Biochemical Pharmacology, Vol. 21, pp. 2457–2462. Pergamon Press, 1972. Printed in Great Britain.

# INFLUENCE OF PHENOBARBITAL TREATMENT ON *p*-NITROPHENOL AND BILIRUBIN GLUCURONIDATION IN WISTAR RAT, GUNN RAT AND CAT

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(*Received* 22 *March* 1972; *accepted* 18 *April* 1972)

**Abstract**—The influence of phenobarbital pretreatment on the glucuronidation of *p*-nitrophenol and bilirubin has been compared in liver microsomes from Wistar rat, homozygous and heterozygous Gunn rat and cat. Both for Wistar rat and cat it appeared that phenobarbital has a different effect on *p*-nitrophenol and bilirubin glucuronidation. No bilirubin glucuronidating activity could be detected in homozygous Gunn rats both before and after exposure to phenobarbital. The *p*-nitrophenol glucuronidating capacity of Gunn rats, however, was strongly enhanced after phenobarbital treatment. These results are arguments in favour of the involvement of different enzymes in the glucuronidation of *p*-nitrophenol and bilirubin.

UDPGLUCURONYLTRANSFERASE (UPDglucuronate glucuronyltransferase, EC 2.4.1.17, acceptor unspecific) catalyzes the transfer of the glucuronyl group from glucuronic acid to aglycones. Ether-, ester-, N- and S-glucuronides are thus formed.

There are many indications that UDPglucuronyltransferase consists of a heterogeneous group of enzymes. Homozygous Gunn rats, for instance, although deficient in bilirubin glucuronidation, have a normal glucuronidating capacity towards *p*-nitrophenol, aniline and tetrahydrocortisone. The cat, however, has been reported to have a normal bilirubin metabolism accompanied by a poor capacity to glucuronidate *p*-nitrophenol, menthol and *o*-aminophenol (summarized by Dutton[1]). In this study Wistar rats, homozygous and heterozygous Gunn rats and cats were treated with phenobarbital. The effect of this treatment on the *in vitro* activity of UDPglucuronyltransferase, with *p*-nitrophenol and bilirubin as substrates, has been studied.

It is known that treatment with phenobarbital stimulates liver growth and increases the amount of microsomal protein in the liver.[2] In order to differentiate between these effects and those on the *p*-nitrophenol and bilirubin glucuronidation, an increase of specific activity was considered to be a prerequisite for enzyme induction.

Mulder[3] and Winsnes[4] have reported that phenobarbital does not stimulate *p*-nitrophenol glucuronidation unless activated enzyme preparations are used. In this investigation microsomes were activated with ultrasonic vibration which has a similar effect as Triton X-100, used by the latter authors.[5]

## METHODS

*Animals.* Male Wistar and Gunn rats, 3–4 months old, weighing about 250 g, and male cats, approx. 1 year old, weighing about 2500 g were used.

Gunn rats were obtained from the University of California in 1958 and bred in our

Animal Laboratory. Icteric homozygous males were mated with nonicteric heterozygous females.

*Phenobarbital treatment.* Wistar and Gunn rats were treated daily with 75 mg and cats with 40 mg phenobarbital/kg body wt. for 6 days.

Wistar rats and cats treated for 12 days successively received daily 20 mg/kg for 3 days, 30 mg/kg for 3 days and 40 mg/kg for 6 days. The Gunn rats received daily, 75 mg phenobarbital/kg body wt. for 12 days. Control animals received saline. The animals were injected intraperitoneally, once daily at 9 a.m. Enzyme activities were determined 24 hr after the last injection.

*Preparation of microsomes.* The rats were killed by stunning and decapitation, the cats by injecting 4 ml sodium pentobarbital (6 per cent) into the heart, immediately followed by decapitation. After the animals were exsanguinated the livers were excised, weighed and kept in ice.

Portions of the livers were weighed, finely minced and transferred into 4 vol. of an ice-cold 0·25 M sucrose solution, containing $10^{-3}$ M sodium ethylenediamine tetraacetate (EDTA–Na) and $5 \times 10^{-2}$ M tris(hydroxymethyl)aminomethane–HCl buffer (pH 7·4).

