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INHIBITION OF ACETAMINOPHEN GLUCURONIDATION BY OXAZEPAM

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Abstract—Glucuronidation of [^{3}H]acetaminophen (pHAA) in mouse liver microsomes is enhanced about 3-fold by 0.025% Triton X-100. Oxazepam inhibits microsomal glucuronidation of pHAA, yielding apparent competitive kinetics in native microsomes. Phenobarbital-pretreatment has no effect on the microsomal glucuronidation of pHAA. MH_1C_1 rat hepatoma cells also glucuronidate pHAA, approximately 40 nmoles per mg cell protein per hr being conjugated at a concentration of 1 mM. Oxazepam also inhibits pHAA glucuronidation in the cell culture system. Intraperitoneal injection of mice with oxazepam 15 min before subcutaneous injection of pHAA significantly increases the plasma half-life of pHAA.

The major metabolites of the analgesic acetaminophen in man and experimental animals are the glucuronide and sulfate derivatives [1–4]; the ratio of glucuronide and sulfate metabolites varies widely between the different species, however. After low doses of acetaminophen, mice excrete more glucuronide than sulfate whereas rats excrete more sulfate than glucuronide [5]. Humans are reported to excrete 63 per cent as the glucuronide and 32 per cent as the sulfate in the urine after oral therapeutic doses of the drug [6].

The urinary excretion of the tranquilizer oxazepam both in man and in laboratory animals is largely as its glucuronide, accounting for at least 95% of urinary metabolites [7, 8]. Oxazepam glucuronide can also be formed after previous demethylation and hydroxylation of diazepam [9]. If acetaminophen and oxazepam are glucuronidated by a similar liver microsomal glucuronyl transferase, they might impede each others metabolism. The present paper is concerned with the action of oxazepam on acetaminophen glucuronidation.

MATERIALS AND METHODS

Chemicals. [^{3}H](G)-Acetaminophen (sp. act. 370 mCi/m-mole) was obtained from New England Nuclear Co. and unlabelled acetaminophen from Eastman Kodak Co. Oxazepam was obtained from Wyeth Laboratories Inc. Uridine 5'-diphosphoglucuronic acid (UDPGA, ammonium salt) was purchased from Sigma Co. Other chemicals used were the best available commercial grades.

Microsome experiments. Livers from male Swiss-Webster mice (20–22 g) were homogenized in two vol. of 1.15% KCl containing 20 mM Tris buffer, pH 7.4. The homogenate was centrifuged at 9000 *g* in a Sorvall RC-2 centrifuge for 20 min and the supernatant was decanted and recentrifuged for 1 hr at 105,000 *g* in a Beckman preparative ultracentrifuge. The microsomal pellet was resuspended in the Tris–KCl buffer. Microsomes from mice pretreated with phenobarbital (75 mg/kg i.p. daily for three days) or with 5,6-benzoflavone (80 mg/kg i.p. 48 hr before sacrifice) in corn oil, were prepared in an identical manner. Control mice were injected with 0.9% sodium chloride or corn oil for similar periods.

The incubation mixture contained (final concentrations) in ice-cold reaction tubes in a final volume of 0.5 ml (unless otherwise stated): 2 mg/ml microsomal protein, 0.5 mM [^{3}H]acetaminophen (approx. 300 dis/min/nmole), 2 mM UDPGA, 40 mM Tris buffer, pH 7.4, 10 mM $MgCl_2$. To the appropriate tubes 0.025% Triton X-100 and oxazepam in 1% DMSO were added; in the oxazepam experiments control tubes received 1% DMSO. Tubes were incubated in a shaking water-bath usually for 20 min; reactions were stopped by immersing the tubes in boiling water for 2 min. Samples were extracted 5 times with 2 ml of ethyl acetate [5], and an aliquot (50 μl) of the remaining water phase was counted in 15 ml of a BBOT scintillation fluid. Values were corrected for blanks (tubes without UDPGA) and expressed as nmoles acetaminophen glucuronide formed per mg of microsomal protein per min. Microsomal cytochrome P-450 was measured according to the method of Omura and Sato [10].

