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EFFECT OF COLCHICINE AND *S,S,S*-TRIBUTYL PHOSPHOROTRITHIOATE (DEF) ON THE BILIARY EXCRETION OF SUCROSE, MANNITOL AND HORSERADISH PEROXIDASE IN THE RAT*

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Abstract—The purpose of this study was to demonstrate the effect of colchicine and *S,S,S*-tributyl phosphorotrithioate (DEF) on vesicular transport of horseradish peroxidase (HRP) into bile at the same time that permeability changes occur in the canalicular membrane. Anesthetized Sprague–Dawley rats were surgically prepared with a bile duct cannulae. Four hours after colchicine pretreatment (10 mg/kg, i.p.), biliary excretion of intravenously injected sucrose and mannitol was increased, while that of HRP was decreased. Administration of these marker compounds from the bile side by segmented retrograde intrabiliary injection (SRII) (40 μl washed in with 110 μl saline) resulted in decreased biliary excretion of all three markers in colchicine-pretreated rats. Twenty minutes after DEF pretreatment by intrabiliary administration (40 μl of DEF washed in with 31 μl of saline and held in place for 0.5 min), the biliary excretion of intravenously injected sucrose and mannitol was decreased but that of HRP was increased; when administered by SRII, the biliary excretion of all three marker compounds was increased. These results demonstrate that colchicine, in addition to having blocked vesicular transport (HRP), also increased canalicular permeability to sucrose and mannitol. DEF, on the other hand, decreased canalicular permeability to sucrose and mannitol and enhanced vesicular transport of HRP.

Because of the limitations of the *in vivo* techniques that are available for studying the mechanisms of biliary excretion of xenobiotics, we have developed a technique called segmented retrograde intrabiliary injection (SRII) [1, 2] whereby xenobiotics are administered into the biliary tree of the rat. By the use of this technique, we have demonstrated that an extract of *Amanita phalloides* (presumed to act through its content of phalloidin) increases biliary tree permeability in the absence of an overt effect on bile flow [3]. Dubin *et al.* [4] used phalloidin and colchicine to implicate the involvement of microfilaments and microtubules in bile salt dependent bile flow. The purpose of the present study was to determine whether colchicine (a microtubule blocker) might also affect biliary tree permeability as did phalloidin (a microfilament blocker). At the same time, we used horseradish peroxidase (HRP) to assess biliary excretory function because Renston *et al.* [5] provided evidence that HRP is transferred from blood to bile by vesicular transport that involves the microtubular system, a process which can be blocked by colchicine. We evaluated *S,S,S*-tributyl phosphorotrithioate for its effect on the permeability of the biliary tree because cholinesterase inhibitors are known to affect the permeability in another system—the blood–brain barrier [6]. We have demonstrated that DEF treatment not only decreased the permeability of the biliary tree for sucrose and mannitol but also appeared to stimulate vesicular transport of HRP.

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MATERIALS AND METHODS

Chemicals and drugs. The sources of the main chemicals and drugs were as follows. D-[1-^{14}C]Mannitol (sp. act. 45 mCi/mmole) and [G-^{3}H]apo horseradish peroxidase (sp. act. 10 Ci/mmole) were obtained from the New England Nuclear Corp., Boston, MA. [6,6′(n)-^{3}H]Sucrose (sp. act. 15.5 Ci/mmole) was obtained from the Amersham/Searle Corp., Arlington Heights, IL. Colchicine (mol. wt 399) and purified horseradish peroxidase (HRP, mol. wt 40,000, Sigma II) were obtained from the Sigma Chemical Co. St. Louis, MO. *S,S,S*-Tributyl phosphorotrithioate (DEF is a defoliant for cotton; mol. wt 314) was obtained from the Mobay Chemical Corp., Kansas City, MO.

Animal preparation. Male Sprague–Dawley rats, weighing 280–400 g, were anesthetized with pentobarbital sodium (45 mg/kg, i.p.). The femoral vein was cannulated with PE50 polyethylene tubing. The common bile duct was cannulated with a 10 cm piece of PE20 tubing, just distal to the bifurcation of the common bile duct near the liver hilus [7]. In i.v. studies the renal pedicles were tied. After surgery, a thermistor probe was inserted into the rectum, with temperature monitored by a tele-thermometer (Yellow Springs Instruments Co., Yellow Springs, OH). The rats were placed near incandescent lamps to maintain body temperature at 37 ± 0.5°.

