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Research Article

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The administration of protoporphyrin sufficient to achieve maximal canalicular secretion was found to significantly reduce bile flow in rats infused with 0, 40, and 80 µmol sodium taurocholate/h. Linear regression analysis defined the relationship between bile flow and biliary bile acid secretion and showed that the bile acid-independent fraction of bile flow was reduced ($P < 0.01$). Bile acid-dependent flow was unaffected and there was no significant difference in biliary bile acid secretion rates between control and protoporphyrin-perfused livers. Perfusion of rat livers with varying concentrations of protoporphyrin demonstrated the reduction of bile flow was dose-related. Analysis of perfusate enzyme activity did not reveal abnormalities that could account for the cholestasis. Studies to evaluate the effect of protoporphyrin on regional hepatic hemodynamics were inconclusive.

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A B S T R A C T The pathogenesis of liver disease in protoporphyria has been presumed to result from the hepatic deposition of protoporphyrin. To examine the effects of protoporphyrin on hepatic bile flow and histopathology, studies were performed employing an isolated, *in situ*, rat liver perfusion system. Rat livers in the control group were perfused with 0–80 μmol sodium taurocholate/h. Rat livers in the experimental group were perfused with sodium taurocholate and (*a*) sufficient quantities of protoporphyrin to produce maximal canalicular secretion and (*b*) perfusate protoporphyrin concentrations of 0.01, 0.1, and 1 μM.

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Histopathological studies of control and protoporphyrin-perfused rat livers did not show ab-

normalities on light microscopy. However, canalicular dilatation, distortion, and loss of microvilli were present in the protoporphyrin-perfused livers examined by transmission electron microscopy. Although ultraviolet microscopy showed diffuse fluorescence of the hepatocytes and canaliculi of protoporphyrin-perfused livers, the deposition of protoporphyrin in amorphous or crystalline forms was notably absent in studies with polarizing and transmission electron microscopy.

These studies provide evidence that protoporphyrin has hepatotoxic properties that affect the canalicular secretory apparatus. The mechanism(s) responsible for the injury require further clarification.

INTRODUCTION

Protoporphyria is an inherited disorder of porphyrin metabolism in which deficiency of heme synthase leads to overproduction of protoporphyrin (1–5). The initial manifestation and dominant symptom of the disorder is a photodermatitis characterized by erythema, edema and pain (1, 6, 7). However, gallbladder and liver disease are now recognized to be the most serious consequences of the illness. Gallstones have often been reported in patients with protoporphyria and have occurred at an unusually early age (7, 8). Cirrhosis and liver failure are singularly responsible for death that results from the disorder (9–11).

15 deaths from liver failure have been reported in patients with protoporphyria involving both sexes ranging in age from 11 to 60 yr (9–15). Histopathologic examination of the liver in these cases has revealed cholestasis, periportal fibrosis, and micronodular cirrhosis. This is associated with brownish-black pigmentation and deposition of granular and globular pigment in hepatocytes, canaliculi and Kupffer cells (9, 10, 16, 17). The pigment has been shown to represent deposits of amorphous and

crystalline protoporphyrin by fluorescent (8, 9), polarizing (9, 16, 18), and electron microscopy (9, 16, 17, 19). These findings have been corroborated by demonstrating protoporphyrin accumulation in massive amounts ranging from 0.2 to 57.5 mg/g wet liver (10).

The pathogenesis of the liver disease in protoporphyria is not clearly understood. In part, this is because of lack of a suitable animal model. Liver disease with histopathology resembling that seen in patients with protoporphyria has been produced by administering porphyrinogenic agents, e.g., griseofulvin, to mice (20–22). Unfortunately this has not proved particularly valuable in addressing the nature of protoporphyrin deposition in the liver as it applies to cases of protoporphyria in humans (23), except in cases in which the liver is primarily responsible for protoporphyrin overproduction (24). The recent demonstration of a bovine form of protoporphyria may provide a more meaningful model to clarify many aspects of the disorder (25).

Because protoporphyrin is primarily eliminated by the liver, characterization of its disposal may provide an understanding of the factors responsible for its accumulation. In an isolated *in situ* liver perfusion system, protoporphyrin was rapidly cleared from perfusate by the liver but biliary excretion was limited (26). In those studies and subsequent investigation of the maximal canalicular secretion of protoporphyrin (27), it was observed that bile flow was reduced in rat livers perfused with protoporphyrin. Therefore, studies were designed to examine the specificity and significance of this phenomenon. Results of these studies provide evidence that protoporphyrin induces cholestasis in the isolated, *in situ*, perfused rat liver unassociated with the use of porphyrinogenic agents or with deposition of amorphous or crystalline protoporphyrin.

MATERIALS

Animals and operative procedure. Nonfasting male Sprague-Dawley rats (Holtzman Laboratories, Madison, Wis.) weighing 250–350 g and fed standard laboratory chow (Simonson Laboratories, Gilroy, Calif.) were used in all experiments. A modification of the techniques of Miller et al. (28) and Hems et al. (29) was used to prepare rats for the isolated *in situ* liver perfusion. After ether anesthesia, the abdomen was opened through a midline incision and the portal anatomy exposed. The common bile duct was cannulated near its entrance into the duodenum with bevelled PE 10 polyethylene tubing (Clay Adams, Parsippany Div., Beckton, Dickinson & Co., N. J.) which was inserted to a point just short of the bifurcation of the duct. The portal vein was cannulated with a 14 gauge (1.8-mm i.d.) Cathlon IV angiocath (Jelco Laboratories, Raritan, N. J.), immediately flushed with 5–10 ml heparinized saline, and secured in place. The anterior thoracic rib cage was then excised and a 14-gauge angiocath inserted into the intra-

thoracic portion of the inferior vena cava via the right atrrium. Residual hepatic blood was removed by injecting 5–10 ml of heparinized saline into the portal vein catheter after ligating the inferior vena cava above the right renal vein. The isolated *in situ* preparation was then moved to a prewarmed plexiglass cabinet and connected to the perfusion apparatus. The time interval between interruption of the portal circulation to onset of artificial perfusion was <3.5 min. Experiments were discontinued if interruption of the portal circulation was prolonged, media leaked from the liver, the gross appearance of the liver suggested nonhomogeneous perfusion, or conditions of the experiment were unexpectedly altered in any way.

