Interruption of the Enterohepatic

Circulation of Digitoxin by Cholestyramine

I. PROTECTION AGAINST LETHAL DIGITOXIN INTOXICATION

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A B S T R A C T Previous studies have demonstrated that considerable amounts of parenterally administered cardiac glycosides are excreted in the bile and reabsorbed across the intestinal mucosa in several species. It is currently believed that the more prolonged action of nonpolar digitalis glycosides is due to their retention and recycling in the enterohepatic circulation. This report describes studies carried out to evaluate the effects of pharmacologic interruption of this enterohepatic cycle with the intraluminal sequestering agent cholestyramine.

Cholestyramine was found to bind substantial quantities of digitoxin-^{3}H and digoxin-^{3}H in vitro and this binding was only modestly inhibited by the presence of bile. Administration of cholestyramine to rats by intragastric catheter before the subcutaneous injection of the LD$_{100}$ dose of digitoxin (10 mg/kg) resulted in a 70% survival rate. Further, oral administration of cholestyramine to rats before the subcutaneous injection of digitoxin-^{3}H resulted in accelerated fecal excretion of radioactivity and lower levels of digitoxin-^{3}H and metabolites in brain tissue compared to controls. Similarly, pretreatment of guinea pigs with cholestyramine orally before the injection of digitoxin in dosages of 10.0 and 4.0 mg/kg resulted in a 25 and 70% survival rate respectively as compared to survival rates of 0 and 30% in control animals. Cholestyramine pretreatment of guinea pigs was also accompanied by lower levels of digitoxin-^{3}H and metabolites in heart and liver 90 min after injection of digitoxin-^{3}H. Cholestyramine therapy did not result in significant changes in serum potassium levels excluding the possibility that drug-induced hyperkalemia might have affected the cardiac uptake of digitoxin.

The data obtained in this study indicate that cholestyramine treatment affords a significant degree of protection against lethal digitoxin intoxication in rats and guinea pigs. It is suggested that cholestyramine binds appreciable amounts of digitoxin in the intestinal lumen resulting in reduced reabsorption, increased fecal excretion, and lower tissue levels of glycoside in critical organs. The protective effects of cholestyramine appear to be mediated by interruption of the enterohepatic circulation of digitoxin.

INTRODUCTION

In 1955 it was first demonstrated that digitoxin-^{14}C was excreted into the small intestine via the biliary tract and digitoxin metabolites subsequently reabsorbed by the intestinal mucosa (1). On the basis of these and other studies (2, 3), it was postulated that digitalis glycosides undergo an enterohepatic circulation in which digitalis metabolites excreted in the bile are modified in the gut to yield nonpolar readily absorbed cardioactive compounds (4). More recently Okita has suggested that the persistent and more prolonged action of nonpolar glycosides, such as digitoxin, is due to their retention and recycling in the enterohepatic circulation (4). This is believed to be due to the greater lipid solubility of nonpolar glycosides which facilitates their reabsorption across the lipoid membrane of the intestinal mucosa and renal tubule. Theoretically it should be possible to decrease the duration of action of digitoxin by interrupting the enterohepatic cycle. This might be accomplished by either continuous aspiration of bile secreted into the duodenum or by the binding of digitalis to an intraluminal sequestering agent such as cholestyramine.

In this investigation studies were carried out to determine the effect of pharmacologic interruption of the enterohepatic circulation of digitalis glycosides. The data

obtained indicate that (*a*) cholestyramine, an anion exchange resin, binds appreciable amounts of digitoxin-³H and digoxin-³H in vitro; and (*b*) cholestyramine treatment of rats and guinea pigs results in accelerated fecal excretion of ³H-digitoxin and affords some degree of protection against lethal digitalis intoxication.

METHODS

Materials. Tritium-labeled digitoxin (SA 1.0 mCi/0.133 mg) and digoxin (SA 1.0 mCi/0.133 mg) were randomly labeled by catalytic exchange[1] and found to be greater than 98% pure by thin layer chromatography as previously described (5). Solutions of digitoxin-³H and digoxin-³H of desired specific activity were prepared by appropriate dilution with nonlabeled digitoxin[2] and digoxin.[2] Digitalis glycoside solutions of 10 µg/ml or less were prepared in aqueous media. Glycoside solutions with a concentration greater than 10 µg/ml required preparation of a stock solution in absolute ethanol and dilution to desired concentration with water.[3] Suspensions of cholestyramine[4] were prepared by mixing the resin with an appropriate amount of Krebs-Ringer bicarbonate buffer or distilled water and continuously stirring on a magnetic mixer.

Measurement of radioactivity. Radioactive samples were counted in a Packard Tri-Carb scintillation spectrometer, model 3375,[5] equipped with automatic external standardization. Duplicate 0.5 ml portions of liquid samples were added to 15.0 ml of a scintillation mixture (Bray's solution) of the following composition: 120 g naphthalene, 8 g PPO (2,5-diphenyloxazole), 400 mg POPOP (1,4-bis[-2-(5-phenyloxazolyl)]-benzene), 200 ml absolute methanol, 40 ml ethylene glycol brought to a final volume of 2000 ml with p-dioxane. Weighed tissue samples were counted by the method of Marks, Dutta, and Hoffman (6). Enough counts were taken on each sample to assure an error less than 3%.

Biliary excretion studies. Female Wistar rats weighing 120–180 g underwent laparotomy under light ether anesthesia. Either the proximal or distal 20 cm of small intestine was isolated between double silk ligatures with care taken to preserve the blood supply. The common bile duct was ligated, partially transected, and cannulated with PE 10 polyethylene tubing. The cannula was tied in place and brought out through a stab wound in the right upper quadrant where it was fixed with a superficial ligature. Before closure of the laparotomy incision, a solution containing digitoxin-³H or digoxin-³H in a dose of 10 µg/2 µCi/ml for each 100 g body weight was injected into the isolated intestinal loop. Collection of bile from the external cannula began immediately and continued while the rat was placed in a restraining cage for 24 hr. Only those preparations yielding a free flow of bile throughout the duration of the experiment were used in the final calculations. Bile was collected quantitatively in tubes kept in the dark and duplicate 0.5 ml portions assayed for radioactivity. Results are expressed as per cent of dose excreted in 4 or 24 hr.

