Biochemical Pharmacology, Vol. 19, pp. 235–244. Pergamon Press. 1970. Printed in Great Britain

THE ROLE OF THE HEPATIC ENDOPLASMIC RETICULUM IN THE BILIARY EXCRETION OF FOREIGN COMPOUNDS BY THE RAT

THE EFFECT OF PHENOBARBITONE AND SKF 525-A (DIETHYLAMINOETHYL DIPHENYLPROPYLACETATE)

W. G. LEVINE,* P. MILLBURN, R. L. SMITH and R. T. WILLIAMS

Department of Biochemistry, St. Mary's Hospital Medical School, London W.2 U.K.

(*Received* 13 *June* 1969; *accepted* 18 *July* 1969)

Abstract—Phenobarbitone pretreatment of rats stimulated the biliary excretion of biphenyl, stilboestrol and phenolphthalein all of which undergo metabolism prior to excretion in the bile. However, this treatment did not affect the biliary elimination of stilboestrol glucuronide, phenolphthalein glucuronide, succinylsulphathiazole and indocyanine green all of which are excreted unchanged. SKF 525-A which inhibited the glucuronide conjugation of stilboestrol and phenolphthalein depressed their excretion in the bile in the form of their *O*-glucuronides. SKF 525-A did not influence the biliary excretion of phenolphthalein glucuronide.

The biliary excretion of compounds such as biphenyl, stilboestrol and phenolphthalein can be considered to occur in at least two steps: (i) metabolism and (ii) transfer of the metabolites to bile. Phenobarbitone and SKF 525-A treatment in rats influences (i) but not (ii).

This suggests that although the endoplasmic reticulum is involved in the metabolism of foreign compounds it does not appear to play a role in their transfer from liver to bile.

THE MOLECULAR size and polarity of foreign compounds appear to influence the extent to which they are excreted in the bile and it has been suggested that, as far as the rat is concerned, appreciable biliary excretion occurs when the compound has a molecular weight of not less than 300–400 and a strongly polar group (see Abou-El-Makarem *et al.*;[1] Millburn *et al.*[2, 3]). These two properties may occur in the compound itself or may be acquired by a metabolic change. Thus biphenyl, an uncharged molecule of molecular weight 154, appears in the bile mainly as two polar glucuronides of hydroxybiphenyls of molecular weight 346 and 362.[2] The metabolic transformations of foreign compounds occur mainly in the endoplasmic reticulum of the liver and it is possible that this structure may also play a role in the transport of foreign compounds or their metabolites from the liver cells to the bile. In order to investigate this possible role of the endoplasmic reticulum, the effect of phenobarbitone and SKF 525-A on the biliary excretion of several other foreign compounds, was studied. These two compounds are known to influence the properties of the endoplasmic reticulum.[4, 5]

To distinguish compounds which are excreted in the bile unchanged from those which are excreted as metabolites, the term "non-metabolized compound" is used

* Present address: Department of Pharmacology, Albert Einstein College of Medicine, Yeshiva University, Bronx N.Y. 10461.

for the former and "metabolized compound" for the latter. Some of the results described in this paper have been briefly reported.[6]

MATERIALS AND METHODS

Chemicals. Biphenyl, m.p. 70°, phenolphthalein, m.p. 258°–260°; sodium phenobarbitone (British Drug Houses Ltd.), sodium phenolphthalein glucuronide, stilboestrol, m.p. 168° (Koch–Light Laboratories Ltd.), succinylsulphathiazole, m.p. 186°–188° (May & Baker Ltd.), indocyanine green (Hynson, Westcott & Dunning Inc.) and [monoethyl-1-^{14}C] stilboestrol (104 μc/mg) (The Radiochemical Centre, Amersham, Bucks.) were purchased. The melting points quoted are uncorrected.

[Monoethyl-1-^{14}C] Stilboestrol monoglucuronide (0·2 μc/mg) was prepared biosynthetically.[7] SKF 525-A hydrochloride was kindly supplied by Smith, Kline & French Laboratories Ltd.

