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EFFECT OF PHENOBARBITAL ON THE EXCRETION OF AN EXOGENOUS BILIRUBIN LOAD*

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Abstract—Treatment of mice with phenobarbital significantly enhanced the disappearance of exogenously administered bilirubin from the plasma. Not only did this enhanced disappearance occur in the absence of biliary excretion but it was accompanied by liver bilirubin concentrations exceeding those found in controls. Both observations suggest enhanced uptake of free bilirubin by the liver as one cause of the faster rate of disappearance of bilirubin from plasma. In rats, the maximal rate of bilirubin excretion was enhanced by treatment with phenobarbital. The bile volume was also greater in phenobarbital-treated animals than in controls. The concentration of bilirubin in the bile was not significantly different, indicating that the increase in bilirubin excretion was probably due to the increase in bile volume. Indirectly, some of the data suggest that increased bilirubin conjugation may play a role in the enhanced uptake and excretion of bilirubin after phenobarbital treatment.

AMONG other drugs, phenobarbital has previously been reported by this laboratory to potentiate α-naphthylisothiocyanate (ANIT)-induced hyperbilirubinemia.[1] Present knowledge of both ANIT and phenobarbital engenders ideas of possible mechanisms responsible for the potentiation: one is that phenobarbital alters liver function in a manner additive to the liver damage resulting from ANIT treatment; a second is that ANIT is biotransformed to a hepatotoxic metabolite, and that this biotransformation is enhanced by phenobarbital pretreatment, owing to the ability of phenobarbital to stimulate certain microsomal drug-metabolizing enzymes.[2]

ANIT has been shown to impair the disappearance of exogenous loads of bilirubin and to decrease the apparent bile transport maximum for bilirubin excretion.[3] Although the effect of phenobarbital on the handling of exogenous loads of bilirubin has not been investigated, Fujimoto *et al.*[4] have shown that sulfobromophthalein (BSP) disappearance is enhanced after phenobarbital pretreatment. These authors felt that the experimental evidence favored an adaptive type of hepatic mechanism as responsible for the enhanced disappearance of BSP. Bilirubin studies with phenobarbital were therefore intiated so that a comparison could be made between the effects of ANIT and phenobarbital on the disappearance pattern of exogenously administered bilirubin. Because the removal of bilirubin from plasma is dependent upon hepatic uptake, biotransformation, and biliary excretion, an attempt has been made to assess the relative contribution of each to the observed effect.

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MATERIALS AND METHODS

Male Swiss–Webster mice (25–40 g) and male Simonsen rats (300–400 g) were used throughout the study. Before use, the animals were randomized 10 per cage and maintained unrestricted on laboratory diet and tapwater. Phenobarbital sodium was given i.p. in 0·9% NaCl; control animals received an identical volume of saline (0·01 ml/g) alone. Bilirubin (Sigma Chemical Co. St. Louis, Mo.) for i.v. injection was prepared by dissolving 10 or 30 mg crystalline bilirubin in 10 ml of an isotonic solution containing 0·5 g of Na_2CO_3 and 0·52 g NaCl/100 ml distilled water. Bilirubin for infusion in the rat was prepared by dissolving 40 mg bilirubin in 10 ml of the isotonic Na_2CO_3–NaCl solution.

Plasma bilirubin disappearance studies. Mice were injected (0·01 ml/g) via the tail vein with bilirubin solution (10 mg/kg). After administration of the bilirubin load, blood samples were obtained at various time intervals from separate groups of animals by cardiac puncture under ether anesthesia. Each blood sample was collected in an individual Kahn tube which had previously been treated with sodium oxalate solution and dried. Mice pretreated with phenobarbital (60 mg/kg) once daily for 3 days, along with the corresponding controls, were tested in this manner.

The plasma disappearance of an exogenous bilirubin load in the absence of biliary excretion was studied by ligating the bile duct; ligation was accomplished in mice by means of a small abdominal incision under pentobarbital anesthesia. The wound was closed with 9-mm clips and the animals allowed to recover from the anesthesia (1–2hr) before bilirubin was administered. Blood samples were collected as described previously. Control mice and mice given phenobarbital (60 mg/kg), once daily for 3 days prior to bile duct ligation and bilirubin injection, were tested in this manner.