In Vitro binding of ³H-digitalis. In vitro binding studies

were carried out according to a modification of the method of Gallo, Bailey, and Sheffner (7). A stock suspension of cholestyramine (50 mg/ml) was prepared in Krebs-Ringer bicarbonate (KRB)[6] buffer of the following composition: 128 mM NaCl, 5.7 mM KCl, 1.2 mM CaCl₂, 1.3 mM KH₂PO₄, 0.7 mM MgSO₄, and 25.6 mM NaHCO₃. After preparation, pH was adjusted to 2, 5, or 8 by addition of appropriate quantities of 0.1 N HCl or 0.1 N NaOH. Stock KRB used as diluent was similarly pH adjusted. Incubation flasks were prepared in duplicate at each pH so that a flask contained 1, 10, 100, or 500 µg (0.01 µCi) of digitoxin-³H or digoxin-³H, and 50, 100, 200, or 400 mg cholestyramine. To each flask was added KRB at the proper pH to a final volume of 10 ml, so that concentration of glycoside ranged from 0.1 to 50 µg/ml and concentrations of cholestyramine ranged from 5 to 50 mg/ml. Each flask was shaken in a Dubnoff metabolic incubator in room air at 23°C for 15 min. The contents of each flask were then centrifuged at 6000 *g* for 15 min and duplicate 0.5 ml portions of the supernatant assayed for radioactivity. By comparison of supernatant radioactivity with control flasks containing identical concentrations of digitalis-³H without cholestyramine, the per cent of available digitalis-³H bound was determined.

In preliminary studies it was determined that incubations carried out at 37°C or for 12 hr did not result in significantly different binding than under the conditions described above. Subsequently the method described was employed for all further in vitro binding studies including an additional study in which water and rat bile were substituted for KRB.

Effect of cholestyramine on lethal digitoxin intoxication. In order to determine whether cholestyramine treatment would protect rats and guinea pigs against lethal digitalis intoxication, our initial studies were carried out in animals given lethal doses of digitoxin and no other treatment.

Digitoxin was dissolved in 40% ethanol, in order to keep the injection volume below 1.0 ml and injected subcutaneously into groups of at least 10 animals of similar age and weight at each dose level tested. Female Wistar rats weighing 140–200 g received digitoxin in doses of 7.5 mg/kg or 10.0 mg/kg subcutaneously. Rats given such toxic doses of digitoxin usually developed characteristic neuromuscular signs within 24–36 hr and died between 48 and 120 hr after injection. Accordingly, digitoxin-treated rats living for 7 days after injection were considered to be survivors. Female guinea pigs weighing 200–350 g were injected with digitoxin in doses ranging from 2.0 to 10.0 mg/kg. In contrast to rats, guinea pigs given toxic doses of digitoxin died within 45–90 min. Therefore guinea pigs living for 24 hr after injection were judged to be survivors.

All animals in the experimental groups received p.o. cholestyramine in a dosage of 80 mg/2.0 ml as a suspension through an olive-tipped catheter. One group of rats received cholestyramine 2 hr after injection of digitoxin, and three additional doses (80 mg each) at 2 hr intervals on the 1st day. Thereafter, they received cholestyramine three times daily for 5 days or until they died. A second experimental group of rats received the first dose of cholestyramine 2 hr before digitoxin, and thereafter, according to the above schedule. A third group represented additional controls and received 2 ml water on the same dosage schedule as the first cholestyramine treatment group.

The experimental design was modified in guinea pigs because of the short survival time following injection of digi-

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[1] New England Nuclear Corp., Boston, Mass.

[2] Mann Research Labs, Inc., New York.

[3] The final solution was similar to the 5% ethanol-water solution used by Saral and Spratt (19) in in vitro binding studies with cholestyramine and digitoxin.

[4] Kindly supplied by Mead Johnson & Co., Evansville, Ind.

[5] Packard Instrument Co., Inc., Downers Grove, Ill.

[6] *Abbreviation used in this paper:* KRB, Krebs-Ringer bicarbonate buffer.

toxin in control animals. Groups of 10 guinea pigs were given 2 ml cholestyramine orally 4 and 1 hr before injection of digitoxin and received no further cholestyramine.

Fecal and urinary excretion of ³H-digitoxin and metabolites. Female Wistar rats weighing 160–220 g were housed in individual metabolic cages permitting separate and accurate collection of feces and urine. Samples were quantitatively collected in separate containers every 24 hr. 2 hr before subcutaneous injection of digitoxin-³H the animals received 2 ml water (control group) or 2 ml (80 mg) of cholestyramine suspension. Collection of specimens began with the subcutaneous injection of digitoxin-³H in a dose of 50 μg (2 μCi) per 100 g body weight. Animals then received water or cholestyramine at 2 hr intervals three times during the first 24 hr and three times daily for the next 4 days. Each daily stool specimen was homogenized in 50% ethyl alcohol with a Potter-Elvehjem teflon homogenizer, the total volume recorded, and duplicate 0.5 ml portions assayed for radioactivity. Each daily urine specimen was filtered to remove residual food particles, the residue washed three times and filtered, the filtrate pooled, and the volume recorded. Duplicate 0.5 ml portions were assayed for radioactivity. The total radioactivity per 24 hr stool or urine was calculated and the result expressed as per cent of the administered digitoxin-³H dose excreted per 24 hr. In preliminary experiments it was determined that 93–97% of digitoxin-³H added to rat feces was recovered. In further preliminary studies it was found that less than 0.5% of the tritium label from the administered digitoxin-³H dose was excreted in daily stool and urine collections after the 4th day. Subsequently, 4 day collection periods were used for all metabolic studies.

Serum and tissue levels of digitoxin-³H and metabolites. To determine whether cholestyramine treatment affected the metabolic disposition of digitoxin, blood and tissue levels of digitoxin-³H were measured in animals given cholestyramine before the injection of digitoxin-³H. As in the toxicity studies, groups of rats received 2 ml water or cholestyramine before injection, three more doses at 2 hr intervals on the 1st day, and three times daily thereafter until sacrifice. Groups of guinea pigs received similar doses 4 hr and 1 hr before injection. In these studies, all animals were injected with digitoxin-³H subcutaneously in a dose of 50 μg (2.0 μCi) per 100 g body weight. At appropriate intervals, a laparotomy was performed under ether anesthesia and blood collected by aortic puncture. Serum was removed after centrifugation and duplicate 0.5 ml portions taken for determination of radioactivity. Tissue studies were performed in the same groups of animals following exsanguination. Rats sacrificed at 24, 48, and 72 hr were decapitated and the heart excised. The parietal and occipital bones of the skull were removed with forceps. The brain was exposed, teased free of cranial nerve and spinal cord, transferred whole to aluminum foil containers, and frozen. The heart was dissected free of noncardiac tissue, the ventricles opened and washed free of gross blood with tap water, and frozen. In guinea pigs, the heart was similarly removed and prepared. In addition, the liver was excised, washed free of gross blood, and frozen. Solid tissues were prepared for counting by thawing, gentle blotting, and weighing. Rat brain was then quartered and all pieces transferred to a counting vial containing 15 ml of Bray's solution. Rat and guinea pig hearts were weighed and similarly placed in counting vials. Approximately 500 mg of the right lobe of the guinea pig livers was excised, weighed, and added to scintillation solution.