Treatment of animals. Female Wistar albino rats (200 $\pm$ 25 g body weight) with free access to food and water were injected intraperitoneally with an aqueous solution of phenobarbitone Na (70 mg/kg) once daily for 4 days; control animals were injected with water (5 ml/kg). Biliary fistulae were established as previously described[1] 24 hr after the last phenobarbitone injection. The phenobarbitone treatment was shown to induce the drug-metabolizing enzymes of the endoplasmic reticulum. Thus, microsomes isolated by differential centrifugation from liver homogenates of treated rats when incubated under suitable conditions, demethylated aminopyrine, as measured by formaldehyde production,[8] four times more rapidly than microsomes from controls.

SKF 525-A (75 mg/kg) was injected intraperitoneally into biliary cannulated rats 30 min before injection of the test substance. Rogers & Fouts[5] have shown that maximal suppression of microsomal drug-metabolizing activity by SKF 525-A is seen at this time. Bile samples were collected at 10, 15 or 20 min intervals for 1–3 hr.

All test compounds were injected i.p. as solutions except indocyanine green which was given i.v. Phenolphthalein and its glucuronide, succinylsulphathiazole and indocyanine green were dissolved in water as their sodium salts, and biphenyl, stilboestrol and stilboestrol monoglucuronide in propane-1, 2-diol. In some experiments, both renal pedicles were ligated to prevent urinary excretion. At the end of the experiment, the rats were killed and their livers removed and weighed and in some cases blood samples were taken prior to sacrifice.

Analytical methods. The determination in bile of several of the compounds studied in this paper has been described.[2, 3] For the determination of indocyanine green, bile samples (0·1–0·3 ml) containing human plasma (0·1 ml) to stabilize the dye, were suitably diluted with 0·9% NaCl and the absorption read at 795 mμ in a Unicam SP.600 spectrophotometer was compared with that of dye standards (0·5–3·5 μg/ml) containing 0·1 ml plasma and 0·1 ml normal rat bile per 10 ml of diluted dye. Bile samples containing indocyanine green were chromatographed on Whatman No. 1 paper using the descending technique with the following solvent systems (all proportions by volume): A, butan-1-ol-acetic acid–water (4:1:2), B, butan-1-ol-aq.NH_3 (sp.gr. 0·88)–water (10:1:1), C, propan-1-ol-aq.NH_3 (sp.gr. 0·88) (7:3). The R_f values of indocyanine green in these solvents were: A, 0·55; B, 0·11; C, 0·58 and 0·75, the dye being detected on chromatograms as a green spot. In solvent C, two spots were observed; the one of R_f 0·58 being green and the one of R_f 0·75 being brown. Bile

from rats treated with indocyanine green, when chromatographed in solvents A, B and C, was found to contain the unchanged dye. It has been reported that indocyanine green is not metabolized.[9] The distribution of succinylsulphathiazole between plasma, liver and bile was determined by methods previously described.[10]

Standard deviations were calculated and the significance of any difference between the results found for control and test animals was examined by Student's *t*-test.

RESULTS

1. *Effect of phenobarbitone*

(a) *Non-metabolized compounds.* Figure 1 shows the extent of biliary excretion of four non-metabolized compounds, namely, succinylsulphathiazole, indocyanine green, stilboestrol monoglucuronide and phenolphthalein glucuronide by control and

