Effect of Ethanol on Sulfobromophthalein and Indocyanine Green Metabolism in Isolated Perfused Rat Liver (34295)

B. KOTELANSKI,[1] R. J. GROSZMANN,[1] J. KENDLER, AND H. J. ZIMMERMAN[2]

Medical Service, Veterans Administration Hospital and Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02130

Cholestatic jaundice is observed occasionally in alcoholic patients, with varying degrees of liver disease, particularly fatty metamorphosis. In some of these patients, the cause of the jaundice appears to be steatonecrosis ("acute alcoholic hepatitis") (1). Others may have obstructive jaundice due to pancreatitis (2). Some alcoholics with fatty metamorphosis and jaundice, however, appear to be instances of intrahepatic cholestasis (3). Since equally marked degrees of fatty metamorphosis due to other causes (diabetes, obesity) are not associated with this form of jaundice, the possibility that alcohol, *per se*, can have an adverse effect on bile secretion warrants consideration.

Lieberman and Childs (4) demonstrated that parenteral administration of ethanol to rats leads to impaired excretion of sulfobromophthalein (BSP). Since their studies indicated that the decreased excretion was the result of impairment of conjugation of the dye rather than of excretion, their data did not provide an explanation for a cholestatic syndrome. Accordingly, efforts to investigate further effects of ethanol on biliary excretion were undertaken. The isolated, perfused liver was selected as the experimental model, since this afforded the opportunity to study, directly, the effects of ethanol on excretory processes without the complicating factors provided by the intact animal.

The results of these studies indicate that ethanol, in some of the concentrations studied, interfered with excretion of BSP and of indocyanine green (ICG) and with bile flow.

Materials and Methods. Animals. Female Sprague-Dawley rats (300–350 g) were used as donors of blood and livers. The animals were kept under standard environmental and dietary conditions and were not fasted prior to experimentation.

Perfusion medium. Blood was drawn from the ventral aorta into heparinized[3] syringes and mixed in a solution of Krebs–Ringer–bicarbonate buffer (pH = 7.4) to form a 17% suspension (v/v) with an hematocrit of 12%. A constant volume of 65 ml was maintained in the system throughout the experiment.

Preparation. The perfusion method of Penhos *et al.* (5) was used with slight modification. Cannulations of the common duct with PE 10 tube and the portal vein with PE 205 (Intramedic, Clay-Adams) were performed under pentobarbital anesthesia (5 mg/kg of body wt). This was followed by hepatectomy, and the isolated liver was then transferred to a perfusion chamber (Metaloglass, Boston). The entire procedure, from initial cannulation until transfer to the chamber required 4–6 min. To avoid transient anoxia during excision of the liver, oxygenated heparinized buffer was passed through the portal cannula before transfer of the liver to the chamber.

Perfusion. The chamber was thermostatically maintained, at 38 ± 0.5° during the experiment. Blood flow, measured through a calibrated bypass was 45–50 ml/min. Care was taken to keep the hydrostatic pressure of

[1] Present address: Veterans Administration Hospital, Section of Haemodynamics, Medical Service, Washington, D.C.

[2] Requests for reprints should be addressed to: Dr. H. J. Zimmerman, Chief, Medical Service, Veterans Administration Hospital, Boston, Massachusetts 02130.

[3] Heparin sodium; Upjohn.

the portal vein at 17 cm of water. Oxygenation was ensured by a constant flow of air mixture (O_2: CO_2/95:5%).

Ethanol (absolute, USP) was added to the perfusion medium in amounts sufficient to provide concentrations of 5, 15, 25, 125, and 250 mmoles/liter. In each experiment, the liver was perfused for 30 min of equilibration before the addition of BSP[4] or ICG[4] to the system. The same interval of time was used for equilibration in control experiments, in which no ethanol was added to the perfusion medium.

The BSP was administered in a concentration of 0.15 mg/ml of perfusion medium and ICG in a concentration of 0.015 mg/ml. The concentration of BSP utilized is similar to that used in some of the perfusion studies of Plaa and Hine (6). The concentration of ICG was 10-fold lower, because higher concentrations had a tendency to precipitate in the system. Blood samples (0.3 ml) were drawn every minute for the first 10 min following the addition of the dye, then at 5-min intervals for 45 min. Bile was collected continuously into 20-μ pipettes, and the periods of collection were carefully timed. A total of 35 perfusions were performed. The number at each concentration of ethanol is listed in Fig. 1.

