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RADIATION INACTIVATION ANALYSIS OF MICROSOMAL UDP-GLUCURONOSYLTRANSFERASES CATALYSING MONO- AND DIGLUCURONIDE FORMATION OF 3,6-DIHYDROXYBENZO(*a*)PYRENE AND 3,6-DIHYDROXYCHRYSENE

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Abstract—Indirect evidence has suggested that multiple subunits of microsomal UDP-glucuronosyltransferases (UGTs) are involved in diglucuronide formation of diphenols of polycyclic aromatic hydrocarbons (Bock *et al.*, *Mol Pharmacol* **42:** 613–618, 1992). To substantiate this suggestion functional target sizes of UGTs catalysing these reactions were determined in microsomes *in situ* by radiation inactivation analysis. Target sizes of UGTs catalysing the glucuronidation of 1-naphthol and 6-hydroxychrysene were found to be 91 ± 29 and 120 ± 27 kDa, respectively. However, target sizes for mono- and diglucuronide formation of 3,6-dihydroxybenzo(*a*)pyrene were 118 ± 33 and 218 ± 24 kDa, respectively. Similarly, using 3,6-dihydroxychrysene as substrate target sizes of 109 ± 21 and 101 ± 23 kDa were found for 6-*O*-monoglucuronide and 3-*O*-monoglucuronide formation and a target size of 192 ± 34 kDa observed for diglucuronide formation. Based on subunit molecular masses of 50–60 kDa for UGTs, these results suggest that UGTs involved in monoglucuronide formation of phenols may function as dimers. In contrast, UGTs involved in diglucuronide formation of diphenols of polycyclic aromatic hydrocarbons may function as tetramers in microsomes *in situ*.

Key words: radiation inactivation analysis; UDP-glucuronosyltransferase; diglucuronide formation; 3,6-dihydroxybenzo(*a*)pyrene; 3,6-dihydroxychrysene

UGTs† represent members of a supergene family of conjugating enzymes catalysing glucuronidation of a wide variety of endo- and xenobiotics [1–4]. They are firmly bound to the membranes of the endoplasmic reticulum and the nuclear envelope. Two enzyme families have been defined based on their amino acid sequence identity. Family 1 consists of multiple phenol UGTs and bilirubin UGTs which are derived from a single gene complex by differential splicing [5, 6]. Family 2 consists of several steroid UGTs. The different UGT isozymes show overlapping substrate specificity.

Evidence has been obtained previously that phenols of PAHs may be more selective substrates of MC-inducible phenol UGTs than widely used simple phenols, such as 1-naphthol [7]. Evidence was based on enzyme purification studies and induction factors, i.e. ratios of enzyme activities in liver microsomes of MC-treated rats versus untreated controls. Particularly high induction factors (40- and 300-fold, respectively) were found for diglucuronide formation of 3,6-dihydroxybenzo(*a*)pyrene [8–10] and 3,6-dihydroxychrysene [11]. Diglucuronides of 3,6-dihydroxybenzo(*a*)pyrene have also been detected *in vivo* [12]. 3,6-Dihydroxychrysene has been selected for detailed studies of mono- and diglucuronide formation because it is stable, easy to synthesize and less toxic than the corresponding benzo(*a*)pyrene derivative [13]. It has been suggested that the unusually high induction factors for diglucuronide formation of PAH diphenols are due to the interaction of multiple UGT subunits in microsomes [11]. To substantiate this hypothesis, radiation inactivation analysis has been utilized to identify functional molecular masses of the membrane-bound enzymes *in situ*. By irradiating microsomal preparations with different irradiation doses, enzyme inactivation curves are obtained from which functional target sizes can be determined even for complex membranes such as microsomes. The larger the structure, the more likely it will be "hit". Since the method relies on measurement of biochemical activity the technique describes a functional unit rather than a structural object. Target sizes of many molecules determined by target analysis have been validated by comparison with studies utilizing conventional techniques [14–18]. Recently, radiation inactivation analysis has been applied to the study of microsomal UGTs. Complex activation–inactivation curves were observed after irradiation of microsomes. The initial activation of UGT activity was eliminated by removing the latency of UGT activity, i.e. by addition of detergent to the assay [19]. Radiation inactivation analysis of mono- and diglucuronidation of bilirubin suggested that UGTs may function as oligomers in microsomes [20]. These studies should be extended to facilitate more general conclusions. Availability of sensitive UGT assays