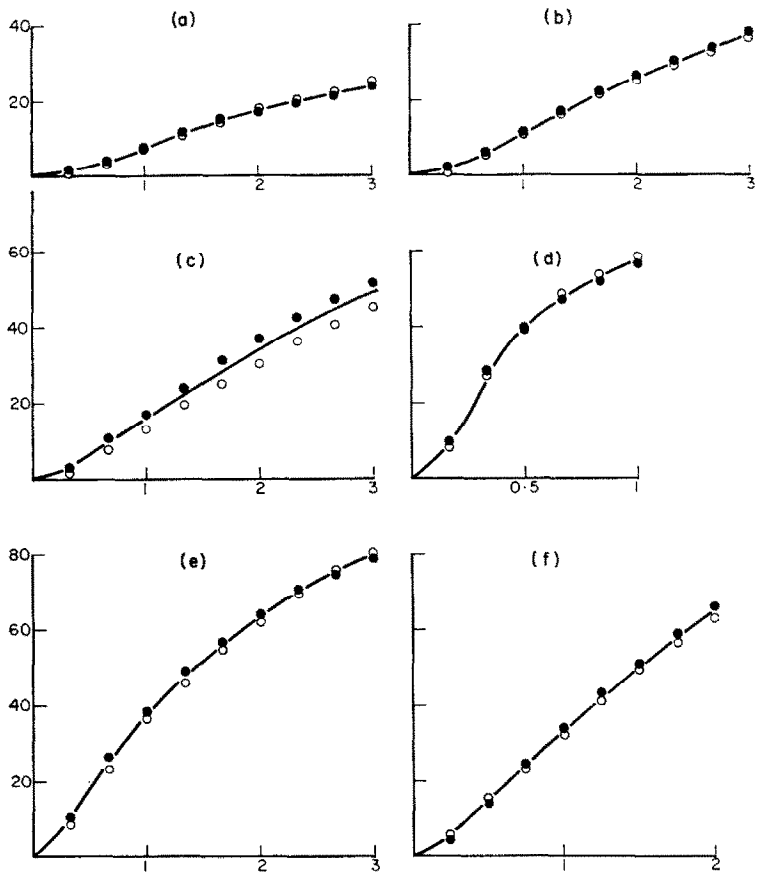

FIG. 1. The biliary excretion of four non-metabolized compounds by phenobarbitone treated rats. Ordinate = % dose in bile; abscissa = time after injection (hr).

The compounds were administered to control and phenobarbitone treated rats and bile collected as described in the text. Mean values for five or more animals are given. In experiments (b) and (c) the renal pedicles were ligated. (a) and (b) succinylsulphathiazole (20 mg/kg i.p.); (c) succinylsulphathiazole (200 mg/kg i.p.); (d) indocyanine green (2·5 mg/kg i.v.); (e) stilboestrol monoglucuronide (18 mg/kg i.p.) and (f) phenolphthalein glucuronide (81·5 mg/kg i.p.).

○ control; ● phenobarbitone treated.

phenobarbitone-treated rats. There is no significant difference in either the total amounts or the rate of excretion of these four compounds in the bile in the two groups of rats.

The biliary excretion of succinylsulphathiazole is a process which exhibits saturation at a dose level of 100 mg/kg in renal ligated rats.[10] Below (20 mg/kg) or above (200 mg/kg) this saturation level, phenobarbitone treatment appears to have no significant effect on its biliary excretion (Fig. 1b, c). As shown previously, succinylsulphathiazole is taken up rapidly by the liver and concentrated in the bile to give bile to plasma ratios of over 10:1.[10] Table 1 shows that phenobarbitone does not significantly alter

TABLE 1. THE DISTRIBUTION OF SUCCINYLSULPHATHIAZOLE BETWEEN PLASMA, LIVER AND BILE IN PHENOBARBITONE-TREATED RATS

	Concentration of succinylsulphathiazole (μg/g or μg/ml) in:		
	plasma	liver	bile
Control	340 $\pm$ 41 (7)	561 $\pm$ 99 (4)	8231 $\pm$ 3052 (7)
Phenobarbitone treated	296 $\pm$ 78 (4)	449 $\pm$ 65 (4)	6938 $\pm$ 955 (4)

Succinylsulphathiazole (200 mg./kg.i.p.) was administered as described in the text; renal pedicles were ligated. After 30 min the concentration of the drug in plasma, liver and bile was determined. Mean values $\pm$ S.D. are given, with numbers of animals in parentheses.

the distribution of succinylsulphathiazole between the plasma, liver and bile of the rat, suggesting that it does not affect the mechanism responsible for the transfer of succinylsulphathiazole from plasma to bile.

Both the protein content and weight of the liver of rats is increased by phenobarbitone treatment.[11] In our experiments, the average liver/body weight ratio for forty-one control rats was 0·030 $\pm$ 0·004, while the ratio for forty-one treated rats was 0·038 $\pm$ 0·005, an increase of 27 per cent. If the rates of excretion of the four non-metabolized compounds are calculated in terms of liver weight (i.e. mg/min/g liver), there appears to be a 10–20 per cent depression of their biliary excretion in the treated animals compared to the controls.