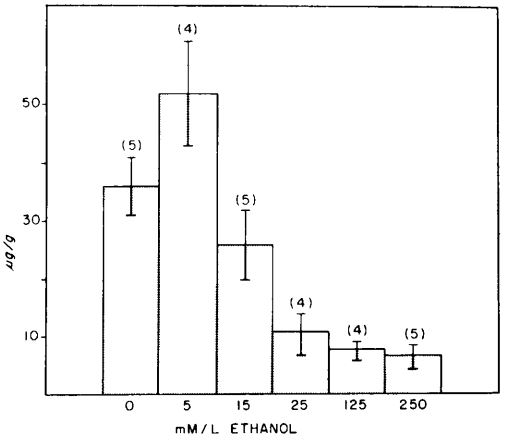


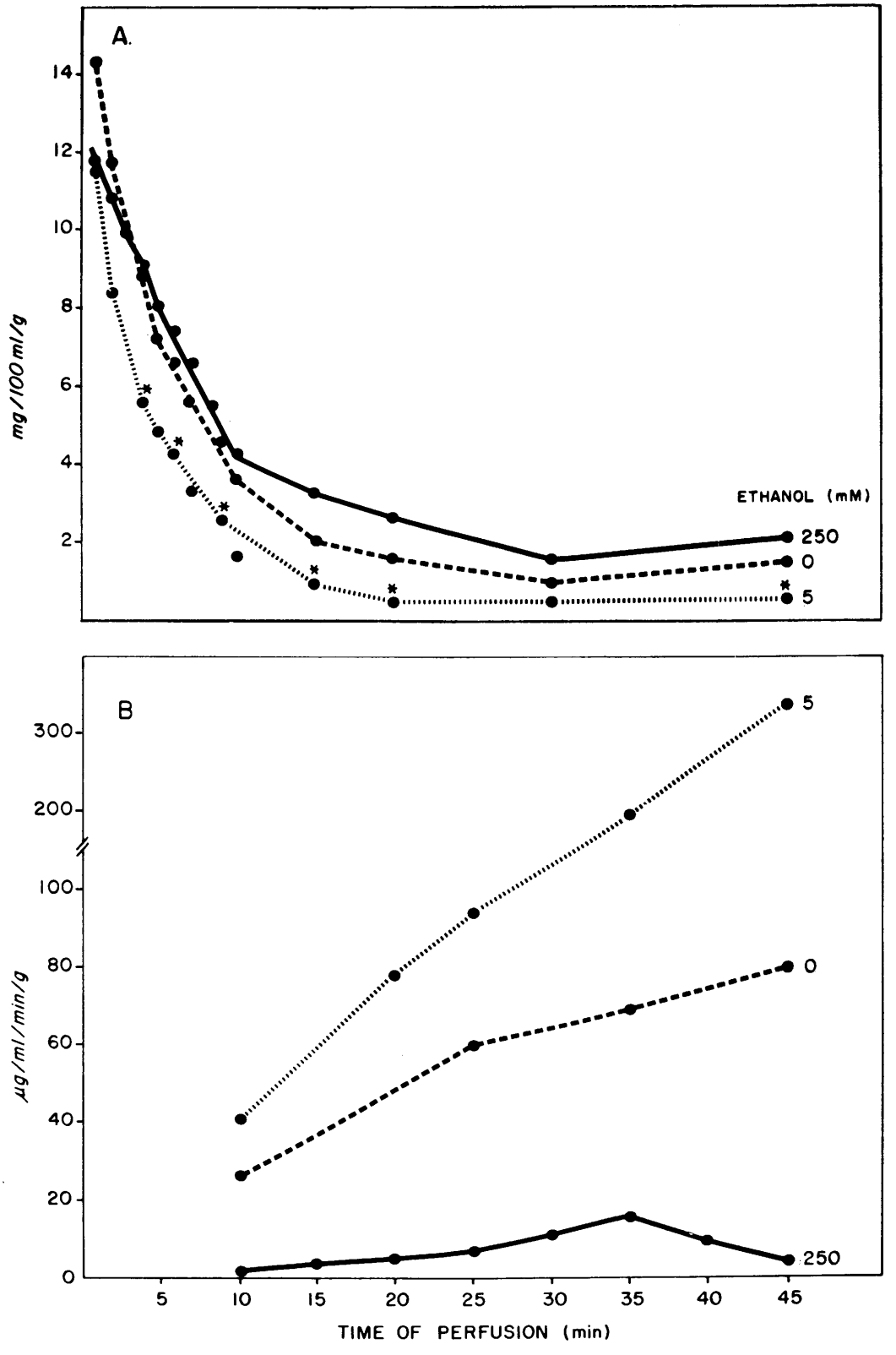
FIG. 1. Total BSP excreted in bile during 45 min of perfusion; Numbers of experiments for each concentration of ethanol is shown in parentheses; vertical lines represent standard deviations. Ethanol concentrations of 25, 125, and 250 m*M* were significantly different from controls and also from 15 m*M* concentration ($p<0.005$–0.01).