Perfusion apparatus, media, and experimental conditions. The entire perfusion apparatus was contained within a plexiglass cabinet (0.9 × 0.6 × 0.6 m). The apparatus was designed to recirculate 100 ml of oxygenated media at a constant rate through the liver. Liver perfusion occurred in the following way: media was pumped (Masterflex multihead peristaltic pump 7545-10, Cole Parmer Instrument Co., Chicago, Ill.) from a receptacle to a membrane oxygenator. Oxygenation of the media was accomplished by passing it through 4.57 m of silastic tubing (i.d. 1.47 mm; o.d. 1.96 mm, Dow Corning Corp., Midland, Mich.) coiled in a glass jar that was suffused with 5% CO_2 and 95% O_2 flowing at 3 liters/min. Before entering the portal vein the oxygenated media was passed through a flowmeter (No. 12, Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.). A constant perfusate flow rate of 40 ml/min was maintained by appropriately setting the pump speed. This system obviated the potentially variable rate of perfusion in gravity-dependent flow systems. Media exiting the liver was directed back to the receptacle for recirculation. An alternate media contained in a separate reservoir, circulated by means of the multichannel peristaltic pump, could be substituted by manipulation of a y-piece connector without interruption of flow to the liver. The plexiglass cabinet containing the perfusion apparatus was humidified, prewarmed, and maintained at 37°C with a fan-heater. The perfusion apparatus and tubing were washed with deionized water before and after each perfusion.

The perfusion media consisted of Krebs-Henseleit solution (30) to which 3% bovine albumin (Fraction V, Sigma Chemical Co., St. Louis, Mo.), protoporphyrin IX (Porphyrin Products, Logan, Utah) and sodium taurocholate (NaTC,[1] Steraloids, Inc., Milton, N. H.) were added. Erythrocytes were not added to the perfusate because of their ability to absorb protoporphyrin. A stock solution of Krebs-Henseleit solution with 3% bovine albumin was prepared by passing it through a 0.45-μm filter (Millipore Corp., Bedford, Mass.), adjusting the pH to 7.43 and adding gentamicin, 1 ml/liter (Schering Corp., Kenilworth, N. J.). The stock solution was refrigerated at 4°C and appropriate amounts warmed prior to use. The protoporphyrin, which was added to the stock solution of perfusion media, was prepared on the day of use from a supply of purified material refrigerated at 4°C and protected from light. A solution of protoporphyrin was made by dissolving 5–7 mg in 0.5 ml 1 N NH_4OH, diluting it to 10 ml with D_5W and filtering it through a 0.45-μm Millipore filter. The osmolarity of this solution was ~280 mosmol. The concentration of protoporphyrin in the filtered solution was determined spectrophotometrically using a Carey model 15 spectrophotometer (Varian Associates Inc., Palo Alto, Calif.).

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[1] *Abbreviations used in this paper*: BAIF, bile acid-independent fraction; NaTC, sodium taurocholate.

Appropriate amounts of this concentrated solution of protoporphyrin (550–650 μg/ml) were added by bolus or constant syringe-pumped infusion (Harvard pump 940, Harvard Apparatus Co., Inc., St. Natick, Mass.) to the perfusion media to obtain concentrations desired in the experimental studies. During circulation of media containing protoporphyrin, glassware and tubing were wrapped in aluminum foil to reduce exposure to light. NaTC was found to be >98% pure by thin layer chromatography (31). After dissolving in normal saline, NaTC was syringe-pumped into the perfusion media at a constant rate varying from 2–80 μmol/h through a port in the receptacle. The media was magnetically stirred and the pH continuously monitored (Radiometer PHM 63, digital pH meter, London Co., Westlake, Ohio) and maintained between 7.41–7.43 by frequent manual addition of minute amounts of concentrated sodium bicarbonate.

The isolated *in situ* liver perfusions were maintained for 90 min. During the initial 20 min, which was considered the basal equilibration period, livers were perfused only with the Krebs-Henseleit solution containing albumin. For the remaining 70 min, the control group of livers were perfused with and without solutions containing NaTC; protoporphyrin was added in the experimental group. Immediately after perfusion, livers were removed from the abdomen, gently blotted, weighed, and processed for histologic and analytic studies.