Cell culture experiments. MH_1C_1 cells, a cell line derived from Morris rat hepatoma No. 7795 (American Type Culture Collection, CCL 144) were grown in Ham's F10 medium containing 15% horse serum (Gibco), 2.5% fetal calf serum (Gibco) and antibiotics. In some experiments H-4-II-E cells (Reuber cells), a cell line derived from Reuber rat hepatoma H-35 (generously given to us by Dr. Snorri S. Thorgeirsson, National Institute of Child Health and Human Development, Bethesda, Md., U.S.A.) and grown in Eagle's medium No. 2 with 10% calf serum (Gibco) and 10% fetal calf serum, were used.

For experiments cells were washed twice with warm serum-free medium and incubated with varying concentrations of [^{3}H]acetaminophen (approx. 300 dis/min/nmole) in 10 ml serum-free medium with or without oxazepam in 0.4% DMSO. Control cultures received only 0.4% DMSO. One-ml samples of the incubation medium were withdrawn at desired time intervals, extracted 5 times with 5.0 ml ethyl acetate and aliquots (100 μl) of the water phase were counted

and corrected for blanks ([^{3}H]acetaminophen medium incubated without cells). Similar samples from cell incubations were applied to DEAE-cellulose thin-layer plates (Avicell, 250 μm thick) and developed in a system of *n*-propanol–0.4 M ammonium hydroxide (80:20), as described by Jollow *et al.* [5]. After development, the chromatograms were scraped off in 1-cm sections and transferred to scintillation vials; each sample was eluted with 0.4 ml water and counted in 15 ml BBOT. After incubation with acetaminophen, the cells were washed twice with 5 ml PBS pH 7.4 and dissolved in 4.0 ml NaOH. The protein concentration in both cell culture and in microsome experiments was determined by the method of Lowry *et al.* [11] using bovine serum albumin as standard.

In vivo *experiments*. The rate of disappearance of acetaminophen from plasma was determined by administering [^{3}H]acetaminophen (50 mg/kg s.c., 0.3 μCi/μmole) to mice 15 min after injection of oxazepam (50 mg/kg in DMSO, 0.05 ml per animal, i.p.) or DMSO alone. Animals were decapitated at desired time intervals and blood was collected in heparinized tubes. The samples were centrifuged, and the [^{3}H]acetaminophen in 0.1 ml of plasma was extracted into 2.0 ml of ethyl acetate. One ml of ethyl acetate was added to 15 ml BBOT scintillation fluid; counts were corrected for background and quench and converted to μg [^{3}H]acetaminophen/ml plasma.

RESULTS

The microsomal glucuronidation of [^{3}H]acetaminophen is shown in Fig. 1 and Table 1. In native microsomes the reaction was linear with respect both to time up to 30 min and protein concentration up to 5 mg/ml. Addition of low concentrations of Triton X-100 increased the amount of [^{3}H]acetaminophen glucuronidated, and activity was maximally enhanced, about 3-fold, by 0.025% Triton X-100 (with 2 mg/ml microsomal protein) when compared with control microsomes (Fig. 1C). Increasing the amount of protein at constant Triton concentration diminished this effect above 1 mg/ml (Fig. 1B).

MH_1C_1 cells also metabolized [^{3}H]acetaminophen (Fig. 2), showing time and substrate concentration dependency in the production of water-soluble metabolites. At higher acetaminophen concentrations there was a deviation from linearity, perhaps due to product inhibition or direct effects of acetaminophen on the cells. Reuber cells, on the contrary, did not show any sign of glucuronidation measured at substrate concentrations between 0.05 mM and 1.0 mM during 6 hr of incubation. Thin-layer chromatography of medium from cells that had been incubated with [^{3}H]acetaminophen gave two sharp peaks with R_F values similar to those reported for acetaminophen glucuronide and acetaminophen respectively [5].

Pretreatment of mice with phenobarbital increased the microsomal content of cytochrome P-450 to 238% of control levels (Table 1), yet it did not alter the rates of microsomal acetaminophen glucuronidation either in the unactivated or Triton-activated state. The polycyclic hydrocarbon inducer, benzoflavone, which in this mouse strain increases cytochrome P-448 levels by only 24%, had a comparable effect on unactivated and Triton-activated microsomal glucuronidation of acetaminophen.

