Biochemical Pharmacology, Vol. 24, pp. 1283–1286. Pergamon Press, 1975. Printed in Great Britain.

PHENOBARBITAL-ENHANCED BILIARY EXCRETION OF ADMINISTERED UNCONJUGATED AND CONJUGATED SULFOBROMOPHTHALEIN (BSP) IN THE RAT*

GREGORY WHELAN† and BURTON COMBES‡

The Liver-Gastrointestinal Unit, Department of Internal Medicine, The University of Texas Southwestern Medical School at Dallas, Dallas, Tex. 75235, U.S.A.

(*Received* 23 *October* 1974; *accepted* 6 *December* 1974)

Abstract—Phenobarbital pretreatment resulted in increases in the maximal rates of biliary excretion of intravenously administered sulfobromophthalein (BSP) and BSP–glutathione. In rats given unconjugated BSP, the rise in excretion was due to an increase in the amount of conjugated dye delivered into bile. These results provide evidence for at least two sites of action of phenobarbital in enhancing maximal rates of BSP excretion into bile: (1) increased transport of conjugated dye from liver cells into bile, and (2) increased intrahepatic metabolism of dye.

Most studies concerned with the effect of phenobarbital pretreatment on biliary excretion of administered substances demonstrate that excretion of compounds which are largely or completely metabolized in the liver prior to transport into bile is enhanced, whereas excretion of compounds that are not metabolized may or may not be increased. For example, biliary excretion of injected bilirubin [1], stilbesterol [2], biphenyl [2], phenolphthalein [2], thyroxine [3] and probenecid [4], compounds which undergo conjugation with glucuronic acid, and of BSP [5–8], which is metabolized with glutathione, is enhanced. By contrast, excretion of administered succinyl sulphathiazole [2, 8], stilbesterol monoglucuronide [2], phenolphthalein monoglucuronide [2], taurocholic acid [8, 9] and indocyanine green (in rats of both sexes [2, 4, 8], except for one study in males [4]), compounds which are not metabolized further prior to transport into bile, is not enhanced in phenobarbital-treated animals. There are some discrepancies with these findings in that biliary excretion of injected phenol red [4, 8], a compound which is partly excreted as a glucuronide [10], and of cholic acid [9], which is largely conjugated with taurine in the rat, is not increased, whereas enhanced bile transport of the nonmetabolized compounds, phenol 3,6-dibromphthalein disulfonate [5, 8], (diBSP), amaranth [8], ouabain [8] and chlorothiazide (in the female but not the male rat [4, 8]) is observed after phenobarbital pretreatment.

These observations suggest that more than one mechanism may be stimulated when phenobarbital enhances the biliary excretion of a compound. One is increased metabolism, with the implication that the metabolite is excreted into bile at a more rapid rate than the nonmetabolized moiety. A second is stimulation of the putative canalicular transport systems involved in biliary excretion of these compounds. In addition, phenobarbital increases the initial rate of hepatic uptake of a number of the compounds listed above, perhaps because of increased hepatic content of the cytoplasmic organic anion binding protein Y in pretreated animals [7]. In circumstances where hepatic uptake limits the overall rate of hepatic transport from blood to bile, this mechanism will also result in enhanced biliary excretion of a compound.

The present studies were designed to give additional insight into the mechanisms by which phenobarbital enhances the biliary excretion of administered BSP. Availability of a method for synthesizing the BSP conjugate, BSP-glutathione, in large amounts [11], made it possible to compare the biliary excretion of dye after the injection of either conjugated or unconjugated BSP in phenobarbital-pretreated rats and in pair-fed controls. It was thus possible to assess the effects of phenobarbital on intrahepatic metabolism and on transport from liver cells into bile in relation to the overall hepatic disposition of BSP. Doses of administered dye were selected that would result in maximal rats of biliary excretion of the compound. Under these circumstances, hepatic uptake does not limit biliary excretion of BSP [11].

MATERIALS AND METHODS

Simonsen Sprague–Dawley female rats (196–240 g) were housed in individual metabolic cages and fed routine laboratory Chow. After 2 days of acclimitization, one group of randomly selected rats was given sodium phenobarbital by subcutaneous injection daily for 7 days in a dose of 7·5 mg/0·5 ml of cottonseed oil/100 g of body weight. The control group of rats, consisting of individually pair-fed animals, was

* This study was supported in part by United States Public Health Service Research Grant AM 13757 and Training Grant AM 05490 from the National Institutes of Health.

