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Role of Manganese, Bilirubin and Sulfobromophthalein in Manganese-Bilirubin Cholestasis in Rats (40189)

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Large doses of manganese administered to rats cause a mild and readily reversible intrahepatic cholestasis; both morphologic and physiologic alterations occur (1, 2). Although the morphologic changes generated include necrosis, other ultrastructural alterations resemble those seen in humans during cholestasis induced by drugs or chemicals. Therefore manganese-induced cholestasis could be a useful model for studying the phenomena associated with cholestasis.

If manganese overload is subsequently followed by infusions of bilirubin the lesion is more severe (3, 4) depending upon the dose of bilirubin infused (5). Klaassen (6) observed that low doses of manganese are not cholestatic in the rat but can decrease bile secretion if followed by a subsequent dose of bilirubin; however manganese is not cholestatic if the bilirubin is administered before the manganese.

Manganese–bilirubin cholestasis is prevented by the simultaneous infusion of sulfobromophthalein (BSP), but this effect is partially reversed by increasing the bilirubin load (7, 8). Measurement of manganese excretion and of bilirubin concentrations in blood, liver and bile, in cholestasis and with BSP protection, indicate that neither bilirubin nor manganese concentrations at these sites is the determinant factor in the induction of cholestasis.

The first aim of the present study was to assure ourselves that both manganese and bilirubin are essential for inducing cholestasis and to study the type of interaction that could exist between those two substances. Secondly, we wanted to determine if protection provided by BSP infusion was limited by the dose of BSP administered or by its subsequent conjugation with glutathione.

Materials and Methods. Animals. Male Sprague–Dawley rats weighing 220–320 g were maintained on Purina Rat Chow and water *ad libitum*. For surgical preparation the animals were anesthetized with sodium pentobarbital (60 mg/kg, ip) and catheters were placed in the common bile duct (PE-10 tubing) and a femoral vein (PE-50 tubing). Pentobarbital-induced anesthesia was maintained throughout the biliary secretion studies. Body temperature was monitored via a rectal probe (YSI Thermoregulator) and maintained at 37° by means of an infrared lamp to eliminate temperature-dependent changes in bile flow (9).

Solutions were injected into the femoral vein. When giving more than one treatment, injection of a small quantity of saline (0.9% NaCl) preceded each treatment to permit washing of the cannula and to prevent contact between the various substances. Bile flow was measured volumetrically with 1.00-ml graduated pipets. The bile was collected over four 15-min periods and one 30-min period. The first sample was always used as the control value and the effects of the treatments were compared to the control. In most cases manganese was injected 15 min after cannulation, BSP was given 5 min later and bilirubin was given 15 min after the manganese. When bilirubin was given alone it was injected 15 min after the cannulation. Administration times of manganese and bilirubin were reversed in one experiment. The effect of longer time intervals between manganese and bilirubin injections was also studied.

Injectable materials. Monohydrated manganese sulphate was dissolved in saline and injected at a dose of 15 mg/kg (0.12 ml/100 g, 1.25 g/100 ml) or 7.5 mg/kg (0.12 ml/100 g, 0.625 g/100 ml) within 3 min.

Freshly prepared solution containing 0.52 g of NaCl, 0.52 g of Na_2CO_3 and 0.250 g of bilirubin per 100 ml was injected (1 ml/100 g) over a 4-min period.

Concentrated BSP solution (5 g/100 ml, Dade Laboratories) as such or after dilution in saline was injected in 1 min. Three doses of BSP were used: 6 mg/kg (0.12 ml/100 g,

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0.5 g/100 ml), 15 mg/kg (0.12 ml/100 g, 1.25 g/100 ml) and 120 mg/kg (0.24 ml/100 g, 5 g/100 ml). The two lower doses, given alone or in combination with manganese, did not cause choleresis but the highest one caused a marked increase in bile flow under the same conditions.