Homogenates of rat liver were prepared using a Teflon-glass Potter–Elvehjem homogenizer. Cat liver was homogenized with an Ultra-Turrax apparatus.

Microsomal fractions were obtained by centrifuging the homogenates at 9000 *g* in an IEC B-20 centrifuge at 2° for 20 min. The 9000 *g* supernatants were centrifuged at 105,000 *g* in a Christ Omega II ultracentrifuge at 2° for 1 hr. The pellets from the latter step were gently resuspended in the homogenization medium with the Potter–Elvehjem apparatus. One ml suspension contained microsomes derived from 1 g liver.

The suspensions were treated with an ultrasonic distintegrator (MSE-100 Watt) for $6 \times 10$ sec, at maximal output.

*Chemicals.* Bilirubin, ascorbic acid, sulfanilic acid and sodium nitrate were obtained from Merck A. G., Germany; *p*-nitrophenol from the British Drug Houses Ltd., England; the disodium salt of UDPglucuronate from C. F. Boehringer, Germany. Bovine serum albumin was obtained from Poviet, The Netherlands.

*Enzyme assay.* Bilirubin was dissolved in calcium-free Tyrode buffer (pH 9·0). The incubation system consisted of: 0·30 ml microsomal preparation, 0·80 ml Tris–HCl buffer (0·05 M, pH 7·4), 0·15 ml UDP glucuronate ($6 \times 10^{-3}$ M, final conc.), 0·05 ml $MgCl_2$ ($3{\cdot}3 \times 10^{-3}$ M, final conc.) and 0·20 ml bilirubin ($20–80 \times 10^{-6}$ M, final conc.). The products were assayed according to the method described by Lathe and Walker[6] using diazotized sulphanilic acid as the diazo reagent. Blanks containing all above mentioned ingredients, except UDPglucuronate, were submitted to exactly the same diazo procedure as the reagent mixtures.

The *p*-nitrophenol incubation system consisted of: 0·05 ml microsomal preparation, 0·10 ml $MgCl_2$ ($0{\cdot}5 \times 10^{-3}$ M, final conc.), 0·05 ml UDPglucuronate ($6 \times 10^{-3}$ M, final conc.) and 0·05 ml *p*-nitrophenol ($0{\cdot}5–6 \times 10^{-3}$ M, final conc.) and the reagents were dissolved in Tris–HCl buffer (0·05 M, pH 7·4). The assay was done as described by Henderson and Kersten.[7] The reaction mixtures were incubated at 37° for 30 min.

*Protein determination.* Protein was determined by the method of Lowry *et al.*[8] with bovine serum albumin as reference.

*Calculations.* Kinetic data were analysed using Lineweaver–Burk plots. Michaelis–

Menten constants ($K_m$) are called apparent because crude microsomal preparations were used. Maximal substrate turnover rates per mg microsomal protein ($t_{max}$) were calculated by extrapolation. Statistical analysis was done with Student's $t$-test.

## RESULTS

The results are summarized in Fig. 1 and Table 1. We ascertained that rates of conversion for both $p$-nitrophenol and bilirubin remained unchanged during the incubation period.

*Wistar rats.* $p$-Nitrophenol glucuronidation appeared to be induced after a 6 day treatment with high doses phenobarbital whereas no induction of the bilirubin glucuronidation was found. Both $p$-nitrophenol and bilirubin glucuronidation were induced after a 12 day treatment with low doses phenobarbital.

Concomitant with increased $t_{max}$-values apparent $K_m$-values were also increased (Fig. 1).