Kinetic analysis of the effect of 0.5 mM oxazepam on the microsomal glucuronidation of acetaminophen is shown in Fig. 3. Reciprocal reaction velocities from incubations with and without Triton were plotted against reciprocal substrate concentrations between 0.1 and 1.0 mM; the points were fitted to the line by the method of least squares, and the kinetic parameters of the Michaelis–Menten equation were calculated. As determined from the plot the K_m for the native microsomes was 0.21 mM, whereas the maximal velocity was 1.00 nmoles/mg protein/min. In Triton-activated microsomes the K_m was increased to 0.62 mM and the V_{max} to 3.70 nmoles/mg protein/min. Oxazepam, 0.5 mM altered the K_m in the native microsomes to 0.41 mM, whereas the V_{max} was unchanged (1.04 nmoles/mg protein/min). In the presence of Triton and oxazepam the K_m and the V_{max} were increased to 1.30 mM and 5.41 nmoles/mg protein/min, respectively. The addition of 1% DMSO did not alter the rates of microsomal glucuronidation.

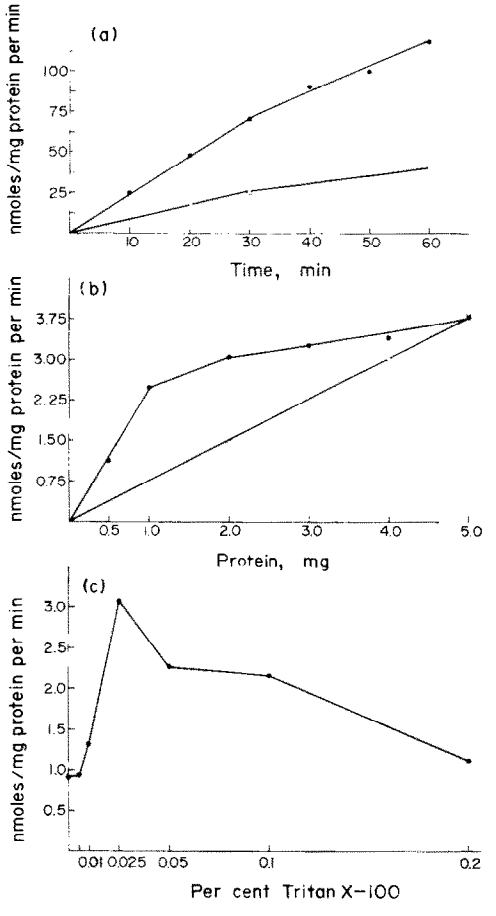

Fig. 1. A,B,C. Glucuronidation of 0.5 mM [^{3}H]acetaminophen in mouse microsomes. A. Time course in unactivated (O——O) and Triton X-100, 0.025% activated (●——●) microsomes, 2 mg protein/ml. B. Varying protein concentrations in unactivated (O——O) and Triton X-100, 0.025% activated (●——●) microsomes. C. Effect of varying Triton X-100 concentrations at microsomal concentration of 2 mg/ml. Values are means of duplicates for each point.

Table 1. Effects of pretreatment on 0.5 mM [^{3}H]acetaminophen glucuronidation by microsomes and content of cytochrome P-450 in mice

Pretreatment	pHAA glucuronidation		Cytochrome P-450 (P-448) (nmoles/mg)
	Unactivated (nmoles/mg/min)	Triton-activated (nmoles/mg/min)	
Control	0.89 ± 0.05	1.89 ± 0.14	0.93 ± 0.10
Phenobarbital	0.97 ± 0.08	1.95 ± 0.08	2.21 ± 0.19
Benzoflavone	1.16 ± 0.10	2.10 ± 0.08	1.15 ± 0.12

Livers from four animals in each treatment group were pooled; values are means ± S.D. of four determinations.

Oxazepam also inhibited the glucuronidation of acetaminophen in MH_1C_1 cells (Fig. 4). Cells without oxazepam metabolized 73.2 nmoles [^{3}H]acetaminophen per mg cell protein in 4 hr starting with a substrate concentration of 0.5 mM. Oxazepam, 0.05 mM and 0.1 mM, reduced this amount to 39.5 and 24.7 nmoles/mg protein, respectively. The final concentration of DMSO was 0.4%; this did not alter the rates of glucuronidation.