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† Abbreviations: UGT, UDP-glucuronosyltransferase (EC2.4.1.17); MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbons.

offered the possibility of investigating microsomal phenol UGTs involved in mono- and diglucuronide formation of PAH diphenols by radiation inactivation analysis.

MATERIALS AND METHODS

Chemicals. 3,6-Dihydroxybenzo(*a*)pyrene was obtained from benzo(*a*)pyrene-3,6-quinone by reduction with ascorbic acid [9]. The quinone was obtained from the Chemical Carcinogen Reference Standard Repository (National Institutes of Health, Bethesda, MD, U.S.A.). 6-Hydroxychrysene and 3,6-dihydroxychrysene were kindly provided by Dr Albrecht Seidel (Institute of Toxicology, University of Mainz, Germany). The chrysene phenols were synthesized and identified as described [11, 13].

Treatment of animals and preparation of liver microsomes. Male Wistar rats (200 g) were used. MC (40 mg/kg, dissolved in olive oil) was given once intraperitoneally, and the animals were killed after 4 days. Liver microsomes were prepared as described [21] and suspended in 0.01 M Tris–HCl/0.25 M sucrose, pH 7.4, at a protein concentration of 3 mg/mL. Microsomal protein was determined according to the method of Lowry *et al.* [22]. Aliquots of 1.5 mL were filled in 2 mL plastic vials (Nunc, Wiesbaden, Germany), bubbled briefly with nitrogen, frozen and kept in liquid nitrogen (−196°) until used for radiation inactivation analysis.

Irradiation of liver microsomes. Liver microsomes from MC-treated rats were stored in polypropylene vessels and kept under liquid nitrogen (−196°) prior to irradiation. Irradiation of microsomal membranes was performed with high energy electrons produced by a 10-MeV linear accelerator at the radiation laboratories, Justus-Liebig University, Giessen, Germany. The temperature of the sample holder was measured with a sensor at the position of the aluminium rack. During one irradiation experiment, a pre-determined temperature between −80° and −120° was constantly maintained. Radiation doses were determined with FWT-60-00 radiachromic detectors (Far West Technology, Goleta, CA, U.S.A.). Internal standard enzymes and membrane carriers were tested under identical conditions: alcohol dehydrogenase, alkaline phosphatase, Na^+/K^+-ATPase and β-galactosidase [23] and hepatocellular bile acid carriers [24]. After irradiation samples were stored at −80° until used for enzyme assays.

UGT assays. With all UGT assays care was taken that (i) the detergent Brij 58 maximally activated UGT activity, and (ii) UGT activity represented initial rates at substrate saturation. Previously described methods were used for assay of UGT activity toward 1-naphthol [21], 6-hydroxychrysene and 3,6-dihydroxychrysene [11] and 3,6-dihydroxybenzo(*a*)pyrene [9, 11], with the following exception; in the assay of UGT activity toward 3,6-dihydroxybenzo(*a*)pyrene the substrate concentration was increased from 25 to 50 μM and microsomal protein was reduced to 0.015 mg per incubation mixture. Reduction of microsomal protein further increases substrate concentration at the active site of UGT since lipid-soluble substrates are sequestered in microsomal membranes [25, 26] and membrane–membrane interactions favour the diffusion of lipophilic substrates to the active site [27].

