CONVERSION OF BENZO[a]PYRENE-3,6-QUINONE TO QUINOL GLUCURONIDES WITH RAT LIVER MICROSOMES OR PURIFIED NADPH-CYTOCHROME *c* REDUCTASE AND UDP-GLUCURONOSYLTRANSFERASE

K. W. BOCK, W. LILIENBLUM and H. PFEIL

Department of Pharmacology and Toxicology, University of Göttingen, Kreuzbergring 57, 3400 Göttingen, FRG

Received 5 September 1980

1. Introduction

Polycyclic aromatic hydrocarbons such as benzo[a]pyrene (BP) exert their toxic effects after metabolic activation. A delicate balance between activating and detoxifying enzyme reactions may be decisive for the accumulation of reactive intermediates in cells and may explain in part the organ specificity of toxic effects. UDP-glucuronosyltransferase (GT, EC 2.4.1.17) is one of the enzymes conjugating intermediary phenols and dihydrodiols of BP [1–3], thus preventing direct cytotoxicity of these intermediates and recycling to ultimate cytotoxins, mutagens, and carcinogens [4]. BP quinones, a third major class of primary metabolites, can be readily conjugated with glucuronic acid after reduction to their quinols [5,6]. BP quinones may cause cytotoxicity in many ways, for example, after reduction to semiquinones or by oxidation–reduction cycles with continuous production of H_2O_2, $O_2^{\cdot-}$ and $OH^{\cdot}$ [7]. In addition quinones strongly inhibit BP oxidation [6]. When inhibitory quinones are removed by stimulation of conjugation of the corresponding quinols, BP monooxygenase activity is increased [6,8].

GT probably consists of a family of closely related but functionally heterogeneous enzyme forms. Two forms are differentially inducible in rat liver by inducing agents such as 3-methylcholanthrene (MC) or phenobarbital [9,10]. They can be separated and purified to apparent homogeneity [11] and show different developmental patterns [12] and tissue distributions [3]. The methylcholanthrene-inducible form of GT is operationally called GT_1 [3]. All enzyme forms are latent in the intact microsomal membrane. They can be activated by UDP-*N*-acetylglucosamine, which is probably a physiological activator, and can be fully activated by various alterations of the membrane structure, e.g., by the addition of detergents [13]. To be able to distinguish induction from activation, enzyme induction is usually studied in the fully activated state.

Because of the complexity of GT activities it was necessary to investigate which form of GT is responsible for the conjugation of BP quinols. We demonstrate that isolated GT_1 and NADPH-cytochrome *c* reductase from rat liver, both purified to apparent homogeneity, readily form BP-3,6-quinol glucuronides from BP-3,6-quinone. Using a sensitive fluorimetric assay for the detection of BP-3,6-quinol glucuronides, rates of conversion obtained with the purified enzymes are compared with those determined with the membrane-bound enzymes from liver microsomes.

2. Materials and methods

Previously described methods were used for treatment of rats in vivo with MC or phenobarbital, for preparation of liver microsomes, for assay of GT activity with 1-naphthol as substrate [14], and for purification of GT_1 to apparent homogeneity [11]. The GT preparation may be similar to that in [15]. Purified GT_1 (6000 mU/mg protein) was stored at 4°C after addition of egg lecithin dispersions (0.1 mg/ml enzyme solution). Under these conditions the enzyme was stable for 4 weeks with 25% loss of enzyme activity. NADPH-cytochrome *c* reductase was isolated from trypsin-treated microsomes and purified according to [16] to spec. act. 4000 mU/mg protein. One unit is the enzyme activity which metabolizes 1 μmol sub-

strate/min at 37°C (25°C for NADPH-cytochrome *c* reductase).

Conversion of BP-3,6-quinone to its quinol glucuronides: BP-3,6-quinone was dissolved in hexamethylphosphoramide and incubated at 0.05 mM with fresh liver microsomes (0.2 mg protein) or purified enzymes (amounts specified in table 1), Tris–HCl (pH 7.4) (0.1 M), $MgCl_2$ (5 mM), NADP (0.5 mM), sodium isocitrate (5 mM), isocitrate dehydrogenase (100 mU; Boehringer, Mannheim), and UDP-glucuronic acid (3 mM) in 0.5 ml. Activators of the microsomal enzyme (Brij 58, 0.05% (v/v) or UDP-*N*-acetylglucosamine, 3 mM) were added when indicated. At 0, 5, 10 and 20 min 0.1 ml aliquots were taken from the incubation mixture. Unmetabolized quinone and quinol were extracted with 1 ml $CHCl_3$. After addition of 0.1 ml methanol and 1.0 ml 0.4 M glycine buffer (pH 10) to 0.05 ml of the aqueous phase, fluorescence of the quinol glucuronides was determined.

