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The Effect of Cycloheximide on Bile Flow in Rats (40594)[1]

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In a series of studies on the toxicity of α-naphthylisothiocyanate (ANIT), cycloheximide (Cx) was found to effectively protect rats against ANIT-induced hyperbilirubinemia and cholestasis (1, 2). Cx produces an almost instantaneous inhibition of hepatic protein synthesis and also of protein synthesis in the gastrointestinal tract and lung (3–5). It is believed that this mechanism protects cells in various tissues from the cytotoxic effects of several agents (6). Moreover, it has been reported (7) that, in the liver of animals given ANIT, Cx will prevent necrosis of the cells lining the small biliary ducts.

It was thought that this inhibition of protein synthesis might modify the metabolism of ANIT by preventing the formation of more toxic metabolites, and in this way afford protection (8, 9). However, during these studies evidence became available suggesting that Cx itself also modified hepatic bile formation. The experiments reported in this communication were designed to examine this phenomenon in more detail.

Materials and methods. Cycloheximide, kindly donated by Upjohn Company, Kalamazoo, Michigan, was dissolved in 0.9% NaCl before use. Hydroxysteroid dehydrogenase was obtained from Worthington Biochemicals; bilirubin, bile salts, and NAD^+ were all obtained from the Sigma Chemical Company and the Monitor Jendrassik Bilirubin Kit was purchased from the American Monitor Corporation.

Male Sprague–Dawley rats weighing between 230 and 280 g were purchased from Bio-Breeding, Ottawa, Ontario. Housed in stainless-steel cages, the animals were maintained on Purina Chow and water *ad libitum*. At the beginning of each experiment all animals received routinely 10 ml/kg of 1.5% carboxymethyl cellulose (CMC) solution by gavage. This vehicle had been used in previous experiments to dissolve ANIT, and its use was maintained in the present study. The administration of CMC was taken to represent zero time.

Rats were first treated i.p. with Cx (2 mg/kg) or saline (0.9% NaCl, 2 ml/kg). About 20 min later they were lightly anesthetized with ether and the bile duct cannulated with PE-10 cannula. The cannula (90 cm long) was passed under the skin and exteriorized at the back of the head. Continuous total bile drainage was maintained for the entire experiment. Exactly 30 min after the Cx, CMC was given. The animals were placed in individual cages without restriction of movement and bile was collected in previously tared tubes. Water was made available *ad libitum* so that the rats would not become dehydrated. Volumes of bile secreted were measured every hour for a total of 14 hr. Biliary bilirubin concentrations were determined (10, 11) on each hourly sample. All bile samples were analyzed for total bile acids (12).

In a second series of experiments the role of an intact enterohepatic circulation was assessed. Rats were treated with Cx or saline, followed 30 min later by CMC. Twenty minutes before collecting bile (4, 8, or 16 hr after CMC) the animals (1 group per time period) were anesthetized with a mixture of phenobarbital (30 mg/kg) and ethyl carbamate (urethan, 400 mg/kg) (13). The trachea was cannulated as were the femoral artery, femoral vein, and bile duct. Body temperature was maintained at 36–37°C by placing the animals on surgical boards which were thermostatically controlled. Bile was collected for 1 hr. Thus these animals had an intact enterohepatic circulation for the entire period up to the time bile was collected (4, 8, or 16 hr). Total bile acids and bilirubin were determined for each bile sample.

The transport maximum (Tm) for bilirubin was determined in Cx-treated and control

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groups between 1 and 24 hr after treatment. Thirty minutes prior to the measurement of Tm the animals were anesthetized with the phenobarbital/urethan mixture. The trachea was cannulated, as were the femoral artery, femoral vein, and the bile duct. Body temperature was monitored and maintained at 36–37°C. After allowing the preparation to stabilize for 15 min, a bolus dose of bilirubin (20 mg/kg) in an isotonic solution of sodium carbonate and sodium chloride at pH 8 was given i.v. This was immediately followed by a continuous infusion of bilirubin (1.5 mg/kg/min). Bile samples were collected every 15 min over a 1-hr period. Aliquots of each sample were analyzed to determine the quantity of bilirubin excreted.