Diglucuronide formation of 3,6-dihydroxychrysene starting from its monoglucuronides. Monoglucuronides of 3,6-dihydroxychrysene were presynthesized by incubating 3,6-dihydroxychrysene at a concentration of 0.8 mM for 1 hr with 38 mg mouse liver microsomal protein in a final volume of 6 mL. Mouse liver microsomes were used since they form the diglucuronide very slowly [28]. The reaction was stopped by extraction of unconjugated substrate with chloroform. The concentration of monoglucuronides in the aqueous supernatant was determined by HPLC methods described previously [11]. Under these conditions 3-*O*-monoglucuronide and 6-*O*-monoglucuronide were formed in equal quantities. Aqueous supernatants (300 μL) containing the monoglucuronides (50 μM) were incubated for 5 min in the presence of 3 mM UDP-glucuronic acid and liver microsomes from MC-treated rats (0.02 mg). Diglucuronide formation was determined as described [11].

Calculation of functional target sizes. The natural logarithm of residual UGT activity (normalized to the non-irradiated controls which were set at 100%) was plotted against the radiation dose. Data points of four irradiation experiments were analysed by linear regression and the dose needed to reach 37% residual activity (D_{37}) was calculated from the slope k, as described by Kempner and Schlegel [14]: $D_{37} = 1/k$. The correlation of the data from each irradiation experiment was $r > 0.9$. Functional molecular masses were calculated according to Kepner and Macey [15] using the temperature correction factor f of Kempner and Haigler [16; for −100° f was found to be 2.3] and the formula: molecular mass $= 6.4 \times 10^{11} \times f/D_{37}$.

RESULTS

Microsomes from MC-treated rats were used for irradiation since diglucuronide formation was low in microsomes from untreated controls. Table 1 shows the microsomal UGT activities set at 100% in Figs 1 and 2. UGT activity toward 1-naphthol, a widely used standard substrate, and toward PAH phenols and diphenols are compared. The data demonstrate the high rate of 3,6-dihydroxybenzo(*a*)pyrene 6-*O*-monoglucuronide formation. With the benzo(*a*)pyrene derivative diglucuronide formation appears to proceed almost exclusively via the 6-*O*-monoglucuronide [9]. The rate of 3,6-dihydroxychrysene diglucuronide formation obtained in the sequential reaction from the aglycone was similar to that found with presynthesized monoglucuronides. Similar results were previously obtained in studies on 3,6-dihydroxybenzo(*a*)pyrene diglucuronide formation [11] suggesting that substrate saturation (V_{max}) was reached under these conditions.

Radiation inactivation of UGT activities toward 1-naphthol and 6-hydroxychrysene was studied for calibration purposes. After removal of enzyme latency by addition of detergent monophasic exponential decay curves were observed (Fig. 1).

Table 1. UGT activities toward 1-naphthol, PAH-phenols and diphenols with microsomes from MC-treated rats

Substrate	UGT activities (nmol/min/mg protein)
1-Naphthol	113 ± 14
6-Hydroxychrysene	55 ± 8
3,6-Dihydroxybenzo(*a*)pyrene:	
(6-*O*-monoglucuronide formation)*	418 ± 110
(diglucuronide formation)	21 ± 7
3,6-Dihydroxychrysene:	
(6-*O*-monoglucuronide formation)	10.7 ± 1.7
(3-*O*-monoglucuronide formation)	2.4 ± 0.8
(diglucuronide formation)	6.0 ± 0.9
(diglucuronide formation from presynthesized monoglucuronides)	6.3 ± 1.3

Data represent means ± SD of UGT determinations with the respective non-irradiated control microsomes of four radiation experiments.
* The corresponding 3-*O*-monoglucuronide is negligible [9].