Pretreatment with colchine and DEF. Colchicine (10 mg/kg body weight, i.p.) in 0.9% saline, was given 4 hr before the start of the experiment; controls received an equal volume of saline. *S,S,S*-Tributyl phosphorotrithioate (DEF) was administered by retrograde injection into the biliary tree. DEF (125 μl) was dissolved in a mixture of 237 μl ethanol and 237 μl Emulphor-620, and the solution was made up to 5 ml by adding a solution of 0.9% sodium chloride. Polyethyleme tubing that contained 40 μl of this solution was attached to the bile duct cannula and the solution was washed in, with 31 μl of 0.9% saline, at a rate of 2.3 μl/sec. After the completion of the injection, the solution was held in place for 0.5 min. After this, the occlusion was released and bile was allowed to flow freely for 20 min before continuing the experiment. In a separate experiment in three rats, the vehicle for DEF was given in a similar fashion. During the waiting periods, bile flow was measured [3].

Administration of marker compounds to assess biliary excretory function. In one procedure, the marker compounds were given intravenously. The radiochemicals (1.25 to 2.5 μCi) were injected into the femoral vein ([^{14}C]mannitol or [^{3}H]sucrose) or the hepatic portal vein ([^{3}H]apo HRP), and their excretion into bile was measured. Purified HRP (10 mg/100 g body wt), dissolved in 0.5 ml of 0.9% saline, was injected into the femoral vein. In another procedure, the marker compounds were administered by the SRII technique of Olson and Fujimoto [1]. In brief, 40 μl of solution in a polyethylene tubing containing 0.4 to 0.5 μCi of radiochemical ([^{14}C]mannitol, [^{3}H]sucrose or [^{3}H]apo HRP) or 3 mg of purified HRP was injected into the biliary tree with 110 μl of 0.9% saline at a rate of 2.3 μl/sec. Through hydrostatic filtration of the marker compound across the hepatobiliary epithelium including the canalicular membrane, a portion of the administered marker compound that, depending on its molecular weight, is left unabsorbed in the biliary tree lumen can be quantified in the recollected bile. Bile collection was started within 5 sec after completion of the SRII, and individual bile drops were collected serially. Mannitol and sucrose were chosen as the marker compounds because they had been used earlier to demonstrate the hydrostatic filtration process and the changes in biliary tree permeability [1–3]. Compared with the recovery of the unabsorbed portion in bile, relatively little of the mannitol and sucrose that enter the systemic circulation is excreted in the bile [2]. As previously found, a drop of bile was equal to 5.7 μl. In the experiment in which the vehicle for DEF was given retrogradely into the biliary tree, the recoveries of sucrose and mannitol (after i.v. and SRII administration) were the same as in the corresponding saline-treated control groups.

Measurement of radioactivity. Bile drops 1 through 10 and the even numbered drops 12 through 40 were collected individually into liquid scintillation vials containing 5.0 ml of medium. The medium consisted of 4 g of 2,5-diphenyloxazole (PPO) and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) dissolved in 1.0 liter of toluene and 0.5 liter of Triton X-100. The radioactive content of ^{3}H and ^{14}C in bile samples was measured by counting in a liquid scintillation spectrometer (model POS/3-ISOCAP/300, Searle Analytical, Inc., Des Plaines, IL).

