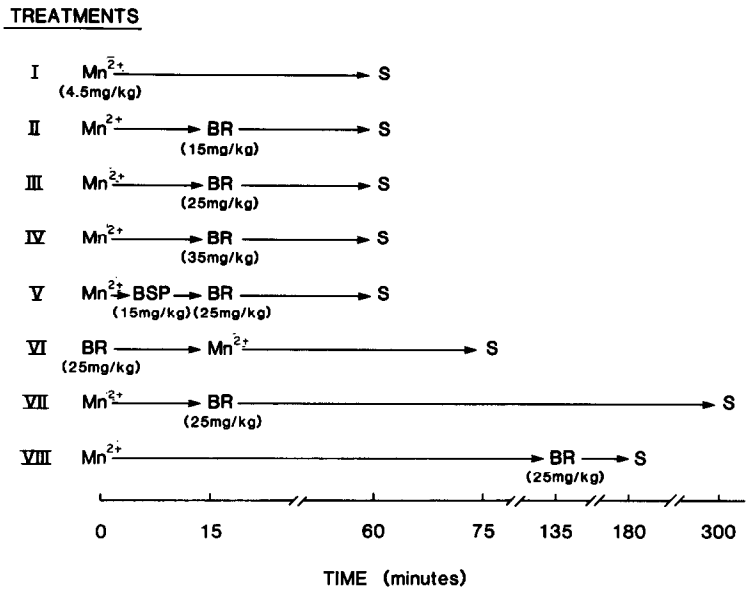

Fig. 5. Treatment protocols. Results obtained using these treatments are shown in Table 3. Abbreviations: Mn^{2+}, manganese; BR, bilirubin; BSP, sulfobromophthalein; and S, sacrifice.

[14] showed that injecting bilirubin before manganese was not effective in producing cholestasis. Under these conditions (treatment VI), manganese level in LCPM-BCM was not different than the one observed with manganese alone. All the non-cholestatic treatments (I, VI, VII, and VIII) were associated with low manganese values in the LCPM-BCM fraction and were significantly smaller ($P < 0.05$) than the values observed with the Mn^{2+}-BR treatments III and IV.

We previously demonstrated [8] a relationship between the severity of the cholestasis and the bilirubin dose administered. With the 15 mg/kg bilirubin dose (treatment II), Mn^{2+} content in LCPM-BCM was higher than with manganese alone, but was not statistically different from non-cholestatic treatments (I, VI, VIII). This bilirubin dose is not sufficient to produce a significant fall in bile flow. Doses of 25 and 35 mg Mn^{2+}/kg (treatments III and IV) both produced high manganese content in the LCPM-BCM fraction ($P < 0.05$ versus I and II); these bilirubin doses produce a severe cholestasis when injected 15 min after manganese [6]. With the 35 mg/kg dose (treatment IV), the selectivity for the LCPM-BCM fraction was lost and manganese content was significantly greater ($P < 0.05$) in the LCPM-PM fraction. These experiments were interpreted to indicate that manganese incorporation in LCPM-BCM is likely to be a key event in Mn^{2+}-BR induced cholestasis.

An additional experiment was later performed to assess whether or not the increase in manganese content produced by the Mn^{2+}-BR treatment in the LCPM-BCM fraction was simply a secondary effect due to the presence of bile stasis, and not a causal event. To respect the time patterns used in the other experiments, four rats were first treated with manganese (4.5 mg/kg), and 15 min later the common bile duct was ligated. After a 45-min period of obstruction the animals were killed, the liver homogenized, and membrane fractions assessed for manganese content. The following results were obtained: LCPM-BCM, 59 ± 5 μg Mn^{2+}/mg protein; LCPM-PM, 55 ± 8 μg/mg protein. These values are comparable to those

