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EFFECT OF PHENOBARBITAL AND 3-METHYLCHOLANTHRENE PRETREATMENT ON GUINEA PIG HEPATIC MICROSOMAL BILIRUBIN GLUCURONYLTRANSFERASE ACTIVITY*

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Abstract—The effect of multiple doses of phenobarbital or of a single dose of 3-methylcholanthrene on bilirubin glucuronyltransferase (EC 2.4.1.17) activity was studied with liver microsomal preparations from guinea pigs. A significant increase in enzyme activity of the liver occurred after 10 days of phenobarbital administration, but enzyme activity per milligram of protein was unchanged. In contrast, a single dose of 3-methylcholanthrene produced an increase in specific activity of the enzyme. Untreated mice, rats, rabbits and cats were also studied and significant differences were found for the apparent K_m and V_{max} values of the various species.

PHENOBARBITAL is one of many drugs that stimulate varied pathways of metabolism by liver microsomes, whereas polycyclic aromatic hydrocarbons such as 3-methylcholanthrene stimulate a more limited number of reactions.[1] Studies in several laboratories have shown that total serum bilirubin is decreased in humans after the administration of phenobarbital.[2–7] Such indirect evidence has been used to support the hypothesis that phenobarbital enhances bilirubin glucuronyltransferase (UDP glucuronate glucuronyltransferase, EC 2.4.1.17) activity in the liver.

The investigations reported here were undertaken to evaluate the role of phenobarbital and 3-methylcholanthrene as stimulators of glucuronyltransferase activity in liver microsomes using bilirubin as substrate. Although the guinea pig microsomal system was studied in more detail, comparisons of enzymatic activity in untreated mice, rats, cats and rabbits were also obtained.

METHODS AND MATERIALS

Animals and drug pretreatment. Adult male rats (Long Evans, 200–400 g), mice (Swiss Webster, 20–40 g), Dutch rabbits (1·0–1·7 kg), guinea pigs (250–500 g) and cats (1·5–3·5 kg) were allowed access to food and water *ad lib.* Animals were maintained in local animal facilities for 3–5 days prior to use. The guinea pigs were divided into groups of equivalent weights and distribution of weight for the drug studies. One group received daily intraperitoneal injections of sodium phenobarbital (80 mg/kg) while the control group received 0·9% saline solution (5 ml/kg/day). The intraperitoneal route was also used for 3-methylcholanthrene (Sigma Chemical Company; 40 mg/kg) and control guinea pigs received a similar intraperitoneal injection of the corn oil vehicle (5 ml/kg).

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Enzyme preparation. The animals were sacrificed by cervical fracture 24 hr after the last injection of phenobarbital or 48 hr after the single dose of 3-methylcholanthrene. Washed microsomes were prepared as previously described.[8] The pellet was diluted with isotonic alkaline KCl to give a suspension of microsomes equivalent to 1 g liver/ml. Microsomal protein concentrations were determined by the method of Lowry *et al.*,[9] with bovine serum albumin (Sigma) serving as the standard.

Enzyme assay. The total incubation volume was 1·22 ml. Expressed as concentration per milliliter of incubate, samples from both treated and untreated animals contained 2·4 mg microsomal protein, 50 μmoles Tris buffer (pH 8·0 at 25°) and bilirubin. The bilirubin (Calbiochem) was prepared by rapidly dissolving 9·5 mg in 2·5 ml of 0·2 N NaOH and diluting with 7·13 ml of distilled water. A volume of 0·2 ml of this bilirubin solution yielded a concentration of 160 μg or 275 mμmoles/ml of incubate. Variable concentrations of uridine-5′-diphosphoglucuronic acid (UDPGA, ammonium salt; Sigma Chemical Company) were added as described in the Results section. An equivalent amount of water was added when UDPGA was deleted. It should be noted that the interspecies comparisons were performed with a total incubate content of washed microsomes from 0·2 g liver without reference to protein content.