Total bilirubin content in the plasma samples was determined by the bilirubin oxidation method of Ferro and Ham.[5] In certain experiments the same plasma samples were also analyzed for free and conjugated bilirubin levels by the diazotization method of Weber and Schalm.[6]

Liver bilirubin studies. These experiments were done on mice whose bile ducts were ligated prior to the injection of bilirubin (30 mg/kg). At selected time intervals after bilirubin administration, the animals were sacrificed by cervical dislocation and the livers removed, sliced, and washed with an isotonic solution containing KCl (11·5 g/l.) and $KHCO_3$ (32 mg/l.). The liver was homogenized with a Teflon–glass homogenizer for 20 sec in sufficient KCl–$KHCO_3$ solution to give a 10% (w/v) homogenate. Total liver bilirubin in the homogenate was estimated by a diazo reaction involving a modification of Hargreave's procedure.[3]

Biliary excretion studies. In general, the method of Weinbren and Billing[7] was used in these experiments. Rats were anesthetized with pentobarbital and the bile duct and external jugular vein cannulated with PE-10 tubing. A priming dose of bilirubin (20 mg/kg) was followed by constant infusion of 0·3 mg/min by means of a Harvard infusion pump (0·07 ml/min) for a 60-min period. Such an infusion rate results in plasma bilirubin levels that rise constantly during the infusion period. Six bile collection periods of 10-min duration each were employed. The volume of bile collected was measured by means of a pipet graduated in 0·01 ml, and the bilirubin concentration of the bile sample was estimated by the method of Weinbren and Billing. The rats were given phenobarbital (60 mg/kg) once daily for 3 days prior to running the experiments.

Liver-weight studies. Changes in liver weight were estimated after 3 days of phenobarbital (60 mg/kg). The animals were sacrificed, the liver removed, blotted free of blood, and weighed. The livers were then placed in a vacuum desiccator and heated to 100° until changes in liver weight were no longer evident with repeated weighing

Phenobarbital analysis. Estimates of phenobarbital levels in the plasma of mice were done essentially by the spectrophotometric method of Goldbaum.[8] Mice were treated with phenobarbital for 3 days (60 mg/kg). On the fourth day blood was obtained by cardiac puncture and immediately analyzed for phenobarbital content.

Statistical analysis. Control and phenobarbital groups were compared statistically by the Student's t test ($P < 0{\cdot}05$ for rejection of the null hypothesis). The best-fitting lines for total plasma bilirubin disappearance were calculated by the method described by Goldstein.[9] The half-life for bilirubin disappearance was calculated as described by Neilands and Stumpf.[10]

RESULTS

Plasma bilirubin disappearance studies

Figure 1 illustrates the results obtained from control mice and mice treated with phenobarbital once daily for 3 days prior to injection of bilirubin. Optimum dosages

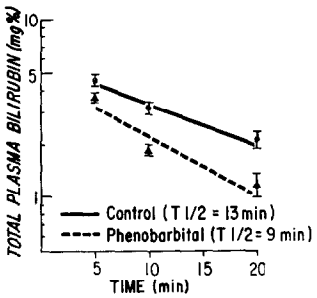

FIG. 1. Disappearance of an exogenous bilirubin load (10 mg/kg, i.v.) from plasma of phenobarbital-pretreated animals (60 mg/kg, i.p. once daily for 3 days prior to bilirubin administration). Bilirubin was determined by oxidation to biliverdin. Each point represents the mean value ± S.E. determined from a group of ten mice. The half-life ($T_{\frac{1}{2}}$) was calculated from the 5- and 20-min values. The 5-, 10-, and 20-min total plasma bilirubin levels were significantly lower in the phenobarbital-treated animals ($P < 0{\cdot}05$).

and sampling times for demonstrating bilirubin disappearance were established in preliminary experiments. Blood samples were obtained from separate groups of mice 5, 10, and 20 min after the injection of bilirubin. The phenobarbital-treated animals exhibited significantly lower total bilirubin levels at all three time periods. The calculated half-life for disappearance of total bilirubin from plasma was 13 min for control mice vs. 9 min for phenobarbital-treated mice. Phenobarbital, therefore, significantly enhanced the disappearance of exogenously administered bilirubin from the plasma.