Although in phenobarbitone-treated rats the bile flow was increased (Fig. 2), the rate at which non-metabolized compounds were excreted was unchanged (see Fig. 1).

(b) *Metabolized compounds.* About 10 per cent of an injected dose of biphenyl appears in rat bile in 24 hr as the glucuronic acid conjugates of the 4-hydroxy and the 4,4′-dihydroxy derivatives.[2] But when one of these conjugates, namely 4-glucuronosidobiphenyl, is injected, 59 per cent of the dose is excreted in the bile in 24 hr.[2] This suggests that the biliary excretion of biphenyl is dependent on its microsomal hydroxylation and conjugation. The 24 hr biliary excretion of biphenyl was therefore measured in phenobarbitone-treated and control rats. Treatment with phenobarbitone enhances the biliary excretion of this compound by 90 per cent (Table 2). Furthermore, this increase is attributable mainly to greater amounts of 4-glucuronosidobiphenyl in the bile. This suggests that the first hydroxylation is the rate-limiting step in the biliary excretion of biphenyl.

Stilboestrol is excreted in the bile largely as its monoglucuronide.[2] Figure 3 shows that phenobarbitone treatment markedly accelerates its biliary excretion and this effect is particularly evident during the early part of the collection period. Phenolphthalein is a drug which is rapidly excreted in the bile, almost entirely as its glucuronide.[2] However, its biliary excretion is only marginally enhanced by treatment with phenobarbitone (Table 3).

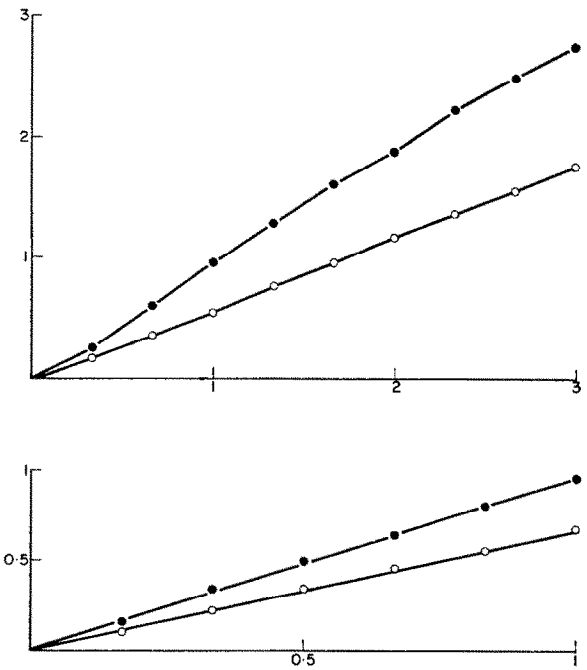

FIG. 2. The effect of phenobarbitone treatment on bile flow in the rat.
Ordinate = bile volume (ml); abscissa = time after injection (hr).
(a) succinylsulphathiazole (20 mg/kg i.p.) or (b) indocyanine green (2·5 mg/kg i.v.) was administered to control and phenobarbitone treated rats and bile collected as described in the text. Average values are given for five or more animals.
○ control; ● phenobarbitone treated.

TABLE 2. THE EFFECT OF PHENOBARBITONE TREATMENT ON THE BILIARY EXCRETION OF BIPHENYL BY THE RAT

	% of dose in bile in 24 hr	Metabolites found in bile (% of dose)
Control	10 ± 3·1	4-Glucuronosidobiphenyl (7) 4-Glucuronosido-4′-hydroxybiphenyl (3)
Phenobarbitone treated	19 ± 3·9	4-Glucuronosidobiphenyl (16) 4-Glucuronosido-4′-hydroxybiphenyl (3)

The methods of phenobarbitone treatment, bile collection and analysis are described in the text. The dose of biphenyl was 100 mg/kg.i.p. Mean values ± S.D. are given for five control and six phenobarbitone treated animals.