Studies of viability and function. The functional integrity of the isolated perfused rat liver was assessed by measurement of bile flow, oxygen consumption, and glucose synthesis. Structural integrity was assessed histologically. Bile flow was determined by measurement of bile volumes at 10-min intervals. Bile volume was measured using calibrated micropipettes (Clay Adams Div.) which were compared with weighed standards and found to be accurate to within ±2 μl. Three control perfusions showed a bile flow of 0.050±0.002 ml/h per g wet liver (mean±SEM). The bile flow rate is consistent with values reported by other investigators employing similar techniques (29, 32). Oxygen consumption was determined from the PO_2 difference between pre- and postliver samples of perfusion media collected anaerobically in heparinized, iced, glass syringes after 30, 45, and 60 min of perfusion. The PO_2 was analyzed with a Radiometer BMS 3 MK 2 blood gas machine (London Co.). Oxygen consumption was determined according to the equation: oxygen consumption = K (O_2) × F, where K = solubility coefficient of oxygen in plasma, 0.00126 mmol/liter; O_2 = portal-caval O_2 difference in mm Hg; and F = flow rate, ml/min per g wet liver (33). The average O_2 consumption was 1.73±0.03 μmol O_2/min per g wet liver (mean±SEM). The O_2 consumption was ~80% of that reported for isolated perfused rat liver studies employing hemoglobin as an O_2 carrier (29, 34). Glucose synthesis by the perfused liver was determined by examining the production of glucose from lactate. This was accomplished by depleting glycogen stores in the liver during 30 min of perfusion, adding 10 mM lactate to the media and measuring glucose concentration in media taken from the reservoir 15 and 30 min later. Glucose concentration was measured with the coupled enzyme method of Slein (35), using hexokinase and glucose-6-phosphate dehydrogenase (Statzyme glucose, Worthington Biochemical Corp., Freehold, N. J.). The glucose synthesis rate in control rat liver perfusions ($n = 3$) was 1.00±0.05 μmol glucose/min per g wet liver (mean±SEM). This synthesis rate is comparable to values reported by Hems et al. (29). Tissue from three control liver perfusions were processed for light, scanning, and electron microscopy as described below in the section on histopathology. Light

microscopy revealed normal architecture and no evidence of edema, cell necrosis, or inflammation. The sinusoids, canalicular apparatus, and hepatocyte organelles did not show significant abnormalities when examined by scanning and electron microscopy. Vacuoles were present in some hepatocytes (36, 37).

Studies of bile flow and bile acid secretion. Bile volumes were determined as previously described in studies on liver viability and function. All livers were perfused with Krebs-Henseleit solution containing albumin for a 20 min basal period. Three types of studies were then performed. In the first study, the control group of livers were perfused with solutions to which 0, 40, or 80 μmol NaTC/h were infused. Livers in the experimental group were perfused with similar solutions with the addition of protoporphyrin. Protoporphyrin was administered as a 1 μmol bolus followed by a constant syringe-pumped infusion of 0.05 μmol protoporphyrin/min to establish maximal canalicular transport (38). Pilot studies indicated that the infused amount of protoporphyrin, after a 1 μmol bolus, was the minimum dosage sufficient to establish a constant or slowly rising perfusate protoporphyrin concentration (3–5 μg/ml) during the last 50 min of perfusion. In the second study, the control group of livers ($n = 31$) were perfused with solutions to which 2–80 μmol NaTC/h was added. Livers in the experimental group ($n = 37$) were perfused with similar solutions but with the addition of the same amounts of protoporphyrin as administered in the first studies. In the third study, the control group of livers ($n = 5$) were perfused with solutions containing no NaTC; the experimental livers ($n = 3$) were perfused with solutions to which a single bolus of protoporphyrin was added to achieve perfusate concentrations of 1, 0.1, and 0.01 μM (5, 0.5, and 0.05 mg/ml, respectively). [^{14}C]erythritol clearances were not used to measure canalicular bile flow because it has been shown previously that bile formation in the isolated perfused rat liver is entirely dependent on canalicular flow (34). To compare bile flow between rats, bile volumes secreted during the experimental period of perfusion were expressed as a percentage of the basal 20 min period, which was assigned a value of 100%.

Bile acid secretion was determined by analyzing the bile acid output for the last 70 min of liver perfusion. Bile acid samples were immediately frozen after collection and stored at −20°C. Bile acid analysis was made according to the method of Iwata and Yamasaki (39) employing 2-hydroxysteroid dehydrogenase (Worthington Biochemical Corp.) and NADH (Sigma Chemical Co.).

Studies of regional hepatic perfusion. Regional hepatic perfusate flow was assessed at the conclusion of perfusions (90 min) with and without protoporphyrin by determining the distribution of technetium-99m-labeled human albumin microspheres, 10–35 μM in Diam (3M Co., St. Paul, Minn.), according to the technique described by Tavoloni et al. (40). A total of 10–15,000 microspheres containing 4–5,000 μCi mixed in 1 ml of perfusion media were injected just proximal to the portal vein within 10 s. 2 min later the perfusion was stopped. The livers were then photographed, *in situ*, using a gamma scintillation camera (Siemens Corp., Iselin, N. J.) with a pinhole collimator, and radioactivity in 50–100 mg tissue samples obtained from peripheral and central areas of left, median, caudate, and right (pyramidal) lobes determined in a well counter (Baird Corp., Bedford, Mass.). The regional hepatic lobar flow was expressed as a ratio of peripheral/central radioactivity.

Perfusate aspartate aminotransferase and lactic dehydrogenase activities. The activities of aspartate aminotransferase and lactic dehydrogenase were determined by
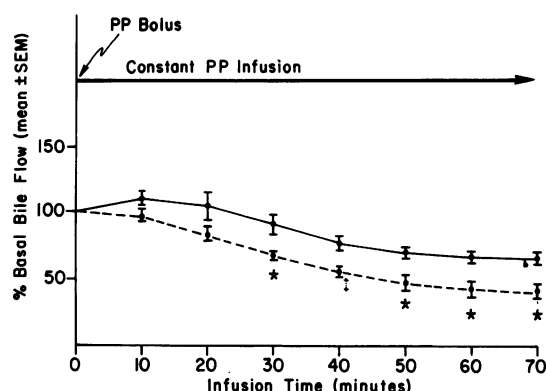
FIGURE 1 Effect of protoporphyrin in perfusion solutions on bile flow in the *in situ*, isolated perfused rat liver without NaTC infusion: control vs. protoporphyrin-perfused rat livers. Protoporphyrin (PP) was administered as a 1-μmol bolus followed by a constant infusion of 0.05 μmol protoporphyrin/min. Each point represents the mean±SEM for a 10-min interval of bile flow, expressed as a percentage of basal bile flow. The basal bile flow rate (microliters per minute per gram liver) determined for a 20-min period (-20-0 min) was assigned a value of 100%. After 30 min of perfusion with protoporphyrin ($n = 5$; broken line), significant inhibition of bile flow occurred when compared to controls ($n = 5$; solid line). Statistical significance is indicated by symbols, *$P < 0.05$; ‡$P < 0.01$.

standard techniques (41–43) in 200 μl samples of perfusate at 0, 10, 20, 40, and 70 min during control ($n = 7$) and protoporphyrin perfusions ($n = 10$). Mean enzyme activity for the total perfusion period was used to assess differences between control and protoporphyrin-perfused livers.