The effect of oxazepam on the metabolism of acetaminophen *in vivo* was also studied (Fig. 5). Plasma concentrations of acetaminophen in control animals and in animals injected i.p. with oxazepam were measured after s.c. injection of [^{3}H]acetaminophen; after $\log_{10}$ transformation of acetaminophen concentrations the lines were plotted by the method of least squares. There is evidently a very rapid absorption of acetaminophen, and a fairly rapid decline of acetaminophen concentration in plasma in the control animals up to 60 min after injection (t_1 = 17.6 min); below 5 μg/ml the elimination proceeds more slowly. This biphasic pattern is also seen in oxazepam treated animals. In this situation, the elimination of [^{3}H]acetaminophen is impeded, the plasma t of the rapid phase being 27.0 min. The rate of decline of acetaminophen concentration in oxazepam-treated mice was significantly different from the control ($P < 0.001$) using the Aspin–Welch procedure to calculate degrees of freedom [12].

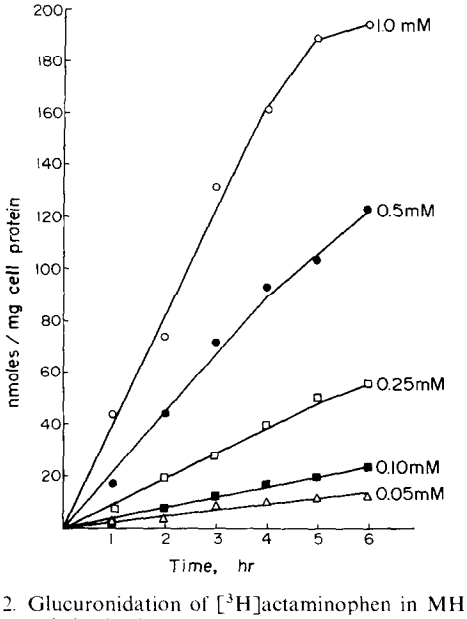

Fig. 2. Glucuronidation of [^{3}H]actaminophen in MH_1C_1 cells. Parallel subcultures were incubated with varying concentrations of [^{3}H]acetaminophen and samples withdrawn at intervals. Values are from two flasks for each concentration of acetaminophen.

DISCUSSION

The microsomal glucuronidation of acetaminophen can be enhanced 3-fold by *in vitro* addition of Triton X-100. Glucuronidation of several phenolic substances is increased by a wide variety of detergents [13, 14, 15], as well as trypsin [16] and phospholipase A [17]. Whether any activation of microsomal UDP-glucuronyl transferase occurs *in vivo* and thus plays a role in the glucuronidation of endogenous substrates or drugs is not known with certainty.

The activity of hepatic glucuronyl transferase in rats is known to increase after pretreatment of animals with phenobarbital, polycyclic hydrocarbons and polychlorinated biphenyls [18, 19, 20]. However, phenobarbital pretreatment does not increase the amount of urinary metabolites of acetaminophen in rats at doses below 400 mg/kg; only at 800 and 1200 mg/kg was an increase in glucuronide excretion and a decrease in sulfate excretion seen [5]. Phenobarbital failed to increase the microsomal glucuronidation of acetaminophen in Swiss–Webster mice; this corresponds well with the effect *in vivo* (Dr. Mitchell, NIH, personal communication). It is also known that

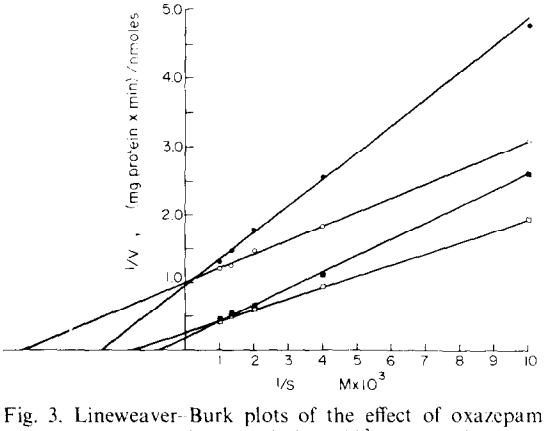

Fig. 3. Lineweaver–Burk plots of the effect of oxazepam on the microsomal glucuronidation of [^{3}H]acetaminophen. Values are means of duplicates for each point. ○---○ Acetaminophen; ●——● acetaminophen + oxazepam, 0.5 mM; □---□ acetaminophen + Triton X-100, 0.025%; ■——■ acetaminophen + Triton X-100, 0.025% + oxazepam, 0.5 mM.