2. *Effect of SKF 525-A*

In preliminary experiments, it was found that SKF 525-A (10–100 mg/kg i.p.) depressed the flow of bile in rats by about 25 per cent compared to controls, although considerable variation was encountered in individual experiments. A dose level of 75 mg/kg of SKF 525-A was used, as this had the least effect on bile flow and markedly depressed the microsomal enzyme activity.

(a) *Non-metabolized compounds.* The effect of SKF 525-A on the biliary excretion of succinylsulphathiazole and phenolphthalein glucuronide is illustrated in Fig. 4a and b.

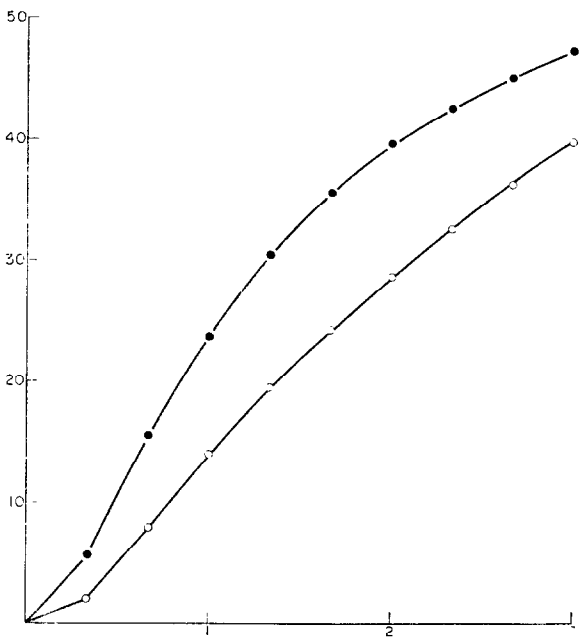

FIG. 3. The effect of phenobarbitone on the biliary excretion of [^{14}C] stilboestrol.
Ordinate = % dose in bile; abscissa = time after injection (hr).
[^{14}C] stilboestrol (10 mg/kg i.p.) was administered to ten control and six phenobarbitone-treated rats and bile collected as described in the text. Mean values are given.
○ control; ● phenobarbitone treated.

TABLE 3. THE EFFECT OF PHENOBARBITONE TREATMENT ON THE BILIARY EXCRETION OF PHENOLPHTHALEIN BY THE RAT

Time after injection (min)	% of dose in bile*	
	Control	Phenobarbitone treated
15	7·4 ± 2·5	12 ± 3·1†
30	20 ± 6·3	29 ± 10†
60	40 ± 12	49 ± 14
120	64 ± 18	72 ± 22

See text for details of dosing, bile collection and analysis. Mean values ± S.D. are given for ten control and six phenobarbitone-treated rats.
* Bile contained only phenolphthaleinglucuronide.
† Significantly different from the control group ($P < 0·05$).

The rate of excretion of both compounds is the same in treated and control animals. However, the biliary excretion of indocyanine green was markedly retarded by SKF 525-A (Fig. 4c). The possible significance of this apparently anomalous result will be discussed later.

(b) *Metabolized compounds.* Figure 5 shows the effect of SKF 525-A on the biliary excretion of stilboestrol and phenolphthalein. The excretion of both drugs was reduced, although for phenolphthalein this is apparent only during the early part of the experiment.

DISCUSSION

The site of metabolism of many drugs appears to be the endoplasmic reticulum of the liver. Phenobarbitone treatment causes a marked proliferation of this reticulum, together with an induction of its associated drug-metabolizing enzymes.[4] SKF 525-A has a depressant effect upon many of these enzymes; it is rapidly taken up by the liver and tightly bound to the endoplasmic membranes.[5] The influence of these two substances on the biliary excretion of two types of compound was investigated. The first type consisted of the "non-metabolized compounds" succinylsulphathiazole, stilboestrol monoglucuronide, phenolphthalein glucuronide and indocyanine green,

