SPECIAL COMMUNICATIONS

Demonstration of bile stasis in the mouse by a direct and an indirect method[1]

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PLAA, GABRIEL L., AND BERNARD A. BECKER. *Demonstration of bile stasis in the mouse by a direct and an indirect method.* J. Appl. Physiol. 20(3): 534–537. 1965.—Cannulation of the common bile duct of the mouse has been effected. Bile flow rates and bile pigment concentrations have been determined in these preparations. These values are lower than comparable values in bile duct-cannulated rats. An indirect test for detection of bile flow in the mouse, using injected fluorescein as an indicator, has been devised. Using all-or-none criteria, results obtained using the indirect technique correlate well with those obtained by the direct (cannulation) method. The use of the indirect test in conjunction with liver function tests, e.g., Bromsulphalein (BSP) retention, is recommended as a means of elucidating causes of liver dysfunction. By this means, a difference has been demonstrated between two hepatotoxic agents which effect BSP retention in the mouse: carbon tetrachloride and alpha-naphthylisothiocyanate (ANIT). The latter agent interferes with BSP excretion as shown by the indirect bile flow test; carbon tetrachloride does not cause blockage of bile flow.

bile flow alpha-naphthylisothiocyanate CCl_4 cannulation of bile duct in mice fluorescein measure of bile flow in mice sulfobromophthalein bile pigment concentration in mice

REMOVAL OF INJECTED sulfobromophthalein sodium (Bromsulphalein, BSP) from the blood stream of the mouse has been employed as a means of assessing the hepatotoxic properties of drugs and chemicals (1, 5–7). One possible cause of BSP retention is cessation of bile flow. In order to test this possibility, one must be able to demonstrate bile flow or cessation of bile flow. Animals larger than the mouse can readily be prepared with a biliary fistula; the size of the mouse common bile duct would appear to preclude use of such a procedure. However, cannulation of the mouse common bile duct has been achieved in this laboratory. One purpose of this report is to present bile flow rates and bile pigment concentrations measured in untreated, bile duct-cannulated mice.

The numbers of animals usually required for obtaining dose-response data in drug studies and the time involved in preparing these animals would appear to limit the routine use of this direct cannulation technique. Accordingly, an indirect method of demonstrating bile flow was sought. Fluorescein is eliminated, in part, via the bile. Hence, if an hepatotoxic agent effected cholestasis, passage of administered fluorescein into the extrahepatic biliary structures would be prevented. The absence of fluorescein in the biliary tree would be evidence of cessation of bile flow. The second purpose of these investigations was the establishment of an indirect method of detecting bile flow in the mouse using fluorescein. Using all-or-none criteria, results obtained by the indirect method were compared to those obtained by the direct method.

The utility of the indirect method for distinguishing between different types of hepatotoxic agents was assessed. For example, both carbon tetrachloride (CCl_4) and alpha-naphthylisothiocyanate (ANIT) produce BSP retention in the mouse. CCl_4 effects degeneration and necrosis of the liver parenchymal cells, which are readily seen with the light microscope, whereas ANIT is a cholestatic agent (3). Accordingly, these two liver poisons were chosen to test the indirect method.

METHODS

Cannulation of the bile duct of the mouse. Mice weighing 35–45 g were anesthetized with pentobarbital sodium, 90 mg/kg, intraperitoneally. The common bile duct was incised with a pair of fine iridectomy scissors about 6 mm below the hilum of the liver. A PE-10 polyethylene tube was passed through the incision and propelled towards the hilum for a distance of about 3 mm. (Care is necessary to avoid tearing of the bile duct as the tubing is larger than the duct.) A restraining ligature was placed above the incision so that the cannula was held firmly in place. The body wall was closed with small Michel wound clips (9 or 11 mm), taking care to avoid constriction of the cannula. The mouse was then re-

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strained in the supine position by binding with adhesive tape to a small wooden splint. Bile was collected in calibrated Wintrobe tubes. Animals were not given food or water during the collection period. In the otherwise untreated animal, bile was delivered at the end of the cannula within a few minutes.

Bile pigment content. Ferro and Ham (2) described a method for determining serum bilirubin by oxidation of bilirubin to biliverdin, and estimation of biliverdin concentration spectrophotometrically. This procedure was adapted for estimation of total bile pigment content of bile. A 0.10-ml sample of bile was treated with 1.2 ml of the Ferro-Ham oxidizing reagent, centrifuged, and the supernatant transferred to a cuvette. The optical density of the supernatant was determined 1 hr after initial treatment at 660 mμ with a Bausch and Lomb Spectronic 20 spectrophotometer, and compared to that of appropriate chloroform solutions of bilirubin treated in the same manner. Results were expressed as mg/100 ml of bilirubin.