The bile flow data obtained in the 14-hr experiment (continuous bile drainage) were transformed to a percentage of the flow recorded during the first hour, to normalize the data and permit statistical analysis. In this experiment, control and Cx-treated means were compared statistically by Student's *t* test (unpaired). Linear regression analysis of bile flow versus bile acid excretion was achieved by the method of least squares (14). The data obtained in the intact enterohepatic circulation experiment were compared statistically by Student's *t* test (paired). In all analyses the null hypothesis was rejected when $P \leq 0.05$.

Results. The data presented in Figure 1 show that in rats Cx produced a marked reduction in bile flow when exteriorized biliary drainage was maintained throughout the experiment. The reduction became apparent as early as 2–4 hr following its injection; after 8 hr the differences between Cx-treated and control animals were statistically significant and continued to fall until the end of the experimental period. At the same time, there was a significant reduction in bilirubin excretion (Fig. 2). The cumulative amounts of bile acids excreted appeared to follow a similar pattern (Fig. 2), but the differences observed between control and Cx-treated animals were not statistically significant.

In order to ensure that changes in body temperature were not responsible for the changes observed in the Cx-treated group, a similar experiment was carried out in which both control and treated animals were kept in a temperature-controlled environment (incubator set at 30°C). Bile flows and biliary excretions of bile acids and bilirubin were essentially identical to those observed when no temperature regulation was involved.

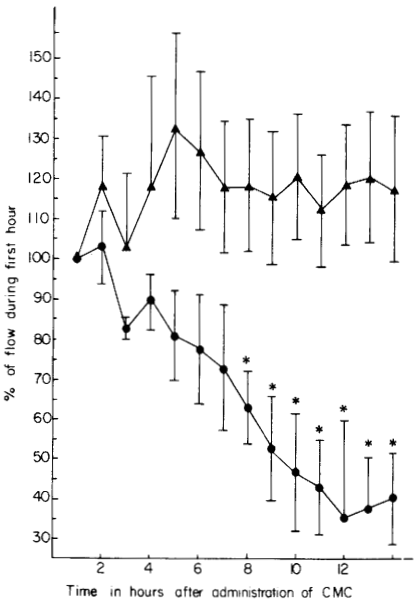

FIG. 1. Changes in bile flow over a 14-hr period following bile duct cannulation. Rats were treated with cycloheximide (2 mg/kg) (●——●) saline (0.9% NaCl, 2 ml/kg) (×——×). Thirty minutes later 1.5% carboxymethyl cellulose (10 ml/kg) was administered to all animals and bile was collected over hourly intervals. Recorded volumes were converted to a percentage of the volume delivered during the first hour (saline, 43.9 ± 9.6 μl/kg/min; cycloheximide, 46.31 ± 5.9 μl/kg/min). Each point represents the mean ± SEM of four animals. Values statistically different from controls ($P < 0.05$) are marked with an asterisk.

The individual bile flow rates (56 for control, 56 for Cx) for the data summarized in Figure 1 and the corresponding bile acid excretion rates for these flows were subjected to a linear regression analysis. The analysis indicated that for the control group there was a statistically significant relationship ($P \leq 0.05$), but the linear correlation was weak ($r = 0.30$). For the Cx-treated animals the relationship was not only statistically significant, but the linear correlation was superior ($r = 0.73$). The graphical representations of these analyses are depicted in Figure 3. The equations for the regression lines are quite different; the slope for the Cx-treated rats is much steeper (about threefold) than that of