Biochemical assay of HRP. Biochemical assay of HRP in bile aliquots was performed spectrophotometrically [8]. Hydrogen peroxide in phosphate buffer, pH 6, was used as the substrate. Ortho-dianisidine dye was used as the hydrogen donor. To 3 ml of substrate (1 ml of 30% H_2O_2 diluted to 100 ml with 0.01 M phosphate buffer) and 0.05 ml of 1% dye in methyl alcohol, 10 μl of bile or buffer was added, and the contents were mixed by inverting the cuvettes. The rate of decomposition of H_2O_2 by peroxidase in the presence of *o*-dianisidine was determined by measuring the absorbancy at 460 nm. A standard curve was constructed; it indicated that the relationship between HRP concentration and absorbance was linear. Later in our experiments on the effect of DEF administration, we used radioactive assay of [^{3}H]HRP because biochemical assay was time-consuming, and [^{3}H]HRP could be measured in a single drop of bile. When statistical comparisons were made, Student's *t*-test was used, with $P < 0.05$ taken to indicate a significant difference between mean values [9].

RESULTS

Effects of colchicine and DEF pretreatment on the biliary excretion of intravenously administered mannitol, sucrose and HRP. In Fig. 1, the pattern of [^{14}C]mannitol excretion illustrates the relationship between the intravenous administration of mannitol and its excretion into bile. Over the period of the experiment, the total recovery of mannitol in bile was low because mannitol diffuses across the canalicular membrane into bile and the amount of bile formed relative to the volume of distribution of

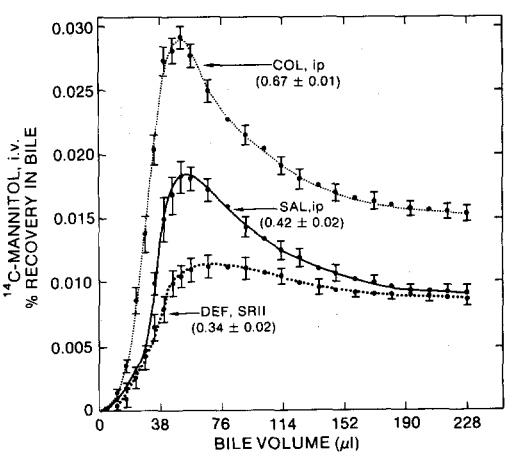

Fig. 1. Effects of colchicine and DEF pretreatment on biliary excretion of intravenously administered [^{14}C]mannitol (1.25 μCi) in the rat. Colchicine (COL), 10 mg/kg, i.p., and sodium chloride (SAL), 0.9% (w/v) i.p., were given 4 hr before [^{14}C]mannitol. DEF (1 μg in 40 μl), washed in with 31 μl saline, was given by intrabiliary administration as described in Materials and Methods. Values are means ± S.E. of six rats per group. Values in parentheses indicate total recovery ± S.E. Recoveries were significantly different from SAL control ($P < 0.05$).

mannitol is small [10]. However, it is evident that both colchicine and DEF pretreatments affected the pattern of excretion of mannitol in bile. In both cases, the rate of bile flow was unchanged from the saline control group (Table 1). Thus, the changes in mannitol excretion seen in Fig. 1 were not due to changes in bile flow. The working hypothesis was that colchicine pretreatment increased the permeability of the canalicular membrane to mannitol and that DEF pretreatment decreased this permeability.

In Fig. 2, the results for the saline control group show that the biliary excretion of [^{3}H]sucrose was lower than that of [^{14}C]mannitol. The reason for this difference could have been that sucrose entry into the canalicular bile was restricted more to extracellular pathways in the liver, while mannitol entered the bile through both extracellular and intracellular pathways [10]. The results in Fig. 2 indicate that colchicine pretreatment increased the biliary excretion of [^{3}H]sucrose. These results are consistent with those for mannitol in Fig. 1 and have been interpreted to indicate that colchicine pretreatment increased the permeability of the canalicular membrane. With DEF pretreatment in Fig. 2, the biliary excretion of intravenously administered [^{3}H]sucrose was not reduced as greatly as that of mannitol in Fig. 1. Thus, the effect of DEF pretreatment in decreasing canalicular permeability was not as great for sucrose as it was for mannitol.

