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Protection against Toxic Redox Cycles between Benzo(*a*)pyrene-3,6-quinone and Its Quinol by 3-Methylcholanthrene-Inducible Formation of the Quinol Mono- and Diglucuronide

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SUMMARY

Cytotoxic effects of quinones are thought to be mediated by redox cycles between quinones and quinols whereby reactive oxygen species are generated. The role of glucuronidation in preventing these toxic redox cycles was investigated by using benzo(*a*)pyrene-3,6-quinone and isolated rat hepatocytes or Reuber hepatoma cells (H4IIE). Inhibition of quinol glucuronidation by salicylamide enhanced quinone-dependent oxygen uptake and cytotoxicity. Conjugation of benzo(*a*)pyrene-3,6-quinol was shown to proceed via the 6-monoglucuronide to the diglucuronide. Diglucuronide formation was low in hepatocytes from untreated controls and phenobarbital-treated rats. However, it was highly stimulated (26-fold) in hepatocytes from 3-methylcholanthrene-treated rats and was also high in Reuber hepatoma cells. Kinetic analysis with liver microsomes indicated that 3-methylcholanthrene-stimulated glucuronidation was due to an increased V_{max} of UDP-glucuronosyltransferase which was enhanced 10- and 40-fold or mono- and diglucuronide formation, respectively. These findings suggest that the investigation of quinol glucuronidation (in particular the formation of benzo(*a*)pyrene-3,6-quinol diglucuronide) is a most useful probe for the 3-methylcholanthrene-inducible isoenzyme(s) of UDP-glucuronosyltransferase. Moreover, this isoenzyme may be particularly suited to protect against toxic redox cycles between benzo(*a*)pyrene quinones and quinols.

INTRODUCTION

Quinones are ubiquitous metabolites of carcinogenic aromatic hydrocarbons (1–3). The toxicity of quinones is thought to be mediated by redox cycling between quinones and quinols with the intermediate formation of semiquinone radicals. During these redox cycles, reactive oxygen species are generated overstoichiometrically such as the superoxide radical ($O_2^{\cdot-}$) and singlet oxygen (1, 2, 4; Fig. 1). Recycling of BP[1] quinones has been shown to be responsible for inhibition of cell growth (2) and DNA strand breaks (1). Suggestive evidence was obtained previously that glucuronidation of quinols may prevent redox cycling and thus protect cells from quinone toxicity (5–8). Conjugation with sulfate would similarly be protective. In addition, glutathione is known to react with semiquinones. However, this reaction may not protect against toxicity since, for example, the glutathione conjugate of menadione (2-methyl-1,4-naphthoquinone) has been shown to undergo redox cycling (9). In the present communication, the role of glucuronidation in protecting against redox cycles has been substantiated by using BP-3,6-quinone and isolated rat hepatocytes and Reuber hepatoma (H4IIE) cells. It is shown that (a) glucuronidation proceeds via the 6-monoglucuronide to the diglucuronide, (b) diglucuronide formation is dramatically stimulated by treatment with MC, and (c) inhibition of glucuronidation leads to enhanced quinone toxicity.

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[1] The abbreviations used are: BP, benzo(*a*)pyrene; MC, 3-methylcholanthrene; GT, UDP-glucuronosyltransferase, EC 2.4.1.17; HMPT, hexamethyl phosphoric acid triamide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

MATERIALS AND METHODS

Chemicals

BP-3,6-quinone and 3-hydroxy-BP were obtained from the Chemical Carcinogen Reference Standard Repository, National Institutes of Health, Bethesda, MD. 6-Acetoxy-BP (m.p. 208–209°) was synthesized from BP (10). β-Glucuronidase from rat preputial glands was purified as described (11). All other chemicals were from commercial sources: UDP-[U-^{14}C]glucuronic acid (260 mCi/mmol) and [^{3}H]thymidine (45 Ci/mmol) from Amersham Buchler, Braunschweig, F. R. G.; Brij 58 (a condensate of hexadecyl alcohol with 20 mol of ethylene oxide/mol) from Atlas, Essen, F. R. G.

Treatment of Animals

Male Wistar rats (200–250 g) were used. MC (40 mg/kg; dissolved in olive oil) was given once intraperitoneally and the animals were killed after 4 days. Phenobarbital, sodium salt (100 mg/kg), was given once intraperitoneally, followed by 0.1% (w/v) of the drug in drinking

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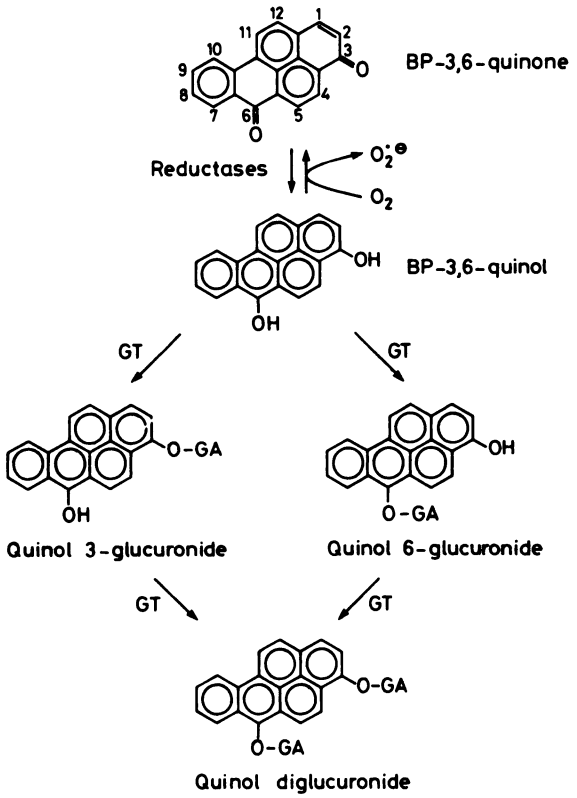