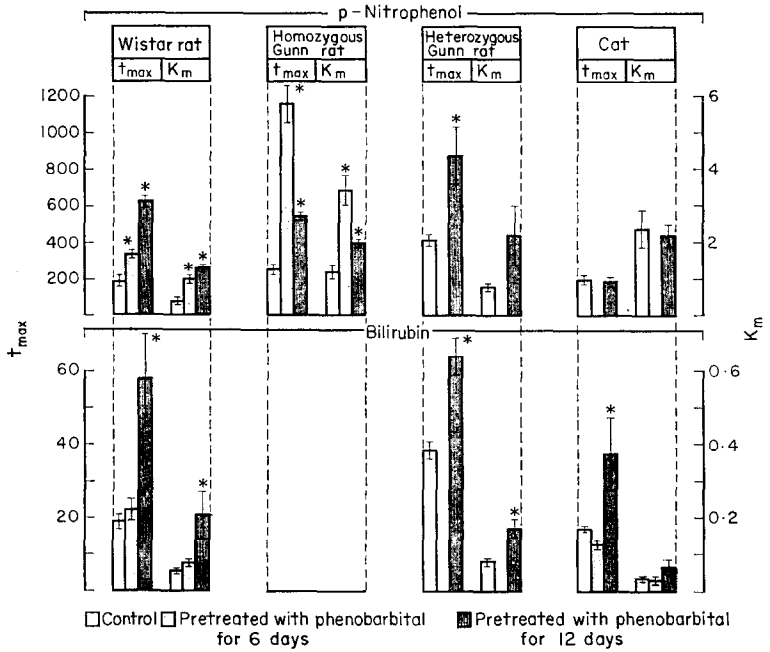


FIG. 1. Influence of phenobarbital treatment upon apparent $K_m$ and $t_{max}$ of $p$-nitrophenol and bilirubin glucuronidation in Wistar rat, homozygous and heterozygous Gunn rat and cat.

The number of animals and dosage are described in Table 1. Blocks represent means ± S.E.

* $P < 0{\cdot}05$ (Student's $t$-test). $t_{max}$ and $K_m$ are expressed as nmoles/hr per mg protein and mMg respectively.

Increments of microsomal protein were found after phenobarbital treatment but liver wt./body wt. ratio was not changed (Table 1).

*Homozygous Gunn rats.* A total deficiency in the glucuronidation of bilirubin was found together with high activities for the glucuronidation of $p$-nitrophenol. After 6 and 12 days phenobarbital treatment the $t_{max}$ for the glucuronidation of $p$-nitro-

TABLE 1. INFLUENCE OF PHENOBARBITAL TREATMENT ON MICROSOMAL PROTEIN AND LIVER WT./BODY WT. RATIO IN WISTAR RAT, GUNN RAT AND CAT

| | Days treated with phenobarbital* | *n* | Microsomal protein† (mg/g wet liver wt.) | $\frac{\text{Liver wt.}}{\text{Body wt.}} \times 100$† |
|---|---|---|---|---|
| Wistar rat | Control | 6 | 22·9 ± 0·7 | 4·3 ± 0·5 |
| | 6 | 6 | 26·4 ± 0·9§ | 5·0 ± 0·5 |
| | 12 | 6 | 25·8 ± 0·4§ | 5·1 ± 0·5 |
| Homozygous Gunn rat | Control | 6 | 18·1 ± 1·3 | 4·0 ± 0·7 |
| | 6 | 6 | 25·5 ± 2·1§ | 5·4 ± 0·9 |
| | 12 | 6 | 26·6 ± 1·8 | 4·5 ± 0·5 |
| Heterozygous Gunn rat | Control | 7 | 18·8 ± 1·0 | 3·7 ± 0·3 |
| | 12 | 4 | 19·8 ± 1·7 | 3·9 ± 0·4 |
| Cat | Control | 6 | 12·5 ± 1·3 | 3·0 ± 0·3 |
| | 6 | 3 | 15·3 ± 2·7 | 3·3 ± 1·2 |
| | 12 | 3 | 13·4 ± 0·8 | 4·2 ± 0·3‡ |

* Wistar rats and homozygous Gunn rats were treated daily with 75 mg phenobarbital/kg body wt for 6 days; cats with 40 mg/kg for 6 days. Wistar rats and cats treated for 12 days received daily, successively, 20 mg/kg for 3 days, 30 mg/kg for 3 days and 40 mg/kg for 6 days. The Gunn rats received daily 75 mg/kg for 12 days. Control animals received saline.
*n*, number of animals in each group.
† means ± S.E.
‡ $0{\cdot}01 < P < 0{\cdot}05$.
§ $P < 0{\cdot}01$.

phenol amounted respectively to about 450 and 200 per cent of the control value. The same pattern was observed for the apparent $K_m$-value (Fig. 1).