Histopathologic studies. Samples of liver tissue from the central area of the left lobe of control and protoporphyrin-perfused livers, without the infusion of NaTC, were processed for light, fluorescent, polarizing, scanning, and electron microscopy. Samples were fixed in either 10% buffered formalin or cold Karnofsky's fixative or frozen at -70°C. Light microscopy was performed on 4-μm sections of liver tissue stained with hematoxylin and eosin. Unstained sections were viewed under ultraviolet light with a Zeiss universal fluorescent microscope (Carl Zeiss, Inc., New York) using BG-38 and UG-1 excitation filters. Tissue sections were also examined under polarization (polarizing lens; Carl Zeiss, Inc.,). Tissue for scanning and electron microscopy was prepared in the laboratory of Dr. F. Moatamed at the Veterans Administration Hospital, Salt Lake City, Utah. Specimens for scanning electron microscopy, obtained from comparable areas of control and protoporphyrin-perfused livers, were hand sectioned (fractured), rinsed in 0.1 M phosphate buffer, and postfixed in 1% osmium tetroxide. After ethanol dehydration, the tissue was dried with liquid CO$_2$, mounted on scanning electron microscopy stubs and coated with gold. Tissue was examined with a JEOL JSM-35 scanning electron microscope (JEOL, USA. Electron Optics Div., Medford, Mass.) at × 130–15,000. For transmission electron microscopy, 1-μm thick sections were stained with toludine blue and used to trim tissue blocks to within a 12 cell radius around a central vein. These areas contained midzonal hepatocytes, as defined by Loud (44), as well as centrolobular cells. 10 sections were prepared from the selected blocks. Five electron micrographs were taken per

block at ×5,000 (JEOL 100 CX transmission electron microscope) and photographically enlarged to ×13,750. The areas photographed were selected randomly with a viewing screen just touching two edges of the copper grid, as described by Weibel et al. (45). The volume density of the biliary space was determined by superimposing a double lattice test system over the micrographs and counting the fraction of points lying within the biliary space (45, 46). Additionally, ultrastructural observations were made at magnifications up to ×181,500. These electron micrographic studies were performed by a pathologist (Dr. Lee) on coded specimens.

Statistical methods. The Student's t test was used to compare the percent change in bile flow between corresponding periods of control and protoporphyrin-perfused livers. It was also employed to compare differences in bile acid secretion, volume density of the biliary space, perfusate enzyme activity, and regional hepatic perfusion between these groups. Linear regression analysis was performed on the straight portion (30–70 min) of the percentage of basal bile flow curves for control and protoporphyrin-perfused livers. The derived y-intercepts and slopes were analyzed by the F test for statistical differences. Linear regression analysis was also used to derive best-fit lines describing the relationship of bile flow and bile acid secretion for control and protoporphyrin-perfused livers. Correlation coefficients were derived from linear regression analysis (47).

RESULTS

Effect of protoporphyrin on bile flow and biliary bile acid secretion with and without NaTC infusion. The effect of perfusion of protoporphyrin on bile flow without infusion of NaTC is illustrated in Fig. 1. Livers perfused with protoporphyrin had a significant reduction of bile flow compared to controls within 30 min and this reduction persisted throughout the remainder of the perfusion. The effect of perfusion of protoporphyrin during infusion of 40 μmol NaTC/h (physiologic amounts) and 80 μmol NaTC/h on bile flow is shown in Figs. 2 and 3. Infusion of NaTC was found to produce the expected increase in bile flow in all perfusions. However, livers perfused with protoporphyrin had significantly less flow when compared to controls. The degree of inhibition of bile flow in protoporphyrin plus NaTC perfused livers was not significantly different from livers perfused with protoporphyrin alone. The results of linear regression analysis of the percentage of basal bile flow (30–70 min) for the control group vs. the protoporphyrin-perfused group is shown in Table I. In each case, the y-intercepts for the livers perfused with protoporphyrin were significantly lower than controls, whereas the slopes did not differ significantly.

Biliary bile acid secretion was measured during perfusions with and without protoporphyrin as described in Methods, and results are shown in Table II. Livers perfused with NaTC had minimal bile acid secretion. Infusion of 40 μmol NaTC/h resulted in biliary bile acid secretion comparable to that reported by Boyer (34), indicating that the isolated

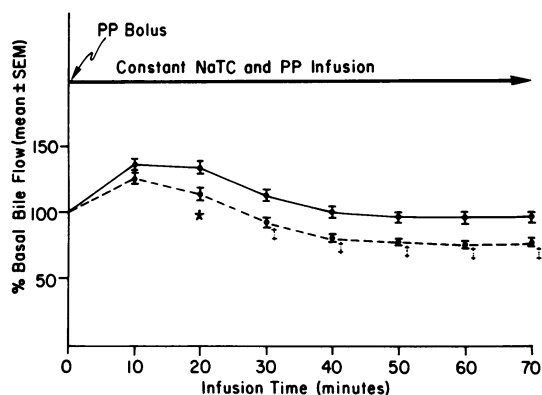*D. L. Avner, R. G. Lee, and M. M. Berenson*