Synthetic BSP–GSH (BSP–glutathione) was prepared by the method of Whelan *et al.* (10) dissolved in saline and injected intravenously. The dose used was 20.8 mg/kg (0.24 ml/100 g, 0.876 g/100 ml) and equimolar to that of the 15-mg/kg dose of BSP.

Bilirubin excretion. Heirwegh *et al.* (11) used a coupling reaction of biliary pigments and diazotized ethyl anthranilate to determine conjugated bilirubin concentrations. This analysis requires a blank which contains total bile pigments and which is not diazotized. We therefore adapted the method to measure total bilirubin concentrations in bile, using this nondiazotized blank. According to Van Roy and Heirwegh (12) nondiazotized conjugated bilirubin possesses an absorption spectrum with a maximum from 420 to 455 nm. We confirmed these findings and obtained a plateau in this region for total bilirubin (Coleman Jr. Spectrophotometer) in bile samples. We selected the working wavelength to be 445 nm because the absorbance is high and in the center of the plateau. A standard curve was established (Table I) from concentrated bile obtained from bilirubin-infused rats. For each diluted bile sample, prepared by the method of Heirwegh *et al.* (11), absorbance at 445 nm of the nondiazotized sample was noted. The actual total bilirubin concentration in the diluted bile sample was determined by Nosslin's modification (13) of Jendrassik and Grof's method (14). The standard curve was linear up to an absorbance of 0.6; the slope was 0.10 $(mg/100\ ml)^{-1}\ cm^{-1}$; the ordinate was 0.015 and the correlation coefficient, r, was 0.999. Total bilirubin concentration of a 0.5-ml sample, treated and then extracted with 2.5 ml of pentan-2-one:*n*-butyl acetate (17:3, v/v) was calculated using the following equation:

$$(A445 - 0.015)/0.10\ (mg/100\ ml)^{-1}\ cm^{-1} \times (dilution/light\ path) = (BR)$$

The presence of manganese or BSP in bile did not interfere with the determination of conjugated or total bilirubin concentration. Manganese added to bile samples in similar or even higher concentrations than that found in bile after a 4.5-mg/kg manganese load (6) gave identical bilirubin concentration values than those of nontreated samples. We repeated the experiment using BSP–GSH, the predominant form of BSP in bile, and we obtained similar results.

Statistics. Statistically significant differences ($P < 0.05$) between control values and treatment values were determined by Student's t test.

Results. Tables II and III summarize the major alterations in bile flow and cumulative bilirubin excretion seen after the various treatments. The first three experiments demonstrated that neither the individual doses of manganese nor the bilirubin used affected bile flow significantly. However combination of the chemical treatments produced severe cholestasis if manganese was administered 15 min before bilirubin; cumulative bilirubin excretion fell not only because bile flow was low but also because the bilirubin concentration in bile was diminished. Therefore, both substances seem to be essential in manganese–bilirubin cholestasis. Furthermore, it seems that a dose-response relationship between manganese and the severity of cholestasis either does not exist or is extremely steep since 15 mg/kg induced a dramatic fall in bile flow whereas 7.5 mg/kg exerted no effect. If the injection times of manganese and bilirubin were reversed, bile flow and bilirubin excretion remained normal. This indicates that the manganese–bilirubin interaction pro-

TABLE I. STANDARD CURVE FOR DETERMINATION OF TOTAL BILIRUBIN CONCENTRATION.

A445[a]	0.577	0.437	0.282	0.195	0.152	0.123	0.075	0.060
(BR)[b] (mg/100 ml)	5.56	4.20	2.78	1.73	1.32	1.10	0.57	0.44

[a] Absorbance of a 0.5-ml bile sample treated, then extracted, with 2.5 ml of pentan-2-one:*n*-butyl acetate (17:3, v/v).

[b] Actual total bilirubin concentration determined by Nosslin's modification (13) of Jendrassik and Grof's method (14).