The influence of phenobarbital treatment upon microsomal protein and liver wt./body wt. ratio was the same as found for the Wistar rat (Table 1).

*Heterozygous Gunn rats.* The $t_{max}$-values of both *p*-nitrophenol and bilirubin glucuronidation were enhanced after 12 days treatment with phenobarbital but only the apparent $K_m$ for the bilirubin glucuronidation was concomitantly increased (Fig. 1). Neither microsomal protein nor liver wt./body wt. ratio were increased after phenobarbital treatment (Table 1).

*Cats. p*-Nitrophenol glucuronidation could not be induced by phenobarbital treatment. The $t_{max}$-value of the bilirubin glucuronidation was increased after a 12 day phenobarbital treatment whereas no induction was found after 6 days. In both cases phenobarbital treatment did not result in increased apparent $K_m$-values.

Phenobarbital treatment had no influence on the microsomal protein of cat liver. Liver wt./body wt. ratio was increased only after 12 days (Table 1).

## DISCUSSION

The question whether or not UDPglucuronyltransferase is one enzyme has never definitely been resolved. Mulder[9] and Storey[10] studying the competition between several aglycones, like *p*-nitrophenol, *o*-aminophenol, phenolphthalein and anthranilic acid, came to contradictory conclusions. Assuming the existence of several glucuronyltransferases however, these enzymes might be quite unspecific towards these

substrates. Tomlinson and Yaffe[11] and Halac and Reff[12] tried to solubilize glucuronyltransferase from crude liver microsomes with detergents and reported that different results were obtained with *p*-nitrophenol and bilirubin as substrates. Moreover a different post-natal development[11] and a different behaviour towards heat denaturation[12] was found with these two substrates.

In the present study it is shown that the hereditary lack of the bilirubin glucuronidation enzyme of the homozygous Gunn rat is total. This is at variance with the data of other authors[14] who reported low activities in these animals. In contrast the *p*-nitrophenol glucuronidating activity was not deficient. This is in agreement with the results of Van Leusden *et al.*,[13] who used Gunn rats of the same breeding colony. The *p*-nitrophenol-glucuronidating capacity of the homozygous Gunn rats increased strongly after phenobarbital treatment. It appeared that the *p*-nitrophenol-glucuronidating enzyme does not accept bilirubin as a substrate since no bilirubin glucuronidation was found after 6 days of phenobarbital treatment whereas the amount of *p*-nitrophenol-glucuronidating enzyme was abundant. This pleads for the existence of a separate bilirubin-glucuronidating enzyme-system which is lacking in the homozygous Gunn rat. This bilirubin-glucuronidating enzyme must also be different from the enzyme(s) glucuronidating aniline, tetrahydrocortisone, *o*-aminophenol, phenoldibromphthalein and diphenylacetic acid, in which the homozygous Gunn rat is not deficient.[15–18]

*p*-Nitrophenol and bilirubin glucuronidation were influenced differently by phenobarbital, both in Wistar rat and cat. In the Wistar rat *p*-nitrophenol glucuronidation was induced after a 6 day treatment with high doses, whereas bilirubin glucuronidation was only found to be induced after a 12 day treatment. This was achieved with relatively low doses phenobarbital.

Our finding that *p*-nitrophenol glucuronidation is elevated in rats after a short treatment with high doses phenobarbital is in agreement with the results of Mulder.[3] As far as bilirubin glucuronidation is concerned, Pilcher *et al.*[19] reported that induction was only found after treating rats for a long period with high doses phenobarbital whereas no increase was found after treatment for a short period. These authors however found a decreased specific activity for the bilirubin glucuronidation after 30 mg phenobarbital/kg for 10 days. As they used for the *in vitro* assay albumin as a carrier for bilirubin the effective substrate concentration might have been much lower than the calculated one, due to the strong binding of bilirubin to albumin.[20] A rise in apparent $K_m$ after phenobarbital treatment might then explain their results.