Incubations were performed for 30 min in a Dubnoff metabolic shaking incubator at 37°. The unconjugated and conjugated bilirubin was determined immediately after incubation according to the method of Weber and Schalm,[10] with the following modifications: (1) samples were centrifuged at 1700 *g* for 20 min and (2) unconjugated bilirubin was determined using the concentrated diazo reagent. Samples were read at 555 nm in a Spectronic 20 colorimeter. The amount of enzymatic conjugation was determined by the difference between the matched samples with and without UDPGA.

Statistics. Data from the drug treatment studies were analyzed by the Student group *t*-test except where F ratios were significant in testing for the equality of variance. A two-sample rank test was used in the latter case. Both procedures are described by Goldstein.[11] The acceptable significance level was $P < 0{\cdot}05$ and one-tail tables were used, since an increase in conjugation of bilirubin was expected with both phenobarbital and 3-methylcholanthrene.

Kinetic constants for the various species were determined by procedures described by Wilkinson[12] and programmed by Cleland[13] to conform to the equation $v = VA/(K + A)$. Statistical variations were evaluated by a completely random design analysis of variance, and significant differences ($P < 0{\cdot}05$) between means were determined by Duncan's new multiple range test.[14]

RESULTS

Parameters of the conjugating system. The amount of bilirubin conjugated was found to be linear with respect to time during a 30-min incubation period for the guinea pig, rat, mouse, rabbit and cat. In addition, there was a linear relationship between the amount of bilirubin conjugated and the concentration of guinea pig microsomal protein (0–2·4 mg/ml) when bovine serum albumin was used to maintain a constant protein concentration. Although it was not pursued in detail, initial pilot studies had indicated that the use of less than 2 mg/ml of protein could result in aberrant data. It was not established whether this influence occurred primarily during incubation, in the separation process, or both. No such effects were noted when total protein concentration was maintained at 2·4 mg/ml. The apparent K_m for UDPGA was 2·7 $\pm$ 1·3 μmoles/ml of

incubate and the V_{max} for the variable UDPGA was 4·5 ± 0·7 mμmoles of bilirubin conjugated per mg protein per 0·5 hr of incubation, when the bilirubin concentration was 275 mμmoles/ml of incubate. Ten μmoles UDPGA/ml of incubate has been the usual concentration used throughout these studies. The apparent K_m for bilirubin was 25 ± 9 mμmoles/ml of incubate and the V_{max} was 4·8 ± 0·3 mμmoles bilirubin conjugated per mg of protein per 0·5 hr of incubation at this concentration of UDPGA. The usual bilirubin concentration used throughout these studies was 275 mμmoles/ml of incubate.

Phenobarbital pretreatment. A significant increase in guinea pig total liver bilirubin conjugating capacity was demonstrated after 10 days of phenobarbital pretreatment (Table 1). Although animals were initially matched for body weight as closely as

TABLE 1. BILIRUBIN GLUCURONYLTRANSFERASE ACTIVITY IN GUINEA PIG HEPATIC MICROSOMAL FRACTIONS AFTER PRETREATMENT WITH PHENOBARBITAL

Days of pretreatment	Bilirubin conjugated*		Phenobarbital/ Saline
	Saline	Phenobarbital	
2	0·98 ± 0·11	1·05 ± 0·18	1·07
4	0·67 ± 0·06	0·76 ± 0·06	1·15
6	0·89 ± 0·07	1·11 ± 0·12	1·24
8	0·90 ± 0·13	1·13 ± 0·09	1·25
10	0·81 ± 0·09	1·04 ± 0·06	1·29†

* Data are shown as the mean ± S.E. and are expressed as μmoles per total liver per 0·5 hr incubation with eight to ten animals per mean for both treated and control groups.

† Phenobarbital-pretreated (80 mg/kg/day) are significantly different from saline-pretreated animals at $P < 0·05$ by the one-tail Student's *t*-test. No significant differences occurred between treated and control liver weights at the stated intervals.

possible, the experimental variability is still quite evident. Even though the daily variation in total activity was large, a comparison of groups at each respective time interval shows a progressively increasing ratio of enzyme activity in phenobarbital-pretreated vs. saline-pretreated animals. There were no significant differences between treated and control groups in terms of bilirubin conjugated per milligram of microsomal portein. Protein content in milligrams per gram of liver for saline-treated animals averaged 19·2 ± 0·3 (mean ± S.E.) over the five time intervals tested as compared to 20·7 ± 1·2 after 2 days and 25·6 ± 0·7 after 10 days of phenobarbital treatment.