TABLE II. Bile Flow and Cumulative Bilirubin Excretion After Manganese and/or Bilirubin Treatments in Rats.

Experiment		Control	Mn[a]	BR[a]	Mn-BR	Mn-BR	BR-Mn
Treatment	15 min	nothing	15 mg/kg $MnSO_4 \cdot H_2O$	25 mg/kg BR	15 mg/kg $MnSO_4 \cdot H_2O$	7.5 mg/kg $MnSO_4 \cdot H_2O$	25 mg/kg BR
	30 min	nothing	nothing	nothing	25 mg/kg BR	25 mg/kg BR	15 mg/kg $MnSO_4 \cdot H_2O$
Time (min) vs bile flow (μl/min/100g)							
0–15		$8.54^b \pm 0.24$	8.43 ± 1.40	6.86 ± 0.54	8.40 ± 0.80	8.48 ± 0.81	8.63 ± 0.33
15–30		8.49 ± 0.20	8.44 ± 0.75	6.79 ± 0.48	8.80 ± 0.90	7.80 ± 0.83	7.89 ± 0.62
30–45		8.52 ± 0.29	8.44 ± 1.40	7.33 ± 0.68	6.90 ± 1.40	8.14 ± 0.95	8.31 ± 0.42
45–60		8.60 ± 0.25	8.36 ± 1.40	7.28 ± 0.59	$1.90^c \pm 0.50$	7.71 ± 1.05	7.74 ± 0.51
60–90		8.34 ± 0.28	8.76 ± 1.90	7.35 ± 0.45	$2.00^c \pm 0.50$	7.98 ± 0.90	6.88 ± 0.62
Animals used		3	4	4	4	4	4
Cumulative Bilirubin excretion (% dose)		–	–	77.7 ± 6.1% in 75 min	21.4 ± 2.4% in 60 min		68.7 ± 1.9% in 60 min

[a] Mn: $MnSO_4 \cdot H_2O$; BR: bilirubin.
[b] Values are means ± SE.
[c] $P < 0.05$ versus 0- to 15-min value.

TABLE III. Bile Flow and Cumulative Bilirubin Excretion Rate in Experiments Using Manganese, Bilirubin, BSP and BSP–GSH in Rats[a].

Experiment Dose of BSP or BSP–GSH	BSP 15 mg/kg	BSP 6 mg/kg	BSP 120 mg/kg	BSP–GSH 20.8 mg/kg without cholestasis	BSP–GSH 20.8 mg/kg with cholestasis
Number of animals	4	4	4	3	5
Time (min) vs Bile flow (μl/min/100g)					
0–15	$8.05^b \pm 0.74$	13.76 ± 1.10	11.81 ± 1.57	11.81 ± 0.60	10.08 ± 1.00
15–30	8.54 ± 1.60	13.35 ± 0.60	7.51 ± 1.15	11.24 ± 0.57	9.73 ± 0.60
30–45	9.98 ± 1.90	13.29 ± 0.62	14.71 ± 2.21	11.30 ± 0.36	6.35 ± 0.84^c
45–60	8.14 ± 1.90	$7.73^c \pm 2.30$	13.92 ± 1.66	9.33 ± 1.88	2.68 ± 0.70^c
60–90	8.00 ± 2.20	9.82 ± 1.20	12.60 ± 1.08	9.24 ± 1.31	1.63 ± 0.41^c
Bilirubin excretion rate (% of dose in 60 min)	59.0 ± 4.9	55.0 ± 8.3	55.6 ± 2.7	60.9 ± 7.6	5.9 ± 7.2

[a] $MnSO_4 \cdot H_2O$ 15 mg/kg was injected at 15 min, BSP or BSP–GSH at 20 min, BR 25 mg/kg at 30 min.
[b] Values are means ± SE.
[c] $P < 0.05$ versus 0- to 15-min value.

ceeds via an intermediate pathway. Lastly, the fact that increasing the period of time between injections of manganese and bilirubin diminished the cholestatic effect (Table IV) permits us to suggest that manganese–bilirubin interaction occurs via a short-lived intermediate.