† Present address: Department of Medicine, St. Vincent's Hospital, Fitzroy 3065, Victoria, Australia.

‡ Recipient of Research Career Program Award 5-K3-AM-18,260 from the National Institutes of Health, United States Public Health Service, during performance of these studies.

injected with cottonseed oil (0·5 ml/100 g). All animals were studied 24 hr after the final injection.

Animals anesthetized with ethyl ether were prepared via a midline abdominal incision with a common bile duct cannula (PE 10 tubing), and the abdominal incision was sutured. A heating pad placed beneath the animal was used to maintain the rat's temperature between 37° and 38°, as monitored by means of a rectal probe attached to an electronic thermometer (Tele Thermometer, Yellow Springs Instrument Co.). Animals were given either unconjugated BSP or synthetic BSP–glutathione (BSP–GSH; prepared by the method of Whelan *et al.* [11]) dissolved in 0·9% saline in a dose of 18 μmoles BSP or 30 μmoles BSP–GSH, each per 100 g of body weight, as a single intravenous injection via a femoral vein in a volume of 0·5 ml saline. Bile was collected into previously tared bottles commencing 10 min prior to injection of dye and continuing for four consecutive 10-min collection periods after injection. Bile volume was considered equivalent to the weight of bile collected during these periods. A blood sample was removed from the aorta at the end of the experiment and the liver was rapidly removed, blotted and weighed. Specimens obtained from any animal which experienced respiratory difficulty or a sharp decline in bile flow during the course of the experiment were not analyzed and such experiments were excluded from the study.

The concentration of BSP in alkalinized bile specimens was determined colorimetrically in a Beckman DU spectrophotometer set at 575 nm. Total BSP excreted in any sample was calculated as the product of BSP concentration and bile volume. The maximal rate of dye excretion into bile for each animal was considered to be the highest value achieved during any bile collection period, or the mean of this value and values obtained during consecutive collection periods that were within 10 per cent of the highest value. BSP concentration in plasma was determined by the method of Gaebler [12].

Aliquots of bile from all animals were applied to Whatman No. 1 filter paper and subjected to descending chromatography as previously described [13]. The bile of animals injected with unconjugated BSP contained a band with mobility indentical to that of injected unconjugated BSP and usually three bands with lesser mobility containing BSP metabolites reported in Results and Table 1 collectively as conjugated BSP.

The Mann–Whitney *U* test for nonparametric analytical procedures was utilized to evaluate statistically the results of the above experiments [14].

RESULTS

Animals injected with unconjugated BSP (*Table* 1). Both the phenobarbital-treated and the control animals lost small amounts of weight during the course of these studies. However, animals in the phenobarbital pretreatment group experienced a marked increase in hepatic size resulting in a higher ratio of liver weight as a percentage of body weight, 3·5 versus 2·4 per cent in their pair-fed controls.

Dye excretion into bile after the injection of unconjugated BSP in control rats reached a mean maximum value of 1·34 μmoles/100 g of body weight/10 min. Pretreatment with phenobarbital resulted in a significant elevation of this apparent T_m to a mean value of 2·06 μmoles/100 g of body weight/10 min. This rise in excretion rate was accompanied by an increase in bile flow from 0·057 to 0·106 ml/100 g/10 min. It should be noted that an increase in bile flow was observed in phenobarbital-pretreated animals prior to the injection of dye. Thus the treated group had a bile volume, prior to injection of BSP, of 0·100 compared to 0·075 ml/100 g/10 min in the pair-fed control group. Maximal BSP concentration in bile did not change with phenobarbital treatment.

Analysis of the chromatograms of bile collected during maximal rates of dye excretion revealed that an average of 56·8 per cent of dye excreted in the control group was in the conjugated form. After pretreatment with phenobarbital, an average of 77·7 per cent of the excreted dye was conjugated. The rise in total dye excretion in bile in the phenobarbital-pretreated animals was accounted for by an increased

Table 1. Effect of phenobarbital pretreatment on the biliary excretion of injected BSP and BSP–GSH*