FIG. 1. *Conversion of BP-3,6-quinone to the corresponding quinol glucuronides*

GA, glucuronic acid.

water for 4 days. Liver microsomes were prepared as described (12). Microsomal protein was determined according to Lowry *et al.* (13).

GT Assay (BP-3,6-quinol or BP-3,6-quinol 6-monoglucuronide as Substrate)

Conditions were based on those described previously (7). All experiments with BP metabolites were carried out under subdued light.

Sequential formation of BP-3,6-quinol mono- and diglucuronide. Incubation mixtures (0.5 ml) contained 0.2 M Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.1 M ascorbic acid, microsomal protein (0.05 to 0.2 mg/ml), Brij 58 (0.5 mg/mg of protein), and 25 μM (final concentration) BP-3,6-quinone (dissolved in 5 μl of HMPT). The mixture was preincubated for 5 min at 37° to reduce the quinone to the quinol (80%). Then the reaction was started by addition of UDP-glucuronic acid (3 mM). The reaction was stopped by removing serial aliquots (0.15 ml) and rapidly mixing them with 5 volumes of chloroform. In this way, the quinone and the quinol were completely extracted (>98%). Fluorescence of quinol glucuronides was determined after mixing an aliquot of the aqueous phase (0.05 ml) with 1 ml of 0.4 M glycine-NaOH, pH 10.3 at the following wavelengths of excitation and emission: quinol 6-monoglucuronide, 468 and 530 nm; quinol diglucuronide, 380 and 433 nm. In blanks, UDP-glucuronic acid was omitted. Detection limits of the quinol glucuronides were 5–10 nM, when defined as 2-fold blank fluorescence. Fluorescence was determined using a Perkin-Elmer model 650-10S fluorescence spectrophotometer fitted with a xenon XBO 150-W lamp. Fluorescence intensity was calibrated with quinine sulfate.

Under anaerobic conditions, the quinone can also be completely reduced by an NADPH-regenerating system (7). In the presence of boiled microsomes, formation of BP-3,6-quinol mono- and diglucuronide was not observed.

Diglucuronide formation from BP-3,6-quinol 6-monoglucuronide. BP-3,6-quinol 6-monoglucuronide was formed using the above reaction mixture and liver microsomes from untreated rats (incubation for 30 min). After extraction of the free quinol with chloroform, the reaction was started again by addition of UDP-glucuronic acid (3 mM) and liver microsomes (0.05–0.2 mg/ml). Diglucuronide formation was measured fluorimetrically as described above.

Isolation and Identification of the Mono- and Diglucuronide of BP-3,6-quinol

Glucuronides were synthesized enzymatically in the incubation mixture described above (30-min incubation) in the presence of UDP-[^{14}C] glucuronic acid. Almost exclusive formation of BP-3,6-quinol 6-monoglucuronide or diglucuronide was achieved by using liver microsomes from untreated controls or from MC-treated rats, respectively. The reaction was stopped by an equal volume of methanol. Separation of the two conjugates was possible by thin layer chromatography in ethanol/0.2 M Tris-HCl, pH 9.0 (88:12, v/v) using silica gel 60 F_{254} plastic sheets (Merck, Darmstadt, F. R. G.) which had been prechromatographed with the eluent. Zones containing the fluorescent mono- and diglucuronide (R_f values of 0.5 and 0.25, respectively) were cut out and extracted with 1 ml of 50 mM glycine-NaOH, pH 10.3. Aliquots of the samples were used for determination of both radioactivity of glucuronic acid and fluorescence of the BP moiety. Glucuronic acid was determined from the specific radioactivity of UDP-[^{14}C]glucuronic acid in the incubation mixture. Fluorescence was calibrated by complete hydrolysis of glucuronides to BP-3,6-quinol. Hydrolysis was carried out by incubating the glucuronides with preputial β-glucuronidase (10,000 Fishman units) in the presence of 0.2 M Tris-HCl, pH 7.4, and 0.1 M ascorbic acid for 30 min at 37° under a nitrogen atmosphere. The resulting BP-3,6-quinol was kept in the reduced state by addition of some grains of sodium dithionite. In turn, fluorescence of the quinol was calibrated with known amounts of BP-3,6-quinone which was completely reduced with sodium dithionite under anaerobic conditions. In several experiments, the molar ratio of BP-3,6-quinol to glucuronic acid was found to be 1:1 and 1:2 for the compounds isolated at R_f values of 0.5 and 0.25, respectively, indicating that the two compounds represented mono- and diglucuronides.