Analytical. The BSP concentration in blood plasma and bile was determined by a colorimetric method, read at 575 nm on a Coleman Junior colorimeter. Since plasma was usually discolored by slight hemolysis, readings were taken after adequate dilution with normal saline (50 μl:10 ml). Each sample, prior to alkalinization, served as its "blank." Solutions were made alkaline by addition of 0.2 ml of 3 *N* NaOH to the 10-ml sample. For bile samples, dilutions were 2.5 times higher. The ICG content was assayed by direct reading of diluted plasma in a Beckman DU spectrophotometer at 805 nm. Construction of a "standard" curve for ICG measurement was done by adding known amounts of dye to diluted (17%) rat plasma. Diluted plasma was also used as the "blank" in the individual determinations. Standards for determination of BSP concentration in bile were prepared separately using bile collected from donor rats. Analysis of variance was carried out according to Snedecor (7).

Results. Effect of ethanol on BSP excretion: The effects of perfusion of the liver, with a medium containing ethanol, on the excretion of BSP is shown in Fig. 1. Concentrations of ethanol, ranging from 25 to 250 m*M* led to a significant and dose-related decrease in the rate of excretion of dye. Figure 2 indicates that the interference with excre-

FIG. 2. Mean rate of BSP disappearance from perfusion medium (A) and BSP appearance in bile (B). (A) Ethanol concentration of 5 m*M* resulted in a statistically significant difference from controls (*), $p<0.01$; 250 m*M* of ethanol led to no significant change in the rate of BSP disappearance. (B) Ethanol concentration of 5 and 250 m*M* showed a significant difference from controls ($p<0.01$–0.05).

[4] Sulfobromophthalein and Cardiogreen, Hynson, Wescott & Dunning, Inc., Baltimore, Maryland.


A.
mg/100ml/g
14
12
10
8
6
4
2
ETHANOL (mM)
250
0
5
B
µg/ml/min/g
300
200
100
80
60
40
20
0
5
0
250
5
10
15
20
25
30
35
40
45
TIME OF PERFUSION (min)