FIGURE 2 Effect of protoporphyrin in perfusion solutions on bile flow in the *in situ*, isolated perfused rat liver with 40 μmol NaTC/h: control vs. protoporphyrin-perfused rat livers. Protoporphyrin (PP) was administered and the percentage of basal bile flow plotted as described in Fig. 1. After 20 min of perfusion with protoporphyrin ($n = 5$; broken line), significant inhibition of bile flow occurred when compared to controls ($n = 5$; solid line). Statistical significance is indicated by symbols, $*P < 0.05$; $\ddagger P < 0.01$.

perfused rat liver was capable of efficient bile acid clearance. Bile acid secretion rates for control and protoporphyrin-perfused livers were not significantly different when adjusted for liver weights.

Effect of protoporphyrin on the relationship of bile flow and biliary bile acid secretion. The relationship of bile flow and biliary bile acid secretion for the groups of livers perfused with and without protoporphyrin is shown in Fig. 4. Linear regression analysis was employed to derive best-fit lines for the two groups. A
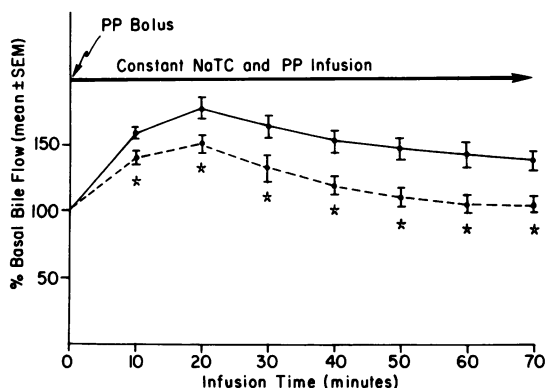
TABLE I

Linear Regression Analysis of the Percentage of Basal Bile Flow: Control vs. Protoporphyrin-Perfused Rat Livers

Perfusion groups	NaTC	n	y-intercept	Slope	r
	$\mu mol/h$				
Control	0	5	98.6	−0.76	−0.68
Protoporphyrin	0	5	77.6*	−0.83	−0.81
Control	40	5	123.5	−0.69	−0.74
Protoporphyrin	40	5	102.9*	−0.64	−0.77
Control	80	5	176.0	−1.00	−0.53
Protoporphyrin	80	5	142.0*	−0.88	−0.66

Linear regression analysis was performed on the points representing the percent of basal bile flow, for 10-min intervals, between 30–70 min (the straight portion of curve) of control and protoporphyrin-perfused livers infused with and without NaTC. Protoporphyrin and NaTC were infused as described in Figs. 1–3. The y-intercepts of the controls were significantly greater than protoporphyrin-perfused rat livers. The asterisks indicate a $P < 0.01$, control vs. protoporphyrin-perfused rat livers.

direct correlation of bile flow and biliary bile acid secretion was indicated by r values of 0.83 and 0.84 for the control group and protoporphyrin-perfused group, respectively. The bile acid-independent fraction (BAIF) of bile flow, indicated by the y-intercept, for the control group was 0.97 μl/min per g liver. In contrast, the BAIF of bile flow for the group treated with protoporphyrin was significantly ($P < 0.01$) reduced to 0.70. There were no significant differences between the slopes of the two lines (control, −0.79; protoporphyrin-perfused, −0.77). The ratio of

FIGURE 3 Effect of protoporphyrin in perfusion solutions on bile flow in the *in situ*, isolated perfused rat liver with 80 μmol NaTC/h: control vs. protoporphyrin-perfused rat livers. Protoporphyrin (PP) was administered and the percentage of basal bile flow plotted as described in Fig. 1. Within 10 min of perfusion with protoporphyrin ($n = 5$; broken line), significant inhibition of bile flow occurred when compared to controls ($n = 5$; solid line). Statistical significance is indicated by the symbols, $*P < 0.05$; $\ddagger P < 0.01$.

TABLE II

Effect of Protoporphyrin on Biliary Bile Acid Secretion with and without NaTC Infusion: Control vs. Protoporphyrin-Perfused Rat Livers

Perfusion groups	n	NaTC	Biliary bile acid secretion
		$\mu mol/h$	$\mu mol/min/g\ liver$
Control	5	0	0.003±0.000
Protoporphyrin	5	0	0.002±0.000
Control	5	40	0.050±0.000
Protoporphyrin	5	40	0.050±0.000
Control	5	80	0.080±0.000
Protoporphyrin	5	80	0.070±0.010

Biliary bile acid secretion (mean±SEM) during isolated, *in situ*, rat liver perfusions. NaTC was infused at 0, 40, and 80 μmol/h with and without perfusion of protoporphyrin sufficient to achieve maximal canalicular secretion (1-μmol bolus plus a constant infusion of 0.05 μmol protoporphyrin/min). At each rate of NaTC infusion, differences between the control and protoporphyrin-perfused groups were not statistically significant.
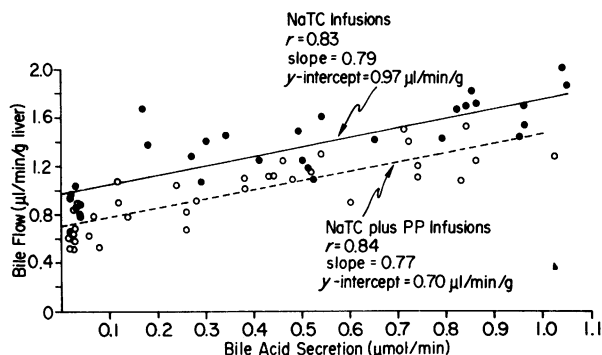
FIGURE 4 Correlation of bile flow and bile acid secretion during 0-80 μmol NaTC infusion in the *in situ*, isolated perfused rat liver with and without protoporphyrin. Each point represents the bile flow rate for a separate 70 min liver perfusion. Solid circles indicate controls without protoporphyrin ($n = 31$). Open circles indicate protopor-phyrin-perfused rats ($n = 37$). Protoporphyrin was administered as described in Fig. 1. Linear regression analysis is shown by the solid (control) and broken lines (protopor-phyrin-perfused). There is a significant difference ($P < 0.01$) between the y-intercepts of the groups; but no difference in slopes. This indicates an inhibitory effect of proto-porphyrin on the BAIF of bile flow.