	BSP (18 μmoles/100 g)			BSP–GSH (30 μmoles/100 g)		
	Control (7)†	Phenobarbital (7)	P‡	Control (6)	Phenobarbital (6)	P‡
Body wt (g)						
Original	223 ± 15	209 ± 22		206 ± 16	207 ± 7	
Final	204 ± 24	204 ± 22		179 ± 13	174 ± 17	
Liver/body wt (%)	2·4 ± 0·17	3·5 ± 0·23	<0·001	2·3 ± 0·46	3·3 ± 0·34	0·004
Bile flow (ml/100 g/10 min)						
Prior to dye	0·075 ± 0·010	0·100 ± 0·014	<0·002	0·073 ± 0·011	0·088 ± 0·008	0·02
During dye output	0·057 ± 0·011	0·106 ± 0·015	<0·001	0·097 ± 0·018	0·145 ± 0·013	0·001
BSP concn in bile (μmoles/100 ml)	2000 ± 160	1960 ± 180	NS	2780 ± 255	2830 ± 150	NS
BSP excretion (μmoles/100 g/10 min)						
Total	1·23 ± 0·22	2·06 ± 0·23	<0·001	2·71 ± 0·63	4·10 ± 0·45	0·002
Unconjugated	0·53 ± 0·11	0·45 ± 0·08	NS			
Conjugated	0·70 ± 0·17	1·61 ± 0·25	<0·001			
Total/g liver	0·51 ± 0·10	0·59 ± 0·06	NS	1·18 ± 0·16	1·23 ± 0·05	NS
Plasma BSP at end (μmoles/100 ml)	60·8 ± 14·8	28·8 ± 8·5	<0·001	63·1 ± 17·1	18·3 ± 6·2	<0·001

* Results are given as mean ± S.D.

† Number of rats in each group is listed in parentheses.

‡ Statistical significance of the difference of the data in phenobarbital-treated and appropriate control rats; NS = not significant.

excretion of conjugated dye, from 0·70 to 1·61 μmoles/100 g/10 min. The amount of unconjugated dye excreted in bile did not change significantly after phenobarbital treatment.

Animals injected with BSP–GSH (*Table* 1). Once again animals in both groups lost weight during the pretreatment period of these studies. Liver weight as a percentage of body weight was greater in the phenobarbital-pretreated group than in the control group: 3·3 versus 2·3 per cent.

Dye excretion into bile after administration of BSP–GSH was higher than that found after injection of BSP. This observation confirms the results found in our previous studies utilizing similar weight-adjusted doses of dye [11, 15]. In addition, the maximal rates of excretion of injected BSP–GSH after pretreatment with phenobarbital were higher than those obtained in the control group, 4·10 versus 2·71 μmoles/100 g of body weight/10 min. The rise in excretion rate of dye was accompanied by an increase in bile flow from 0·097 to 0·145 ml/100 g/10 min. Phenobarbital treatment in this group of rats also resulted in a rise in bile volume, prior to injection of BSP–GSH, from 0·073 to 0·088 ml/100 g/10 min. BSP concentration in bile was higher in animals receiving BSP–GSH rather than BSP intravenously. Biliary concentration of dye was comparable in control and phenobarbital-treated rats injected with BSP–GSH.

DISCUSSION

Three major processes are involved in the movement of BSP from blood to bile: uptake into liver cells where dye is stored, conjugation with glutathione and subsequent further partial metabolism to cysteinylglycine and cysteine metabolites, and transport of conjugated and unconjugated BSP compounds from liver cells into bile. In the present studies, hepatic uptake did not limit BSP movement across the hepatic cells. Thus the increase in the maximal rate of dye excretion into bile observed in phenobarbital-treated rats infused with unconjugated BSP could result from enhanced metabolism, enhanced biliary transport or a combination of both.

The increment in dye excretion we observed was accounted for completely by a marked rise in the amount of conjugated BSP compounds appearing in bile. In the studies of Klaassen and Plaa [5], increased excretion of both unconjugated and conjugated BSP accounted for the rise in BSP T_m observed in phenobarbital-treated rats. The composite observations provide evidence that BSP conjugation *in vivo* is enhanced in phenobarbital-treated rats, a conclusion that has also been suggested in such rats, by the findings *in vitro*, in homogenates of liver, of increased activity of glutathione s-aryl transferase [5], the enzyme that catalyzes BSP–glutathione conjugation. Previous observations in our laboratory have provided evidence that conjugated BSP compounds are excreted into bile at a greater maximal rate than unconjugated BSP [11, 15] and that excretion of conjugated BSP is inhibited by the presence in liver of free BSP [16]. It is not surprising therefore that stimulation of hepatic metabolism of BSP by phenobarbital pretreatment would result in enhancement of the biliary excretion rate of the dye.

