activities under the conditions employed in this study. To further clarify this area, we are also currently examining the ability of a non-carcinogenic "P-448 type" inducer, β-naphthoflavone (BNF), to neonatally program the hepatic PSMOS. Preliminary findings indicate that neonatally administered BNF did not alter adult PSMOS activities.

Medical College of Virginia
Virginia Commonwealth University
Department of Pharmacology and Toxicology
Richmond, VA 23298, U.S.A.

DANIEL M. BAGLEY
JOHNNIE R. HAYES*

REFERENCES

1. P. Demoor and C. Denef, *Endocrinology* **82**, 480 (1968).
2. W. Levin, D. Ryan, R. Kuntzman and A. H. Conney, *Molec. Pharmac.* **11**, 190 (1975).
3. P. Skett and J. A. Gustafsson, in *Reviews in Biochemical Toxicology* (Eds. E. Hodgson, J. Bend and R. Philpot), Vol. 1, p. 27. Elsevier North Holland, New York (1979).
4. D. M. Bagley and J. R. Hayes, *Biochem. biophys. Res. Commun.* **114**, 1132 (1983).
5. R. A. Faris and T. C. Campbell, *Cancer Res.* **43**, 2576 (1983).
6. V. J. Simpson and L. W. K. Chung, *Proc. west. Pharmac. Soc.* **25**, 257 (1982).
7. T. Cresteil, J. P. Flinois, A. Pfister and J. P. Leroux, *Biochem. Pharmac.* **28**, 2057 (1979).
8. J. R. Hayes, M. U. K. Mgbodile and T. C. Campbell, *Biochem. Pharmac.* **22**, 1005 (1973).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
10. F. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
11. J. A. Peterson, R. E. Ebel and D. H. O'Keefe, in *Methods in Enzymology* (Eds. S. Fleischer and L. Packer), Vol. 52, p. 221. Academic Press, New York (1978).
12. M. U. K. Mgbodile, J. R. Hayes and T. C. Campbell, *Biochem. Pharmac.* **22**, 1125 (1973).
13. V. Ullrich and P. Weber, *Hoppe-Seyler's Z. physiol. Chem.* **353**, 1171 (1972).
14. D. W. Nebert and H. V. Gelboin, *J. biol. Chem.* **243**, 6242 (1968).
15. A. H. Conney, *Pharmac. Rev.* **19**, 317 (1967).

*Author to whom correspondence should be addressed.

Biochemical Pharmacology, Vol. 34, No. 14, pp. 2573–2575, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00
Pergamon Press Ltd.

Factors influencing drug sulfate and glucuronic acid conjugation rates in isolated rat hepatocytes: significance of preincubation time

(*Received* 12 *December* 1984; *accepted* 1 *March* 1985)

Certain phenolic drugs such as salicylamide and acetaminophen are metabolized by glucuronidation and sulfation *in vivo*. Both conjugations are bisubstrate reactions with UDP-glucuronic acid (UDPGA) in glucuronidation and with 3′-phosphoadenosine-5′-phosphosulfate (PAPS) in sulfation. To study these reactions *in vitro*, isolated hepatocytes apparently retaining the essential properties of intact liver for cosubstrate biosynthesis and enzyme activities are often used [1–9]. Under the best experimental conditions reflecting reactivity *in vivo*, it should be possible to determine the pharmacokinetics in drug conjugative metabolism from kinetic data in hepatocytes.

Hepatocytes, following preparation, are generally stored at 0–4° to maintain high viability [3, 5, 9, 10]. The cells are preliminarily incubated at 37° just before the reaction but the preincubation time may vary from 0 min until 20 min [1, 3, 4, 6, 7, 9]. Preincubation not only enhances the reaction temperature to 37° but activates various cells to function physiologically. In glucuronidation and sulfation, the extent of activation of the biosynthesis of UDPGA and PAPS in enzyme systems by preincubation should have important influence on reaction rates.