Experiments with Isolated Hepatocytes and Reuber Hepatoma Cells

Hepatocytes were isolated as described previously (14, 15) except that $MgSO_4$ in Krebs-Henseleit buffer was replaced by $MgCl_2$, to practically abolish sulfate conjugation. Hepatocytes (1×10^6 cells) or Reuber hepatoma cells (5×10^6 cells) were incubated in 1 ml of sulfate-free Krebs-Henseleit buffer, pH 7.4, containing 5 mM HEPES and 5 mM glucose. BP-3,6-quinone (dissolved in 10 μl of HMPT) was added and glucuronide formation was analyzed in aliquots of the incubation mixture as described under GT assay.

Determination of Cellular Oxygen Uptake

Oxygen uptake was measured polarographically at 37° using a 1-ml water-jacketed cell fitted with a Clark oxygen electrode. Cell concentrations of hepatocytes and Reuber hepatoma cells were 1×10^6 and 5×10^6 cells/ml, respectively. Antimycin A (25 μM final concentration, dissolved in 10 μl of dimethyl sulfoxide) was added to inhibit mitochondrial oxygen uptake. After 2-min preincubation, determination of uptake was started by addition of menadione (in 10 μl of dimethyl sulfoxide) or BP-3,6-quinone (in 10 μl of HMPT).

Culture Conditions of Reuber Hepatoma Cells (H4IIE) (16, 17)

Cells were grown on plastic culture dishes (21 cm^2) at 37° in a humidified atmosphere of 5% CO_2 in air. They were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum, penicillin G (100 units/ml), and streptomycin (100 μg/ml).

Cytostatic Effects of BP-3,6-quinone (2)

Reuber hepatoma cells (1.5×10^5 cells in 3 ml of growth medium) were labeled by addition of [^{3}H]thymidine (0.07 μCi/ml) in the logarithmic phase of growth. At the same time, various amounts of BP-3,6-

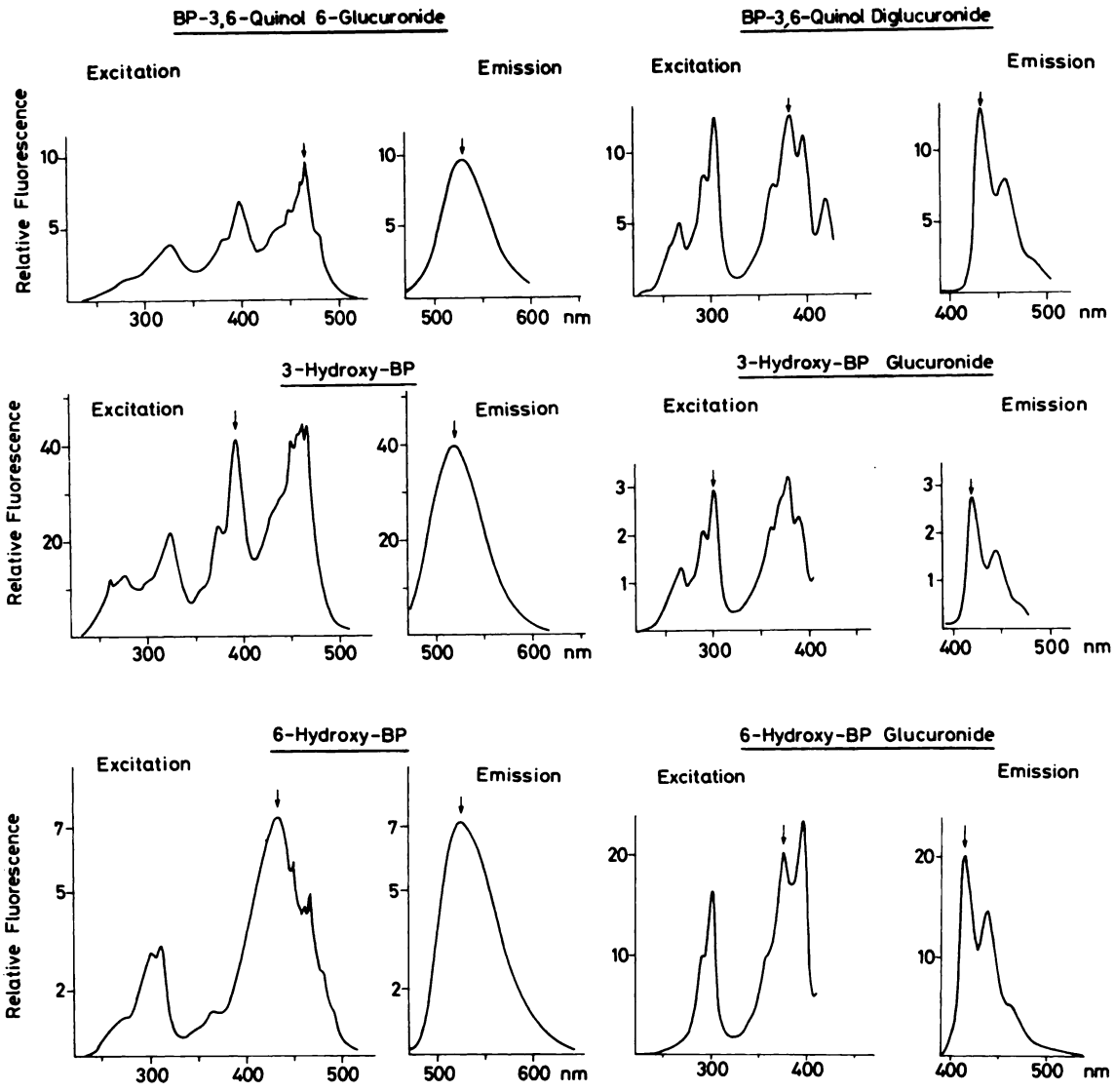

FIG. 2. *Fluorescence spectra of BP-3,6-quinol glucuronides and of corresponding phenols*