The studies in which BSP–GSH was administered intravenously provide important evidence that phenobarbital also stimulates the transport process by which conjugated dye compounds are excreted into bile. The maximal rate of dye excretion into bile was higher in control rats receiving already synthesized BSP–GSH, in confirmation of earlier studies reported from this laboratory [11, 15]. This rate of excretion was enhanced substantially by phenobarbital pretreatment. It seems likely therefore that stimulation of both biliary transport and intrahepatic metabolism accounts for the increased rate of dye excretion into bile when unconjugated BSP is administered.

In confirmation of the findings of others [1, 4–6], we observed that the rate of bile flow recorded prior to dye injection was already higher in phenobarbital-treated than in control rats. After injection of unconjugated BSP, bile flow decreased during the period of dye excretion in control rats, but remained elevated in phenobarbital-treated rats. Depression [15, 17–19] and stimulation [5] of bile flow have been reported by others after administration of unconjugated BSP to the rat. During dye excretion, bile flow increased above resting values in control rats given BSP–GSH. Bile flow increased even further in phenobarbital-treated rats. Regardless of whether BSP or BSP–GSH was administered, enhanced maximal excretion rates of dye into bile were the consequence mathematically of increased rats of bile flow, since the concentration of dye in bile did not change in phenobarbital-treated animals. Others have also found the phenobarbital-related increased excretion of dye in rats given unconjugated BSP [5, 6], and the increased excretion of bilirubin in phenobarbital-pretreated rats is accounted for by an increase in bile flow. Nevertheless, these findings should not be construed to indicate that enhanced bile flow *per se* in phenobarbital-treated rats results in the increase in the rate of excretion of these compounds into bile.

The rise in bile flow found in phenobarbital-treated rats is considered to result from stimulation of the canalicular bile-salt-independent fraction of bile flow [20, 21]. Increased canalicular flow could enhance biliary excretion of dye if: (a) maximum activity of the carrier that transports dye into bile is regulated by intracanalicular concentration of BSP, or (b) back-diffusion of BSP compounds from the canaliculus to liver cells or plasma along substantial concentration gradients is a major determinant of net BSP excretion. Substantial evidence against these possibilities has been provided by the observations that increases in the canalicular bile-salt-independent fraction of bile flow induced acutely by theophylline [22–24], or by β-(2,4-diamethoxy-5-cyclohexylbenzoyl) propionic acid (SC-2644) [25] do not result in enhanced BSP excretion in the dog. Moreover, an analysis of steady state BSP kinetics achieved at two levels of sodium taurocholate infusion has also led to the conclusion that canalicular choleresis *per se* is not an important determinant of BSP excretion in the rat [26]. Additional evidence that the increased bile flow induced by phenobarbital is not responsible for enhanced biliary excretion of compounds like BSP is provided by studies in which phenobarbital administration stimulated bile flow but did not enhance the biliary excretion of a number of compounds actively transported from liver cells into cana-

licular bile [2, 4, 8, 9]. These considerations suggest that rather than being the cause of the increased rate of dye excretion, the enhanced bile flow recorded during dye excretion in phenobarbital-treated rats is the consequence of a combination of the increment in bile flow generated by the osmotic activity of the greater amount of BSP transported into bile per unit time, and the independent stimulation of the canalicular bile-salt-independent fraction of bile flow.

Stimulation of dye transport capacity by phenobarbital could result from increased activity of existing or accretion of new, presumably canalicular-based, transporting units. Although a definitive answer is not available, the proportionate increases in the maximum excretory rates of dye and in liver weight observed in the present study when either BSP or BSP–GSH was administered (Table 1), without a change in the concentration of dye in bile, favors accretion of new transporting units as the liver enlarges. Increase in liver size is a well recognized feature of the hepatic response to the microsomal enzyme inducer, phenobarbital [27]. Hepatomegaly is accounted for mainly by hypertrophy but also by hyperplasia of hepatocytes when phenobarbital is administered to adult rats for up to 7 days [28–31]. Synthesis of new transporting units is not simply a consequence of increasing liver size, however, since the hepatomegaly induced by a number of other microsomal enzyme inducers is not accompanied either by increased capacity for BSP transport or increased bile flow [6, 32]. Phenobarbital appears to have a unique property, therefore, which results in synthesis of at least some new canalicular transport units.