In this paper, the effects of preincubation time on K_m and V_{max} of the glucuronidation and sulfation rates of acetaminophen (APAP) in hepatocytes are studied and the best reaction conditions are proposed.

Materials and methods

APAP was purchased from Wako Pure Industries Co. Ltd. (Osaka, Japan). APAP glucuronide (APAP-Glu) and APAP sulfate (APAP-Sul) were synthesized by the methods of Shibasaki *et al.* [11] and Burkhardt and Wood [12], respectively. All other chemicals and reagents were of analytical grade or better. Hepatocytes were isolated from male Wister rats weighing 230–285 g by a slightly modified method of Baur *et al.* [10] reported previously [9]. After isolation, the cells were suspended in a reaction medium containing 4% bovine serum albumin at 2×10^6 cells/ml and stored in ice-water. The reaction medium consisted of 137 mM NaCl, 5.2 mM KCl, 0.9 mM $MgSO_4 \cdot 7H_2O$, 0.12 mM $CaCl_2 \cdot 2H_2O$ and 5 mM glucose buffered with 3 mM Na_2HPO_4 and 15 mM HEPES (*N*-2-hydroxyethylpiperazone-*N*′-2-ethanesulfonic acid). The final pH was adjusted to 7.4 with NaOH. Trypan blue exclusion was more than 94% immediately after preparation and endogeneous cellular respiration was normal [9]. One and a half ml of the cell suspension stored in ice-water were first incubated in a 30 ml Erlenmeyer flask at 37° for 0, 5, 10, 20, or 30 min before starting the reaction. Following this, 1.5 ml of the APAP solution of the same reaction medium (0.04–10 mM) were added. The mixture was then incubated for 11 min at 37° since both conjugation reactions at all APAP concentrations are linear until 11 min. Incubation was performed in a metabolic shaker (90 oscillations/min) to ensure a complete mixing of the reaction medium. A supply of 95% O_2–5% CO_2 to the incubation mixture was unnecessary since this gas has no significant effect on the extent of conjugation [9]. The conjugation reaction was brought to a stop by adding 0.5 ml of the incubation mixture to 0.1 ml of 25% perchloric acid containing 1.8 mM 4-fluorophenol as the internal standard (IS). Then 0.2 ml of 0.8 M $BaCl_2$ solution was added to the mixture followed by centrifugation for 10 min at 3000 rpm. The supernatant was used for the reversed-phase HPLC assay. The HPLC apparatus and conditions were virtually the same as reported previously [13, 14]. The mobile phase was water:methanol:acetic acid (81:17.5:1.5 v/v/v) containing 100 mg/l KNO_3 and 26.4 mg/l tetrabutylam-

monium bromide as an ion pair agent and eluted at 1.5 ml/min. Ultraviolet absorption was measured at 250 nm. The capacity factors (k') of APAP, APAP-Glu, APAP-Sul and IS were 3.6, 2.0, 6.5 and 9.5, respectively. The values of K_m and V_{max} were obtained from the nonlinear least squares fit (MULTI program [15]) of the data to Michaelis–Menten equation.

Results and discussion

Figures 1(a) and (b) show the effects of preincubation time on the sulfation and glucuronidation rates at 0.02–5 mM APAP in the isolated hepatocytes, respectively. Only the sulfation rate at the lowest APAP concentration (0.02 mM) was slightly smaller without preincubation but the preincubation time hardly influenced the sulfation rate at more than a 0.06 mM APAP concentration (Fig. 1a). The glucuronidation rate increased with preincubation time (Fig. 1b). Figures 2(a) and (b) also show the effects of preincubation time on K_m and V_{max} obtained from the results in Figs 1(a) and (b), respectively. The ordinate values are the ratios of values obtained from each preincubation time to those from 5 min preincubation shown in Table 1. The value of V_{max} for sulfation ($V_{max,S}$) was independent of preincubation time, but K_m ($K_{m,S}$) tended to decrease for 10 min and attained a constant value (Fig. 2a). The value of K_m for glucuronidation ($K_{m,G}$) decreased for 10 min in the same manner as $K_{m,S}$(Fig. 2b). V_{max} of glucuronidation ($V_{max,G}$) increased asymptotically up to 20 min (Fig. 2b).