Cholestasis was prevented (Table III) by a small dose of BSP (15 mg/kg) injected 5 min after manganese. This dose is smaller than that of Witzleben and Boyce (7) but it is not choleretic; the cholestasis is prevented, not masked by BSP-induced choleresis. Protection was abolished by reducing the dose of BSP to 6 mg/kg but not by a dose increase to 120 mg/kg. These three doses of BSP gave similar (60 min) cumulative bilirubin excretion values (Table II) but lower than those obtained with bilirubin alone (70.7 ± 6.8% of dose in 60 min), BSP and bilirubin being competitors for biliary excretion.

Protection against cholestasis seems more related to BSP than to its principal metabolite BSP-GSH. In one experiment using BSP–GSH we obtained two cases of severe cholestasis (bile flow less than 2 μl/min/100 g) one moderate cholestasis (bile flow less than 5 μl/min/100 g) and three rats with normal bile flow and bilirubin excretion rates. We questioned the homogeneity of our BSP–GSH. In a second experiment, in which we assured homogeneity, administration of BSP–GSH failed to prevent cholestasis in the two animals employed. Bilirubin excretion

TABLE IV. BILE FLOW SEEN AFTER MANGANESE–BILIRUBIN TREATMENT FOR LONGER TIME INTERVAL BETWEEN TWO INJECTIONS IN RATS.

	Time	15–30	30–45	45–60	60–75	75–90	90–105
Exp 1[a]	Bile flow, μl/min/100 g	9.02[b] ±1.09	8.68 ±1.21	5.05 ±1.36	3.63 ±0.99	3.75 ±0.99	4.18 ±1.08
	Time	45–60	60–75	75–90	90–105	105–120	120–150
Exp 2[c]	Bile flow, μl/min/100 g	8.02[d] ±0.16	7.15 ±0.31	7.19 ±0.19	6.91 ±0.31	6.83 ±0.69	6.99 ±0.39

[a] $MnSO_4 \cdot H_2O$ (15 mg/kg) was injected at time zero and bilirubin (25 mg/kg) was injected 30 min later.
[b] Values are means ± SE for two animals.
[c] $MnSO_4 \cdot H_2O$ was injected at time zero and bilirubin was injected 60 min later.
[d] Values are means ± SE for four animals.

rates in these cases were similar to those observed during manganese–bilirubin cholestasis.

Discussion. We used a dose of manganese much smaller than that employed by Witzleben (60 mg/kg) to be sure that it would not be the only cause of cholestasis at the time of bilirubin injection. Since Gartner *et al.* (15) demonstrated that bilirubin could cause cholestasis in the rhesus monkey, we selected a dose of bilirubin which did not affect bile flow. Combination of these treatments produces severe cholestasis if the order and injection times are respected. Therefore manganese and bilirubin both seem to be essential for the induction of cholestasis under our experimental conditions.

Turbidity appears in bile collected during cholestasis and the presence of yellow acellular material within the lumen of many bile ducts (3) suggests the formation of a precipitate as a cause of cholestasis. The precipitate cannot be a manganese–bilirubin complex nor bilirubin precipitated because of a lowered solubility caused by the presence of manganese since Witzleben and Boyce (7, 8) demonstrated that the precipitate does not contain large amounts of manganese and that manganese concentration is not a determinant factor for the induction of cholestasis. The precipitate is probably not as important as previously postulated; it also appears with a dose of manganese too low to induce cholestasis (7.5 mg/kg, $MnSO_4 \cdot H_2O$); contact with air and time are necessary for its production and there is no evidence that it can be formed in the liver.