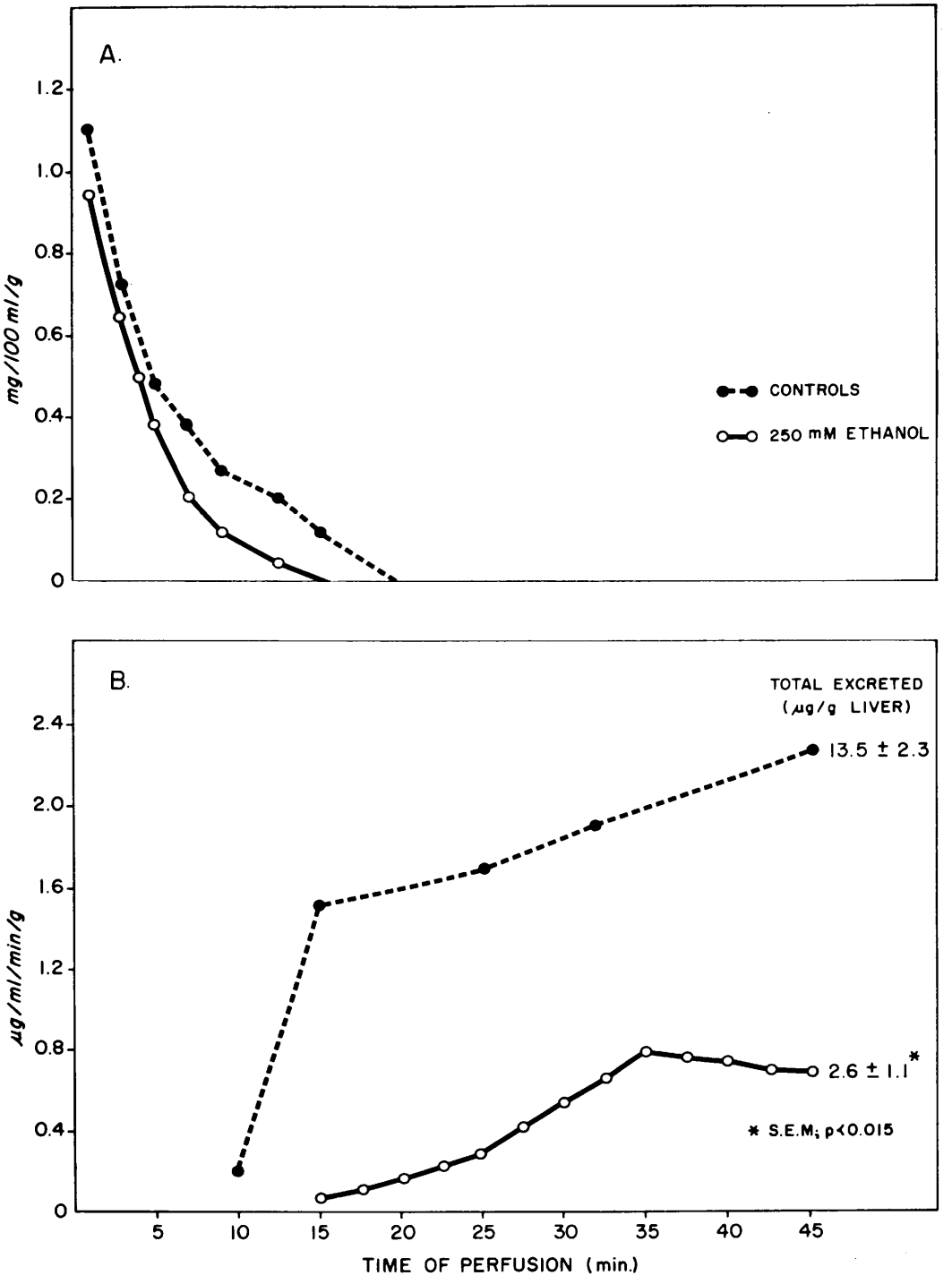


FIG. 3. Mean rate of ICG disappearance from perfusion medium (A) and ICG appearance in bile (B); each point of the curves represent the mean of 4 experiments.

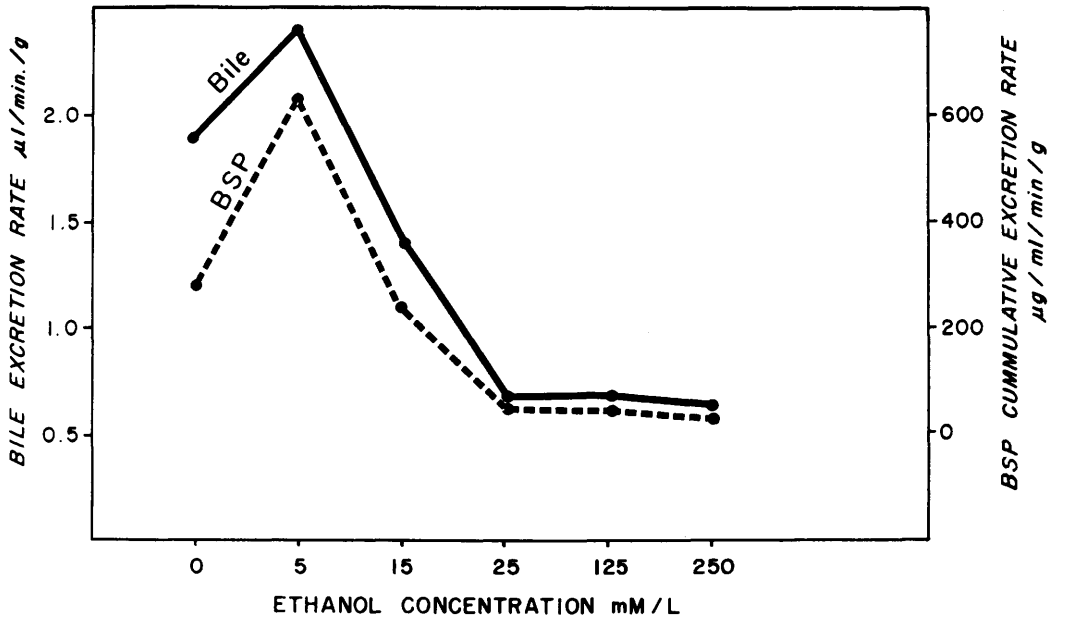


FIG. 4. Effect of ethanol on the rate of bile excretion and on the biliary excretion of BSP.

tion continued during the entire period of ethanol perfusion.