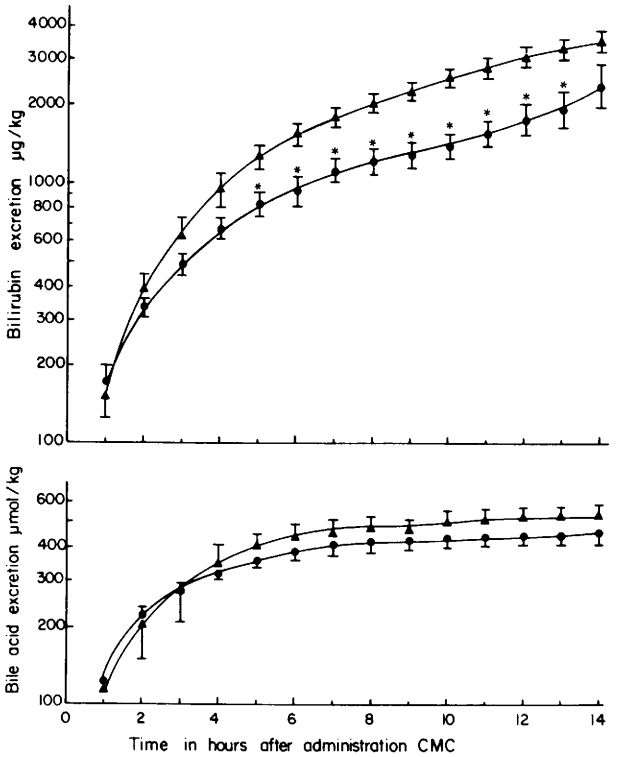

FIG. 2. Cumulative excretion of bilirubin and bile acids over a 14-hr period. Rats were treated with cycloheximide (●——●) or saline (×——×) as in Figure 1. Each point represents the mean ± SEM of four animals. Values statistically different from control ($P < 0.05$) are marked with an asterisk.

the controls. Similar analyses were performed using the bile flow and bilirubin excretion data; no correlation was observed.

Closer examination of Figure 3 indicates that although a single linear regression line can be calculated, the data from the Cx-treated rats suggest that at least two different linear patterns exist for this group, one for bile acid excretion rates ranging from 0 to 0.25 µmole/kg/min, and another for values between 0.25 and 4.0 µmole/kg/min. The linear regression analyses for the 0.25–4.0 µmole/kg/min data are presented in Figure 4. A statistically significant relationship was found for both the saline-treated and the Cx-treated rats; the equations for both regression lines were virtually identical. Figure 5 depicts the results obtained when the 0–0.25 µmole data were subjected to linear regression analysis. For the control group, no statistically significant relationship between bile flow and bile acid excretion was demonstrable ($P = 0.27$). However, for the Cx-treated animals the relationship was statistically significant ($P < 0.05$). Furthermore, the slope of the 0- to 0.25-µmole line in the Cx group was steeper (about ninefold) than that obtained for the 0.25- to 4.0-µmole line. These linear regression analyses indicate that although there was an overall diminution in bile flow in the Cx-treated rats, the amount of bile secreted per unit of bile acid excreted in the 0–0.25 µmol/kg/min range was greater than that observed in control rats.

When the enterohepatic circulation remained intact up to the moment of bile collection (4, 8, or 16 hr), Cx treatment no longer produced a decrease in bile flow (Table I); at 4 hr there was a significant increase in flow, but this was not observed at 8 or 16 hr. While there were slight reductions in the mean values for bilirubin excretion rate at 8 or 16 hr the differences were not statistically significant. There was also no decrease in bile acid excretion rate following Cx, except at 16 hr, when a statistically significant decrease was