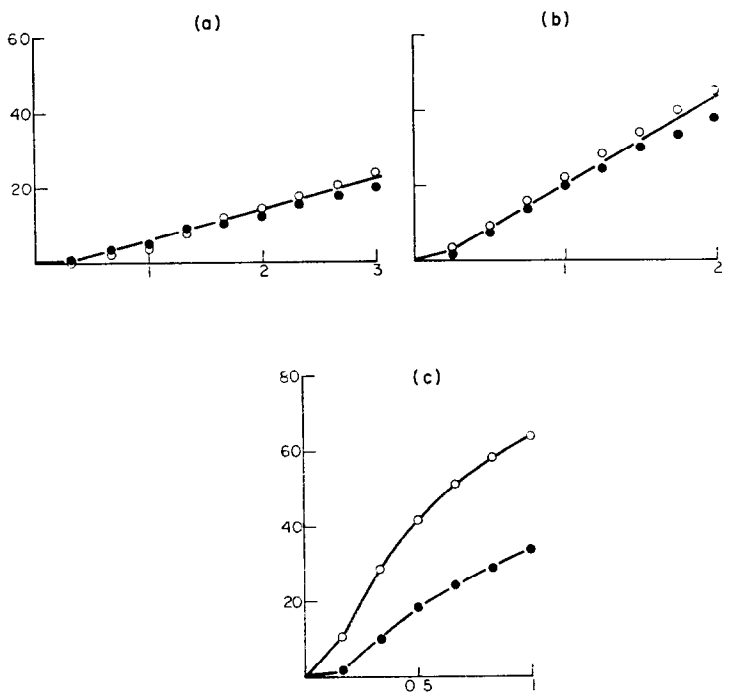

FIG. 4. The biliary excretion of three non-metabolized compounds by SKF 525-A-treated rats.
Ordinate = % dose in bile; abscissa = time after injection (hr).
The compounds were administered to control and SKF 525-A-treated rats and bile collected as described in the text. Mean values for at least five animals are given.
(a) succinylsulphathiazole (20 mg/kg i.p.); (b) phenolphthalein glucuronide (81·5 mg/kg i.p.) and (c) indocyanine green (2·5 mg/kg i.v.).
○ control; ● SKF 525-A treated.

which allowed the study of the effect of phenobarbitone and SKF 525-A treatment on the biliary secretion process without the complication of metabolism. The second type consisted of the "metabolized compounds" stilboestrol, phenolphthalein and biphenyl. Both stilboestrol and phenolphthalein are conjugated with glucuronic acid before appearing in bile, whereas biphenyl undergoes both aromatic hydroxylation and glucuronic acid conjugation before excretion, thus allowing the study of the significance of metabolism in biliary excretion.

(a) *Non-metabolized compounds.* The biliary excretion of succinylsulphathiazole, stilboestrol monoglucuronide, phenolphthalein glucuronide and indocyanine green