the BAIF of bile flow to total bile flow in the control group was comparable to that reported by Boyer (34).

Effect of varied protoporphyrin concentrations on bile flow. The effect of liver perfusion with varying concentrations of protoporphyrin on bile flow is presented in Fig. 5. The protoporphyrin bolus that achieved 0.01 μM reduced bile flow but was not significantly different from controls. The protoporphyrin bolus that achieved 0.1 μM significantly reduced bile flow compared with the control group within 20 min and persisted throughout the perfusion. Similarly, the protoporphyrin bolus that achieved a concen-
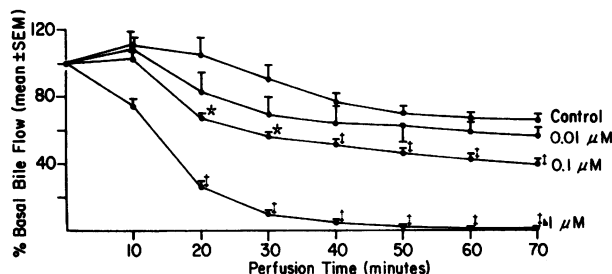

FIGURE 5 Effect of varying perfusate concentrations of protoporphyrin (without NaTC) on bile flow. Protoporphyrin was administered as a single bolus to achieve perfusate concentrations of 0.01, 0.1, and 1 μM. Bile flow was plotted as the percentage of basal bile flow as described in Fig. 1. Bile flow was reduced in rat livers perfused with proto-porphyrin ($n = 3$/concentration) compared to controls ($n = 5$), and decreased as a function of protoporphyrin concentration. However, it was significantly different than controls only at protoporphyrin concentrations of 0.1 and 1 μM as indicated by the symbols, *$P < 0.05$; ‡$P < 0.01$.
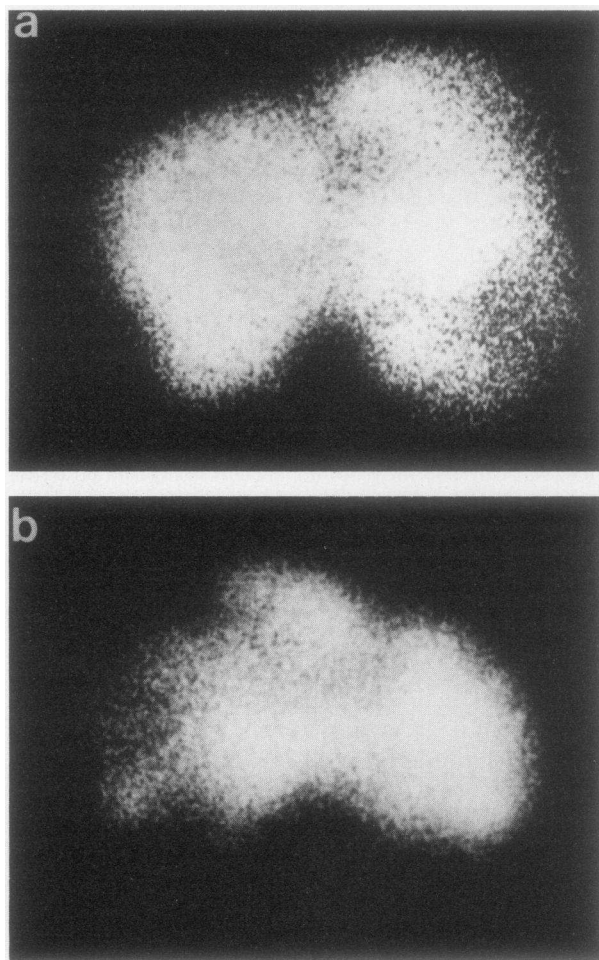

FIGURE 6 2 min after injection of radiolabeled micro-spheres, livers, *in situ*, were positioned 5 cm beneath a scintillation camera for 200,000 counts. (a) Control liver: relative homogeneity is apparent. (b) Protoporphyrin-per-fused liver: both central and peripheral defects can be seen.

tration of 1 μM markedly inhibited bile flow within 20 min when compared to the control group and essentially stopped all bile flow after 40 min of perfusion.

Effect of protoporphyrin on regional hepatic perfusion. Representative technetium-99m-labeled human albumin microsphere scans for control and protoporphyrin-perfused livers are shown in Fig. 6. The scans of control livers were relatively homo-geneous, although both central and peripheral defects were variably observed. Livers perfused with pro-toporphyrin showed more nonhomogeneity than con-trols, but neither a preponderance of central nor peripheral defects could be appreciated. Results of the quantitative analysis of radiolabeled microsphere distribution is presented in Table III. Comparison of the mean ratio of peripheral/central radioactivity failed to discriminate significant differences be-

tween the lobes of control and protoporphyrin-perfused livers.