The concentration of 15 m*M* yielded values lower than those of the control, but the differences were not statistically significant. The lowest concentrations (5 m*M*) indeed appeared to increase the rate of excretion of the dye (Fig. 1).

Measurement of the rate of disappearance of BSP from the perfusion medium showed no impairment of dye removal by the liver at the concentrations of ethanol that had been found to interfere with excretion of BSP. At the concentration of ethanol of 5 m*M* there was a significant increase in the rate of BSP removal from the perfusate, similar to the observed increase in the excretion of BSP in the bile (Fig. 2).

Effects of ethanol on excretion of ICG. Measurements of the rate of excretion of ICG during the perfusion with ethanol, at the concentration of 250 m*M*, demonstrated a striking reduction in excretion of the dye in the bile (Fig. 3). This was accompanied by no significant decrease in the rate of removal of ICG from the perfusate (Fig. 3).

Effects of ethanol on excretion of bile. Ethanol, in concentrations of 15 m*M* or more led to a significant and dose-related decrease in the rate of bile flow. In all of these experiments BSP was also infused and its excretion measured. The decrease in rate of bile flow paralleled closely the decrease in rate of BSP excretion in the same experiments (Fig. 4). In order to test the effect of ethanol alone, 250 m*M* concentration was infused without BSP. This concentration of ethanol induced a significant reduction ($p < 0.01$) in the rate of bile excretion equivalent to the effect of a lower (25 m*M*) concentration of ethanol when BSP was also infused.

Discussion. The results of these studies utilizing an *in vitro* model, are similar in part to those of Lieberman and Childs (4). These authors utilizing an *in vivo* model, observed that ethanol administration impaired excretion of BSP without interference with hepatic removal of the dye from the perfusing "blood." The studies of Lieberman and Childs (4), however, led them to conclude that the chief effect of ethanol was to inhibit the hepatic conjugation of the dye, a known requirement for its excretion (8).

Results of the present study in an *in vitro* model, however, appeared to demonstrate a different or additional mechanism. Ethanol,

in concentrations ranging from 25 to 250 m*M* interfered with the excretion of BSP, ICG, and bile. The interference with the excretion of ICG, in contrast to that of BSP could not be attributed to impairment of conjugation (9), since ICG is not conjugated prior to excretion. Furthermore, the most important physicochemical determinant of bile flow appears to be the concentration of bile salts in the canalicular fluid (10), a phenomenon not directly relatable to the conjugating capacity of the liver; since Stone (11) has shown that interference with excretion of BSP, by administering an agent that reduces BSP conjugation and excretion (probenecid), does not appear to decrease bile flow.

The difference betwen the results of the present study and those of Lieberman and Childs (4) may relate to differences in metabolism of ethanol in the perfused liver *in vitro*, as compared to the intact, *in vivo* model. Furthermore, the concentration of ethanol in blood perfusing the liver in the intact animal is not known and can only be estimated. It is possible, that at some concentrations, impairment of conjugation is the chief defect, whereas at other concentrations, impaired excretion may be the important one.

Nevertheless, the results of the present study would seem to indicate that ethanol in the concentrations studied does interfere with excretion of these two foreign dyes. It has been proposed (12) that ICG may depend on a "slightly" different pathway of excretion than does BSP. Accordingly, it is also conceivable that impairment of BSP excretion might depend on interference with conjugation, yet the impairment of ICG excretion might result from defective transfer across the canalicular membrane. That the effect of ethanol may indeed be at the excretory process also is suggested by the decrease in bile flow. While BSP and ICG may themselves lead to decrease in the rate of bile excretion (13, 14), a decrease in bile flow seems ascribable to the ethanol. With a constant BSP load in all experiments, a progressive decrease in rate of bile flow could be correlated with the progressive increase in ethanol concentration.