Acknowledgement—The authors are indebted to Ruthie Rose for technical help and to Dorothy Bass for assistance with the manuscript.

REFERENCES

1. R. J. Roberts and G. L. Plaa, *Biochem. Pharmac.* **16,** 827 (1967).
2. W. G. Levine, P. Millburn, R. L. Smith and R. T. Williams, *Biochem. Pharmac.* **19,** 235 (1970).
3. J. A. Goldstein and A. Taurog, *Biochem. Pharmac.* **17,** 1049 (1968).
4. L. G. Hart, A. M. Guarino and R. H. Adamson, *Am. J. Physiol.* **217,** 46 (1969).
5. C. D. Klaassen and G. L. Plaa, *J. Pharmac. exp. Ther.* **161,** 361 (1968).
6. C. D. Klaassen, *Biochem. Pharmac.* **19,** 1241 (1970).
7. H. Reyes, A. J. Levi, Z. Gatmaitan and I. M. Arias, *J. clin. Invest.* **50,** 2242 (1971).
8. C. D. Klaassen, *J. Pharmac. exp. Ther.* **175,** 289 (1970).
9. G. Paumgartner, K. Sauter, A. P. Schwarz and R. Herz, *Gastroenterology* **64,** 161 (1973).
10. L. G. Hart and L. S. Schanker, *Proc. Soc. exp. Biol. Med.* **123,** 433 (1966).
11. G. Whelan, J. Hoch and B. Combes, *J. Lab. clin. Med.* **75,** 542 (1970).
12. O. H. Gaebler, *Am. J. clin. Path.* **15,** 452 (1945).
13. B. Combes and G. S. Stakelum, *J. clin. Invest.* **39,** 1214 (1960).
14. S. Siegel, *Nonparametric Statistics for the Behavioral Sciences.* McGraw-Hill, New York (1956).
15. G. Whelan, J. Hoch and B. Combes, *Proc. Soc. exp. Biol. Med.* **132,** 704 (1969).
16. G. Whelan and B. Combes, *J. Lab. clin. Med.* **78,** 230 (1971).
17. R. J. Groszmann, B. Kotelanski, J. Kendler and H. J. Zimmerman, *Proc. Soc. exp. Biol. Med.* **132,** 712 (1969).
18. B. G. Priestly and G. L. Plaa, *Proc. Soc. exp. Biol. Med.* **135,** 373 (1970).
19. D. Dhumeaux, P. Berthelot, A.-M. Preaux, S. Erlinger and R. Fauvert, *Revue Étud. clin. biol.* **15,** 279 (1970).
20. P. Berthelot, S. Erlinger, D. Dhumeaux and A.-M. Preaux, *Am. J. Physiol.* **219,** 809 (1970).
21. C. D. Klaassen, *J. Pharmac. exp. Ther.* **176,** 743 (1971).
22. J. Barnhart, D. Ritt, A. Ware and B. Combes, in *The Liver. Quantitative Aspects of Structure and Function,* p. 315. Karger, Basel (1973).
23. J. L. Barnhart and B. Combes, *Am. J. Physiol.* **227,** 194 (1974).
24. S. Erlinger and M. Dumont in *The Liver. Quantitative Aspects of Structure and Function,* p. 306. Karger, Basel (1973).
25. G. E. Gibson and E. L. Forker, *Gastroenterology* **66,** 1046 (1974).
26. E. L. Forker and G. Gibson, in *The Liver. Quantitative Aspects of Structure and Function,* p. 326. Karger, Basel (1973).
27. A. H. Conney, *Pharmac. Rev.* **19,** 317 (1967).
28. W. Kunz, G. Schaude, H. Schimasser, W. Schmid and M. Siess, *Proc. Eur. Soc. Study Drug Toxicity* **7,** 138 (1966).
29. T. Barka and H. Popper, *Medicine, Baltimore* **46,** 103 (1967).
30. T. S. Argyris, *J. Pharmac. exp. Ther.* **164,** 405 (1968).
31. W. Stäubli, R. Hess and E. R. Weibel, *J. Cell Biol.* **42,** 92 (1969).
32. C. D. Klaassen, *J. Pharmac. exp. Ther.* **168,** 218 (1969).