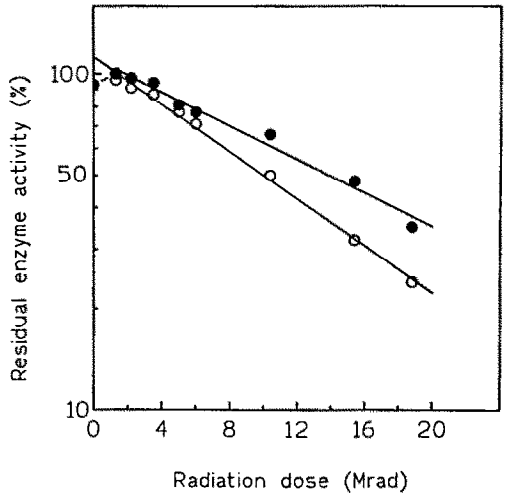

Fig. 1. Radiation inactivation analysis of UGTs catalysing glucuronidation of 1-naphthol and 6-hydroxychrysene. The decrease in UGT activities toward 1-naphthol (●) and 6-hydroxychrysene (○) has been plotted semilogarithmically versus radiation dose. Data represent means of duplicate UGT assays of four independent radiation experiments.

With 1-naphthol as substrate a slight increase in enzyme activity was found after irradiation at doses < 2 Mrad. Above this dose an exponential decay of UGT activity was found. For UGTs catalysing glucuronidation of 1-naphthol and 6-hydroxychrysene target sizes of 91 ± 29 and 120 ± 27 kDa were calculated (Table 2). However, when UGT activity toward 3,6-dihydroxybenzo(*a*)pyrene was investigated two different decay curves were found for mono- and diglucuronide formation (Fig. 2A) for which target sizes of 118 ± 33 and 218 ± 25 kDa, respectively were calculated. Notably, with non-saturating substrate concentration both decay curves were less steep, leading to erroneous target sizes (not shown). Similar to the benzo(*a*)pyrene derivative, different decay curves were observed for mono- and diglucuronide formation of 3,6-dihydroxychrysene (Fig. 2B). For 6-*O*-monoglucuronide, 3-*O*-monoglucuronide and diglucuronide formation target sizes of 109 ± 21, 101 ± 23 and 192 ± 34 kDa were calculated, respectively (Table 2). Interestingly, when diglucuronide was formed from presynthesized monoglucuronides a target size of 171 ± 14 was found.

DISCUSSION

Exponential decay curves of UGT activities were obtained after irradiation of microsomes for which functional target sizes were calculated. Target theory is based on the assumption that if critical structures of the enzyme are randomly hit once by primary ionizations, enzyme function is completely destroyed and the remaining activity is due to units which have escaped ionization (single-target, single-hit model). Therefore, after exposure to different radiation doses, surviving enzyme activity will decrease as a simple exponential function [14–18]. This model implies that care has to be taken to reach substrate saturation in UGT assays.

Interestingly, the target size for diglucuronide formation of PAH diphenols appears to be about twice (171–218 kDa) that calculated for monoglucuronide formation (101–118 kDa; Table 2), similar to previous findings with bilirubin mono- and diglucuronide formation [20]. It is intriguing that the functional target size is unaltered when diglucuronides are formed from presynthesized monoglucuronides (Table 2). The reason for the high target size of 171 kDa is still unknown.

What is known about the UGT isozymes involved in mono- and diglucuronide formation of PAH diphenols? One rat phenol UGT of family 1 (UGT1.6, previously termed UGT1A1) has been stably expressed in hamster lung fibroblast (V79) cell cultures [11]. The nomenclature system of UGTs used is based on that proposed by Burchell *et*