The effects of phenobarbital on other hepatic microsomal drug-metabolizing systems in the guinea pig have not been studied in detail. The potential effect of phenobarbital treatment on the metabolism of aniline, benzphetamine and aminopyrine by guinea pig microsomes was therefore investigated using methods described by Gram *et al.*[15] for the rat and rabbit. These results (Table 2) indicate that all three pathways were stimulated in guinea pig liver microsomes after phenobarbital treatment.

3-methylcholanthrene pretreatment. Experiments performed 48 hr after a single dose of 3-methylcholanthrene showed an increase in enzyme activity in terms of bilirubin conjugated per milligram of microsomal protein. This effect was studied at several

TABLE 2. EFFECT OF PHENOBARBITAL PRETREATMENT* ON GUINEA PIG HEPATIC DRUG-METABOLIZING ENZYME ACTIVITIES*

Substrate	Activity (mμmoles/mg microsomal protein/0·5 hr) Control		Phenobarbital	
Aniline	42 ± 4	(3)	125 ± 77†	(3)
Benzphetamine	285 ± 70	(2)	880 ± 104†	(3)
Aminopyrine	460 ± 5	(2)	1080 ± 137†	(3)

* Guinea pigs were treated with phenobarbital (80 mg/kg/day, i.p.) for 3 days. Data are expressed as mean ± S.E. with number of animals in parentheses.
† Means are significantly different from control by Student's *t*-test at $P < 0.05$.

TABLE 3. BILIRUBIN GLUCURONYLTRANSFERASE ACTIVITY IN GUINEA PIG HEPATIC MICROSOMAL FRACTIONS AFTER PRETREATMENT* WITH 3-METHYLCHOLANTHRENE FOR VARIABLE BILIRUBIN CONCENTRATIONS*

Bilirubin concn (mμmoles/ml incubate)	Bilirubin conjugation	
	Control	3-MC
17	1·66 ± 0·10	2·20 ± 0·11†
34	2·25 ± 0·15	3·85 ± 0·21†
68	2·55 ± 0·14	4·50 ± 0·27†
138	2·92 ± 0·22	5·67 ± 0·27†
275	3·79 ± 0·32	7·85 ± 0·49†

* Data are shown as the mean ± S.E. and are expressed as mμmoles of bilirubin conjugated per mg of microsomal protein per 0·5 hr incubation. Nine corn oil control and nine 3-MC-(40 mg/kg) treated animals were used 48 hr after intraperitoneal injection, with each animal studied at each substrate concentration.
† Means are significantly different from control by Student's *t*-test at $P < 0.05$.

TABLE 4. BILIRUBIN GLUCURONYLTRANSFERASE ACTIVITY IN GUINEA PIG HEPATIC MICROSOMAL FRACTIONS AFTER PRETREATMENT* WITH 3-METHYLCHOLANTHRENE FOR VARIABLE UDPGA CONCENTRATIONS*

UDPGA concn (μmoles/ml incubate)	Bilirubin conjugation	
	Control	3-MC
0·62	1·21 ± 0·14	2·70 ± 0·24†
1·25	1·72 ± 0·13	3·56 ± 0·25†
2·5	2·09 ± 0·15	4·56 ± 0·35†
5·0	2·77 ± 0·31	6·29 ± 0·51†
10·0	3·96 ± 0·34	8·15 ± 0·52†

* Data are shown as the mean ± S.E. and are expressed as mμmoles of bilirubin conjugated per mg of microsomal protein per 0·5 hr incubation. Eight control and eight treated animals were used, with each animal studied at each substrate concentration.
† Means are significantly different from control by Student's *t*-test at $P < 0.05$.

different concentrations of bilirubin, and in each case the preparations from 3-methylcholanthrene-pretreated animals showed significantly greater activity as compared to control (Table 3). Similar increases in conjugating activity were found when the bilirubin concentration was maintained at 275 mμmoles/ml of incubate and the concentration of UDPGA was varied (Table 4). The 3-methylcholanthrene pretreatment also increased the liver/body weight ratio (3·7 ± 0·1–4·0 ± 0·1) and the protein content of the washed microsome preparations (22·1 ± 0·6–24·3 ± 0·5).