Preincubation time for the conjugation reaction in isolated hepatocytes may vary. Periods of 0 min [1], 2–3 min [4], 5 min [9], 10 min [3], 12 min [7] and 20 min [6] have been reported. The effects of preincubation from 0 to 30 min on glucuronidation and sulfation rates were thus examined in this paper. No effect on the sulfation rate could be distinctly observed except at the lowest APAP concentration (0.02 mM) (Fig. 1a). The difference in the effects of preincubation time on $V_{max,S}$ and $V_{max,G}$ is considered to be related to the PAPS and UDPGA levels in hepatocytes, respectively. $V_{max,S}$ values which were constant independent of preincubation time (Fig. 2a) suggest that the PAPS pool in hepatocytes is small, as described by Mulder and Scholtens [16] and that an equilibrium between PAPS and inorganic sulfate is quickly established. The values of $V_{max,G}$ which increased with preincubation time

Table 1. K_m and V_{max} values for the sulfation and glucuronidation of acetaminophen in isolated hepatocytes

	K_m (mM)	V_{max} (nmole/10^6 cells/min)
Sulfation	0.030 ± 0.002	1.09 ± 0.11
Glucuronidation	2.133 ± 0.805	1.30 ± 0.27

Each value represents the mean ± S.E. of three experiments in 5 min of preincubation.

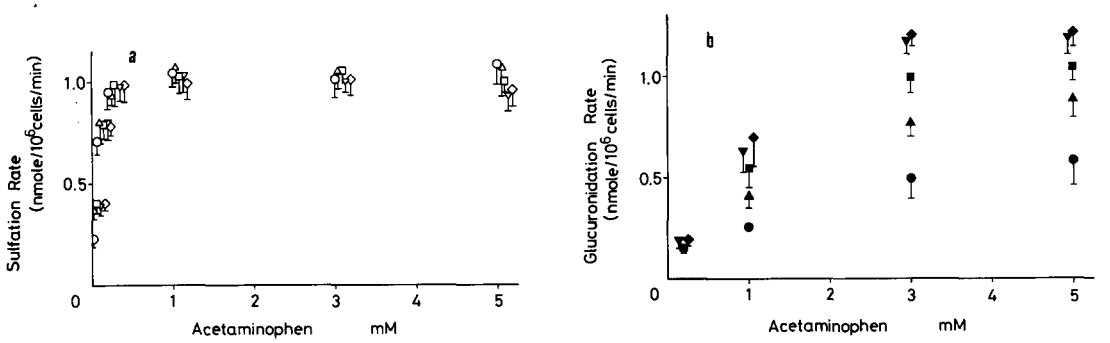

Fig. 1. Effects of preincubation time on the sulfation rate (a) and glucuronidation rate (b) of acetaminophen in isolated hepatocytes. The symbols indicate the following preincubation times: ○●, 0 min; △▲, 5 min; □■, 10 min; ▽▼, 20 min; ◇◆, 30 min. Each datum is the mean ± S.E. of three experiments.