Analysis of ³H-digitoxin and metabolites in excreta and tissues by thin-layer chromatography. Thin-layer chroma-

tography was carried out using a modification of the method of Fauconnet and Waldesbühl (8, 9). 30 g of silica gel H were mixed with 73 ml of distilled water and spread on 20 × 20 cm glass plates to a thickness of 0.2 mm. Plates were activated by heating to 110°C for 1 hr. Chromatograms were run using ascending technique in a saturated chamber at 25°C, with a solvent system of dichloromethane:methanol:water (84:15:1). Highly purified samples of digitoxigenin,[7] digitoxin,[7] digoxigenin,[7] and digoxin[7] were spotted on each plate as controls. The solvent front was allowed to ascend 15 cm from the starting line, which usually required 30–35 min. Plates were then removed, heated to 110°C for 5 min, and sprayed with 33% trichloroacetic acid in chloroform which also contained a few drops of 10% hydrogen peroxide. The plates were again heated at 110°C for 10 min, cooled, and examined under ultraviolet light. Using the spray reagent described and ultraviolet light, digitoxin and its derivatives fluoresce yellow-brown and digoxin and its derivatives appear pale blue (9).

Samples of the original stool homogenates suspended in a 50% ethanol-water mixture were prepared for chromatography by extraction three times with 3 volumes of chloroform. 1 mg of cold carrier digitoxin was added to each stool homogenate before extraction. The pooled extracts were dried, redissolved in 1.0 ml with chloroform, and duplicate 5.0 μl portions spotted on thin-layer chromatography plates. Whole rat brains were homogenized with 10 volumes of chloroform, filtered through Whatman PS paper, and the residue washed three times with chloroform. The filtrates were pooled, dried, and redissolved in 1.0 ml chloroform. Replicate 20 μl portions of samples of brain extract were spotted and developed as described above. After the control spots of digitoxigenin, digitoxin, and digoxin, and digoxigenin and the unknown spots were identified with the spray reagent, corresponding areas of the unknown samples were quantitatively scraped from the plates into counting vials containing 15 ml of Bray's solution and assayed for radioactivity.

In addition to studies similar to those described, serum from control and cholestyramine-treated rats was collected 2, 4, 6, and 24 hr after injection of digitoxin for determination of serum electrolytes. Specimens were analyzed for sodium and potassium by flame photometry and for calcium by the method of Diehl and Ellingboe (10). Statistical analyses were carried out using the Student *t* test (11).

RESULTS

Biliary excretion of radioactivity after intraluminal injection of digitoxin-³H and digoxin-³H. The biliary excretion of the tritium label, 4 and 24 hr after injection of digitoxin-³H or digoxin-³H into either proximal or distal intestinal loops, is shown in Table I. 24 hr after administration of digitoxin-³H, 58.2 ±14.9% (Mean ±1 SD) of the tritium label injected into proximal loops, and 41.4 ±7.3% of the tritium injected into distal intestinal loops was excreted in the bile. Similarly, after administration of digoxin-³H 46.2 ±2.2% and 41.5 ±9.0% of the tritium injected into proximal and distal intestinal loops respectively, was excreted in the bile (Table I). These results indicate that appreciable amounts of digitoxin and digoxin and their

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[7] Kindly supplied by Dr. S. P. Dutta, Columbus, Ohio.

Biliary Excretion of Tritium after Intraluminal Injection of Digitoxin-³H or Digoxin-³H
into either Proximal or Distal Intestinal Loops in Rats*

| | Administered radioactivity excreted in bile | | | |
| | Digitoxin | | Digoxin | |
Site of administration	Proximal loop	Distal loop	Proximal loop	Distal loop
		%		
Time				
4 hr	13.6 ±5.2(4)‡	9.3 ±1.4(3)	20.2 ±0.9(4)	18.0 ±9.1(4)
24 hr	58.2 ±14.9(3)	41.4 ±7.3(3)	46.2 ±2.2(3)	41.5 ±9.0(3)

* Animals received an intraluminal injection of either digitoxin-³H or digoxin-³H (10 μg and 2 μCi)
for each 100 g body weight. Biliary fistula were constructed just before intraluminal injection of
glycoside. Proximal loops included the proximal 20 cm and distal loops the distal 20 cm of small
bowel. For details see text.
‡ Mean ±1 SD.
Numbers in parenthesis indicate number of animals in each group.

metabolites are excreted in the bile of the rat following
absorption from either proximal or distal intestine.

*In Vitro binding of digitalis-³H glycosides by chole-
styramine.* To determine whether significant amounts
of cardiac glycosides are bound by cholestyramine, in
vitro binding studies were carried out (Table II, Fig.
1). It can be seen that at cardiac glycoside concentra-
tions ranging from 0.1 to 50.0 μg/ml, digitoxin was
bound to a greater degree than digoxin. Binding was
directly proportional to the amount of cholestyramine
and inversely proportional to the amount of glycoside
present for both digitoxin-³H and digoxin-³H. The con-
centration of cholestyramine was a more important
factor in influencing digitalis glycoside binding than
was the concentration of the glycoside. At high cho-

lestyramine concentration, per cent digitalis binding was
almost insensitive to variation in digitalis concentra-
tion. For example, at 40 mg per ml cholestyramine,
there was only 6 and 16% maximum difference in
binding of digitoxin and digoxin over a 500-fold range
of glycoside concentration. Digitalis glycoside binding
was found to be independent of pH with virtually iden-
tical results obtained at pH 2, 5, or 8. Additional stud-
ies were carried out to determine the effect of bile
salts and other ions on the binding of cardiac glyco-
sides (Table III). Although a small but statistically
significant difference was noted between binding of
digitoxin-³H in KRB as compared to water (67.8
±0.5% vs. 62.8 ±0.3%; $P < 0.01$), suspending the digi-
toxin and cholestyramine in bile resulted in a more