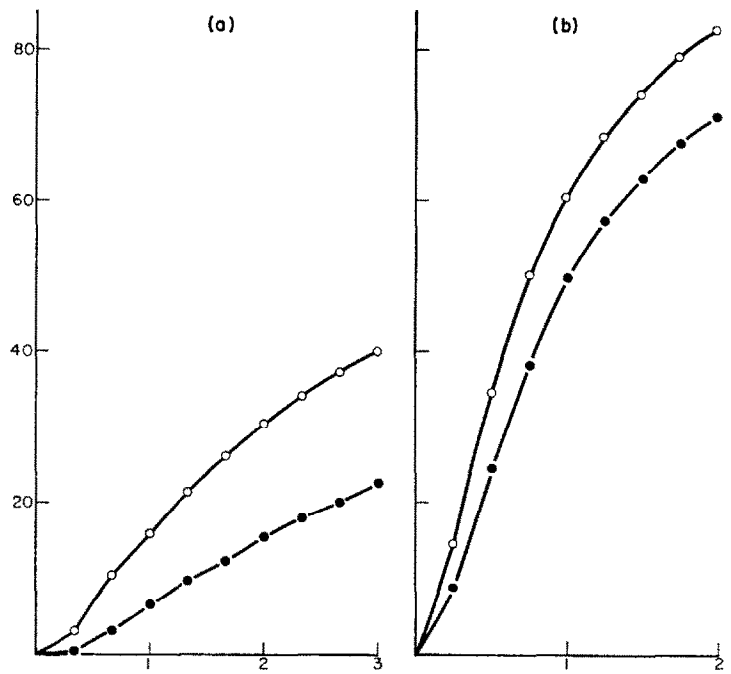

FIG. 5. The effect of SKF 525-A on the biliary excretion of [^{14}C] stilboestrol and phenolphthalein. Ordinate = % dose in bile; abscissa = time after injection (hr).
(a) [^{14}C] stilboestrol (10 mg/kg i.p.) or (b) phenolphthalein (50 mg/kg i.p.) was administered to control and SKF 525-A-treated rats and bile collected as described in the text. Mean values for at least five animals are given.
○ control; ● SKF 525-A treated.

was not affected by phenobarbitone treatment even though this increased both liver weight and bile flow by 27 and 45 per cent respectively. Since phenobarbitone stimulates the formation of new endoplasmic reticulum, our findings suggest that the new membranes are not associated with the transport of the non-metabolized compounds from liver to bile. It cannot be concluded, however, that the original endoplasmic reticulum is not involved in biliary excretion since it appears that the newly induced membranes may differ in character from the original reticulum. This is because phenobarbitone treatment induces some but not all microsomal enzymes.[12] However, the findings with SKF 525-A suggested that the endoplasmic reticulum is not involved

in the transfer of substances from liver to bile, as it did not suppress the biliary excretion of succinylsulphathiazole and phenolphthalein glucuronide.

An apparent discrepancy is the finding that the rate of biliary excretion of indocyanine green, a non-metabolized compound, is depressed by SKF 525-A. This agrees with a similar observation by Hargreaves.[13] It suggests that the excretory mechanism for this substance may differ from that of the other non-metabolized compounds. Indocyanine green differs chemically from succinylsulphathiazole and phenolphthalein glucuronide in that it contains in its structure a positively charged quaternary ammonium group, besides two polar sulphonic acid functions. The liver appears to have independent mechanisms for the excretion of organic cations and anions.[14] It is possible therefore that indocyanine green is excreted by both pathways, whereas succinylsulphathiazole and phenolphthalein glucuronide are excreted by the anionic route. SKF 525-A does not affect the biliary excretion of these anions, but it may influence the cationic pathway and this could explain the apparent discrepancy in our findings. Further work is needed to elucidate the pathway(s) by which indocyanine green is excreted in bile. The relative lack of specificity in the biological effects of SKF 525-A has recently become apparent,[15, 16] and caution is needed in the interpretation of results when using this compound.

(b) *Metabolized compounds.* Phenobarbitone treatment enhances the biliary excretion of stilboestrol and phenolphthalein by rats. Both compounds are converted to glucuronides prior to their appearance in bile.[2] Phenobarbitone treatment is known to enhance hepatic glucuronide synthesis.[17] Since it has been shown above that phenobarbitone does not stimulate the transport into bile of administered stilboestrol monoglucuronide and phenolphthalein glucuronide, the accelerated rate of biliary excretion of their aglycones could be due to an enhanced rate of formation of their glucuronides. The situation is similar in the case of biphenyl, which, before it can be conjugated, has to be converted to its 4-hydroxy and 4,4′-dihydroxy derivatives. Aromatic hydroxylation reactions are also induced by phenobarbitone.[18] The increased biliary excretion of biphenyl after phenobarbitone treatment could be due to an increase in the rate of formation of its hydroxylated derivatives.

That the biliary excretion of some compounds depends on their metabolism is supported by the findings with SKF 525-A, which reduces the biliary excretion of stilboestrol and phenolphthalein. SKF 525-A is known to inhibit glucuronide synthesis[19] and its ability to depress the biliary excretion of stilboestrol and phenolphthalein may be due to reduced formation of their glucuronides.

The biliary excretion of "metabolized compounds" can be regarded as occurring in at least two steps: (i) metabolism and (ii) transfer of the metabolites from liver to bile. Phenobarbitone and SKF 525-A influence (i) but not (ii). This suggests that although the endoplasmic reticulum is involved in the metabolism of foreign compounds, it does not appear to play a role in their transfer from liver to bile.