Next, the effects of colchicine and DEF on vesicular transport of HRP given i.v. were examined. The results from the saline-pretreated control group in Fig. 3 indicated that HRP was excreted slowly into bile. We assumed that this excretion occurred through vesicular transport of HRP from the hepatocyte into bile. Colchicine pretreatment decreased the biliary excretion of HRP. This result confirmed the finding of Renston *et al.* [5]. In Fig. 4, the finding with DEF pretreatment was unexpected; DEF pretreatment increased the biliary excretion of [^{3}H]HRP. If DEF were to act only to decrease canalicular permeability, we would have expected DEF pretreatment either to decrease [^{3}H]HRP excretion, if any [^{3}H]HRP were diffusing from blood into bile, or to have no effect. Since HRP is excreted into bile by vesicular transport, we concluded that DEF increased vesicular transport.

Effects of colchicine and DEF pretreatment on biliary excretion of mannitol, sucrose and HRP given by the SRII procedure. Since the colchicine and DEF pretreatments produced, respectively, increased and decreased permeability presumably of the canalicular membrane, we wondered whether the same permeability changes could be measured by giving mannitol and sucrose from the canalicular side. Therefore, in the next set of experiments, we injected these marker compounds into the biliary tree by the SRII technique. In Figs. 5 and 6, in the saline control group, more sucrose than mannitol was recovered in bile, as expected from the molecular weight relationship [1, 2]. Colchicine pretreatment reduced this recovery of mannitol from 14 to 4% (Fig. 5) and of sucrose from 40 to 11% (Fig. 6). These results were interpreted to indicate that colchicine pretreatment increased the permeability of the canalicular membrane, an effect which was demonstrable from the canalicular side in these SRII experiments.

The result of SRII experiments with [^{14}C]mannitol (Fig. 5) after DEF pretreatment showed an increased recovery of mannitol. This result was interpreted to

Table 1. Lack of effect of colchicine and *S,S,S*-tributyl phosphorotrithioate (DEF) pretreatment on the rate of bile flow in the rat*

	Bile flow rate (μl/min)
Saline, i.p.	21.2 ± 0.7
Colchicine, i.p.	19.7 ± 0.6
DEF, intrabiliary	20.4 ± 0.4

* Colchicine (10 mg/kg body wt, i.p.) in 0.9% saline was given 4 hr before the bile flow was recorded. Saline controls received an equal volume of 0.9% sodium chloride, i.p. DEF was given by intrabiliary administration as described in Materials and Methods. Values are means ± S.E.; N = 6 per group.

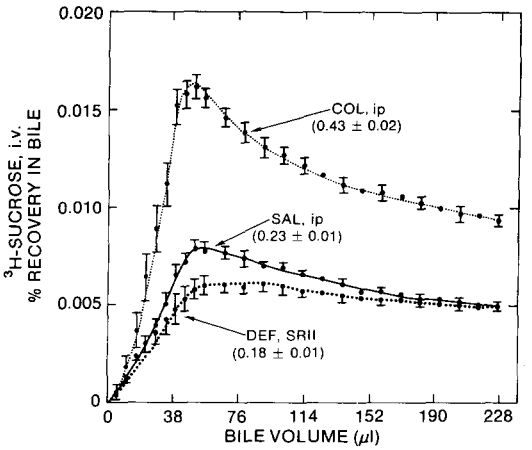

Fig. 2. Effects of colchicine and DEF pretreatment on biliary excretion of intravenously administered [^{3}H]sucrose (2.5 μCi) in the rat. The details are described in the legend to Fig. 1. N = 6 per group. Recoveries were significantly different from SAL control ($P < 0.05$).

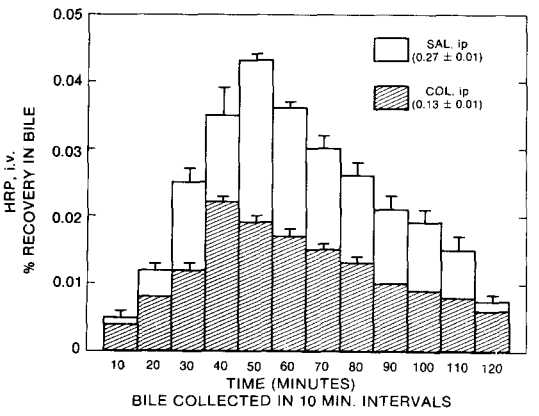

Fig. 3. Effect of colchicine pretreatment on the biliary excretion of intravenously administered HRP in the rat. Colchicine was given as described in the legend to Fig. 1. Bile samples were collected in 10 min intervals, and the HRP content was determined by biochemical assay of enzyme activity. N = 6 per group. The recovery was significantly different from SAL control ($P < 0.05$).