In Vitro Binding of Digitalis-³H Glycosides by Cholestyramine*

| | Glycoside-³H bound | | | | | | | |
| | Digoxin-³H concentration, μg/ml | | | | Digitoxin-³H concentration, μg/ml | | | |
Cholestyramine concentration	0.1	1.0	10.0	50.0	0.1	1.0	10.0	50.0
mg/ml				%				
5.0	11.0	19.7	8.6	2.2	52.6	45.2	46.8	39.4
10.0	28.6	27.3	18.7	12.7	66.3	64.9	63.3	53.2
20.0	46.5	44.1	33.4	22.5	79.4	79.5	75.5	69.6
40.0	57.5	60.4	51.3	41.8	88.2	88.3	86.4	82.0

* The incubations were carried out by a modification of the method of Gallo (5). The incubation flasks
contained 0.01 μCi of glycoside-³H and appropriate concentrations of cholestyramine in a total volume
of 10 ml. The flasks were incubated for 15 min, the suspension centrifuged at 6,000 g for 15 min, and
the supernatant assayed for radioactivity and compared with control flasks which contained no
cholestyramine.
The values shown represent the mean of replicate determinations at pH 5. Nearly identical values were
obtained at pH 2 and 8. For details, see text.

TABLE III

*Effect of Bile Salts and Buffers on the In Vitro Binding of Digitoxin-³H by Cholestyramine**

	Suspending medium for cholestyramine and digitoxin-³H		
	Krebs-Ringer bicarbonate	Water	Bile
Binding, %	67.8 ±0.5‡(4)	62.8 ±0.3(4)	43.0 ±1.0(4)

* The incubation studies were carried out as described in the text and Table II using a cholestyramine concentration of 20 mg/ml and digitoxin concentration of 50 μg/ml. The Krebs-Ringer bicarbonate solution was replaced with either 10 ml distilled water or 10 ml undiluted rat bile. The numbers in parentheses refer to the number of experiments performed.
‡ Mean ±1 SD.

marked decrease in digitoxin-³H binding (67.8 ±0.5% vs. 43.0% ±1.0%; P < 0.001). The data indicate that substantial amounts of the cardiac glycosides digitoxin and digoxin can be bound by cholestyramine in vitro and that the presence of bile causes some reduction in binding.

Effect of cholestyramine on digitoxin toxicity. Since appreciable amounts of digitoxin-³H and metabolites are excreted in bile (Table I), and because significant amounts of digitoxin are bound in vitro by cholestyramine (Table II, Fig. 1), studies were carried out to determine the effect of cholestyramine treatment on
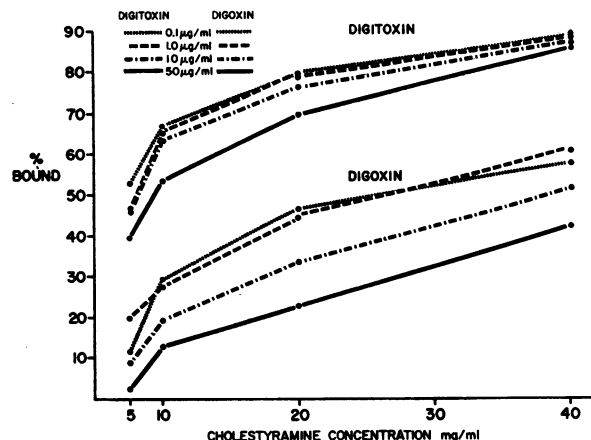

FIGURE 1 Binding of digitoxin-³H and digoxin-³H by cholestyramine in vitro. For details of incubation conditions see text.

lethal digitalis intoxication in rats and guinea pigs (Table IV). In rats receiving 10.0 mg/kg of digitoxin subcutaneously, 100% of control and water-treated rats died. By contrast, 22% of rats receiving cholestyramine after injection of digitoxin survived. More striking was the 70% survival rate in rats who received the initial dose of cholestyramine 2 hr before the LD₁₀₀ dose of digitoxin (Table IV). The effect of cholestyramine pretreatment on digitoxin toxicity in guinea pigs also is shown in Table IV. Because of the wide dose range

TABLE IV

Effect of Cholestyramine on Survival in Animals Given Toxic Doses of Digitoxin

		No. animals surviving/No. animals treated		
			Treatment regimens	
Species	Digitoxin dose given subcutaneously	No treatment	Cholestyramine* after digitoxin	Cholestyramine‡ before digitoxin
	mg/kg			
Rat	7.5	10/12 (83%)§		
	10.0	0/10 (0%)	4/18 (22%)	7/10 (70%)
	10.0	0/10 (0%)‖		
Guinea pig	2.0	10/10 (100%)		
	3.0	9/10 (90%)		
	4.0	3/10 (30%)		7/10 (70%)
	7.5	2/10 (20%)		5/10 (50%)
	10.0	0/10 (0%)		4/16 (25%)

* Cholestyramine given in dose of 80 mg/2 ml 2 hr after injection of digitoxin and three times daily thereafter for 5 days.
‡ In rats, cholestyramine was given by gavage in a dose of 80 mg/2 ml 2 hr before injection of digitoxin and three times daily thereafter for 5 days. In guinea pigs, cholestyramine was given in a dose of 80 mg/2 ml 4 hr and 1 hr before injection of digitoxin.
§ Numbers in parenthesis represent per cent survival.
‖ This control group received water by gavage in the same dosage as the cholestyramine group.

Table V

TABLE V

Effect of Cholestyramine on Fecal and Urinary Excretion of Digitoxin-³H* and Metabolites

Group	No. of rats	Feces					Urine					Total recovery
		Days 1	2	3	4	1–4	Days 1	2	3	4	1–4	
		%										
Control	5	17.0‡ ±2.5	24.3 ±2.7	9.4 ±2.3	1.7 ±0.8	52.4 ±4.8	12.3 ±2.0	3.7 ±0.8	1.0 ±0.4	0.4 ±0.1	17.4 ±2.3	69.8 ±6.5
Cholestyramine	8	25.3 ±6.6	24.3 ±5.3	3.8 ±1.1	1.3 ±0.5	54.6 ±4.2	15.4 ±3.7	3.9 ±1.3	1.0 ±0.4	0.4 ±0.2	20.7 ±5.0	75.3 ±3.2
P Value		<0.025	NS	<0.001	NS	NS	NS	NS	NS	NS	NS	<0.1

* The digitoxin dose was 50 μg (2 μCi) per 100 g body weight given subcutaneously. Cholestyramine-treated animals received a cholestyramine suspension (80.0 mg/2.0 ml) 2 hr before injection of digitoxin and three times daily thereafter for 5 days.
‡ Mean ±1 SD.
NS, no significant difference.

between the LD_{70} (4.0 mg/kg) and the LD_{100} (10.0 mg/kg) for digitoxin in guinea pigs, cholestyramine pretreatment was evaluated at three dose levels in this range, 4.0 mg/kg, 7.5 mg/kg, and 10.0 mg/kg. Cholestyramine administered 4 and 1 hr before injection resulted in increased survival at all three dose levels (30% vs. 70%, 20% vs. 50%, and 0 vs. 25%, respectively). These results indicate that cholestyramine administered orally prior to the parenteral injection of lethal doses of digitoxin attenuates the toxic effects and affords appreciable protection against mortality due to the glycoside.