Free and conjugated bilirubin were estimated by differential analysis for each in the same plasma samples. The results are shown in Table 1. Free bilirubin levels in the phenobarbital-treated groups were significantly lower than in the control group at all

three sampling periods. Conjugated bilirubin levels were lower in the phenobarbital treated animals at the 5- and 10-min sampling periods, but not at the 20-min period. From these data it is apparent that phenobartital treatment has a significant influence on the disappearance of free bilirubin from the plasma.

TABLE 1. CONCENTRATION OF FREE AND CONJUGATED BILIRUBIN IN PLASMA AFTER AN EXOGENOUS BILIRUBIN LOAD

Groups*	Bilirubin concentration† 5 min	10 min (mg/100 ml)	20 min	$T_{\frac{1}{2}}$‡
Free bilirubin				
Control	3·7 ± 0·2	1·9 ± 0·2	1·1 ± 0·1	9
Phenobarbital	2·3 ± 0·1§	0·9 ± 0·1§	0·5 ± 0·1§	7
Conj. bilirubin				
Control	2·3 ± 0·2	1·3 ± 0·3	0·9 ± 0·2	11
Phenobarbital	1·5 ± 0·2§	0·8 ± 0·1§	0·6 ± 0·1	11

* Phenobarbital was given i.p. (60 mg/kg) once daily for 3 days; bilirubin was given i.v. (10 mg/kg).

† Blood samples were obtained 5, 10, and 20 min. after bilirubin administration. Bilirubin was determined by a diazotization reaction. Values shown are mean values ± S.E.obtained from a group of ten mice.

‡ The half-life was calculated from the 5- and 20-min values.

§ Significantly different from respective control ($P < 0·05$).

Table 2 shows the results of a phenobarbital dose–response study. Each of the three different doses of phenobarbital was administered for 3 days to different groups of mice. Although 60 mg phenobarbital/kg was most effective in reducing plasma bilirubin levels, a dose as small as 7·5 mg/kg significantly lowered the total plasma bilirubin concentration 5 and 10 min after bilirubin administration.

TABLE 2. DOSE–RESPONSE STUDY OF PHENOBARBITAL-ENHANCED DISAPPEARANCE OF EXOGENOUS BILIRUBIN LOAD

Groups*	Bilirubin concentration † 5 min	10 min (mg/100 ml)	20 min	$T_{\frac{1}{2}}$‡
Control	5·8 ± 0·1	3·7 ± 0·2	2·3 ± 0·2	11
Phenobarbital (mg/kg)				
7·5	4·6 ± 0·3§	2·5 ± 0·2§	1·9 ± 0·1	12
30	4·3 ± 0·1§	2·9 ± 0·1§	1·5 ± 0·1§	10
60	3·7 ± 0·2§	2·7 ± 0·3§	1·2 ± 0·1§	9

* Phenobarbital was given i.p. once daily for 3 days; bilirubin was given i.v. (10 mg/kg).

† Blood samples were obtained 5, 10, and 20 min after bilirubin administration. Total bilirubin was determined by an oxidation reaction. Values shown are the mean ± S.E. obtained from a group of five mice.

‡ See Table 1.

§ Significantly different from the control group ($P < 0·05$).