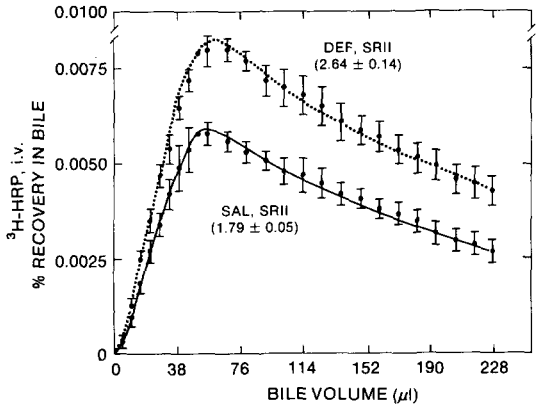

Fig. 4. Effect of DEF pretreatment on the biliary excretion of intravenously administered [^{3}H]HRP (2.5 μCi) in the rat. DEF pretreatment is described in the legend to Fig. 1. ^{3}H-Content was measured in individual bile drops. N = 6 per group. The recovery was significantly different from SAL control ($P < 0.05$).

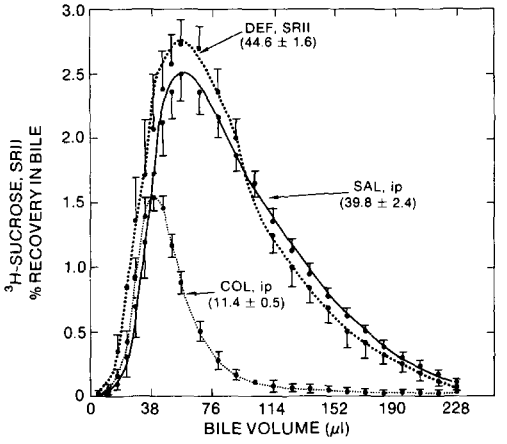

Fig. 6. Effects of colchicine and DEF pretreatment on the biliary excretion of [^{3}H]sucrose (0.4 to 0.5 μCi) administered by SRII in the rat. The remainder of the details are described in the legend to Fig. 1. N = 6 per group. Except for the DEF, the recovery was significantly different from SAL control ($P < 0.05$).

indicate that DEF pretreatment decreased canalicular permeability to SRII mannitol. This decrease in permeability was not evident for sucrose given by SRII (Fig. 6).

The effects of colchicine and DEF pretreatment on HRP and [^{3}H]HRP administered by SRII are given in Figs. 7 and 8 respectively. Recovery of HRP in Fig. 7 was like that of sucrose given by SRII (Fig. 6). For compounds with molecular weights larger than sucrose, the recovery should be similar to that of sucrose [1, 2]. The colchicine pretreatment decreased this recovery for HPR. This decrease was attributed to an increased permeability of the canalicular membrane produced by the colchicine pretreatment.

The results in Fig. 8 demonstrate that DEF pretreatment increased the recovery of [^{3}H]HRP given by SRII. This increased recovery could be attributed to either a decrease in permeability of the canalicular membrane produced by DEF so that more [^{3}H]HRP remained unabsorbed in the lumen of the biliary tree or to an increase in re-excretion of the [^{3}H]HRP into bile.

DISCUSSION

Colchicine affects the secretion of proteins in a variety of tissues [11–17]. Since colchicine inhibits tubulin polymerization into microtubules [18, 19], the effects of this drug on several secretory systems have been ascribed to its interaction with the microtubules [20, 21]. In addition to the work of Renston *et al.* [5] on HRP transport, Mullock *et al.* [22]