Effect of cholestyramine on fecal and urinary excretion of digitoxin-³H. It was postulated that the protective effects of cholestyramine against lethal digitoxin intoxication might be due to accelerated excretion of digitoxin and its cardioactive metabolites resulting in reduced tissue levels in target organs. To evaluate this hypothesis the effect of cholestyramine on the fecal excretion of digitoxin-³H and metabolites was studied (Table V and Fig. 2). In control animals peak fecal excretion of tritium radioactivity (24.3 ±2.7%) occurred during the 2nd day after injection and over a 4 day period 52.4 ±4.8% of the dose was excreted. By contrast, in cholestyramine-treated animals peak fecal excretion occurred within 24 hr after injection, and such animals excreted significantly greater amounts on the 1st day than the controls (25.3 ±6.6% vs. 17.0 ±2.5%, P < 0.025). However, at the end of the 4 day balance study, fecal excretion of tritium radioactivity was comparable in both controls and cholestyramine-treated rats (52.4 ±4.8 vs. 54.6 ±4.2%). No significant differences in urinary excretion of radioactivity were found between control and cholestyramine-treated animals (Table V). The fecal and urinary excretion data suggest that cholestyramine accelerates fecal excretion of digitoxin-³H and metabolites.

To determine whether the tritium activity measured in the stool extracts reflected the presence of intact digitoxin-³H or its metabolites, thin layer chromatography of chloroform extracts of stool collected during the first 24 hr period was carried out (Table VI). With chloroform extraction, 69 ±5% of the total radioactivity excreted in the stool in this period was recovered. In Table VI, it can be seen that all of the recovered CHCl₃ soluble radioactivity migrated as digitoxin, digoxin, or their aglycones. Cholestyramine treatment resulted in significantly greater excretion of unchanged digitoxin as compared with controls (59 ±5 vs. 47 ±3%; P < 0.01). When the excretion of digitoxin and digitoxigenin is compared with the excretion of digoxin and digoxigenin in both groups it is evident that significantly more digitoxin and digitoxigenin was excreted in the stool of cholestyramine-treated animals. By contrast, less digoxin and digoxigenin was excreted
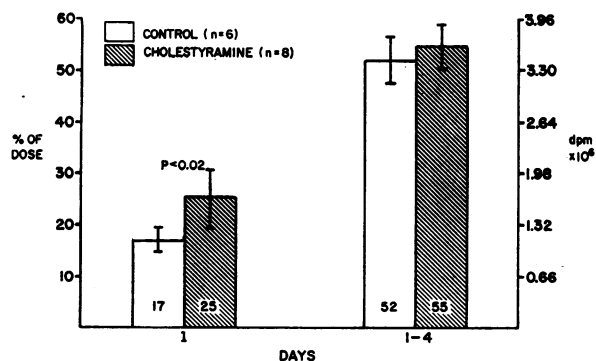

FIGURE 2 Effect of cholestyramine on fecal excretion of digitoxin-³H and metabolites after subcutaneous injection of digitoxin-³H. Rats received 50 μg (2 μCi) digitoxin-³H per 100 g body weight. Cholestyramine-treated rats received 80 mg cholestyramine three times daily for 4 days. For further details see text. Values shown represent the mean ±1 SD.

TABLE VI

Excretion of Digitoxin-³H and Metabolites in Feces in Control and Cholestyramine-Treated Rats*

Group	³H label recovered in				³H label in	
	Digitoxigenin	Digitoxin	Digoxin	Digoxigenin	Digitoxin digitoxigen‡	Digoxin digoxigenin‡
	%				%	
Control (n = 4)	3.8 ±0.4‡	47.4 ±2.6	36.2 ±6.6	12.6 ±3.9	51.3 ±3.0	48.7 ±3.0
Cholestyramine (n = 5)	2.7 ±1.2	59.4 ±4.6	28.9 ±3.3	9.0 ±1.8	62.1 ±4.3	37.9 ±4.3
P value	<0.20	<0.01	<0.10	<0.20	<0.01	<0.01

* The dosage of digitoxin-³H and cholestyramine is described in the text and Table V. The feces collected during the first 24 hr were homogenized in a 50% ethanol-water mixture and extracted three times with 3 volumes of CHCl₃. The CHCl₃ extracts were pooled, dried, resuspended in CHCl₃, and applied to TLC plates along with highly purified samples of digitoxigenin, digitoxin, digoxin, and digoxigenin. The plates were run in a solvent system containing dechloromethane:methanol:water (84:15:1) for 30 min and the spots identified by spraying with a solution containing 33% trichloroacetic acid in CHCl₃ with a few drops of 10% H₂O₂ added. For further details, see text.
‡ Mean ±1 SD.

by treated animals. The remaining water soluble radioactivity was also partially characterized. The aqueous residue was dried and reconstituted with ethanol and thin-layer chromatography carried out as previously described. Preliminary studies show that approximately 50% of the aqueous radioactivity represents radiolabeled digitoxose. The remainder of the activity in this fraction has not been completely identified by currently available chromatographic technique but possibly represents water soluble conjugates of digitoxigenin.