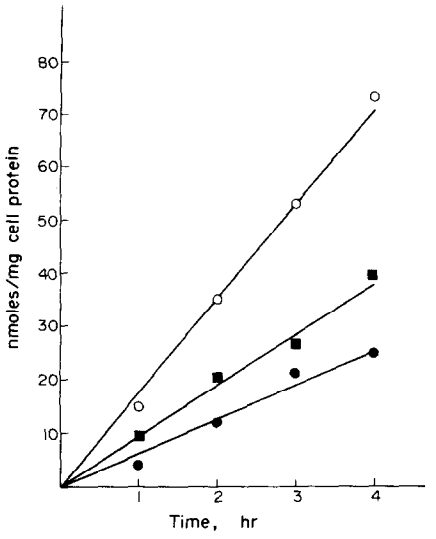

Fig. 4. Effect of oxazepam on the glucuronidation of 0.5 mM [^{3}H]acetaminophen in MH_1C_1 cells. Values represent means from two control cultures (O— —O), two with 0.05 mM oxazepam (■— —■), and two with 0.1 mM oxazepam (●— —●).

benzoflavone is a poor inducer of acetaminophen glucuronidation *in vivo* in this strain (Dr. Mitchell, personal communication).

The finding that MH_1C_1 cells are able to glucuronidate acetaminophen means that these cells must possess both a glucuronyl transferase and enzymes for the synthesis of UDPGA. These cells have previously been shown to glucuronidate bilirubin [21] and phenols [22, 23]. Reuber cells, however, although containing an inducible aryl hydrocarbon hydroxylase [24], were not able to conjugate acetaminophen.

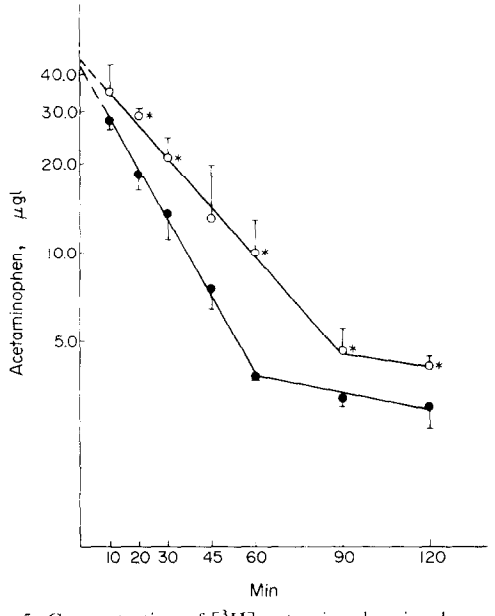

Fig. 5. Concentration of [^{3}H]acetaminophen in plasma of control mice (●— —●) and in oxazepam treated mice (O— —O). (50 mg/kg i.p. 15 min prior to acetaminophen) at various times after the subcutaneous injection of 50 mg/kg. Each point is the mean ± S.D. from four animals. *Significantly different from control value. $P < 0.05$.

Oxazepam is clearly seen to inhibit the microsomal glucuronidation of acetaminophen in mice; the kinetic plots of oxazepam vs. acetaminophen in non-activated microsomes suggest that both drugs have affinity for the same glucuronyl transferase. In activated microsomes the situation is more complex; perhaps the glucuronidation of oxazepam is not enhanced as readily as that of acetaminophen. Most studies with glucuronyl transferase suggest that there is a multitude of similar enzymes with overlapping substrate specificities [25], so that oxazepam and acetaminophen might interact at the level of several transferases.

In the cell culture system also, oxazepam inhibited acetaminophen glucuronidation indicating that the two drugs are eliminated by similar pathways in this whole cell preparation. *p*-Nitrophenol has been shown to inhibit the glucuronidation of *p*-aminophenol and bilirubin both in cultures of MH_1C_1 cells and in homogenates from the same cells fortified with UDPGA [26].

At similar doses oxazepam also inhibited the *in vivo* metabolism of acetaminophen in mice, measured by the rate of disappearance of acetaminophen from plasma, showing that the interaction seen in the two *in vitro* systems has a bearing on the *in vivo* situation. Whether a similar interaction will take place at therapeutic concentrations of the two drugs in humans is not known; it could play a role in cases with large overdoses of either of the two compounds when elimination is of importance for the patient. The very rapid absorption of acetaminophen after subcutaneous injection is presumably due to the presence of DMSO.

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