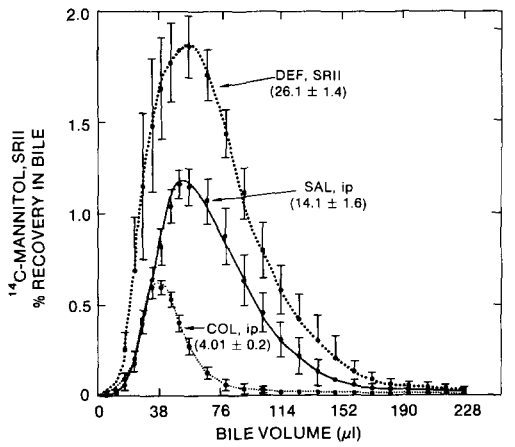

Fig. 5. Effects of colchicine and DEF pretreatment on biliary excretion of [^{14}C]mannitol (0.4 to 0.5 μCi) administered by SRII in the rat. The SRII of [^{14}C]mannitol consisted of 40 μl of [^{14}C]mannitol plus 110 μl of saline. N = 6 per group. Recoveries were significantly different from control ($P < 0.05$).

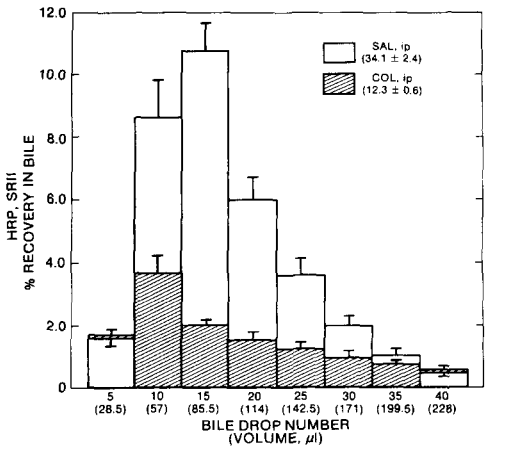

Fig. 7. Effect of colchicine pretreatment on the biliary excretion of HRP (3 mg) administered by SRII in the rat. Colchicine pretreatment is described in the legend to Fig. 1. Bile was collected in 5-drop (28.5 μl) samples. HRP content was determined by biochemical assay of enzyme activity. N = 6 per group. Recovery was significantly different from SAL control ($P < 0.05$).

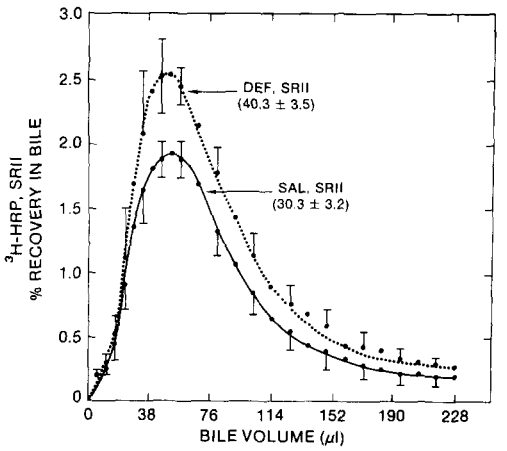

Fig. 8. Effect of DEF pretreatment on biliary excretion of [^{3}H]HRP (0.4 to 0.5 μCi) administered by SRII in the rat. DEF pretreatment is described in the legend to Fig. 1. ^{3}H-Content was measured in individual bile drops. N = 6 per group. The recovery was significantly different from SAL control ($P < 0.05$).

demonstrated that colchicine inhibited the movement of vesicles containing IgA. Thus, in the present study, our finding that colchicine decreased biliary excretion of intravenously administered HRP was taken as evidence that vesicular transport of HRP was inhibited by colchicine.

A new finding for colchicine is the increase in canalicular permeability. Our evidence to support this contention is that, in the colchicine-pretreated rat, the biliary excretion of both [^{14}C]mannitol and [^{3}H]sucrose after i.v. administration was increased. Since mannitol and sucrose are known to enter bile by diffusion, together with formation of canalicular bile [10], our findings in the absence of a change in bile flow were interpreted to mean that colchicine pretreatment increased the permeability of the canalicular membrane to mannitol and sucrose. The concepts regarding the use of mannitol and sucrose as marker compounds for assessment of biliary excretory function and changes thereof are reviewed by Forker [10].