Fluorescence was detected at 433 nm with excitation at 400 nm using a Perkin-Elmer 650-10 S fluorescence spectrophotometer (fig.1). Interference in the fluorescence spectrum by BP-3,6-quinol at pH 10 (obtained by addition of sodium dithionite to a BP-3,6-quinone solution) is not possible since the quinol spectrum is quite different (maxima of excitation and emission at 430 nm and 535 nm, respectively). Zero time blanks were subtracted. The fluorescence was quantified in a parallel incubation using [^{14}C]BP-3,6-quinone prepared by enzymatic conversion of [7,10-^{14}C]BP (Amersham) followed by isolation of [^{14}C]BP-3,6-quinone by HPLC [17]. The HPLC gave a clean separation of BP-3,6-quinone from BP-1,6-quinone. Conversion rates increased linearly up to 0.2 mg microsomal protein and up to 20 min. No change of the fluorescence spectrum was observed during 2 h incubation and after storage of the incubation mixture overnight at 0°C. It remains to be established at which position of the benzo[a]pyrene ring (3-, 6- or both) the glucuronic acid moiety is attached.

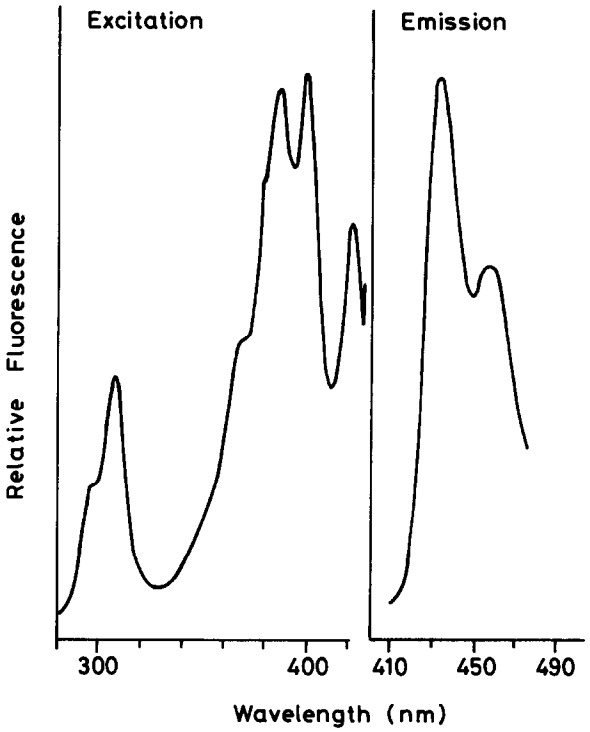

Fig.1. Fluorescence spectrum of benzo[a]pyrene-3,6-quinol glucuronides. The fluorescence spectrum of quinol glucuronides in the water/methanol phase at pH 10 was recorded as in section 2. The excitation spectrum was recorded at 433 nm emission wavelength, the emission spectrum at 400 nm excitation wavelength.

3. Results and discussion

With rat liver microsomes BP-3,6-quinone could be converted to its quinol glucuronides to the extent of 75–98% (fig.2, I). Addition of purified NADPH-cytochrome *c* reductase to microsomes enhanced the conversion rate with microsomes, whether activated by detergent (cf. b with a) or by UDP-*N*-acetylglucosamine (cf. d with c). The metabolic pathway is unusual since the intermediary quinol is rapidly autoxidized in the presence of oxygen [7]. Hence the steady state concentration of the quinol should be rather low. Since NADPH-cytochrome *c* reductase was isolated from trypsin-treated microsomes it had lost the hydrophobic protein segment which is essential for attachment to the microsomal membrane [18]. Nevertheless quinol generated by the soluble reductase could be readily conjugated by the membrane-bound GT. Conversion of the quinone to its quinol glucuronides could also be demonstrated with isolated GT_1 and NADPH-cytochrome *c* reductase, purified to apparent homogeneity (fig.2, II; table 1). The reaction was linear for a remarkably long time period. Decreasing the quantity of reductase only slightly affected glucuronide formation; increasing GT_1 proportionally enhanced it. Hence GT activity appeared to be the major determinant of the overall conversion rate.