Indirect bile flow (fluorescein) method. Groups of 10–15 mice (18–25 g) were administered the selected hepatotoxin, dissolved in corn oil, by stomach tube. A solution of fluorescein was prepared shortly before use by suspending 20 mg fluorescein in 10 ml 0.17 N $NaHCO_3$; after 30 min the suspension was filtered and the clear filtrate employed for injection. After a predetermined interval, the fluorescein solution was injected into the mice via the tail vein, 0.1 ml/10 g body wt. Fifteen minutes later, the animals were sacrificed by cervical dislocation and the viscera exposed. The gall bladder and bile duct were examined in a darkened room under ultraviolet light for fluorescence. Those animals in which fluorescence was not seen in the extrahepatic biliary passages were considered to exhibit cholestasis, i.e., bile flow strongly diminished or absent, and were recorded as "blocked."

BSP retention. Groups of 10 male Swiss-Webster-type mice (18–25 g) were pretreated with the desired hepatotoxic agent. After the predetermined interval, BSP, 75 mg/kg, was administered via the tail vein. Thirty minutes later, blood was collected by cardiac puncture under light ether anesthesia. Plasma BSP was then determined spectrophotometrically (5). Percent retention was calculated and compared to retention obtained in control mice under the same conditions.

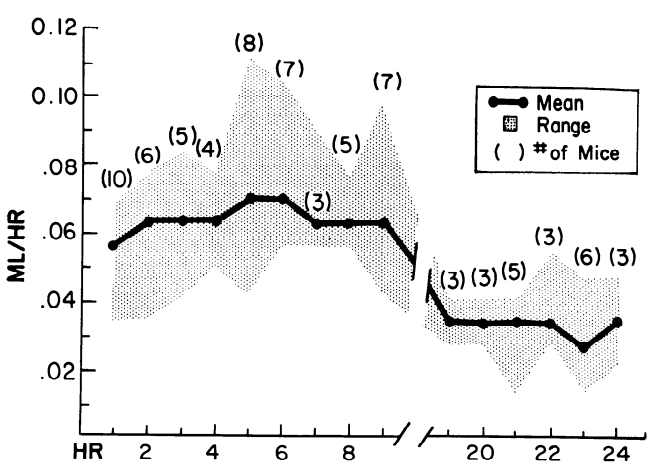

FIG. 1. Bile flow in the bile duct-cannulated mouse. Figures in parentheses represent number of animals employed.

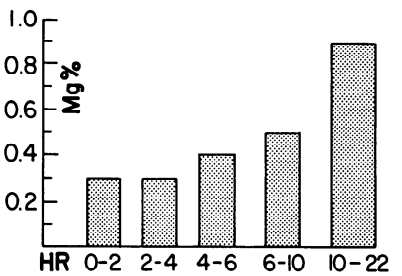

FIG. 2. Bile pigment content of bile from bile duct-cannulated mouse.

RESULTS

Bile flow in the untreated mouse. Bile flow is usually apparent 5–15 min following cannulation of the bile duct and closing of the body wall, even though the mouse is still anesthetized. The initial flow rate is depressed, perhaps due to the depressant effects of the anesthetic. By 2 hr, flow is well established and the rate (approximately 0.06–0.07 ml/hr) tends to remain relatively constant over the next 7 hr. Figure 1 shows the results of a typical experiment. The flow rate declines gradually to about 0.03–0.04 ml/hr at the 19th hr. This level of bile flow then remains relatively stable. Three animals were observed to produce bile at this rate for 48 hr.

Bile pigment content in the untreated mouse. Bile was collected from bile duct-cannulated mice and analyzed for total bile pigments, which were expressed as bilirubin. Figure 2 shows the results obtained in one experiment. The data are the average of three animals. Analyses were performed at the end of the collection period.

BSP retention. The doses of CCl_4 (1 ml/kg, p.o.) and ANIT (150 mg/kg, p.o.) chosen were found to effect approximately equivalent retention of BSP (Table 1). Any animal which retained more than 3.7% of the administered BSP was considered to exhibit significant retention. The value, 3.7% retention, represents the mean retention of 84 control mice +3 standard deviations.

Indirect (fluorescein) method. In a preliminary study, fluorescein was administered orally to groups of three mice. Groups were sacrificed 10, 15, 20, 60, 120, and

TABLE 1. *BSP retention in mice*

	Percent BSP Retained	No. Significant*/ No. Tested
Control	1.2 ± 0.1	0/84
CCl_4, 1 ml/kg	12.0 ± 1.6	9/9
ANIT, 150 mg/kg	13.3 ± 3.7	8/8

Values are means ± standard error. * >3.7% retention ($P < 0.01$).

240 min later and the gall bladders and common bile ducts were examined under ultraviolet light. In all cases, the gall bladders and bile ducts were strongly fluorescent. Fluorescence was also seen in the liver, kidney, ureters, urinary bladder, and at the urethral orifice of the penis. The stomach and small intestine were also fluorescent in the groups sacrificed before and at 60 min. In the 120-min group, however, fluorescence was no longer seen in the stomach.