Compounds were present at the following concentrations: BP-3,6-quinol monoglucuronide (0.38 μM); BP-3,6-quinol diglucuronide (0.18 μM); 3-hydroxy-BP (0.84 μM); 3-hydroxy-BP glucuronide (0.043 μM); 6-hydroxy-BP (1.0 μM); 6-hydroxy-BP glucuronide (0.34 μM). Spectra were recorded in 0.1 N NaOH/methanol (1:1, v/v). 6-Hydroxy-BP was generated by alkaline hydrolysis of 6-acetoxy-BP under anaerobic conditions. Arrows indicate the wavelengths of excitation and emission selected for recording the spectra and for determination of the compounds.

quinone (dissolved in 5 μl of HMPT) were added. When indicated, 2 mM salicylamide (dissolved in 5 μl of acetone) was also present. Cells were incubated for 22 hr at 37°. Afterwards, the medium was removed. Cytostatic effects were then determined in two ways, (a) by direct determination of cell number and (b) by determining [^{3}H]thymidine incorporation into cellular DNA. In the former, cells were treated with trypsin (0.05%, w/v). After removal of trypsin, the cells were resuspended in phosphate-buffered saline and counted in a hemocytometer. In the latter, cells were washed twice with phosphate-buffered saline. They were subsequently lysed with 0.5% sodium dodecyl sulfate. The lysate was cooled on ice and an equal volume of 20% trichloroacetic acid was added. The ice-cold precipitate was collected on glass filters (Sartorius, Göttingen, F. R. G.). The filters were washed with cold 5% trichloroacetic acid, dried, and counted for radioactivity.

Statistical evaluations were done using Student's *t* test.

RESULTS

Identification and determination of BP-3,6-quinol mono- and diglucuronides. Conjugation of BP-3,6-quinol in both the cellular and microsomal system led to the formation of two quinol glucuronides which could be separated by thin layer chromatography. Labeling of the conjugates with [^{14}C]glucuronic acid revealed that the two conjugates, R_f values of 0.5 and 0.25, contained quinol and glucuronic acid at a molar ratio of 1:1 and 1:2, respectively, indicating that the two compounds represented mono- and diglucuronides. These findings are similar to those reported by Lind (18).

Theoretically, BP-3,6-quinol diglucuronide can be formed either via the 3- or 6-monoglucuronide (Fig. 1). The mono- and diglucuronides could be distinguished and identified by analysis of their fluorescence spectra in alkaline solution in comparison with those of corresponding phenols. As shown in Fig. 2 (left panel), BP-3,6-quinol monoglucuronide as well as 3-hydroxy- and 6-hydroxy-BP show maxima of fluorescence emission at 520–540 nm (phenolate spectra). Conjugation of the free