Serum and tissue levels of digitoxin-³H. To test whether the accelerated fecal excretion of digitoxin-³H

produced by cholestyramine treatment would be reflected in lower serum or tissue levels of digitoxin-³H, the studies summarized in Tables VII and VIII and Fig. 3 and 4 were carried out. Serum radioactivity at intervals from 30 min to 72 hr after injection of digitoxin-³H into control and cholestyramine-treated rats is shown in Table VII and Fig. 3. No differences in peripheral blood radioactivity were found between control and cholestyramine-treated rats. Similarly, there were no differences in tritium levels in the heart between the two groups (Fig. 3). Levels of radioactivity in brain were also determined 24, 48, and 72 hr after

TABLE VII

Effect of Cholestyramine on Serum Levels of Radioactivity after Injection of Digitoxin-³H into Rats*

Time after injection	Controls			Cholestyramine-treated		
	No. of rats	dpm/ml serum	% dose per total blood volume‡	No. of rats	dpm/ml serum	% dose per total blood volume
30 min	5	1,884 ±4.6§	1.0	5	1,740 ±95	0.9
60 min	5	3,174 ±720	1.8	5	4,506 ±893	2.5
2 hr	5	11,394 ±666	7.6	5	11,570 ±1218	7.5
4 hr	5	11,250 ±1225	7.3	5	10,615 ±426	6.9
6 hr	5	8,620 ±1357	5.3	5	9,050 ±562	5.9
24 hr	5	1,953 ±170	1.4	5	1,998 ±554	1.4
48 hr	5	665 ±125	0.5	5	667 ±93	0.5
72 hr	5	605 ±69	0.4	5	585 ±152	0.4

* Rats weighing 150–170 g were given cholestyramine (80 mg/2.0 ml) 2 hr before injection of 50 µg (0.5 µCi) ³H-digitoxin per 100 g body weight and three times daily thereafter for 3 days. Rats from both control and cholestyramine-treated groups were sacrificed at the time intervals shown and blood collected by aortic puncture.
‡ % of dose per total blood volume was calculated assuming that blood volume was 8% of body weight.
§ Mean ±1 SD.

injection of ³H-digitoxin into control and cholestyra-mine-treated rats (Fig. 4). Importantly, brain radio-activity was significantly lower in cholestyramine-treated rats at 48 and 72 hr, as compared with controls (2680 ±450 vs. 3435 ±644 dpm/g, and 1472 ±154 vs. 1742 ±70 dpm/g; P < 0.01). To further characterize the tritium activity in the brain, an additional series of experiments was carried out in which rats received a subcutaneous injection of digitoxin-³H in a dosage of 50 μg and 5 μCi per 100 g body weight. The animals were randomized into control and cholestyramine-treated groups and sacrificed after 3 days. Characteriza-tion of brain radioactivity by thin layer chromatography revealed only one sharply circumscribed band of radio-activity and this had the mobility characteristics of digitoxigenin. Significantly greater radioactivity in this digitoxigenin fraction was present in the controls as compared to the cholestyramine-treated rats (4800 ±1500 vs. 2680 ±800 dpm/g brain; P < 0.05). The low specific activity of this extract could have been re-sponsible for failure to detect minute quantities of other ³H-labeled digitoxin metabolites. However, our finding that only digitoxigenin could be identified in rat brain is in accord with observations indicating that the pres-ence of sugar residues on the glycoside nucleus impedes passage of the digitalis moiety into the brain (12). Thus the radioactivity due to the highly lipid soluble digitoxigenin seems to accumulate in rat brain at higher concentrations than in heart and to dissipate more slowly than do serum or tissue levels (Figs. 3 and 4).

Levels of radioactivity in serum, heart, and liver 90 min after injection of digitoxin-³H into control and

TABLE VIII
Effect of Cholestyramine on Serum and Tissue Levels of Radio-activity after Injection of Digitoxin-³H into Guinea Pigs*

Group	No. of animals	³H radioactivity recovered in		
		Serum	Heart	Liver
		dpm/ml	dpm/g	dpm/g
Control	4	5405 ±1303‡	5020 ±570	14140 ±1860
Cholestyramine	4	4437 ±764	4000 ±470	10770 ±2050
P value		>0.1	<0.05	<0.05

* Guinea pigs were given cholestyramine (80 mg/2.0 ml) 4 hr and 1 hr before injection of 50 μg (0.5 μCi) of digitoxin-³H per 100 g body weight. 90 min after injection the animals were exsanguinated by aortic puncture and the tissues removed as described in the text.
‡ Mean ±1 SD.

cholestyramine-treated guinea pigs are shown in Table VIII. It can be seen that levels of total radioactivity in serum were not significantly lower in treated animals as compared with controls (4437 ±764 vs. 5405 ±1303 dpm/ml; P > 0.3). However, levels of tritium label in heart and liver were lower in cholestyramine-treated animals as compared with controls (4000 ±470 vs. 5020 ±570 dpm/g and 10770 ±2050 vs. 14140 ±1860 dpm/g respectively, P < 0.05). These studies indicate that cho-lestyramine pretreatment resulted in significantly lower tissue levels of tritium in both rats and guinea pigs.

Serum electrolytes in cholestyramine-treated rats. It has been demonstrated that changes in serum electro-lytes, especially change in serum potassium, may influ-ence the cardiac uptake of certain digitalis glycosides (13). To exclude the possibility that the protective
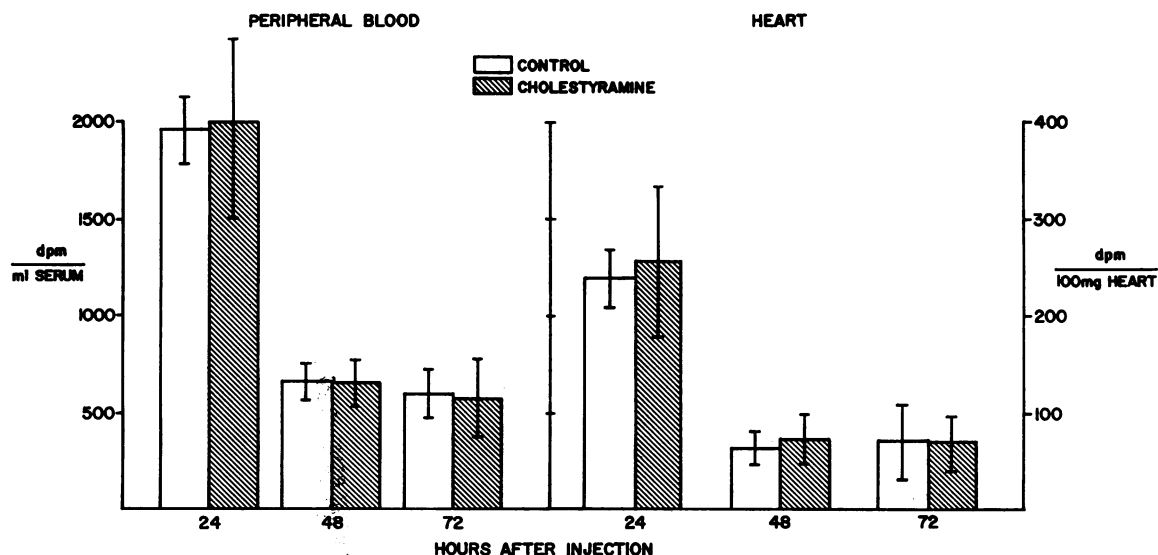