Table 3. Manganese content of the liver cell plasma membrane fractions*

	Manganese (μg/g protein)							
	Treatment							
Liver fraction	I	II	III	IV	V	VI	VII	VIII
Homogenate	200 ± 16	220 ± 10	195 ± 15	222 ± 4	200 ± 14	145 ± 4	38 ± 5	56 ± 4
LCPM-BCM	28 ± 6†‡	46 ± 4†§	93 ± 5‖	82 ± 4‖	59 ± 3§	32 ± 4†‡	19 ± 3‡	39 ± 5†§
LCPM-PM	45 ± 1†‡	48 ± 3†‡	64 ± 7§	86 ± 4‖	57 ± 7†§	37 ± 3‡	32 ± 4‡	41 ± 3†‡
Cholestasis	Absent	Absent	Severe	Severe	Mild	Absent	Absent	Absent

* Values are expressed as the mean ± S.E. determined in four experiments. See Fig. 5 for treatment protocols.
†,‡,§,‖ Significant differences ($P < 0.05$) exist between LCPM means identified with different symbols. Comparisons are made horizontally.

obtained with non-cholestatic regimens (Groups II and V, Table 3) and are smaller than those observed in LCPM-BCM during cholestasis (Groups III and IV, Table 3). Thus, the accumulation of manganese in LCPM-BCM observed in the earlier experiments does not appear to be a secondary effect of cholestasis itself.

DISCUSSION

These studies on manganese distribution in the hepatocyte were undertaken in order to localize the site of action of this metal, leading to the putative intermediate that would react with bilirubin and cause cholestasis [6], or, if administered in sufficient quantity, cause cholestasis and necrosis on its own [1].

In the first series of experiments involving manganese alone, only the membrane fractions (LCPM-BCM and LCPM-PM) showed modification of manganese content correlating with the biological effect of the manganese dose administered. We feel that the disproportionate increase of manganese content with time in both fractions when the 18 mg/kg dose is used could be critical in the sequence of events leading to both cholestatic and necrotic phenomena. Manganese accumulation is already important at 1 hr; it may be a primary event since both cholestasis and necrosis are evident only 4 hr after administration [1]. Following i.v. administration, manganese is readily taken up by the liver and is mainly excreted by the biliary route [14, 32]. We might speculate that with low manganese dose, most of Mn^{2+} is bound to intracellular sites. Raising the dose to 18 mg/kg might cause saturation of intracellular sites and free cytoplasmic Mn^{2+} should then accumulate. The free cation could interact with both the canalicular and the sinusoidal membranes and induce structural and/or functional changes, leading to both necrosis and cholestasis.

It is possible that the presence of Mn^{2+} at the membrane level would change membrane fluidity, thereby affecting its permeability. This possibility appears interesting since other divalent cations have been shown to modify membrane function. Most important is calcium ion. It can interact directly with phospholipids in model bilayers [33, 34] and restricts motional freedom or lipid fluidity. It was observed recently that incubating hepatocyte plasma membranes in calcium solutions ranging from 0 to 4 mM alters membrane fluidity as indicated by polarized fluorescence studies, by both direct (Ca^{2+} binding), and indirect mechanisms (Ca^{2+} triggered lipid modification) [35, 36]. Other divalent cations were studied, and it was concluded that this effect was specific for calcium. However, manganese was not investigated, and it is possible that manganese could have a similar action on membrane lipid dynamics.

Recently, Oelberg *et al.* [37] related bile salt capacity to induce cholestasis to their equilibrium constant of complex formation with calcium. They hypothesize that cholestatic bile salts could carry and release Ca^{2+} in the hepatocyte, thus leading to cytotoxic phenomena. In another study, Oelberg *et al.* [38] observed that cholestatic bile salts caused *in vitro* augmentation of Ca^{2+} content in red blood cells (by opening Ca^{2+} permeating channels), and that Ca^{2+} content and hemolysis correlated consistently. They postulated this mechanism for other forms of bile salt toxicity. Excess Ca^{2+} uptake is a postulated general mechanism for toxicity. Since Mn^{2+} is concentrated in the liver, it may mimic rises in intrahepatic calcium.