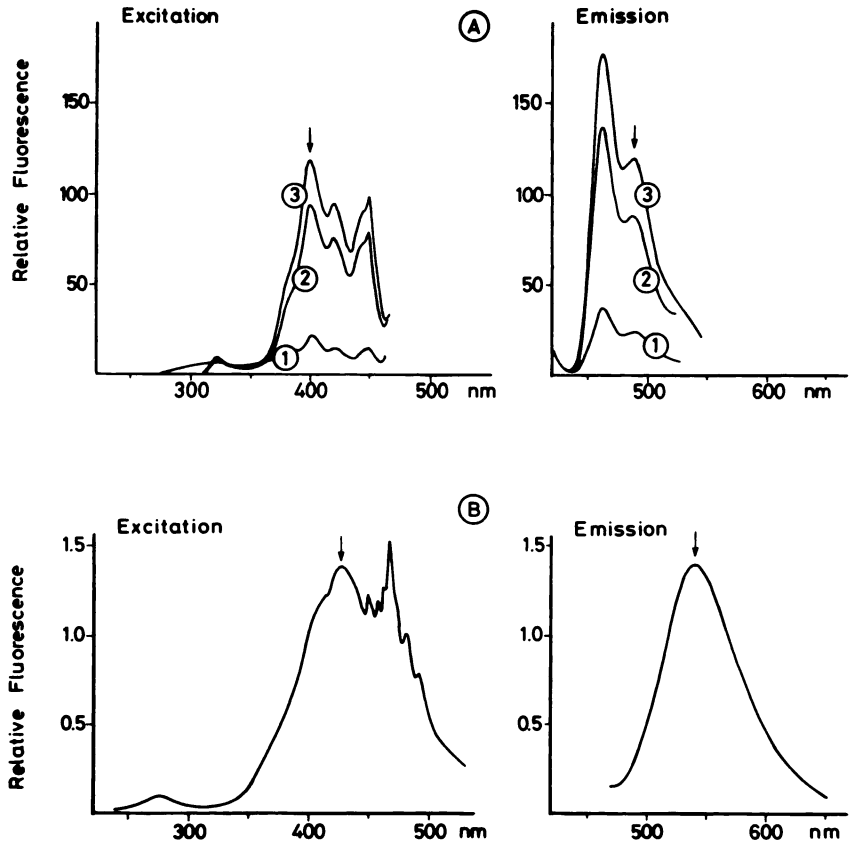

FIG. 3. *Fluorescence spectra of BP-3,6-quinol at pH 7.4 (A) and pH 10.3 (B)*

A, BP-3,6-quinone (25 μM) was reduced in the presence of liver microsomes (0.2 mg of protein/ml). *1*, under aerobic conditions by an NADPH-regenerating system; 2, under aerobic conditions by 0.1 M ascorbate; *3*, under anaerobic conditions by addition of some grains of sodium dithionite. B, BP-3,6-quinone (1.7 μM) was added in 5 μl of HMPT to methanol/0.4 M glycine-NaOH, pH 10.3 (1:1, v/v), and reduced by sodium dithionite as described under A, *3*.

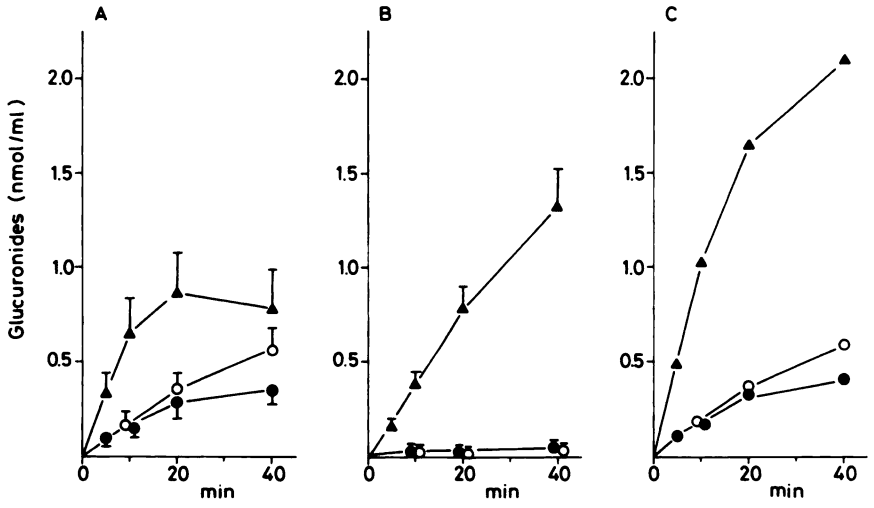

FIG. 4. *Conversion of BP-3,6-quinone to the corresponding quinol mono- (A) and diglucuronide (B) in isolated hepatocytes*

The sum of BP-3,6-quinol mono- and diglucuronide formation is listed in C. Hepatocytes (1×10^6 cells/ml) from untreated controls (●) and from phenobarbital-treated (○) and MC-treated rats (▲) were incubated with BP-3,6-quinone (25 μM). Values represent the means ± standard deviation of four experiments.

phenolic group leads to a blue shift of fluorescence emission (Fig. 2, right panel). This blue shift is also seen when the fluorescence spectrum of BP-3,6-quinol is recorded at pH 7.4 instead of pH 10.3 (Fig. 3, A and B, respectively). By comparing the excitation spectra, it is obvious that the spectrum of BP-3,6-quinol monoglucuronide resembles that of 3-hydroxy-BP but differs from that of 6-hydroxy-BP since in the spectrum of 6-hydroxy-BP the excitation maxima at about 375 and 395 nm are lacking. Therefore, it is conceivable that the 6-hydroxy

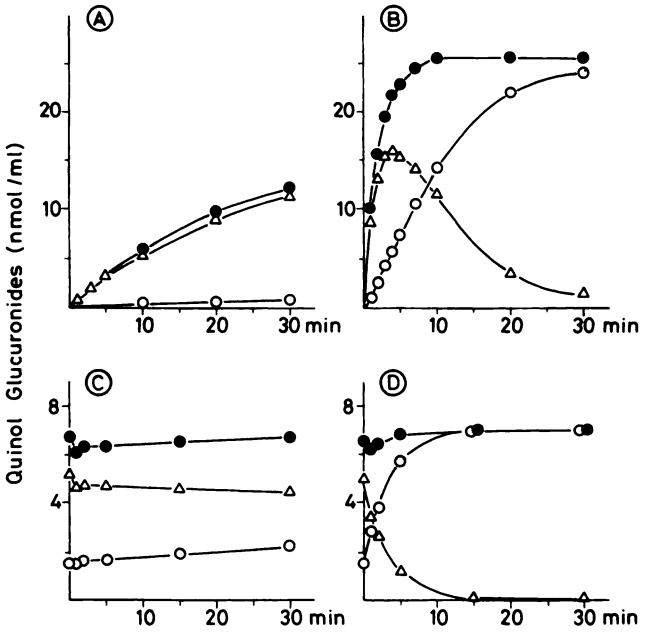

FIG. 5. *Glucuronidation of BP-3,6-quinol to the mono- and diglucuronide (A and B) and of BP-3,6-quinol monoglucuronide to the diglucuronide (C and D) in liver microsomes from untreated controls (A and C) and from MC-treated rats (B and D)*

Symbols indicate BP-3,6-quinol monoglucuronide (Δ), BP-3,6-quinol diglucuronide (○), and total glucuronides (●). Incubation mixtures contained 0.2 mg of microsomal protein/ml.