TABLE 5. BILIRUBIN KINETICS

Species	No. of animals	Apparent K_m (mean ± S.E.)*	Apparent V_{max} (mean ± S.E.)†
Rat	5	77 ± 22	10·7 ± 1·2
Rabbit	4	35 ± 11	7·3 ± 0·6
Guinea pig	4	25 ± 9	4·8 ± 0·3
Mouse	4	37 ± 9	4·2 ± 0·3
Cat	5	23 ± 20	1·6 ± 0·3

* Apparent K_m is expressed as mμmoles/ml incubate. Rat is significantly different from all other species at $P < 0·05$; no other significant comparisons. UDPGA concentration used was 10 μmoles/ml of incubate.

† Apparent V_{max} is expressed as mμmoles/mg microsomal protein/0·5 hr incubation. All species are significantly different from each other at $P < 0·05$, except between the guinea pig and mouse.

Interspecies comparisons. A compilation of the bilirubin conjugating capacity of microsomal preparations from various species is presented in Table 5. These were performed with washed microsomes from a total of 0·2 g of liver with a UDPGA concentration of 10 μmoles/ml of incubate. The apparent K_m and V_{max} values were highest for the rat among the five species tested.

DISCUSSION

Several recent studies have implied that phenobarbital may enhance the hepatic capacity for bilirubin conjugation as a result, in part, of the induction of bilirubin glucuronyltransferase.[2–7] "These findings suggest that the decrease in bilirubin concentration may be the result of drug-mediated enhancement of pigment conjugation in the liver" but "a demonstrable increase in the hepatic glucuronyltransferase system, using bilirubin as substrate, would seem to be a minimum requirement for the validation of this concept".[16] This position stems from the indirect nature of many phenobarbital studies and the possibility that more than one glucuronyltransferase may exist, since variations in substrate specificities have been found.[17,18]

Daily injections of phenobarbital produced an increase in guinea pig hepatic glucuronyltransferase activity and an increased protein content of the liver microsomes in the present study. However, the enzyme activity per unit of protein was not significantly different in phenobarbital-treated guinea pigs and saline-treated controls. These results indicate that bilirubin glucuronyltransferase specific activity is not altered by phenobarbital treatment. Other workers using different techniques with

rat liver homogenates[19] or slices[20] have also reported increased bilirubin conjugation after barbiturates. Only one laboratory has reported an increase in activity per milligram of hepatic protein.[21] The last study mentioned was performed with mouse liver homogenates.

Differing results following phenobarbital pretreatment using *p*-nitrophenol as glucuronide acceptor have also been reported. Zeidenberg *et al.*[22] reported increased *p*-nitrophenol conjugation with rat liver microsomes, whereas Gram *et al.*[23] found no increase using *p*-nitrophenol, *o*-aminophenol or phenolphthalein with either rough or smooth-surfaced rabbit liver microsomes. The 3-methylcholanthrene studies (Tables 3 and 4) showed a rapid enhancement of bilirubin glucuronyltransferase activity per unit of protein. Stimulation of other glucuronyltransferase activities by 3-methylcholanthrene have also been shown. Inscoe and Axelrod[24] used *o*-aminophenol with rat liver slices and Howland and Burkhalter[25] used *o*-aminophenol and morphine with rat and guinea pig microsomes.

The potential therapeutic application of 3-methylcholanthrene in hyperbilirubinemic conditions is obviously doubtful due to its carcinogenic properties. However, the demonstration of this effect with bilirubin as substrate may still be therapeutically relevant, since less hazardous agents may also possess this action on bilirubin glucuronyltransferase. Such agents could prove to be much more efficacious than the delayed and less impressive effect of phenobarbital on bilirubin glucuronyltransferase activity.