Since these results suggested an enhanced excretion of bilirubin by the liver, a similar experiment was performed in the absence of biliary excretion. Table 3 shows the effect of phenobarbital pretreatment on the disappearance pattern of exogenous bilirubin

TABLE 3. CONCENTRATION OF FREE AND CONJUGATED BILIRUBIN IN PLASMA OF BILE DUCT-LIGATED MICE AFTER AN EXOGENOUS BILIRUBIN LOAD

Groups*	Bilirubin Concentration†			$T_{\frac{1}{2}}$‡
	5 min	10 Min (mg/100 ml)	20 min	
Free bilirubin				
Control	6·1 ± 0·3	4·0 ± 0·2	3·0 ± 0·1	15
Phenobarbital	3·8 ± 0·2§	2·4 ± 0·2§	1·8 ± 0·1§	14
Conj. bilirubin				
Control	1·8 ± 0·2	2·0 ± 0·1	2·1 ± 0·3	
Phenobarbital	2·3 ± 0·2	2·6 ± 0·2§	2·9 ± 0·3§	

* Phenobarbital was given i.p. (60 mg/kg) once daily for 3 days prior to bile duct ligation and bilirubin administration (10 mg/kg).

† See Table 1.

‡ See Table 1.

§ Significantly different from respective control ($P < 0·05$).

on mice with ligated bile ducts. Ligation of the bile duct just prior to bilirubin administration failed to alter the enhanced disappearance pattern for bilirubin in the phenobarbital-treated mice. Lower levels of free bilirubin were found in the phenobarbital-treated animals at each sampling time. This suggests enhanced uptake of free bilirubin by the liver. However, conjugated bilirubin levels in the phenobarbital-pretreated animals were significantly greater than controls at the 10- and 20-min time periods suggesting a faster rate of conversion of free to conjugated bilirubin.

Estimates of total liver bilirubin levels under the condition of bile duct ligation were done to establish whether or not an enhanced uptake of bilirubin was actually occurring after phenobarbital treatment. The results are shown in Table 4. As in previous

TABLE 4. EFFECT OF PHENOBARBITAL ON LIVER BILIRUBIN LEVELS AFTER EXOGENOUS BILIRUBIN LOAD IN BILE DUCT-LIGATED MICE

Groups*	Total liver bilirubin concentration†		
	5 min	10 min (μg/g)	20 min
Control	71 ± 5	74 ± 3	125 ± 9
Phenobarbital	81 ± 3‡	90 ± 3‡	159 ± 8‡

* Phenobarbarbital (60 g/kg) was given once daily for 3 days prior to bilirubin administration (30 mg/kg, i.v.).

† Liver bilirubin levels were estimated 5, 10, and 20 min after bilirubin administration. Values shown are mean values ± S.E. obtained from a group of ten mice.

‡ Significantly different from respective control ($P < 0·05$).

studies, phenobarbital (60 mg/kg) was administered once daily for 3 days. Phenobarbital treatment resulted in significantly higher concentrations of total bilirubin in the livers at all three sampling times. These results support the contention that phenobarbital enhances the uptake of bilirubin by the liver.

Biliary excretion studies

Experiments in the rat had revealed that phenobarbital affected bilirubin disappearance in a manner similar to that found in the mouse. Transport maximum (Tm) studies for bilirubin were therefore conducted in the rat. The results are shown in Fig. 2.

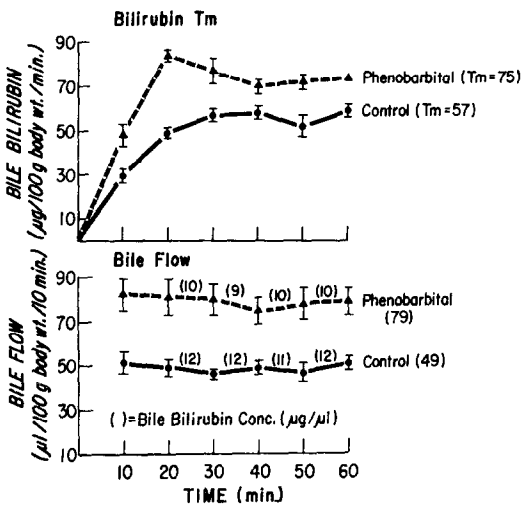

FIG. 2. Effect of phenobarbital (60 mg/kg, i.p., once daily for 3 days prior to bilirubin administration) on the apparent transport maximum (Tm) for bilirubin, bile flow, and bile bilirubin concentration in the rat. Bilirubin was infused at a rate of 0·3 mg/min during the entire 60-min collection period. Infusion was preceded by a priming dose of 20 mg bilirubin/kg i.v. Under these conditions plasma bilirubin levels showed a steady increase during the experiment. Each point represents the mean value ± S.E. determined from six rats. All phenobarbital values are significantly different from control values ($P < 0.05$), with the exception of bile/ bilirubin concentrations, which were not significantly different.