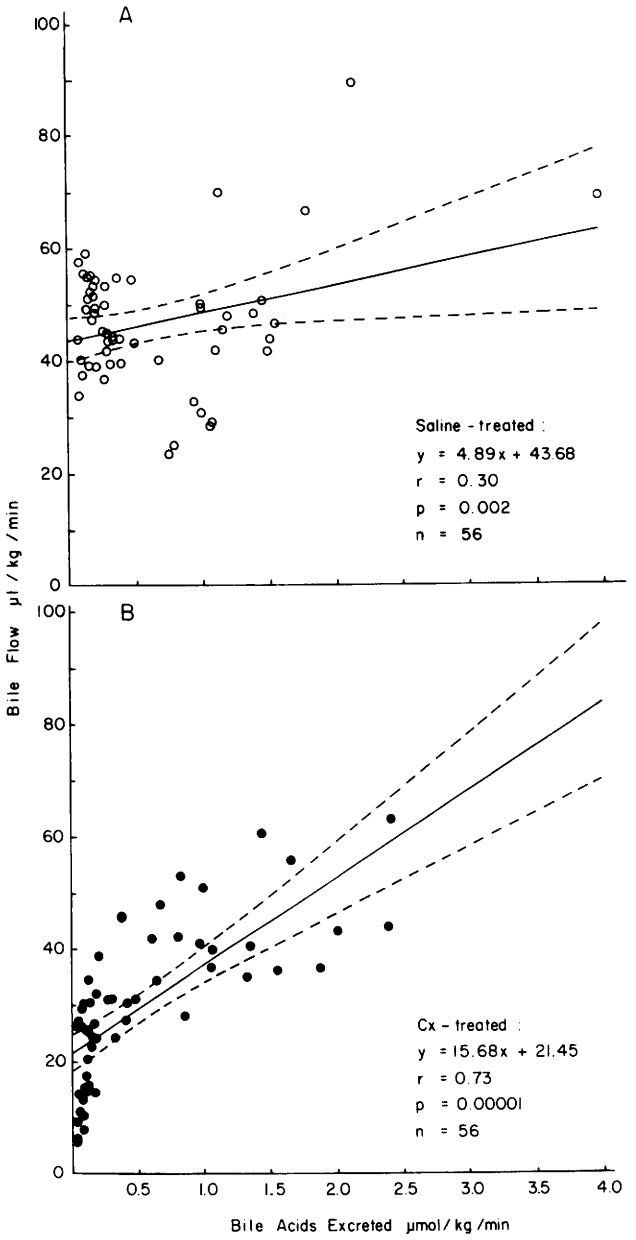

FIG. 3. Relationship of bile flow to bile acid excretion (0–4.0 µmole/kg/min) in the bile produced over a 14-hr period in rats treated with (A) saline or (B) cycloheximide. The relationships were calculated from the experiments summarized in Figures 1 and 2.

observed. From the differences observed between this experiment and the results obtained in the previous one, it is evident that the Cx effect on bile flow was observed only when there was continuous bile drainage to the exterior.

We also examined whether Cx would affect the ability of the liver to remove bilirubin from the blood and excrete it. Since Cx diminished the biliary excretion of endogenously derived bilirubin in the 14-hr experiment (Figure 2), we examined its possible effect on the biliary transport maximum (Tm) of bilirubin. From 1 to 24 hr following a