For mannitol (Fig. 5) and sucrose (Fig. 6) given by SRII, colchicine pretreatment decreased their recovery in the recollected bile by reducing the area under the curve on the right side as compared to the saline control curve. We interpreted this change to indicate that colchicine had increased permeability more at the canalicular that at the ductular or bile duct areas. The drops of bile re-collected in the early (designated by the lower cumulative bile volumes) sample would represent the solution contained in the bile duct and ductiles. The later drops would represent solution collected from deeper sites in the biliary tree corresponding more to the canalicular area (right side of curve).

Since, with an increase in canalicular permeability, we would have expected the recovery of i.v. administered HRP in bile to increase, the observed decrease (Fig. 3) was attributed entirely to the inhibition of vesicular transport of HRP by colchicine. When HRP was given by SRII, colchicine pretreatment decreased the recovery of HRP in the re-collected bile (Fig. 7). Because the recoveries of sucrose and HRP were similar (39.8 ± 2.4% and 34.1 ± 2.4% respectively) under the SRII condition of the experiment, very little of the HRP which gained access to the hepatocyte from its SRII administration was re-excreted into bile. Thus, in the present experiment, we concluded that the decreased recovery of HRP after SRII (but not after i.v.) in the colchicine-pretreated rats was due to increased permeability of the canalicular membrane to HRP. The effect of colchicine on canalicular permeability may be related to one of its other membrane actions. Colchicine binds to isolated membrane fractions [23, 24], affects the distribution of intramembrane particles in *Tetrahymena pyriformis* [25], causes a redistribution of lectin-binding sites on the membrane of leukocytes [26], and affects nucleoside transport [27, 28]. Colchicine may accumulate and expand the plasmalemma [29] since colchicine has been shown to be extensively excreted in the bile of the rat [30].

DEF pretreatment decreased biliary excretion of i.v. mannitol and increased biliary excretion of SRII mannitol. These results indicate that the main effect of DEF was to produce a decrease in the permeability of the canalicular membrane.

With DEF pretreatment, the i.v. curve for sucrose (Fig. 2) did not show as large a decrease in recovery as for mannitol (Fig. 1). The SRII curve for sucrose in the presence of DEF (Fig. 6) also did not show as large an increase in recovery as for mannitol (Fig. 5). Therefore, DEF pretreatment produced a more pronounced decrease in permeability of canalicular membrane toward mannitol compared to sucrose. According to Forker [10], sucrose enters bile through extracellular pathways and its entry is more restricted than that of mannitol. Mannitol ultimately reaches a bile to plasma concentration ratio of 1 at equilibrium but this ratio is less than 1 for sucrose. Thus, it is possible that, when canalicular permeability to systemically administered compounds is decreased by DEF, there is a greater effect on permeability of mannitol than sucrose. Considered from the SRII point of view, it has been shown [1, 2] that recovery increased in bile with an increase in molecular weight of the marker compound (sucrose > mannitol). Also, for compounds larger than sucrose, the recovery was no greater than for sucrose. Thus, a decrease in permeability of the canalicular membrane produced by DEF would be expected to affect mannitol more than sucrose recovery in the SRII experiments. The SRII curve for [^{3}H]HRP recollection in bile (Fig. 8) could be interpreted as indicating a decrease in canalicular permeability to [^{3}H]HRP. In this particular case, the major contributing factor for the increased recovery of [^{3}H]HRP in DEF-treated rats could be either decreased permeability of the canalicular membrane or increased vesicular transport.

Better evidence exists for stimulation of vesicular transport. The results in Fig. 4 indicated that, after DEF treatment, the recovery of intravenously administered [^{3}H]HRP increased in bile. Since HRP is excreted into bile by vesicular transport through

the hepatocyte, it is likely that DEF stimulated vesicular transport of HRP.

In conclusion, colchicine increased canalicular permeability at the same time that vesicular transport was inhibited. On the other hand, DEF decreased canalicular permeability at the same time that vesicular transport was stimulated. DEF is the first agent we have encountered to have these effects. We do not know whether the changes in canalicular permeability are related to, or independent of, the mechanism involved in vesicular transport.

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