Phenobarbital treatment significantly elevated the apparent Tm for bilirubin excretion. The phenobarbital-treated animals had an average bilirubin Tm of 75 μg/100 g body weight/min while the control animals had a Tm of 57 μg/100 g/min.

The bile flows and bile bilirubin concentrations from the same experiments are shown in the lower portion of Fig. 2. The phenobarbital-treated rats had a significantly greater bile flow than the controls, 79 vs. 49 μl/100 g body weight/ 10 min. The bile flow was greater in the phenobarbital-treated rats even before the bilirubin infusion was started. Calculation of bile bilirubin concentration in terms of μg/μl indicated no significant difference between control and phenobarbital-treated animals. This suggests that the enhanced excretion of bilirubin into the bile seen in phenobarbital-treated animals is due to an increase in bile volume rather than an increase in the capacity to secrete bilirubin.

Changes in wet and dry liver weight after phenobarbital treatment were followed in rats and mice in an attempt to find a correlation between per cent increase in liver weight and per cent increase in apparent liver function. The results are shown in Table 5. In both rats and mice there was a significant increase in liver weight. The increase in wet or dry liver weight was approximately 30 per cent in rats and 15% in mice.

TABLE 5. INCREASE IN LIVER WEIGHT AFTER PHENOBARBITAL TREATMENT

Groups*	Liver weight†	
	Wet weight (g liver/100 g body weight)	Dry weight (g liver/100 g body weight)
Mouse		
Control	9·7 ± 0·3	5·9 ± 0·1
Phenobarbital	11·4 ± 0·3‡	6·7 ± 0·2‡
Rat		
Control	4·3 ± 0·2	1·5 ± 0·1
Phenobarbital	5·5 ± 0·2‡	1·9 ± 0·1‡

* Phenobarbital was given i.p. (60 mg/kg) once daily for 3 days. Analysis of liver weight was completed on the fourth day.

† Liver weights, expressed as g liver/100 g total body weight ± S.E., were determined immediately after removal from the animal (wet weight) and after repeated heating to constant weight (dry weight).

‡ Significantly different from the respective control group ($P < 0·05$).

The concentration of phenobarbital in blood was determined to establish the relative influence phenobarbital could have on the plasma binding of injected bilirubin. Phenobarbital could not be detected in the blood obtained from mice which had been treated with phenobarbital once daily for 3 days (60 mg/kg) prior to analysis. Control experiments demonstrated that the analytical procedure used was capable of extracting 95 per cent of the phenobarbital added to mouse blood and of detecting a concentration of phenobarbital of 0·1 mg/100 ml.

DISCUSSION

The data presented indicate that phenobarbital affects the disappearance of exogenously administered bilirubin in a manner opposite to that described for ANIT.[3] Phenobarbital enhances the disappearance of exogenously administered bilirubin from plasma, increases the concentration of bilirubin in the liver, and increases the apparent transport maximum for bilirubin excretion into bile. In light of these results it seems unlikely that the potentiation of ANIT by phenobarbital is a matter of additive deleterious effects of phenobarbital and ANIT on bilirubin clearance. If such were the case, phenobarbital and ANIT would be expected to produce similar effects on the disappearance of exogenous loads of bilirubin.

The results of this experiment permit conjecture on the possible mechanisms responsible for the enhanced removal of bilirubin from plasma after phenobarbital treatment. The altered plasma bilirubin disappearance pattern produced by phenobarbital occurred even in the absence of biliary excretion (Table 3) and was accompanied by liver bilirubin concentrations exceeding those found in control animals

(Table 4). Both suggest enhanced uptake as one cause of the faster rate of disappearance of bilirubin from the plasma.