The interference with excretion of a foreign dye may be indirect and related to the metabolism of ethanol. The lowering of intracellular pH (15) or the alteration in the concentration of coenzymes (16) that accompany the oxidation of ethanol, could interfere with the energetics of the membrane transport. Alternatively alcohol might interfere with excretion by a direct effect on the canalicular membrane structure and function.

The enhancement of BSP excretion at low concentrations of ethanol remains to be explained. Similar biphasic effects of chemical compounds are present in other biological systems, where in low concentrations a drug may have a "favorable" effect and at somewhat higher concentrations, have an "unfavorable" effect. For example, chlorpromazine in concentrations of 10^{-5} *M* "protects" cells against carbon tetrachloride toxicity, yet concentrations of 10^{-4} *M* can be shown to have a demonstrably adverse effect on cells *in vitro* (17).

The relevance of the *in vitro* effects of ethanol on bile flow and excretion of foreign dyes to the *in vivo* syndromes observed in alcoholic patients remains to be demonstrated. Nevertheless, a "cholestatic" effect appears to have been induced in the perfused liver by concentrations of ethanol in a range that can be attained *in vivo*.

Summary. Perfusion of the rat liver *in vitro* with concentrations of ethanol ranging from 25 to 250 m*M* in a medium of diluted rat blood was found to interfere with bile flow and with excretion of sulfobromophthalein (BSP) and indocyanine green (ICG). This impairment of excretion could not be related to interference with removal from the blood, since this was found to proceed normally during the course of the perfusion. Impaired excretion of these foreign dyes also could not solely be the results of impaired conjugation, since ICG does not require conjugation while BSP does. It was inferred that ethanol interferes with transport into the bile of its constituents, at concentrations similar to those that can be attained *in vivo*. The effect of ethanol on bile flow and dye excretion appears to be biphasic in that a low concentration of ethanol (5 mmoles/liter) appeared to increase the flow of bile and both

excretion and clearance of BSP.

The authors are grateful to Mrs. A. Harinasuta for technical assistance and to Dr. J. Penhos for advice.

1. Harinasuta, U., Chomet, B., Ishak, K., and Zimmerman, H. J., Medicine **46**, 141 (1967).

2. Weinstein, B. R., Korn, R. J., and Zimmerman, H. J., Ann. Internal Med. **58**, 245 (1963).

3. Goldberg, M. and Thompson, C. M., Ann. Internal Med. **55**, 416 (1961).

4. Lieberman, A. H. and Childs, H. W., Am. J. Physiol. **213**, 353 (1967).

5. Penhos, J. C., Wu, C. H., Daunas, J., Reitman, B. A., and Levine, R., Diabetes **15**, 740 (1966).

6. Plaa, G. L. and Hine, C. H., A. M. A. Arch. Ind. Health **21**, 114 (1960).

7. Snedecor, G. W., "Statistical Methods," 5th ed. Iowa State Univ. Press, Ames, Iowa (1956).

8. Combes, B., J. Clin. Invest. **44**, 1214 (1965).

9. Wheeler, H. O., Cranston, W. I., and Meltzer, J. E., Proc. Soc. Exptl. Bill. Med. **99**, 11 (1958).

10. Hargreaves, T., "The Liver and Bile Metabolism," p. 39. Appleton, New York (1968).

11. Stone, S. L., *in* "The Biliary System" (W. Taylor, ed.), p. 277. Davis, Philadelphia, Pennsylvania (1965).

12. Hargreaves, T., Quart. J. Exptl. Physiol. **51**, 184 (1966).

13. Schonfield, L. J., McGill, D. B., and Foulk, W. T., J. Clin. Invest. **43**, 1424 (1964).

14. Klaassen, C. D. and Plaa, G. L., J. Pharmacol. Exptl. Therap. **161**, 316 (1968).

15. Seligson, D., Waldstein, S. S., Giges, B., and Meroney, W. H., Clin. Res. Proc. **1**, 86 (1953).

16. Isselbacher, K. J. and Greenberg, N. J., New Engl. J. Med. **270**, 351 (1964).

17. Zimmerman, H. J., Mao, R., and Israsena, S., Proc. Soc. Exptl. Biol. Med. **123**, 893 (1966).

Received June 25, 1969. P.S.E.B.M., 1969, Vol. 132.