Effect of protoporphyrin on perfusate transaminase and lactic dehydrogenase activity. The activity of aspartate aminotransferase in perfusate during constant infusion of protoporphyrin was 6.39 ± 1.79 U/liter (mean$\pm$SEM) and after a bolus of 1 μM protoporphyrin was 6.30 ± 1.66 U/liter. This did not differ significantly from control values of 6.47 ± 2.10 U/liter. The activity of lactic dehydrogenase in perfusate during constant infusion of protoporphyrin was 15.76 ± 5.41 U/liter and after the bolus dose of protoporphyrin, 33.36 ± 14.49 U/liter. This was not significantly different from the control value of 17.53 ± 11.80 U/liter.

Histopathological studies. The histopathology of tissue perfused with and without protoporphyrin was studied by light, fluorescent, polarizing, scanning, and transmission electron microscopy. Light microscopic examination of tissue sections stained with hematoxylin and eosin did not reveal significant differences between the control and protoporphyrin-perfused livers. The lobular architecture was preserved and neither parenchymal necrosis nor edema were present. Ultraviolet microscopy of unstained tissue sections did not reveal fluorescence in livers perfused without protoporphyrin. In contrast, tissue sections from livers perfused with protoporphyrin showed a diffuse, bright, red fluorescence in the cytoplasm of hepatic parenchymal cells and canaliculi. Examination of tissue sections with polarizing microscopy did not reveal birefringent material in livers perfused with or without protoporphyrin. Scanning electron microscopy of tissue sections from livers perfused with protoporphyrin demonstrated normal sinusoidal and parenchymal architure. However, when compared with control livers, microvilli in the canaliculi appeared less numerous. Transmission electron microscopy showed minor mitochondrial distortions, prominent pericanalicular autophagic lysosomal bodies, and occasional cytoplasmic vacuoles in both groups. The
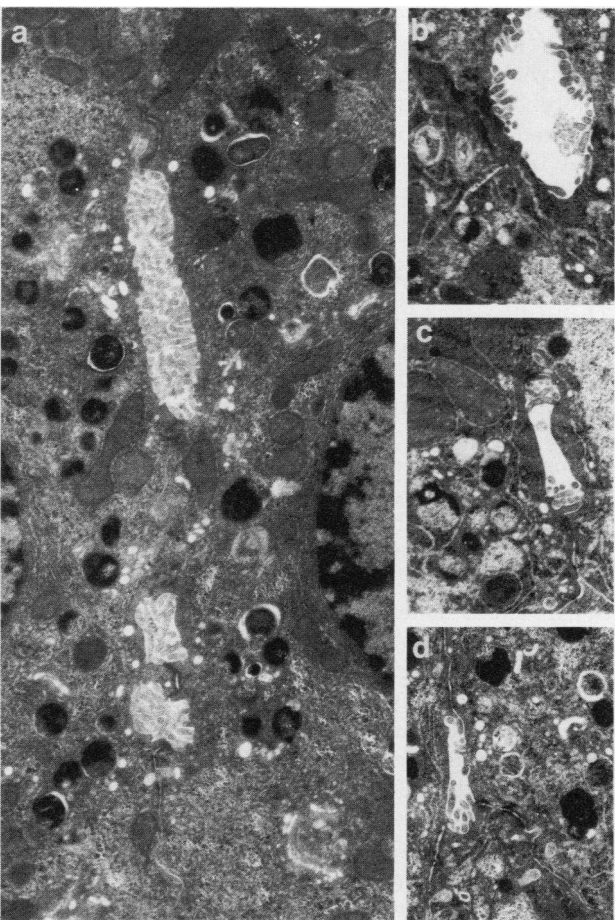

FIGURE 7 Transmission electron photomicrographs that show bile canaliculi from *in situ* rat liver perfusions without NaTC, as described in Methods. (a) Section from a control liver perfused for 70 min without protoporphyrin. Canaliculi appear in longitudinal (top) and cross-sections (bottom). Microvilli are prominent and fill the lumen ($\times$13,750). (b, c, and d) Sections from protoporphyrin-perfused rat livers during maximal canalicular protoporphyrin secretion which represent the range of abnormalities observed. The canaliculus in section b displays less pronounced changes with moderate distortion and loss of microvilli. Section d shows the least change but loss of microvilli is still evident ($\times$13,750).

TABLE III
Regional Lobar Distribution of Radiolabeled Microspheres

Liver Lobe	Control ($n = 4$) P/C	Protoporphyrin P/C ($n = 4$)	P
Left	0.73 ± 0.21	0.73 ± 0.26	<0.99
Median	0.74 ± 0.18	1.41 ± 0.32	<0.20
Caudate	0.73 ± 0.12	0.87 ± 0.20	<0.60
Right (pyramidal)	0.71 ± 0.17	1.10 ± 0.15	<0.20

The ratio of peripheral/central (P/C) radiolabeled microspheres was determined as described in Methods. Tissue samples were counted for 1 min and expressed per milligram liver. The P/C ratio represents the mean$\pm$SEM. No differences were demonstrated between the two groups as indicated by the P values.

portal tracts, hepatic veins, and large bile ducts appeared normal. In contrast, the canaliculi of protoporphyrin-perfused livers, compared to controls, were dilated and distorted (Fig. 7). The microvilli of the canaliculi often appeared short, and irregularly shaped; occasionally they were almost totally absent. These observations were confirmed by a quantitative assessment of the volume density of the biliary space in which 33 canaliculi from controls ($n = 3$) and 37 canaliculi from protoporphyrin-perfused livers ($n = 3$) were compared. The relative canalicular volume, expressed as a percentage of lobular space, was significantly different ($P < 0.01$) between control

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(0.9%) and protoporphyrin-perfused livers (2.0%). These findings are consistent with canalicular abnormalities reported in cholestasis (48–50). No amorphous or crystalline material comparable to that reported in protoporphyric patients (9, 16, 17, 19) or mice treated with griseofulvin (20–22) was identified in the hepatocytes, canaliculi, or Kupffer cells.