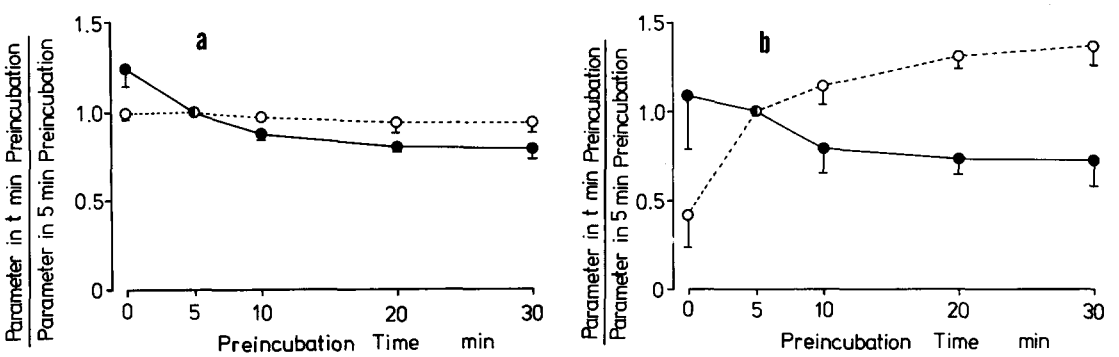

Fig. 2. Effects of preincubation time on K_m (closed circle) and V_{max} (open circle) of sulfation (a) and glucuronidation (b) of acetaminophen in isolated hepatocytes. The ordinate value is the ratio of K_m or V_{max} in each preincubation time to that in 5 min preincubation symbolized by the semi-closed circle. Each symbol represents the mean of the ratio of K_m or V_{max} with the S.E. values.

(Fig. 2b) suggest that the cellular UDPGA level increases with time as reported by Moldeus *et al.* [2] and its biosynthesis rate attains a steady state after 20 min preincubation (Figs 1b and 2b). Since the ratio of glucuronide to the total conjugates increases with substrate concentration (Figs 1a and b), the effect of preincubation time may be greater at higher substrate concentrations.

In summary, our data indicate the effects of preincubation time on the glucuronidation and sulfation rates of acetaminophen in isolated rat hepatocytes. The preincubation time had hardly any effect on $V_{max,S}$, but $K_{m,S}$ tended to decrease with preincubation time, reaching a constant value in 10 min. $K_{m,G}$ decreased with preincubation time in the same way as $K_{m,S}$. More than 20 min were required to attain a constant $V_{max,G}$. These results indicate that the biosynthesis rate of UDPGA to attain a steady state may be slower than that of PAPS. Thus, strictly speaking, for the conjugation reaction in hepatocytes, a preincubation period exceeding 20 min is preferable.

Acknowledgement—The authors thank Miss Takako Ito for her competent technical assistance.

Department of Biopharmaceutics
Tokyo College of Pharmacy
1432-1 Horinouchi, Hachioji
Tokyo 192-03, Japan

TAKASHI MIZUMA
MASAHIRO HAYASHI*
SHOJI AWAZU

* Author to whom all correspondence should be addressed.

REFERENCES

1. P. Moldeus, H. Vadi and M. Berggren, *Acta pharmac. toxic.* **39**, 17 (1976).
2. P. Moldeus, B. Andersson and A. Norling, *Biochem. Pharmac.* **27**, 2583 (1978).
3. L. R. Schwarz, K-H. Summer and M. Schwenk, *Eur. J. Biochem.* **94**, 617 (1979).
4. P. I. Eacho, D. Sweeny and M. Weiner, *Biochem. Pharmac.* **30**, 2616 (1981).
5. M. Koike, K. Sugeno and M. Hirata, *J. Pharm. Sci.* **70**, 308 (1981).
6. H. Koster, I. Halsema, E. Scholtens, M. Knippers and G. J. Mulder, *Biochem. Pharmac.* **30**, 2569 (1981).
7. J. Singh and L. R. Schwarz, *Biochem. Pharmac.* **30**, 3252 (1981).
8. R. R. Erickson, P. Yu-Drent and J. L. Holtzman, *J. Pharmac. exp. Ther.* **220**, 35 (1982).
9. T. Mizuma, M. Machida, M. Hayashi and S. Awazu, *J. Pharmacobio-Dyn.* **5**, 811 (1982).
10. H. Baur, S. Kasperek and E. Pfaff, *Hoppe-Seylers Z. Physiol. Chem.* **356**, 827 (1975).
11. J. Shibasaki, E. Sadakane, R. Konishi and T. Koizumi, *Chem. Pharm. Bull.* **18**, 2340 (1970).
12. G. N. Burkhardt and H. Wood, *J. chem. Soc.* 141, (1929).
13. M. Machida, Y. Morita, M. Hayashi and S. Awazu, *Biochem. Pharmac.* **31**, 787 (1982).
14. T. Mizuma, M. Hayashi and S. Awazu, *J. Pharmacobio-Dyn.* **7**, 784 (1984).
15. K. Yamaoka, Y. Tanigawara, T. Nakagawa and T. Uno, *J. Pharm. Dyn.* **4**, 879 (1981).
16. G. J. Mulder and E. Scholtens, *Biochem. J.* **172**, 247 (1978).