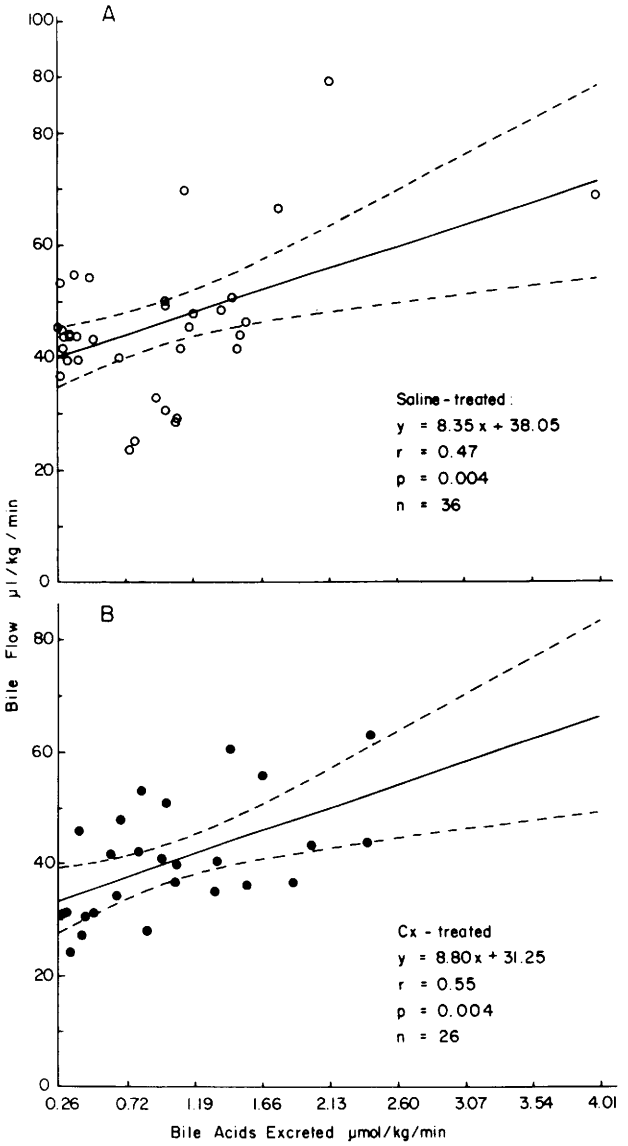

FIG. 4. Relationship of bile flow to bile acid excretion in rats treated with (A) saline or (B) cycloheximide. Data points from values spanning the range 0.25–4.0 µmole/kg/min depicted in Figure 3.

single dose of Cx, the Tm for bilirubin was not adversely affected (Table II).

Discussion. The experiment performed in rats with continuous biliary drainage demonstrates that a single dose of Cx produces a progressive decrease in bile flow. The reduction appears within the first 5 hr although it does not become statistically significant until 8 hr. Bile flow in the Cx-treated animals is reduced by about 60% at 14 hr. Moreover this effect of Cx is not observed if the enterohepatic circulation is maintained until the time bile flow is measured, even up to an overall treatment period of 16 hr (Table I).

When the bile duct of an animal is cannulated, the enterohepatic circulation is interrupted. This results in a depletion of bile acids available for reabsorption from the intestine and an overall depletion of the bile acid pool. When bile flow is compared to biliary bile acid excretion in the 14-hr experiment, it appears that the major determinant

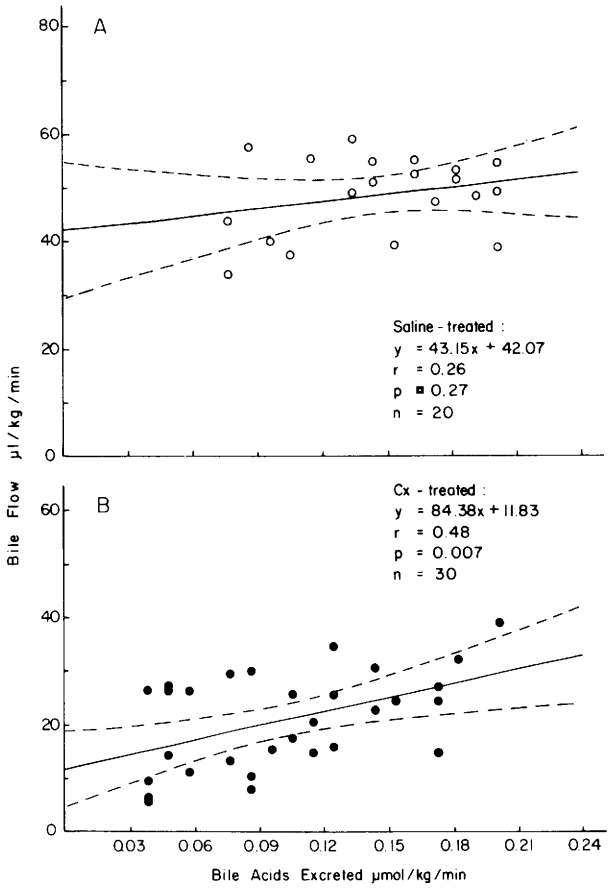

FIG. 5. Relationship of bile flow to bile acid excretion in rats treated with (A) saline or (B) cycloheximide. Data points from values spanning the range 0–0.25 μmole/kg/min depicted in Figure 3.