In the second series of experiments, we saw that, when bilirubin was injected following the proper dose and time requirements, there was a striking elevation of manganese content in LCPM-BCM. Furthermore, experiments conducted under various conditions (cholestatic and non-cholestatic) suggest that a relation exists between manganese content in the LCPM-BCM fraction and the presence of cholestasis. Thus, bilirubin appears to facilitate manganese incorporation into the canalicular membrane selectively. When bilirubin was injected at a high dose (35 mg/kg), this selectivity was lessened and LCPM-PM fraction showed higher manganese content. Bile duct ligation failed to produce comparable increases in manganese content in the LCPM-BCM fraction. The mechanical obstruction produced only a moderate increase of manganese content, in both LCPM-BCM and LCPM-PM fractions. Thus, manganese accumulation in LCPM-BCM observed with the Mn^{2+}-BR treatment does not appear to be an effect secondary to bile stasis.

Previous studies in our laboratory have also provided evidence that the canalicular membrane is the likely site where the manganese–bilirubin interaction takes place. The more important observations are bilirubin incorporation in LCPM-BCM fraction [12], a shift in protein recovery from LCPM-BCM to LCPM-PM observed with the Mn^{2+}-BR treatment [11], an alteration in membrane lipids [12] and protection afforded by phalloidin [11], which produces a thickening of the pericanalicular microfilament network. Ideally, one would wish to use pure bile canalicular and sinusoidal membranes for such studies. Unfortunately, while a number of different fractionation procedures are available, none can be said with certainty to provide pure isolated intact membranes representative of the situation *in vivo*. Although our LCPM-BCM preparations are merely "enriched" in regard to the canalicular complexes, and not pure fractions, we elected to utilize this procedure so that comparisons could be effected with our previous studies [11, 12]. We assume that pure canalicular membranes would react like our LCPM-BCM fractions, but we recognize the inherent limitations of our procedure.

One possible mechanism that could account for the observed manganese incorporation in the LCPM-BCM fraction when bilirubin is administered would be the formation of a Mn^{2+}-BR complex, as suggested previously by Klaassen [14]. Bilirubin is known to form complexes with divalent cations, including manganese [3, 39]. Fifteen minutes after manganese administration, there would be enough manganese in the hepatocyte to assure sufficient Mn^{2+}-BR complex to form; this could be transported to the canalicular locus of the hepatocyte membrane, where it can bind or incorporate, leading to alteration in membrane function. Alternatively, the complex could be formed directly at the canalicular membrane

level, where it would precipitate and modify membrane function. Another possibility would be that bilirubin interferes with manganese excretion or enhances uptake by the liver, which would raise the effective concentration of manganese in the hepatocyte. The latter seems unlikely since manganese content in total homogenate is not modified by bilirubin administration (Table 2). BSP could exert its protective effect by either preventing the formation of the Mn^{2+}-BR complex, or by blocking its action on the canalicular membrane. We observed that BSP blocked partially manganese incorporation in the LCPM-BCM fraction. Previous studies demonstrated a similar effect of BSP on bilirubin incorporation in this fraction [12]. Their presence in the canalicular membrane thus seems closely related. Studies are presently underway to evaluate the effect of these treatments on membrane fluidity and membrane permeability.

The experimental results demonstrate that manganese administration at cholestatic doses produced accumulation of the metal in both membrane fractions. A direct effect of this divalent cation on the membrane could be related with the necrosis and cholestasis. When bilirubin was administered after a small (non-cholestatic) dose of manganese, the incorporation of this metal into the LCPM-BCM fraction was enhanced markedly, without modification in other subcellular fractions. BSP partially inhibited this effect. We propose that the cholestatic effect of the Mn^{2+}-BR combination could be caused by a Mn^{2+}-BR complex acting at the canalicular membrane level.

Acknowledgements—This work was supported by grants from the Medical Research Council of Canada, and studentships from the Fonds F.C.A.C. Québec and from the Université de Montréal. A preliminary communication was presented to the Society of Toxicology in March 1983.

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