The Chemical Forms in Which Phenol Red is Secreted into the Bile Of Rats. (31506)

LARRY G. HART AND LEWIS S. SCHANKER
Laboratory of Chemical Pharmacology, National Heart Institute, National Institutes of Health, Bethesda, Md.

Although the sulfonic acid dye, phenol red, has been reported to be excreted in high concentrations in the bile of a variety of species (1-6), there does not appear to have been any investigation of whether it is excreted as the unchanged compound, as metabolites, or both. In the course of our studies on the biliary excretion of organic compounds, a paper chromatogram of bile from a rat that had been injected with phenol red revealed the presence of two colored substances. This observation led to the following study of the forms in which phenol red is secreted into bile.

Methods and materials. Male Sprague-Dawley rats, weighing 300-320 g, were anesthetized with pentobarbital. Through a midline abdominal incision, the renal pedicles were ligated to prevent renal excretion of phenol red and thus allow maintenance of a fairly constant plasma level of the dye after a single intravenous injection. The bile duct was cannulated with a blunt 23-gauge hypodermic needle shaft attached to a short length of plastic tubing and the incision then closed. Phenol red (27-42 mg/kg) was administered intravenously (femoral vein) over a 1-minute period and bile collected for four 30-minute periods. At the end of an experiment, the animal was decapitated and blood collected in a heparinized beaker.

Phenol red in bile was measured colorimetrically at 555 mμ after appropriate dilution with water and final dilution with 0.3 N NaOH solution. At these dilutions, the "blank" due to colored substances in normal bile was essentially zero. Protein-free filtrates of plasma were prepared by the method of Marshall and Vickers(7) and the dye measured as described above after dilution with NaOH solution. The recovery of known amounts of phenol red that had been added to normal rat plasma was 96%.

For the chromatography of bile, samples were applied to sheets of Whatman no. 1 filter paper and the chromatograms developed ascendingly with several different solvent systems: *n*-butanol-acetic acid-water (40:12:34); pyridine-isoamyl alcohol-water (7:7:6); and *n*-propanol-isoamyl alcohol-acetic acid-water (4:1:1:3). Phenol red appeared as a yellow spot which became red on exposure to ammonia vapor.

Phenol red (phenolsulfonphthalein) was obtained from the Hartman-Leddon Co. Beta-glucuronidase (bovine liver) with a potency of 3000 Fishman units/mg was obtained from the Sigma Chemical Co.

Results. Paper chromatograms of the bile from rats that had been injected with phenol red revealed the presence of two yellow spots. The larger spot became red on exposure to ammonia vapor and had an R_f value identical with that of phenol red in each of the chromatographic systems. The other spot remained yellow in the presence of ammonia and had an R_f value lower than that of phenol red in all the systems. These results indicated that the dye was excreted in at least two forms: as the unchanged compound and as a colored metabolite.

To determine the nature of the metabolite, bile samples were subjected to acid hydrolysis (1 N HCl at 100°C for 30 min) and aliquots of the resulting mixture subjected to paper chromatography. All chromatograms revealed only the phenol red spot, suggesting that the metabolite was a conjugated form of phenol red that could be readily converted to the parent compound on acid hydrolysis. Direct evidence for this view was provided by the increase in the biliary concentration of phenol red that resulted on subjecting the samples to the hydrolytic procedure. Thus, the phenol red content of numerous bile samples was increased 1.4-1.7-fold after hydrolysis. An indication that the conjugate had been completely hydrolyzed under these conditions was

TABLE I. Biliary Excretion of Phenol Red in the Rat.*

Time period after injection (min)	Excretion as % of injected dose		Excretion of conjugate as % of total colored material excreted
	Phenol red	Conjugate	
0- 30	27.3 ± 5.6	10.5 ± 2.1	26.1
30- 60	16.6 ± .6	8.8 ± 1.1	34.3
60- 90	7.3 ± .6	4.6 ± .4	40.7
90-120	4.2 ± .5	3.0 ± .5	43.5
Total	55.4	27.0	

* Rats with ligated renal pedicles received phenol red intravenously, and bile was collected for four 30-min periods. Results are expressed as the mean ± S.E. for 3-4 animals.

obtained on autoclaving several bile samples for 1 hour at 124°C in the presence of 1 N HCl. With this method, the increase in the biliary concentration of phenol red was exactly the same as that obtained under the milder hydrolytic conditions.

The biliary excretion of phenol red and its conjugate over a 2-hour period is shown in Table I. During the first 30 minutes, 27% of the injected dye was excreted as the unchanged compound, and about 11% as the conjugate. By the end of 2 hours, 55% of the dose had been excreted as unchanged phenol red, and 27% as the conjugate. It can be seen from the Table that, as time progressed, the conjugate accounted for an increasing proportion of the total colored material excreted, the percentage rising from 26 after 30 minutes to about 44 after 2 hours.

At the end of the 2-hour experiments, the concentration of phenol red in bile was, on the average, 34 times the concentration in plasma; and the conjugate had a bile-to-plasma concentration ratio even higher, 57.