TABLE I. BILIARY EXCRETION PARAMETERS MEASURED IN RATS TREATED WITH SALINE OR CYCLOHEXIMIDE AT VARIOUS TIMES JUST PRIOR TO BILE DUCT CANNULATION

	4 hr		8 hr		16 hr	
	Saline	Cx	Saline	Cx	Saline	Cx
Number of animals	5	5	3	3	5	5
Bile flow (μl/kg/min)	46.9 ± 7.7[a]	65.9 ± 5.0[b]	55.3 ± 5.6	55.0 ± 2.1	63.9 ± 4.9	51.1 ± 7.3
Biliary bilirubin (mg/kg/min)	2.00 ± 0.13	2.00 ± 0.09	3.06 ± 0.60	2.72 ± 0.36	1.99 ± 0.30	0.97 ± 0.47
Bile acids (μmole/kg/min)	1.43 ± 0.22	2.00 ± 0.41	1.57 ± 0.45	1.56 ± 0.32	1.73 ± 0.12	0.81 ± 0.11[b]

[a] Mean ± SEM.
[b] Significantly different from control values $P < 0.05$ using t test for paired data.

of bile flow in the control rats is the bile salt-independent fraction. This is understandable since bile acid depletion is occurring continuously. In the Cx-treated animals, however, a marked correlation between bile flow and biliary bile acid excretion is observed. By extrapolating the overall regression line to a bile acid excretion rate of zero, it can be seen (Figure 3) that the value for the bile salt-independent fraction for the Cx-treated animals is considerably smaller than that observed for the controls (21 vs 44 μl/kg/min).

TABLE II. TRANSPORT MAXIMUM (Tm) VALUES FOR BILIRUBIN BETWEEN 1 AND 24 HR AFTER TREATMENT WITH SALINE OR CYCLOHEXIMIDE

Time after injection (hr)	TM values ± SEM (mg/kg/min)	
	Saline	Cx
1	0.68 ± 0.01	0.71 ± 0.03
2	0.58 ± 0.10	0.45 ± 0.07
4	0.71 ± 0.06	0.75 ± 0.05
8	0.70 ± 0.01	0.66 ± 0.02
16	0.65 ± 0.04	0.67 ± 0.04
24	0.64 ± 0.04	0.61 ± 0.04

The 53% reduction in bile flow, arrived at by calculating the extrapolated bile salt-independent fractions, agrees reasonably well with the 60% reduction in bile flow actually observed by 14 hr (Figure 1). From these data we conclude that the reduced bile flow observed in the Cx-treated rats is most likely due to changes in the bile salt-independent component of bile flow.

This conclusion is supported when one considers the data in Figure 5, where only the bile flows measured at low bile acid excretion rates (0–0.25 μmole/kg/min) are considered. Balabud *et al.* (15) have demonstrated that the relationship between bile flow and bile acid excretion in the rat is poorly represented by only one overall regression line, and that calculation of the bile salt-independent fraction should not be measured by extrapolation of a single regression line which covers a broad range of bile acid excretion rates. With low bile acid excretion we found that the extrapolated value for the bile salt-independent fraction for the control animals was hardly affected (42 μl/kg/min), but the value (12 μl/kg/min) for the Cx-treated rats was much smaller than that of the control rats and of the value determined from Figure 3 for Cx-treated animals (21 μl/kg/min). The calculated bile salt-independent fraction of the Cx-treated rats was about 30% of the value calculated for control rats.