TABLE 1

Kinetic analysis of BP-3,6-quinol mono- and diglucuronide formation in liver microsomes from untreated and MC-treated rats

BP-3,6-quinone was reduced (>95%) to the corresponding quinol in the presence of NADPH and microsomes under a nitrogen atmosphere. Thereafter, glucuronidation was started by the addition of UDP-glucuronic acid (UDPGA). $K_{m,quinol}$ and $K_{m,UDPGA}$ were determined at a fixed concentration of 3 mM UDPGA and 25 μM BP-3,6-quinol, respectively.

Reaction and treatment	Kinetic constants		
	$K_{m,quinol}$	$K_{m,UDPGA}$	V_{max}
	mM		*nmol/min/mg protein*
Monoglucuronide formation			
None	0.01	0.3	5.8
MC	0.02	0.1	60
Diglucuronide formation			
None	0.05	2.1	0.17
MC	0.04	1.4	6.4

position of BP-3,6-quinol monoglucuronide is conjugated and the 3-hydroxy position is free. Hence, the reaction sequence appears to proceed almost exclusively via the 6-monoglucuronide to the diglucuronide.

Fluorescence of BP-3,6-quinol glucuronides was calibrated on the basis of BP-3,6-quinol liberated by complete hydrolysis of the conjugates. In turn, calibration of BP-3,6-quinol fluorescence was achieved by complete reduction of the corresponding quinone which was used as standard. This was only possible with sodium dithionite under anaerobic conditions (1). It was desirable, however, to achieve almost complete reduction of the quinol under aerobic conditions at pH 7.4. This could be accomplished by addition of ascorbate to the reaction mixture (Fig. 3A). Only under anaerobic conditions, an NADPH-regenerating system led to complete reduction. Calibration of fluorescence was supported by the experiments with labeled UDP-glucuronic acid.

Influence of phenobarbital and MC on BP-3,6-quinol glucuronidation in isolated hepatocytes and Reuber hepatoma cells. The formation of BP-3,6-quinol mono- and diglucuronide was measured fluorimetrically using the wavelengths of excitation and emission indicated in Fig. 3. As shown in Fig. 4A, formation of BP-3,6-quinol monoglucuronide was clearly detectable in hepatocytes from untreated controls (control hepatocytes) and from phenobarbital-treated rats (phenobarbital hepatocytes) but it was markedly stimulated (4-fold) in hepatocytes from MC-treated rats (MC hepatocytes). Surprisingly, diglucuronide formation was low in control and phenobarbital hepatocytes but it was dramatically stimulated (26-fold) in MC hepatocytes (Fig. 4B). Total glucuronide formation is shown in Fig. 4C. It was increased 6-fold in MC hepatocytes. Reuber hepatoma cells formed both mono- and diglucuronides at a rate comparable to that found in MC hepatocytes (not shown).

Kinetic analysis of BP-3,6-quinol glucuronidation in rat liver microsomes. Similar to the observations with isolated hepatocytes, formation of BP-3,6-quinol diglucuronide was low in microsomes from untreated controls (Fig. 5A). Both mono- and diglucuronide formation was markedly stimulated in microsomes from MC-treated rats (Fig. 5B). The rapid decline in the concentration of the monoglucuronide and the concomitant increase of the diglucuronide are indicative of a precursor-product relationship. Interestingly, there was a linear increase of the concentration of the diglucuronide without any lag phase, suggesting that the apparent K_m for diglucuronide formation was very low. From data similar to those listed in Fig. 5, initial rates of mono- and diglucuronide formation were determined and analyzed kinetically. As shown in Table 1, MC treatment did not markedly alter the apparent K_m of the quinol and of UDP-glucuronic acid but highly stimulated V_{max} for monoglucuronide formation (10-fold) and diglucuronide formation (38-fold). The strong stimulation of diglucuronide formation can also be calculated from the data listed in Fig. 5, C and D. With BP-3,6-quinol monoglucuronide as substrate, GT activity was 0.11 and 5.0 nmol/min/mg of protein in microsomes from untreated controls and from MC-treated rats, respectively. Despite the low concentration of the monoglucuronide (about 7 μM), GT activities were close to the values of V_{max} for diglucuronide formation listed in Table 1.

Toxicological implications of BP-3,6-quinol glucuronidation. Quinone-dependent redox cycles can be estimated from cellular extra-oxygen uptake (Fig. 6). Quinone-dependent oxygen uptake appeared to be higher in the absence than in the presence of antimycin A, suggesting that BP-3,6-quinone may stimulate mitochondrial respiration (Fig. 6A). Quinone-dependent oxygen uptake was therefore measured in the presence of antimycin A. Based on cell counts, quinone-dependent oxygen uptake appeared to be higher in hepatocytes (Fig. 6B) than in