Although different strains were used, the apparent K_m values in the species comparison experiments (Table 5) are consistent with those reported for the rat[26,27] and rabbit.[28] It should also be noted that the cat has a very limited ability to form bilirubin glucuronide. This observation is consistent with other reports on glucuronide formation by the cat using other substrates.[29,30]

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REFERENCES

1. A. H. CONNEY, *Pharmac. Rev.* **19**, 317 (1967).
2. S. J. YAFFE, G. LEVY, T. MATSUZAWA and T. BALIAH, *New Engl. J. Med.* **275**, 1461 (1966).
3. M. J. WHELTON, L. P. KRUSTEV and B. H. BILLING, *Am. J. Med.* **45**, 160 (1968).
4. M. J. KREEK and M. H. SLEISENGER, *Lancet* **2**, 73 (1968).
5. H. M. MAURER, J. A. WOLFF, M. FINSTER, P. J. POPPERS, E. PANTUCK, R. KUNTZMAN and A. H. CONNEY, *Lancet* **2**, 122 (1968).
6. D. TROLLE, *Lancet* **2**, 705 (1968).
7. R. P. H. THOMPSON, A. L. W. F. EDDLESTON and R. WILLIAMS, *Lancet* **1**, 21 (1969).
8. G. L. LAGE and J. L. SPRATT, *Archs Biochem. Biophys.* **126**, 175 (1968).
9. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
10. A. PH. WEBER and L. SCHALM, *Clinica chim. Acta* **7**, 805 (1962).
11. A. GOLDSTEIN, in *Biostatistics*, p. 51, MacMillan, New York (1964).
12. G. N. WILKINSON, *Biochem. J.* **80**, 324 (1961).
13. W. W. CLELAND, *Adv. Enzymol.* **29**, 1 (1967).
14. R. G. D. STEEL and J. H. TORRIE, in *Principles and Procedures of Statistics*, p. 99, McGraw-Hill, New York (1960).
15. T. E. GRAM, L. A. ROGERS and J. R. FOUTS, *J. Pharmac. exp. Ther.* **155**, 479 (1967).
16. H. S. MARVER and R. SCHMID, *Gastroenterology* **55**, 282 (1968).
17. K. J. ISSELBACHER, M. F. CHRABAS and R. C. QUINN, *J. biol. Chem.* **237**, 3033 (1962).
18. G. J. DUTTON and J. LAWES, *Biochem. J.* **98**, 30P (1966).
19. A. DELEON, L. M. GARTNER and I. M. ARIAS, *J. Lab. clin. Med.* **70**, 273 (1967).

20. B. P. F. Adlard, R. G. Lester and G. H. Lathe, *Biochem. Pharmac.* **18**, 59 (1969).
21. C. Catz and S. J. Yaffe, *Pediat. Res.* **2**, 361 (1968).
22. P. Zeidenberg, S. Orrenius and L. Ernster, *Trans. N.Y. Acad. Sci.* **29**, 310 (1967).
23. T. E. Gram. A. R. Hansen and J. R. Fouts, *Biochem. J.* **106**, 587 (1968).
24. J. K. Inscoe and J. Axelrod, *J. Pharmac. exp. Ther.* **129**, 128 (1960).
25. R. D. Howland and A. Burkhalter, *Fedn Proc.* **28**, 417 (1969).
26. E. Halac and A. Reff, *Biochim. biophys. Acta* **139**, 328 (1967).
27. F. P. Van Roy and K. P. M. Heirwegh, *Biochem. J.* **107**, 507 (1968).
28. G. A. Tomlinson and S. J. Yaffe, *Biochem. J.* **99**, 507 (1966).
29. G. J. Dutton and C. G. Greig, *Biochem. J.* **66**, 52P (1957).
30. D. Robinson and R. T. Williams, *Biochem. J.* **68**, 23P (1958).