Direct interaction between manganese and bilirubin can be suspected by the necessity of the short time required between the two treatments, but this hypothesis must be rejected since manganese has to be injected first. We must also reject the possibility that manganese is first modified before it interacts with bilirubin since Witzleben and Boyce (8) demonstrated, with total manganese (not only Mn II) measurements, that manganese concentration is not a determinant factor for the induction of cholestasis.

Manganese probably modifies something else that afterwards interacts with bilirubin. Manganese perhaps impairs the bilirubin excretory system by hindering conjugation or inducing synthesis of a toxic or poorly excreted metabolite; that possibility is now under investigation. Manganese might also block bile formation or secretion by acting on bile salts or by blocking energetic metabolism.

We used a lower dose of BSP than that employed by Witzleben and Boyce (7, 8) to be sure the cholestasis would be prevented and not only masked by the known choleretic effect of high doses of BSP. Since the protection is partially reversed by increasing the bilirubin dosage or by reducing the BSP dosage, an interaction between BSP and bilirubin is a possible mechanism for the protection. On the other hand BSP might exert its action on manganese, preventing the modification that leads to cholestasis at the time of bilirubin injection.

Even if BSP can be cholestatic under certain circumstances (16) a high dose of BSP (120 mg/kg) still protects against manganese-bilirubin cholestasis.

Administration of BSP-GSH (equimolar quantity to 15 mg/kg of BSP) failed to prevent cholestasis in five rats out of eight. The results obtained before and after homogenization of BSP–GSH lead us to believe that the protection is caused by BSP, before its

conjugation with glutathione.

Summary. Combination of noncholestatic doses of manganese and bilirubin produced a dramatic fall in bile flow. Furthermore the order and injection times were critical, suggesting that the manganese–bilirubin interaction is complex and proceeds via a short-lived intermediate pathway. Protection against cholestasis seems assured by BSP before its conjugation. Because the protection is abolished either by increasing the bilirubin dosage or lowering the BSP dosage a BSP–bilirubin interaction appears to be a possible mechanism for the protection seen in manganese–bilirubin cholestasis.

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1. Witzleben, C. L., Pitlick, P., Bergmeyer, J., and Benoit, R., Amer. J. Pathol. **53**, 409 (1968).
2. Witzleben, C. L., Amer. J. Pathol. **57**, 617 (1969).
3. Witzleben, C. L., Amer. J. Pathol. **62**, 181 (1971).
4. Witzleben, C. L., Amer. J. Pathol. **66**, 588 (1972).
5. Boyce, W., and Witzleben, C. L., Amer. J. Pathol. **72**, 427 (1973).
6. Klaassen, C. D., Toxicol. Appl. Pharmacol. **29**, 458 (1974).
7. Witzleben, C. L., and Boyce, W. H., Arch. Pathol. **99**, 492 (1975).
8. Witzleben, C. L., and Boyce, W. H., Arch. Pathol. **99**, 496 (1975).
9. Roberts, R. J., Klaassen, C. D., and Plaa, G. L., Proc. Soc. Exp. Biol. Med. **125**, 313 (1967).
10. Whelan, G., Hoch, J., and Combes, B., J. Lab. Clin. Med. **75**, 542 (1970).
11. Heirwegh, K. P. M., Van Hees, G. P., Leroy, P., Van Roy, F. P., and Jansen, F. H., Biochem. J. **120**, 877 (1970).
12. Van Roy, F. P., and Heirwegh, K. P. M., Biochem. J. **107**, 507 (1968).
13. Nosslin, B., Scand. J. Clin. Lab. Invest. **12**, Supp. 49, 1 (1960).
14. Jendrassik, L., and Grof, P., Biochemische Zeitschrift **297**, 81 (1938).
15. Gartner, L. M., Lane, D. L., and Cornelius, C. E., Amer. J. Physiol. **220**, 1528 (1971).
16. Priestly, B. G., and Plaa, G. L., Proc. Soc. Exp. Biol. Med. **135**, 373 (1970).

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