Biochemical Pharmacology, Vol. 34, No. 14, pp. 2575–2577, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

Cisplatin-induced alteration of the copper and zinc content of the rat kidney

(*Received* 1 *November* 1984; *accepted* 8 *March* 1985)

The administration of Cd^{2+} to rats results in an increase in renal Cu concentration [1, 2] which is probably stimulated by the Cd^{2+}-induced synthesis of renal metallothionein, the low molecular weight metal-binding protein which normally functions in Zn and Cu homeostasis [3]. Treatment of rats with Hg^{2+}, Bi^{2+} [2], Au(I) [4] and Au(III) salts [5] has a similar effect on kidney Cu concentrations. In contrast, the anticancer drug *cis*-dichlorodiammine platinum II (cisplatin), which accumulates in the kidneys and produces proximal tubular necrosis [6, 7], reduces the concentrations of Cu and Zn in renal metallothionein and other soluble proteins in the rat kidney [8]. In this paper we have further investigated this interaction by comparing the time course and dose dependence of the cisplatin-induced change in renal Cu and Zn content with the renal uptake and toxicity of Pt in the rat.

Materials and methods

Cisplatin was synthesized by the method of Kauffman and Cowan [9]. Solutions of the drug were prepared in isotonic saline immediately before use. Six groups of male Wistar rats (N = 4, 130–150 g) received a s.c. injection of 5 mg cisplatin/kg (0.2 ml/100 g). Saline-treated controls (N = 4) were included with each group and the animals weighed and killed by decapitation 1, 2, 4, 8, 17 or 27 days after the administration of cisplatin. The kidneys were excised, weighed and a portion analysed for Pt, Cu and Zn, as described below. In a second experiment, 4 groups of rats (N = 4) were given a s.c. injection of 0, 1, 3 or 5 mg cisplatin/kg and then killed 7 days after the administration of cisplatin. Blood was collected from the severed cervical vessels, the serum creatinine concentration determined [10] and the kidneys removed.

Samples of whole kidney (50–100 mg) were digested to dryness with 0.5 ml conc. nitric acid (Aristar grade, BDH Chemicals Ltd, Poole, U.K.) and then analysed in 5 ml of 5% HCl (Aristar grade, BDH Chemicals Ltd) for Pt, Cu and Zn by flameless (Pt) or flame atomic absorption spectrometry (Cu and Zn).

Results and discussion

Cisplatin treatment produced a decrease in the Cu and Zn content of the kidney in relation to controls of the same age (Fig. 1). Thus, in the controls the Cu and Zn content of the kidney increased with age but decreased slightly in the cisplatin-treated animals. The percentage change in total renal Cu content was significantly greater than that of Zn. For example, 27 days after the administration of cisplatin, total renal Zn content in the cisplatin-treated animals was 63 ± 7% of the corresponding age-matched control level, whereas the total Cu content was only 28 ± 6% of the control value.

The total Pt content of the kidney increased to a maximum after 2 days and then declined slowly (Fig. 2A). The relative kidney weights of the cisplatin-treated animals were similar to those of the controls until 2 days after which they increased to a maximum at 8 days and then decreased slowly to the control value at 27 days (Fig. 2B). The