FIGURE 3 Effect of cholestyramine treatment on radioactivity in serum and heart of rats after subcutaneous injection of 50 μg (2 μCi) digitoxin-³H per 100 g body weight. Cholestyramine was given as described in the text and Fig. 2. Values shown represent the mean ±1 SD.
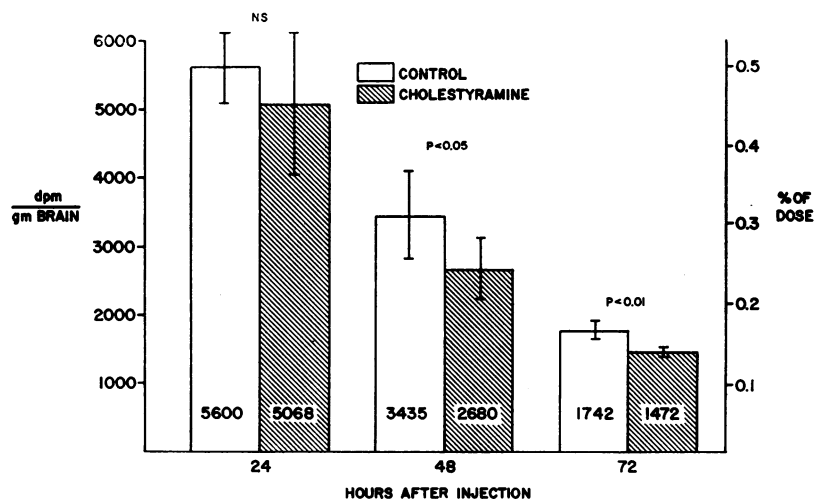
FIGURE 4 Effect of cholestyramine treatment on radioactivity in brain after subcutaneous injection of digitoxin-^{3}H (50 μg; 2 μCi) into rats. Values shown represent the mean $\pm$1 SD.

effects of cholestyramine might be due to induction of hyperkalemia, serum electrolyte concentrations were measured in control and cholestyramine-treated rats 2, 4, 6, and 24 hr after treatment (Table IX). No significant differences in concentrations of sodium, potassium, or calcium were noted at any time interval after cholestyramine treatment.

DISCUSSION

The concept that digitalis glycosides undergo an enterohepatic circulation was proposed by Okita, Talso, Curry, Smith, and Geiling in the course of the first

TABLE IX

Effect of Cholestyramine on Serum Electrolytes after Toxic Doses of Digitoxin

Group	Time after injection	Na+	K+	Ca++
	hr	mEq/liter	mEq/liter	mEq/liter
Control	2	137 ±1.0‡	5.6 ±0.6	4.8 ±0.3
	4	135 ±2	5.2 ±0.8	4.5 ±0.2
	6	135 ±2	4.8 ±0.3	5.0 ±0.1
	24		4.7 ±0.4	
Cholestyramine	2	137 ±1	5.9 ±1.1	4.8 ±0.1
	4	134 ±1	5.3 ±1.3	4.4 ±0.2
	6	132 ±1	5.1 ±1.3	4.7 ±0.2
	24		5.1 ±0.1	

* Rats received 10.0 mg/kg digitoxin subcutaneously. Cholestyramine-treated animals received 80 mg of cholestyramine 2 hr before injection of digitoxin.
‡ Mean ±SD of four determinations.

studies of radiolabeled cardiac glycosides in humans (1). In these studies it was demonstrated that (*a*) high concentrations of digitoxin-C^{14} and metabolites are found in the gastrointestinal tract and its contents after intravenous administration; (*b*) the ratio of unchanged digitoxin: metabolites increased as samples were taken from proximal to distal bowel, suggesting preferential reabsorption of metabolites or metabolic conversion in the course of reabsorption; and (*c*) only 17% of the intravenous dose of digitoxin-C^{14} was excreted in the feces in intact subjects despite postmortem studies showing that a much larger part of the dose could be recovered from the digestive tract.

A decade later, Katzung and Meyers extended the concept of the enterohepatic circulation of cardiac glycoside (2). These investigators demonstrated that in the dog, large amounts of radioactivity (approximately 39% of an intravenous dose of digitoxin-^{3}H) are excreted in the bile in an 8 hr period. Furthermore, they demonstrated a marked decrease in the metabolic half-life of digitoxin-^{3}H from 14 to 6 hr in the bile-fistula dog and this was accompanied by a reduced urinary excretion of digitoxin-^{3}H. It was concluded that the data were best explained by the existence of an enterohepatic cycle, and it was postulated that water-soluble metabolites of digitoxin in bile were hydrolyzed to native digitoxin or other nonpolar glycosides, which in turn, are more likely to be reabsorbed than the water-soluble metabolites. In a further test of this postulate, Katzung and Meyers demonstrated that at least two water-soluble metabolites of digitoxin were easily hydrolyzed to a product chromatographically indistinguishable from digitoxin (3). In spite of this evidence, the enterohepatic cycle of digi-

toxin has been largely neglected. This is due, in part, to the fact that in humans renal excretion of digitoxin and its metabolites ultimately accounts for 60–80% of the total digitoxin lost from the body (14).

We elected to carry out the majority of our studies in rats because of the established existence of biliary secretory mechanisms for cardiac glycosides in this species. Previous studies have shown that in the rat a large fraction of a dose of digitoxin is excreted in the bile following intravenous injection (15). Our studies confirm these observations and further indicate that appreciable amounts of digitoxin and digoxin and their metabolites are excreted in the bile in a 24 hr period after absorption from either the proximal or distal small bowel (Table I). These findings have important implications with regard to the possibility of continuing biliary excretion of glycoside after intestinal reabsorption of the drugs. It is of interest that other investigators have suggested that the enterohepatic circulation of cardiac glycosides could be interrupted by continuous aspiration of bile (3).