Among the possible explanations for enhanced removal of bilirubin from plasma is that phenobarbital decreases the binding of bilirubin to plasma proteins. However, since a 24-hr period was interposed between the last dose of phenobarbital and the administration of bilirubin, only a very small amount of phenobarbital would be present in the blood to affect the binding of bilirubin to plasma proteins. Actual analysis of the mouse blood demonstrated that phenobarbital was essentially absent at the time the bilirubin was administered. Another alternative would be that, although plasma binding is unaffected, uptake of bilirubin by nonhepatic tissues has been increased. This possibility has not been assessed.

A more likely possibility is that phenobarbital alters directly the metabolism of bilirubin by the liver, thereby increasing the capacity of liver to take up and excrete exogenous bilirubin loads. In mice with ligated bile ducts, more conjugated bilirubin appeared in the serum of the phenobarbital-treated mice than in the controls. Remmer, in a study involving the elimination of sulfadimethoxin (SDM) in the rat, stated that phenobarbital treatment seemed to stimulate glucuronide synthesis in the liver thereby increasing three-fold the rate of elimination of SDM.[11] Catz and Yaffe have investigated the adaptive increases in enzymic activity of the liver occurring after treatment of mice with barbital sodium.[12] They reported that the barbital treatment resulted in a 2·5-fold increase in hepatic bilirubin-conjugating activity. Such direct evidence for enhanced bilirubin-conjugating activity after phenobarbital treatment remains to be accomplished.

In the rat, the rate of bilirubin excretion is enhanced by phenobarbital treatment, but the concentration of bilirubin in the bile is similar to that of controls (Fig. 2). Since bile volume is also increased by phenobarbital treatment, the overall increase in bilirubin excretion may be due to an effect of phenobarbital on bile production rather than on active bilirubin secretion into the bile. The observation that the phenobarbital increases liver weight,[13] which has also been observed under our experimental conditions, could easily account for the greater bile production. Indeed, correction of the bile volume and bilirubin Tm data by an amount equal to the per cent increase in liver weight seen after phenobarbital treatment nullifies the differences actually observed. The increase in liver weight, however, does not explain the fact that bilirubin concentrations in the liver were significantly greater in phenobarbital-treated mice than in controls.

In summary, these results indicate that the enhanced bilirubin disappearance from plasma and excretion are a consequence of an increased uptake of free bilirubin by the liver. What remains to be resolved is the mechanism responsible for the increased uptake of bilirubin. Indirect evidence gathered in this study favors the conclusion of enhanced bilirubin conjugation, but direct evidence is lacking.

REFERENCES

1. R. J. ROBERTS and G. L. PLAA, *J. Pharmac. exp. Ther.* **150**, 499 (1965).
2. A. H. CONNEY and J. J. BURNS, *Adv. Pharmac.* **1**, 31 (1962).
3. R. J. ROBERTS and G. L. PLAA, *J. Pharmac. exp. Ther.*, in press.
4. J. M. FUJIMOTO, W. F. EICH and H. R. NICHOLS, *Biochem. Pharmac* **14**, 515 (1965).
5. P. R. FERRO and A. B. HAM, *Am. J. clin. Path.* **44**, 11 (1965).
6. A. P. WEBER and L. SCHALM, *Clin. chim. Acta* **7**, 805 (1962).

7. K. WEINBREN and R. H. BILLING, *Br. J. exp. Path.* **37**, 199 (1956).
8. L. R. GOLDBAUM, *Analyt. Chem.* **24**, 1604 (1952).
9. A. GOLDSTEIN, *Biostatistics*. Macmillan, New York (1964).
10. J. B. NEILANDS and P. K. STUMPF, *Outlines of Enzyme Chemistry*. Wiley, New York (1955).
11. H. REMMER, *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **247**, 461 (1964).
12. C. CATZ and S. J. YAFFE, *Am. J. Dis. Child.* **104**, 516 (1962).
13. A. H. CONNEY, C. DAVIDSON, R. GASTEL and J. J. BURNS, *J. Pharmac. exp. Ther.* **130**, 1 (1960).