Interesting speculations can be made regarding the relative roles of the bile salt-dependent and bile salt-independent components of bile flow in rats with continuous exteriorized biliary drainage. When a poor correlation between bile flow and biliary bile acid excretion is observed (as we observed for control animals in Figures 3 and 5), one tends to conclude that the bile salt-dependent component is inoperative. However, the observation that in the Cx-treated rats bile continues to flow at a reduced rate, but that the rate of flow can be correlated linearly with bile acid excretion (Figure 5), suggests that in the rat even low bile flow rates can be regulated by a bile salt-dependent component. Balabaud *et al.* (15) demonstrated that rats depleted of bile acids, and subsequently infused with sodium taurocholate, exhibited bile salt-dependent bile flow, even at low bile acid excretion rates. They noted that the slopes of the bile flow–bile acid excretion regression lines increased as the bile acid concentrations decreased. They speculated that below the critical micellar concentration, bile acids as monomers created a higher osmotic gradient than when forming micelles. In the present study, we also observed a 10-fold increase in slope in the Cx-treated rats when comparing low and high bile acid excretion rates (Figures 4 and 5). While the data suggest a similar finding in the control groups, a valid comparison cannot be made since the apparent relationship between bile flow and bile acid excretion at low bile acid excretion rates (Figure 5) was not statistically significant. Our findings are consistent with those of Balabaud *et al.* (15). It is interesting that a linear relationship between bile flow and bile acid excretion is still observed in the Cx-treated rats at low bile acid excretion rates, while the relationship is not statistically significant in control animals (Figure 5). Perhaps Cx treatment may only have unmasked a component of bile flow which is normally present, but usually overlooked in control animals (15).

While the data obtained in rats with continuous exteriorized biliary drainage are consistent with the proposed effect of Cx on the bile salt-independent component of bile flow, some of the results (Table I) observed in the rats with an intact enterohepatic circulation are puzzling. If Cx affects only the bile salt-independent component, one would expect to see some decrease in bile flow in these experiments since some fraction of the bile flow should be bile salt independent. Yet, none was observed. We are unable to explain these results. One complicating feature is that these experiments required the use of anesthesia, whereas the others were performed in con-

scious animals. The data in Figure 1 suggest that the residual effects of anesthesia may modify bile flow in control rats since the bile flows measured after 4 hr are 15 to 30% higher than those measured at 1 hr. However, other data in Table I are consistent with the previous observations (Fig. 5). When one calculates the bile flow:bile acid excretion ratios (ml/μmole) from the data in Table I it is evident that Cx exerted an effect at 16 hr (control, 38 ml/μmole; Cx, 64 ml/μmole); more bile was secreted per unit of bile acid excreted.

The mechanisms of action of Cx on bile flow cannot be elucidated from these experiments. The bile salt-independent component of bile flow is thought to be related to the biliary excretion of sodium (16). It is possible that Cx exerts its effect on bile flow by inhibiting the activity or synthesis of Na^+, K^+ ATPase, which is responsible for sodium transport. However, Cx could affect other parameters of bile formation.

In addition to an effect on bile flow, it appears that Cx may possibly affect bilirubin excretion. In the rats with continuous exteriorized biliary drainage, a significant diminution in cumulative bilirubin excretion was observed. A suggestion of such an effect was also seen in the experiment where biliary drainage into the intestine was permitted, but the decreases at 8 and 16 hr were not statistically significant. The bilirubin Tm values, however, were unaffected. This indicates that Cx does not alter bilirubin excretion, but that it may affect some other part of the bilirubin metabolic pathway.

When the enterohepatic circulation was maintained a significant reduction in bile acid excretion was observed at 16 hr, but with continuous exteriorized biliary drainage, the differences in cumulative bile acid excretion were not statistically significant. These results may be indicative of a Cx-induced impairment of bile acid reabsorption from the intestine rather than an effect on bile acid depletion. It is known that inhibitors of protein synthesis can impair intestinal transport (17, 18). However, it is evident that this property of Cx is not the cause of the reduction in bile flow observed when the enterohepatic circulation is interrupted.

Summary. Cycloheximide (2 mg/kg i.p.) was shown to cause a significant reduction in bile flow in rats, when total bile drainage to the exterior was continuous over a period of 14 hr; the reduction was statistically significant after 8 hr. Biliary bilirubin excretion was significantly reduced (after 5 hr). However, when an intact enterohepatic circulation was maintained up to the beginning of a 1-hr collection period, no decrease in bile flow was recorded 4, 8, or 16 hr following cycloheximide. Reductions in bilirubin and bile acids were noted as being slight. Biliary bilirubin Tm was unaffected 1–24 hr after a single dose of cycloheximide. It is concluded that the cycloheximide affects the bile salt-independent component of bile flow in rats with continuous exteriorized total bile drainage.

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