In the cat phenobarbital treatment did not effect *p*-nitrophenol glucuronidation nor microsomal protein whereas bilirubin glucuronidation was induced. Compared with the Wistar rat, *p*-nitrophenol glucuronidation by the cat appears to be less efficient: beside an equal $t_{max}$ a remarkably higher apparent $K_m$ was found for the glucuronidation of this compound in the cat. This might explain the absence of glucuronides in the urine of cats after injection of glucuronidogenic compounds.[21] Presumably in the cat glucuronidation is not a preferential pathway for the elimination of foreign compounds.

These distinct effects of phenobarbital on the glucuronidation of *p*-nitrophenol and bilirubin in both Wistar rat and cat add to the evidence that *p*-nitrophenol and bilirubin are glucuronidated by different enzymes. Compared with the influence of phenobarbital treatment on the microsomal oxydative enzymes,[2] *p*-nitrophenol

glucuronidation and microsomal protein,[2] the induction of the bilirubin-glucuronidating enzyme is a slow effect. The question arises whether this is still a direct effect of phenobarbital treatment or rather some secondary effect. It is known that bilirubin production is increased after phenobarbital treatment.[22] Prolonged exposure to a higher bilirubin level might induce the bilirubin-glucuronidating enzyme, as was found in newborn rats.[23]

*Acknowledgements*—The authors are indebted to Professor Dr. E. J. Ariëns, Institute of Pharmacology, Nijmegen, for valuable advice during this study. Thanks are also due to Miss G. H. M. Van Kuppevelt for skilled assistance.

Financial support was obtained from the Stichting voor Medisch Wetenschappelijk Onderzoek (FUNGO).

## REFERENCES

1. G. J. DUTTON, in, *Glucuronic acid, free and combined* (Ed. G. J. DUTTON), p. 230. Academic Press, New York (1966).
2. A. H. CONNEY, *Pharmac. Rev.* **19,** 317 (1967).
3. G. J. MULDER, *Biochem. J.* **117,** 319 (1970).
4. A. WINSNES, *Biochem. Pharmac.* **20,** 1853 (1971).
5. P. TH. HENDERSON, *Life Sci.* **9,** 511 (1970).
6. G. H. LATHE and M. WALKER, *Biochem. J.* **70,** 705 (1958).
7. P. TH. HENDERSON and K. J. KERSTEN, *Biochem. Pharmac.* **19,** 2343 (1970).
8. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
9. G. J. MULDER, *Biochem. J.* **125,** 9 (1971).
10. I. D. E. STOREY, *Biochem. J.* **95,** 209 (1965).
11. G. A. TOMLINSON and S. J. YAFFE, *Biochem. J.* **99,** 507 (1966).
12. E. HALAC and A. REFF, *Biochem. biophys. Acta* **139,** 328 (1967).
13. H. A. I. M. VAN LEUSDEN, J. A. J. M. BAKKEREN, F. ZILLIKEN and L. A. M. STOLTE, *Biochem. biophys. Res. Commun.* **7,** 67 (1962).
14. A. E. WHITE, in, *Bilirubin metabolism* (Ed. I. A. D. BOUCHIER), p. 183. Blackwell Scientific, Oxford (1967).
15. I. M. ARIAS, *Biochem. biophys. Res. Commun.* **6,** 81 (1961).
16. W. D. DRUCKER, *Proc. Soc. exp. Biol. Med.* **129,** 308 (1968).
17. I. STEVENSON, D. GREENWOOD and J. MCEWEN, *Biochem. biophys. Res. Commun.* **32,** 866 (1968).
18. N. B. JAVITT, *Am. J. Physiol.* **211,** 424 (1966).
19. C. W. T. PILCHER, R. P. H. THOMPSON and R. WILLIAMS, *Biochem. Pharmac.* **21,** 129 (1972).
20. D. BRATLID and J. FOG, *Scand. J. clin. Lab. Invest.* **25,** 257 (1970).
21. D. ROBINSON and R. T. WILLIAMS, *Biochem. J.* **68,** 23P (1958).
22. R. J. ROBERTS and G. J. PLAA, *Toxicol. Appl. Pharmac.* **15,** 483 (1969).
23. A. F. BAKKEN, *Pediat. Res.* **3,** 205 (1969).