When fluorescein was administered intravenously, the same general distribution of fluorescence was seen (except for stomach). Intensity of fluorescence in the bile duct and gall bladder was judged to be greatest 10–30 min after injection and to decline after 30 min. Accordingly, a 15-min period between injection and sacrifice was considered optimal for purposes of standardization. Under these conditions, control mice invariably show well-demarcated gall bladders and bile ducts (Fig. 3).

ANIT was administered orally to groups of 10 or 15 mice. The animals were given fluorescein at various time intervals after treatment, sacrificed, and examined for fluorescence in extrahepatic bile passages. There was no evidence of blockage of bile flow in the animals sacrificed 2 hr after ANIT treatment. However, complete blockade was seen in 7/10 of the animals sacrificed 24 hr after ANIT. From these data (Table 2), the median time to effect (ET_{50}) was calculated by the method of Litchfield (8), with correction for upper truncation, and found to be 16 hr (95% confidence limits: 15–17 hr).

FIG. 3. Indirect bile flow method; control animal. Photograph taken under ultraviolet light shows strongly fluorescent gall bladder, bile duct, and duodenal segment.

TABLE 2. *Effect of alpha-naphthylisothiocyanate (ANIT), 150 mg/kg, p.o., on bile flow in the mouse*

Indirect (Fluorescein) Method		Direct (Cannulation) Method	
Hours*	No. blocked/no. treated	Hours	No. blocked/no. treated
2	0/15	2	0/5
6	2/15	14	3/9
14	3/10	17	6/10
19	7/10	24	6/7
26	7/10		
ET_{50}: 16 (15–17) hr		ET_{50}: 16 (14–18) hr	
s: 1.34 (1.26–1.42)		s: 1.42 (1.39–1.56)	

s = slope function of regression line. Figures in parentheses represent 95% confidence limits. * Time after administration of ANIT.

Direct (cannulation) method. Groups of mice were similarly treated with ANIT and operated at the requisite times. Those animals in which bile flow was not seen within 1 hr after cannulation were considered blocked. Hence, quantal data were obtained and treated by the Litchfield method (8) (Table 2). The calculated ET_{50} was 16 hr (95% confidence limits: 14–18 hr). Agreement between the two methods was excellent. The regression lines did not deviate from parallelism.

Effect of CCl_4 on bile flow. Eleven mice were given CCl_4 orally; 24 hr later, the mice were injected with fluorescein and sacrificed at 15 min. Examination of the extrahepatic bile passages under ultraviolet light showed fluorescence in all of the bile ducts and in all of the gall bladders except one. Hence, CCl_4 is seen to effect BSP retention when bile flow is not completely blocked. The livers of all 11 mice showed gross signs of liver damage.

DISCUSSION

Detection of reduced bile flow can be readily accomplished in large animals by cannulation of the bile duct. Cannulation of the mouse common bile duct is also possible, as reported here, but is sufficiently inconvenient and difficult to rule out the procedure as a routine practice. Recently, Pedreira and Tepperman (9) succeeded in fixing a cannula into the gall bladder of the

TABLE 3. *Bile flow and bilirubin content of rat and mouse*

	Rat*	Mouse
Plasma bilirubin, mg/100 ml	0.6	0.1†
Biliary bilirubin, mg/100 ml	3.6	0.7
Bile flow, ml/hr	0.7	0.07

* Goldfarb et al. (3). † Becker and Plaa (unpublished).

mouse. Their preparation differs from that described above in the exclusion of the gall bladder. Hence, whatever powers of bile concentration or reabsorption from bile that the mouse gall bladder may have, are lost. They obtained a mean flow rate of about 0.10 ml/hr (calculated from their data). This value falls within the upper part of the bile flow rate range observed in our bile duct cannulated mice.

The bile flow and bile pigment data obtained on mouse fistula bile are limited but submitted here because such data are not included in tables of comparative bile composition (4). Goldfarb et al. (3) have published similar data for the rat. These data are compared to the mouse data reported here (Table 3). Mouse values are lower than comparable rat values, perhaps reflecting the smaller size of the mouse and lower circulating blood levels of bilirubin.

The indirect method, employing fluorescein as an indicator, can be easily performed even by personnel unskilled in surgical techniques. The method is rapid and reproducible, especially if interpretations are made on an all-or-none basis, i.e., the extrahepatic biliary structures fluoresce or they do not. The data in Table 2 show that the results obtained by the indirect method are in excellent agreement with those obtained by the direct method. The indirect bile flow technique can, therefore, be used to determine the possible role of cholestasis when liver dysfunction has been demonstrated by other means, e.g., BSP retention test. In the case of the two hepatotoxic agents selected, the indirect bile flow method correctly detected the cholestatic agent. The method is simple and inexpensive enough to be used as a routine screening test in drug evaluation studies.

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