Other studies have indicated the importance of metabolism in biliary excretion. Thus, Goldstein and Taurog[20] found that the biliary excretion of thyroxine as its glucuronide was increased by treatment with the inducing agent 3,4-benzopyrene. This increase was attributed to an enhanced activity of glucuronyl transferase. Similarly, Yaffe *et al.*[21] found that phenobarbitone treatment decreased serum bilirubin in an infant with congenital non-haemolytic jaundice and this was ascribed to increased bilirubin glucuronide formation.

It is of interest to speculate on the possible mechanisms by which phenobarbitone and SKF 525-A enhance and depress respectively bile flow. It is believed that the excretion of bile is intimately associated with the transport of bile salts from the hepatocytes into the bile canaliculi.[22] Before excretion, bile salts are conjugated by mechanisms which may be similar to those involved in the conjugation of foreign compounds. Thus, phenobarbitone and SKF 525-A could affect the conjugation of bile salts and this would determine the quantity of bile salts available for excretion and, therefore, influence the bile flow rate.

Acknowledgement—W. G. L. is grateful to the United States Public Health Service for a Research Career Development Award.

REFERENCES

1. M. M. ABOU-EL-MAKAREM, P. MILLBURN, R. L. SMITH and R. T. WILLIAMS, *Biochem. J.* **105**, 1269 (1967).
2. P. MILLBURN, R. L. SMITH and R. T. WILLIAMS, *Biochem. J.* **105**, 1275 (1967).
3. P. MILLBURN, R. L. SMITH and R. T. WILLIAMS, *Biochem. J.* **105**, 1283 (1967).
4. A. H. CONNEY, *Pharmac. Rev.* **19**, 317 (1967).
5. L. A. ROGERS and J. R. FOUTS, *J. Pharmac. exp. Ther.* **146**, 286 (1964).
6. W. G. LEVINE, P. MILLBURN, R. L. SMITH and R. T. WILLIAMS, *Biochem. J.* **109**, 35 (1968).
7. L. J. FISCHER, P. MILLBURN, R. L. SMITH and R. T. WILLIAMS, *Biochem. J.* **100**, 69 (1966).
8. J. COCHIN and J. AXELROD, *J. Pharmac. exp. Ther.* **125**, 105 (1959).
9. G. R. CHERRICK, S. W. STEIN, C. M. LEEVY and C. S. DAVIDSON, *J. clin. Invest.* **39**, 592 (1960).
10. M. M. ABOU-EL-MAKAREM, P. MILLBURN and R. L. SMITH, *Biochem. J.* **105**, 1295 (1967).
11. A. H. CONNEY, C. DAVISON, R. GASTEL and J. J. BURNS, *J. Pharmac. exp. Ther.* **130**, 1 (1960).
12. S. ORRENIUS, J. L. E. ERICSSON and L. ERNSTER, *J. cell Biol.* **25**, 627 (1965).
13. T. HARGREAVES, *Biochem. Pharmac.* **16**, 1481 (1967).
14. L. S. SCHANKER and H. M. SOLOMON, *Am. J. Physiol.* **204**, 829 (1963).
15. R. D. MAGUS and J. R. FOUTS, *Biochem. Pharmac.* **16**, 1323 (1967).
16. F. N. MARSHALL and H. E. WILLIAMSON, *J. Pharmac. exp. Ther.* **143**, 395 (1964).
17. P. ZEIDENBERG, S. ORRENIUS and L. ERNSTER, *J. cell Biol.* **32**, 528 (1967).
18. P. J. CREAVEN and D. V. PARKE, *Biochem. Pharmac.* **15**, 7 (1966).
19. J. R. COOPER, J. AXELROD and B. B. BRODIE, *J. Pharmac. exp. Ther.* **112**, 55 (1954).
20. J. A. GOLDSTEIN and A. TAUROG, *Biochem. Pharmac.* **17**, 1049 (1968).
21. S. J. YAFFE, G. LEVY, T. MATSUZAWA and T. BALIAH, *New Engl. J. Med.* **275**, 1461 (1966).
22. I. SPERBER, *Pharmac. Rev.* **11**, 109 (1959).