Evidence that the conjugated form of phenol red is a glucuronide was obtained on treating the bile from injected animals with beta-glucuronidase. Samples were incubated with the enzyme (1 mg/ml in acetate buffer, pH 4.5) at 38°C for varying periods up to 24 hours. After incubation, the enzyme protein was precipitated with cold 10% trichloroacetic acid solution, and aliquots of the supernatant fluid were chromatographed. Enzymatic treatment resulted in complete disappearance of the conjugate spot. When the incubated samples were assayed colorimetrically, it was found that enzymatic cleavage of the conjugate was complete after 45 minutes. The amount of phenol red recovered after enzyme treatment was the same as that recovered after acid hydrolysis. The conjugate was not split in control experiments in which bile was carried through all the above procedures in the absence of enzyme.

Discussion. The results of the foregoing study strongly suggest that there is only one metabolite of phenol red excreted in the bile of rats, and that this metabolite is a glucuronide conjugate. The possibility exists that there may also be some colorless metabolities; however, chromatograms of bile revealed no spots that absorbed ultraviolet light other than those of the two colored compounds and those due to the normal components of bile. Thus, the hepatic disposition of phenol red appears to be much simpler than that of certain other phthalein dyes, for example bromsulphalein. The latter compound appears in the bile of rats and other species in the form of a number of colored metabolites (8).

Since the glucuronide of phenol red is highly concentrated in the bile, it is likely that this substance is actively secreted into bile, perhaps by the same acid-transporting process that handles the parent compound as well as a wide variety of organic acids(4).

Summary. Rats with ligated renal pedicles and a cannulated bile duct received an intravenous injection of phenol red. Paper chromatography of the bile revealed the presence of two colored substances, one of which was chromatographically identical with phenol red. The other substance could be converted to phenol red by acid hydrolysis and was thus a conjugated form of the dye. The conjugate appeared to be a glucuronide, since it was completely converted to phenol red on treatment with beta-glucuronidase. Within 2 hours after the injection of phenol red, 55% of the dose was excreted in bile as the unchanged compound, and 27% as the glucuronide conjugate. The conjugate, like the parent compound, appeared to be actively secreted into bile.

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Lysine Deficiency and Leukocyte Metabolism. (31507)

JOHN D. WOJCIK AND IRVING GRAY
Department of Biology, Georgetown University, Washington, D.C.

In a recent publication(1) studies on the mechanism of host resistance to infectious disease were reported. The altered resistance was developed by placing rats on a lysine deficient diet. The interrelationship between altered diets and bacterial infection has been reviewed by Dubos and Schaedler (2) and Schneider(3). Lysine deficiency and its effect on anthrax infection was selected because of the long recognized(4) decreased resistance of herbivorous animals to this infection(5). The results of our earlier studies (1) demonstrated the reduced ability of the reticuloendothelial (RES) to carry out its phagocytic responsibilities. Additional studies (6) showed that under the conditions of the lysine deficiency which caused the lowered resistance to anthrax, there was a decreased ability to mobilize leukocytes. This paper reports continuing studies on the mechanism of the host resistance to infection. It has been shown(7,8) that there is increased oxygen (O_2) utilization by leukocytes during phagocytosis which can be used as a measure of this cellular activity(9). Therefore, the effect of lysine infection on the oxidative metabolism of leukocytes has been studied and is reported here.

Materials and methods. Female rats of the Sprague-Dawley strain were received as weanlings, 22 days old and between 40-50 g, from the supplier. They were randomly divided into control (CON) and experimental (LYS) groups and placed 2 to a cage with a screened floor. The animals were immediately placed on the appropriate diets. These were the same as those used in the previous studies (1) with a slightly different method of preparation. The lysine deficiency was produced by replacing the casein of the control diet with gluten, a protein deficient in this essential amino acid. The two diets were isocaloric. They were prepared by mixing the dry powder with distilled water to a dough-like consistency, placing in aluminum foil moulds and heating in a 250° oven until set. The food was removed from the oven, cooled, cut into squares approximately $2'' \times 2''$, frozen, and held at 0°F until use. The animals were allowed food and water *ad libitum* and kept on the diet for 28 days before use and were not used after 42 days. The weight for each group after 28 days was 77 ± 7 g LYS, 180 ± 3 g CON. These data compare most favorably with the previous report(1).

Peritoneal exudates were stimulated by injecting a 10% solution of glycogen, intraperitoneally, 12 hours and 4 hours before use. The LYS animals received 9 ml and 5 ml and the CON 15 ml and 5 ml at the respective periods. To collect the leukocytes, the rats were anesthetized with ether, the abdomen opened and the fluid in the peritoneal cavity aspirated with a Pasteur pipette. The peritoneal fluid was transferred to a graduated centrifuge tube containing 0.5 ml heparinized Hanks' solution. The peritoneum was washed once with 5 ml (LYS) and 7 ml (CON) of Hanks' solu-