In addition to NADPH-cytochrome *c* (P450) reduc-

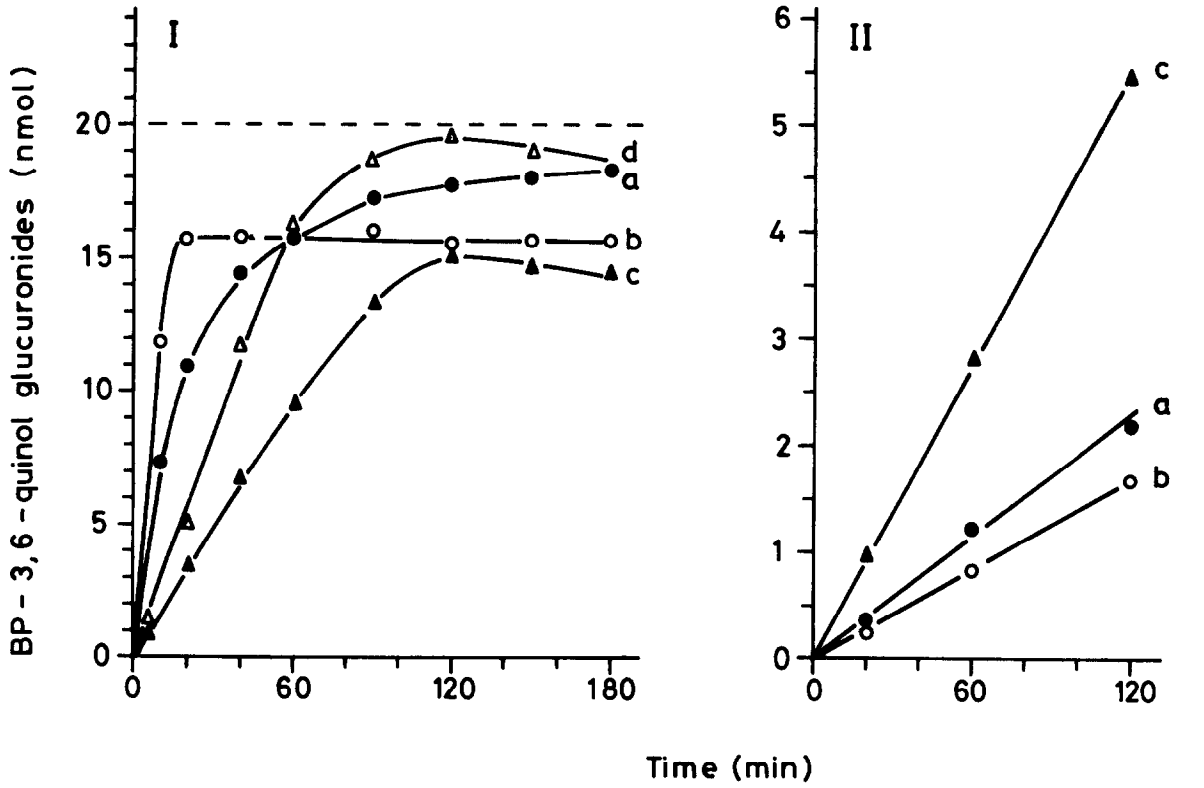

Fig.2. Time course of the conversion of benzo[a]pyrene-3,6-quinone to its glucuronides with rat liver microsomes (I) or purified NADPH-cytochrome *c* reductase and UDP-glucuronosyltransferase (II). Microsomes from MC-treated rats, activated by Brij 58 (a,b) or UDP-*N*-acetylglucosamine (c,d), were incubated with benzo[a]pyrene-3,6-quinone (20 μM). In b and d, purified NADPH-cytochrome *c* reductase (700 mU) was also present. The initial amount of benzo[a]pyrene-3,6-quinone present in the incubation mixture is indicated by the dashed line. In II, purified NADPH-cytochrome *c* reductase and GT_1 (amounts specified in table 1) were incubated with 50 μM benzo[a]pyrene-3,6-quinone (a); in (b) the quantity of the reductase was reduced to 1/5, and in (c) the amount of GT was increased 2.5-fold.

tase (EC 1.6.2.4) liver microsomes (and cytosol) contain another quinone reductase, DT diaphorase (19, EC 1.6.99.2). In contrast to NADPH-cytochrome *c* reductase (inducible by phenobarbital), DT diaphorase, like GT_1, is preferentially inducible by MC [20]. Hence the quantities of the two reductases and of GT_1 can be markedly altered by treatment with inducing agents. Rates of conversion of BP-3,6-quinone to its quinol glucuronides are correlated with, but not proportional to, GT_1 activity after treatment with inducers and activators of GT (table 1). The conversion rate found with microsomes from MC-treated rats was over-proportionally increased, suggesting that not only GT_1 but also DT diaphorase may be involved in the stimulated conversion. The significance and mechanism of GT activation is still under debate [13].

Our results demonstrate that GT_1, the MC-inducible form of GT, is mainly responsible for the conjugation of BP-3,6-quinol and probably other BP quinols. The activity of GT_2 towards BP-3,6-quinol has not yet been tested. MC-inducible enzymes such as BP monooxygenase, DT diaphorase, and GT_1 are metabolically coordinated in the formation and further metabolism of BP quinones. As shown with inbred strains of mice, MC-inducibility of these three enzymes is inherited as a single genetic trait [21,22]. Coordinated induction of these enzymes may facilitate BP detoxication and elimination. It remains to be shown, however, to what extents these enzyme reactions occur under in vivo conditions.