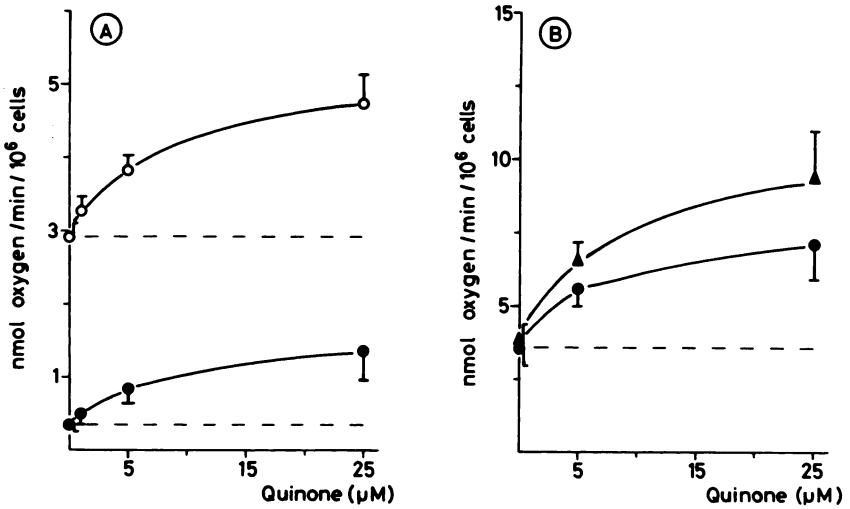

FIG. 6. *Quinone-dependent oxygen uptake in Reuber hepatoma cells (A) and isolated hepatocytes (B)*
Oxygen uptake was determined with BP-3,6-quinone (○,●) and menadione (▲) and in the absence (open symbols) and presence of antimycin A (closed symbols). Values represent the means ± standard deviation of four experiments.

TABLE 2

BP-3,6-quinol-dependent oxygen uptake and quinol conjugation in Reuber hepatoma cells: effects of inhibition of glucuronidation by salicylamide

Reuber hepatoma cells (5×10^6 cells/ml) were incubated for 1 hr with BP-3,6-quinone (5 μM) in the presence or absence of salicylamide (4 mM). Subsequently, aliquots of the incubation mixture were used for determination of oxygen uptake in the presence of antimycin A (25 μM) and for the determination of quinol glucuronides. Values represent the means ± standard deviation of three experiments.

Conditions	Oxygen uptake		Formation of BP-3,6-quinol glucuronides		
	Total	Quinone dependent	6-Monoglucuronide	Diglucuronide	Total
	nmol/min/10⁶ cells		*nmol/ml/hr*		
−Quinone	0.4 ± 0.1				
+Quinone	0.6 ± 0.1	0.2	1.5 ± 0.3	1.8 ± 0.1	3.3
−Quinone, +salicylamide	0.5 ± 0.1				
+Quinone, +salicylamide	1.1 ± 0.2	0.6	1.3 ± 0.4	0.4 ± 0.1	1.7

Reuber hepatoma cells. However, this does not take into account the 8-fold greater volume of hepatocytes. Oxygen consumption was also determined in the presence of menadione, a quinone which is often used in studies of redox cycling (4). Menadione-dependent oxygen consumption was slightly but significantly higher than that found with BP-3,6-quinone.

The role of glucuronidation in protecting against the toxic effects of quinones was studied using salicylamide, which is a selective inhibitor of glucuronidation and sulfation in BP metabolism studies without affecting total BP metabolism (3, 19). Since sulfate formation cannot be detected either in hepatocytes incubated in the absence of sulfate or in Reuber hepatoma cells (20), effects of salicylamide mostly reflect inhibition of glucuronidation. When hepatoma cells were incubated for 1 hr in the presence of salicylamide, glucuronide formation was lower due to inhibition of diglucuronide formation (Table 2). Monoglucuronide formation was not significantly inhibited. Quinone-dependent oxygen uptake was higher in the presence of salicylamide, probably due to the higher quinone concentration in cells after 1-hr incubation under conditions of reduced glucuronidation.

Quinone-dependent oxygen uptake does not necessarily implicate an accumulation of superoxide radicals because they may be efficiently inactivated, for example, by superoxide dismutase and catalase. Therefore, the toxic effect of BP-3,6-quinone was measured directly by studying possible cytostatic effects in Reuber hepatoma cells (Fig. 7). Cytostatic effects were measured both by measuring the incorporation of [^{3}H]thymidine into cellular DNA and by direct cell counting (2). Under our conditions, BP-3,6-quinone in the absence of salicylamide did not exhibit cytostatic effects in the dose range studied (Fig. 7). However dose-dependent cytotoxicity was obvious when glucuronidation of the quinol was inhibited by salicylamide. This effect was supported by direct cell counting, showing that cell numbers were reduced by 50% when cells were grown in the presence of both BP-3,6-quinone (1 μg/ml) and salicylamide (2 mM).