However, because of the known efficacy of cholestyramine in binding bile salts and interrupting the enterohepatic circulation of bile acids (16), we elected to carry out studies to evaluate the binding of cardiac glycosides by cholestyramine. This insoluble anion exchange resin has been used in clinical medicine chiefly in those conditions in which it is desirable to bind bile acids in the intestinal lumen (16). In addition, it has also been shown to bind thyroxine (17) and several drugs (7). Gallo and coworkers (7) showed that 33% of a digoxin solution was weakly bound by cholestyramine when combined in ratios which might be expected if average doses of the two drugs were taken together. Assuming average doses of 4 g for cholestyramine and 0.25 mg for digoxin, the cholestyramine: digoxin ratio on a weight basis would be 16,000:1. Inspection of Table II reveals that similar values are obtained in the dose ratio of 10,000–20,000:1 in our studies (27.3 and 44.1% binding respectively). It can be seen that for digitoxin, similar ratios for cholestyramine and glycoside yield binding values ranging from 64.9 to 79.5%. In addition, our studies show that relatively greater amounts of glycoside may be bound by cholestyramine with larger ratios up to 40,000:1 (88% of a 10 μg dose). Considering that at any given time following parenteral digitalis administration, the concentration of glycoside in the intestinal lumen might be smaller than that found immediately after an oral dose, it is possible that very high ratios of cholestyramine: glycoside (greater than 40,000:1) were present in cholestyramine-treated rats. However, whether such favorable ratios result in equivalent binding of cardiac glycosides in vivo is open to question in view of the moderate inhibitory effects of

bile on glycoside binding by cholestyramine (Table III). This is possibly due to competition between glycosides and bile salts for the available cholestyramine. However, we have no additional data bearing on this point.

In regard to the mechanism of binding of glycosides and other neutral compounds by cholestyramine, the recent studies of Johns and Bates are of interest (18). In detailed investigations of the interaction between bile salts and cholestyramine, these workers found that the primary electrostatic (anion exchange) mechanism is reinforced by a second component. This secondary mechanism is nonelectrostatic and increases as the hydroxylation of the bile salt molecule decreases. For example, cholestyramine has an equal molar capacity for deoxycholate (dihydroxy bile acid) or lithocholate (monohydroxy bile acid), but more lithocholate is actually bound than deoxycholate. The similarity to the greater affinity of cholestyramine for digitoxin (monohydroxy nucleus) than digoxin (dihydroxy nucleus) is apparent. Although our studies do not permit a direct conclusion as to the mechanism of binding of digitalis by cholestyramine, it is possible that the nonelectrostatic binding described by Johns and Bates is at least one of the factors involved.

That cholestyramine may reduce digitoxin toxicity when given concurrently with an oral dose would not be unexpected and has, in fact, been previously reported (19). The mechanism of this effect, however, is fundamentally different from that demonstrated in our studies (Table IV), in that the resin directly interferes with the *initial* absorption of the dose of glycoside. In that study, cholestyramine did not protect mice from death due to intraperitoneally administered digitoxin, presumably because the two were administered concurrently (19). It is possible that a large fraction of the intraperitoneal dose of digitoxin already was excreted into the intestine and reabsorbed before the orally administered cholestyramine reached the small intestinal lumen in amounts sufficient to block reabsorption.

There are several lines of evidence indicating that cholestyramine influences the enterohepatic circulation of parenterally administered digitoxin. First, fecal excretion of radioactivity is significantly increased during the first 24 hr after injection of digitoxin-^{3}H in cholestyramine-treated rats compared to controls (Tables V and VI). This indicates that a greater fraction of the digitoxin-^{3}H and metabolites excreted in the bile soon after injection is rendered unavailable for reabsorption by cholestyramine. After the 1st day the total body glycoside pool is greater in control rats and consequently greater amounts are excreted in the bile, and ultimately in the feces, on days 2 to 4 (Table V). Thus cholestyramine appears to accelerate the fecal excretion of digi-

toxin. It will also be recalled that in the first 24 hr after injection of digitoxin-^{3}H there was significantly greater fecal excretion of digitoxin-^{3}H and digitoxigenin-^{3}H and less excretion of digitoxin-^{3}H and digoxigenin-^{3}H in cholestyramine-treated rats compared to controls (Table VI). This is due in part to the sequestration of digitoxin by cholestyramine within the intestinal lumen which renders the digitoxin unavailable for absorption, metabolic degradation, and enterohepatic circulation.

Second, lower tissue levels of radioactivity are found after the injection of ^{3}H-digitoxin into cholestyramine-treated rats and guinea pigs as compared with controls (Tables VII and VIII). Although no differences in peripheral blood or heart levels of tritium were found in control or cholestyramine-treated rats, significant differences were found in the brain, where concentrations of tritium were considerably higher than in the other tissues examined. In this regard, significantly greater amounts of digitoxigenin were found in the brains of control as compared to cholestyramine-treated rats. This is thought to be especially significant since rats do not die a cardiac death from digitoxin toxicity, but rather experience a characteristic convulsive disorder following lethal doses of digitoxin or digitoxigenin, with the latter being the most potent convulsant agent among digitoxin and its derivatives (20). Further, decreased levels of digitoxin-^{3}H and metabolites were found in the heart and liver of cholestyramine-treated guinea pigs as compared to controls (Table VIII).

Third, no significant changes in serum electrolytes, known to influence digitalis uptake, were found in the rats treated with cholestyramine (Table IX). This is in accord with the product literature available which states that no changes in hemogram, glucose, blood urea nitrogen, pH, or electrolytes occurred in rats or dogs fed cholestyramine for 1 yr (21). The failure to demonstrate hyperkalemia rules out the possibility that cholestyramine caused a potassium-induced alteration in digitalis uptake and binding (13). Fourth, the only alternative to biliary excretion by which parenterally administered digitoxin might reach the intestinal lumen and appear in the feces would be direct transmucosal excretion. There is no clear-cut evidence that this occurs in intact animals, although it may occur in bile fistula animals in whom the normal mucosal to serosal gradient of digitoxin would be absent (22).

The data obtained in this study indicate that cholestyramine treatment affords a significant degree of protection against lethal digitoxin intoxication in rats and guinea pigs. It is suggested that cholestyramine binds appreciable amounts of digitoxin in the intestinal lumen, resulting in reduced reabsorption, increased fecal excretion, and lower tissue levels of glycoside in critical organs. The protective effects of cholestyramine thus appear to be mediated by interruption of the enterohepatic circulation of digitoxin.

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