Table 1
Initial rates of conversion of benzo[a]pyrene-3,6-quinone to its glucuronides under various conditons

Experimental conditions	Conversion of BP-3,6--quinone to its quinol glucuronides	GT_1 activity towards 1-naphthol	NADPH-cytochrome *c* reductase
Microsomes		(mU/mg protein)	
Untreated controls:			
Native	0.009 ± 0.001	2.4 ± 0.6	40 ± 3
+ UDP-*N*-acetylglucosamine	0.014 ± 0.001	7.9 ± 1.3	
+ Brij 58	0.039 ± 0.003	61 ± 15	
Phenobarbital-treatment:			
Native	0.013 ± 0.003	3.6 ± 0.9	94 ± 20
+ UDP-*N*-acetylglucosamine	0.020 ± 0.005	9.4 ± 3.6	
+ Brij 58	0.063 ± 0.015	85 ± 20	
MC-treatment:			
Native	0.26 ± 0.10	8.1 ± 2.0	49 ± 3
+ UDP-*N*-acetylglucosamine	0.43 ± 0.10	14.6 ± 3.1	
+ Brij 58	2.00 ± 0.21	249 ± 46	
NADPH-cytochrome *c* reductase + GT_1:		(mU/ml)	
Complete	0.04	11.8	181
Reductase, 1/5	0.03	11.8	36
GT_1, 2.5-fold	0.10	29.7	181

GT_1 activity towards 1-naphthol and NADPH-cytochrome *c* reductase activity are given for comparison. The experiment with the purified enzymes was repeated once with similar results. The mean ± SD of 4 expt with microsomes is listed

Acknowledgements

We thank Miss S. Zachmann and Miss U. Heine for expert technical assistance and Dr R. F. Volp for help in preparing the English manuscript. Supply of benzo-[a]pyrene-3,6-quinone by the ITT Research Institute (Chicago, IL) and financial support by the Stiftung Volkswagenwerk are gratefully acknowledged.

References

[1] Nemoto, N. and Gelboin, H. V. (1976) Biochem. Pharmacol. 25, 1221–1226.
[2] Bock, K. W. and Lilienblum, W. (1979) Biochem. Pharmacol. 28, 695–700.
[3] Bock, K. W., Clausbruch, U. C. v., Kaufmann, R., Lilienblum, W., Oesch, F., Pfeil, H. and Platt, K. L. (1980) Biochem. Pharmacol. 29, 495–500.
[4] Sims, P., Grover, P. L., Swaisland, A., Pal, K. and Hewer, A. (1974) Nature 252, 326–328.
[5] Lind, C., Vadi, H. and Ernster, L. (1978) Arch. Biochem. Biophys. 190, 97–108.
[6] Fahl, W. E., Shen, A. L. and Jefcoate, C. R. (1978) Biochem. Biophys. Res. Commun. 85, 891–899.
[7] Lorentzen, R. J. and Ts'o, P. O. P. (1977) Biochemistry 16, 1467–1473.
[8] Bock, K. W. (1978) Naunyn-Schmiedeberg's Arch. Pharmacol. 304, 77–79.
[9] Bock, K. W., Fröhling, W., Remmer, H. and Rexer, B. (1973) Biochim. Biophys. Acta 327, 46–56.
[10] Wishart, G. J. (1978) Biochem. J. 174, 671–672.
[11] Bock, K. W., Josting, D., Lilienblum, W. and Pfeil, H. (1979) Eur. J. Biochem. 98, 19–26.
[12] Wishart, G. J. (1978) Biochem. J. 174, 485–489.
[13] Dutton, G. J. (1975) Biochem. Pharmacol, 24, 1835–1841.
[14] Bock, K. W. and White, I. N. H. (1974) Eur. J. Biochem. 46, 451–459.
[15] Burchell, B. (1978) Biochem. J. 173, 749–757.
[16] Omura, T., Siekevitz, P. and Palade, G. E. (1967) J. Biol. Chem. 242, 2389–2396.
[17] Selkirk, J. K., Croy, R. G., Roller, P. P., Gelboin, H. V. (1974) Cancer Res. 34, 3473–3480.
[18] Ito, A. and Sato, R. (1969) J. Cell, Biol. 40, 179–189.
[19] Ernster, L., Danielson, L. and Ljunggren, M. (1962) Biochim. Biophys. Acta 58, 171–188.
[20] Lind, C. and Ernster, L. (1974) Biochem. Biophys. Res. Commun. 56, 392–400.
[21] Owens, I. S. (1977) J. Biol. Chem. 252, 2827–2833.
[22] Kumaki, K., Jensen, N. M., Shire, J. G. M. and Nebert, D. W. (1977) J. Biol. Chem. 252, 157–165.