DISCUSSION

The pathogenesis of liver disease in protoporphyric patients is poorly understood. Protoporphyrin has been presumed to be the culpable agent. However, based on available information, it is not possible to establish a pathophysiologic correlation between the amount of protoporphyrin accumulated in the liver and histopathological abnormalities. Severe protoporphyric liver disease is characterized by an apparent rapid onset and progression, with unusually high erythrocyte, plasma, and hepatic protoporphyrin concentrations. These features suggest that at some point, perhaps related to the insidious deposition of protoporphyrin, hepatic impairment restricts elimination of protoporphyrin, facilitates its rapid accumulation and accelerates the liver disease. Eventually, protoporphyrin accumulation may be accelerated by a reduction in bile flow.

Cholestasis is a major histopathologic feature of protoporphyric liver disease; indeed, it has been referred to as "protoporphyrastasis" (19) because crystalline deposits of protoporphyrin have been observed to occlude canaliculi. This finding is emphasized by some investigators who hypothesize that precipitation of protoporphyrin in canaliculi may initiate development of the liver injury (8). Data to support this hypothesis, however, have not been presented. Therefore, the unexpected observation that bile flow was reduced in rat livers perfused with solutions containing protoporphyrin during experiments designed to determine the threshold of canalicular transport was of particular interest and stimulated further studies to examine this phenomenon.

The isolated perfused rat liver studies presented in this report employed a recirculating, membrane-oxygenated, hemoglobin-free perfusate system with precisely controlled conditions. Metabolic and histologic studies assured viability and maintenance of function. Relative homogeneity of hepatic perfusion was demonstrated by technetium-labeled microsphere studies. Ultraviolet microscopy revealed diffuse red fluorescence in hepatic cells and canaliculi, which indicated that protoporphyrin was not sequestered within sinusoidal cells.

Protoporphyrin was found to significantly reduce bile flow, even during infusion of NaTC. That the inhibition of bile flow was a result of reduction of the BAIF of bile flow was indicated by two observations. Firstly, biliary bile acid secretion was not significantly different between control and protoporphyrin-perfused livers. Secondly, linear regression analysis, which correlated biliary bile acid secretion and bile flow, revealed a significant difference between the y-intercepts of the control group and protoporphyrin-perfused group without altering their slopes (Fig. 4). The degree of inhibition of bile flow was shown to be dose-related. However, further kinetic analyses are necessary to determine the relationship of protoporphyrin load to hepatic accumulation and cholestasis.

The mechanisms mediating the reduction of BAIF of bile flow in these studies is unknown. Based on the lack of significant difference in perfusate enzyme activities between control and protoporphyrin-perfused livers, it seems clear that cholestasis did not result from a nonspecific cellular injury. The contribution of altered regional hepatic perfusion to the inhibition of bile flow is less clear. Reduction of perfusate flow rate has been associated with both inhibition of bile flow (40) and a transient, relative decrease of peripheral hepatic flow (51). In the current studies, flow rate was maintained by a pump system and did not vary. The radionuclide liver scans of protoporphyrin-perfused livers appeared less homogeneous than controls. However, the peripheral/central ratio of hepatic flow was not different. Normally, regional distribution of hepatic flow is quite variable (52) and unfortunately, the convenience of a peripheral/central flow ratio does not necessarily reflect effective acinar perfusion critical to bile formation. Therefore, on the basis of these data alone, the possibility that altered hepatic flow contributed to the decrease in bile flow cannot be excluded. Decreased perfusion rates, however, have not produced canalicular abnormalities (53), a prominent finding in the present studies.

Recent investigations suggest that the regulation of BAIF of bile flow may be governed by a multiplicity of complex, interdependent factors including liver plasma membrane NaK ATPase (54–56), bile acids (57, 58), canalicular membrane fluidity and viscosity (59, 60), and the microfilament apparatus of the canalicular secretory system (61). In this regard, a decrease in liver surface membrane NaK and Mg ATPase activity in protoporphyrin-perfused livers has recently been observed (62). Since protoporphyrin is known to penetrate and alter the physiochemical characteristics of monolayers of a variety of proteins and lipids (63), it seems reasonable to speculate that it may similarly affect lipoprotein components of the canalicular secretory apparatus.

The histopathologic studies of protoporphyrin-

perfused livers revealed three findings that deserve comment. Firstly, light microscopy was essentially normal. This finding was not unexpected in view of the acute nature of these experiments. Secondly, electron microscopy revealed dilatation and distortion of canaliculi with effacement of microvilli. This occurred in conjunction with the reduction of bile flow. Thirdly, neither amorphous nor crystalline deposits of protoporphyrin were found in hepatocytes or canaliculi. This observation is remarkable because it indicates that the deleterious effects of protoporphyrin do not simply result from precipitation within the hepatocytes and canalciuli.

In summary, the present studies provide evidence that perfusion of rat livers with solutions containing protoporphyrin significantly inhibited the BAIF of bile flow. This cholestatic effect was associated with dilatation, distortion, and effacement of hepatic canaliculi in the absence of demonstrable cytoplasmic or canalicular deposits of protoporphyrin. The precise mechanisms for these phenomena are unknown. These observations should not be construed to explain the pathogenesis of the hepatic disease in protoporphyria. Rather, they provide direct evidence that protoporphyrin has hepatotoxic properties that affect the canalicular secretory system, and suggest that the pathophysiologic basis of hepatic lesions in protoporphyria is more complicated than previously thought.

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