DISCUSSION

Previous studies suggested that a MC-inducible isoenzyme of GT is particularly suited to protect against toxic redox cycles between BP-3,6-quinone and the cor-

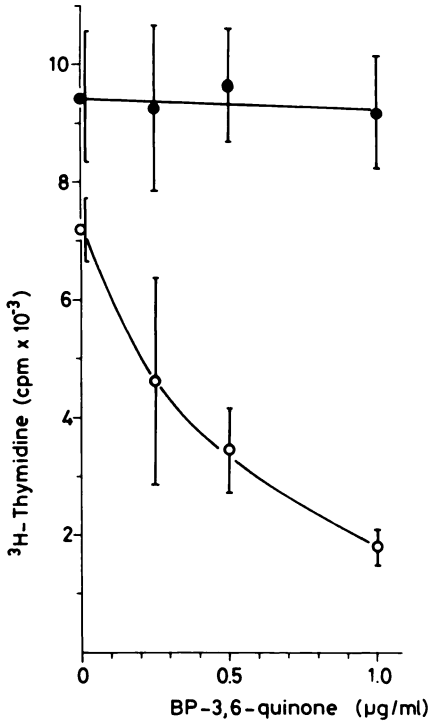

FIG. 7. *Effects of BP-3,6-quinone on [^{3}H]thymidine incorporation into DNA of Reuber hepatoma cells*

Effects were measured in the presence (○) and absence (●) of 2 mM salicylamide. Values represent the means ± standard deviation of four experiments.

responding quinol (6, 7). These suggestions have been substantiated in the present investigation. Inhibition of glucuronidation by salicylamide led to enhanced quinone-dependent oxygen uptake and enhanced cytotoxicity of BP-3,6-quinone. Sulfate formation, which is also inhibited by salicylamide, cannot account for the inhibition of redox cycles since under our conditions (incubation of hepatocytes in the absence of sulfate or use of Reuber hepatoma cells) sulfate ester formation cannot be demonstrated (20).[2]

Surprisingly, glucuronidation of BP-3,6-quinol proceeded in a reaction sequence via the 6-monoglucuronide to the diglucuronide. The preference of the 6-hydroxy position may be due to the higher reactivity of this group, which is also seen when comparing 3-hydroxy-BP and 6-hydroxy-BP as substrates of rat liver microsomal GT (21). In isolated hepatocytes from untreated controls and from phenobarbital-treated rats, the monoglucuronide accumulated. However, the monoglucuronide was readily converted to the diglucuronide in liver microsomes from MC-treated rats (Fig. 5). Kinetic analysis of the reaction indicated that increased diglucuronide formation was mostly due to an increased V_{max} (Table 1), suggesting that increased glucuronidation was not due to alterations of the architecture of the enzyme in the membrane but due to an increased enzyme level. A MC-inducible isoenzyme of GT, conjugating mostly planar phenols, such as 4-nitrophenol or 1-naphthol, has been characterized previously (22, 23). Suggestive evidence was obtained that the MC-inducible isoenzyme may be particularly suitable for the elimination and inactivation of phenolic metabolites of BP (19, 24). Induction factors observed with simple phenols were in the range of 3- to 5-fold (22, 23). However, V_{max} of diglucuronide formation was increased about 40-fold. This high induction factor was observed when diglucuronide formation was studied both with BP-3,6-quinol or BP-3,6-quinol monoglucuronide as substrate (Fig. 5). It has been demonstrated previously that phenols such as 1-naphthol are overlapping substrates for several isoenzymes of GT (23). Hence, BP-3,6-quinol monoglucuronide may be a more selective substrate for the MC-inducible isoenzyme. On the other hand, there may be more than one MC-inducible isoenzyme of GT.

In fact "immunoblotting" of GT isoenzymes using polyclonal rabbit antibodies against the MC-inducible isoenzyme suggests that two polypeptides at M_r = 54,000 and 56,000 are MC-inducible (25). However, more work is needed to correlate functional and molecular properties of these proteins. In any case, the assay of quinol glucuronidation (in particular the formation of BP-3,6-quinol diglucuronide) may be a most useful probe to characterize the MC-inducible isoenzyme(s) of GT.

As demonstrated by the high rates of diglucuronidation by Reuber hepatoma cells, these cells appear to express a GT isoenzyme with functional properties similar to those of the MC-inducible isoenzyme of rat liver, operationally termed GT_1 (22). These findings are in line with a permanent increase of GT_1 activities in putative preneoplastic liver foci, hyperplastic nodules, and hepatomas (26, 27). MC inducibility of this isoenzyme may also be interesting with regard to other MC-inducible drug-metabolizing enzymes. It may not be fortuitous that three functionally related enzymes, involved in the metabolism of aromatic hydrocarbons, appear to be regulated coordinately by the *Ah* locus (28), namely cytochrome P_1-450 (responsible for the oxidation of BP to phenols and quinones), DT diaphorase or quinone reductase (responsible for 2-electron reduction of quinones to quinols), and GT, which is efficiently conjugating quinols.

Similar to GT isoenzymes involved in the conjugation of steroid hormones (29), the MC-inducible isoenzyme of GT appears to be remarkably regioselective in the conjugation of BP phenols. Whereas the conjugation of 3-hydroxy-BP was stimulated 5-fold in liver microsomes from MC-treated rats, the conjugation of 9-hydroxy-BP was only moderately (1.6-fold) increased (30). Regioselectivity is also apparent in the conjugation of BP-3,6-quinol. This compound may be an overlapping substrate for several isoenzymes of GT. It is conceivable that the MC-inducible isoenzyme first catalyzes the conjugation of BP-3,6-quinol at the 6-hydroxy position and then, after a change of the position of the molecule, conjugates the 3-hydroxy position. The glucuronic acid moiety at the 6-hydroxy position obviously does not hinder further conjugation. Hence, BP quinols may be fascinating substrates not only because of their functional importance but also as most useful probes to identify MC-inducible isoenzymes of GT and for the exploration of the properties of the substrate-binding site.

[2] Unpublished results.

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