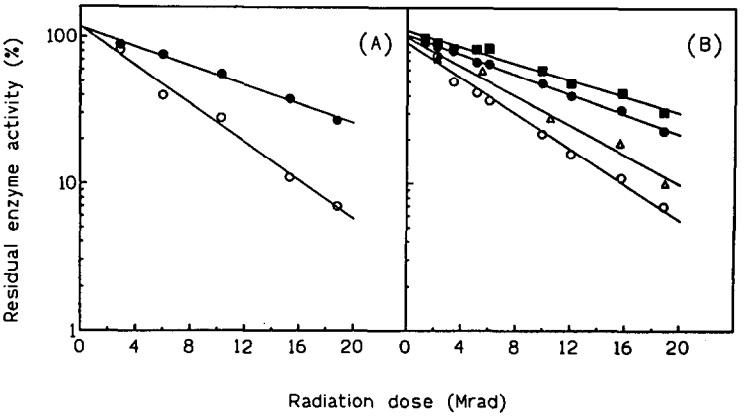

Fig. 2. Radiation inactivation analysis of UGTs catalysing mono- and diglucuronide formation of benzo(*a*)pyrene 3,6-diol (A) and chrysene 3,6-diol (B). The decrease in UGT activities has been plotted semilogarithmically versus radiation dose. (A) Monoglucuronide and diglucuronide formation are represented by full and open circles, respectively; (B) 6-*O*-monoglucuronide and 3-*O*-monoglucuronide are represented by full circles and full squares, respectively; diglucuronide formation from the aglycone and from presynthesized monoglucuronides are represented by open circles and open triangles, respectively. Data represent means of four UGT assays per radiation dose of four independent radiation experiments.

Table 2. Functional molecular masses of UGTs catalysing mono- and diglucuronide formation of PAH phenols determined by radiation inactivation analysis

Substrate	Functional target size (kDa)
1-Naphthol	91 ± 29
6-Hydroxychrysene	120 ± 27
3,6-Dihydroxybenzo(*a*)pyrene:	
(6-*O*-monoglucuronide formation)	118 ± 33
(diglucuronide formation)	218 ± 24
3,6-Dihydroxychrysene:	
(6-*O*-monoglucuronide formation)	109 ± 21
(3-*O*-monoglucuronide formation)	101 ± 23
(diglucuronide formation)	199 ± 36
(diglucuronide formation from presynthesized monoglucuronides)	171 ± 14

Data represent means ± SD of four independent radiation experiments.

al. [4]. Utilizing cell-expressed UGT1.6 it was demonstrated that the enzyme catalyses both mono- and diglucuronide formation of 3,6-dihydroxybenzo(*a*)pyrene, but only monoglucuronide formation of 3,6-dihydroxychrysene. It is concluded that UGT1.6 may be responsible for both mono- and diglucuronide formation of 3,6-dihydroxybenzo(*a*)pyrene [11]. Similar conclusions have been reached for mono- and diglucuronide formation of bilirubin by V79 cell-expressed human bilirubin UGT [29]. However, in the case of 3,6-dihydroxychrysene phenol UGTs other than UGT1.6 may be responsible for diglucuronide formation.

Subunit molecular masses of UGTs between 50 and 60 kDa have been determined [1–4]. Based on these molecular masses the results of radiation inactivation analysis can be interpreted as follows: monoglucuronides of phenols may be formed in microsomes *in situ* by dimeric complexes whereas tetrameric enzyme complexes are necessary for diglucuronide formation. The tetramers may represent homo- and/or hetero-oligomers. The functional units responsible for mono- and diglucuronide formation appear to differ in their properties. For example, diglucuronide formation of 3,6-dihydroxybenzo(*a*)pyrene shows a higher K_m for UDP-glucuronic acid [9], and is more sensitive to inhibition by detergents (T. Hartung and K.W. Bock, unpublished) and by dicoumarol [30].

In conclusion, radiation inactivation studies suggest that phenol UGTs appear to be functional as oligomeric complexes in microsomes while dimers and tetramers appear to be responsible for mono- and diglucuronide formation of PAH polyphenols. However, it remains to be elucidated to what extent these oligomers represent homo- or hetero-oligomers, static or dynamic complexes, the latter formed by lateral movement of phenol UGTs within the plane of the microsomal membrane.

The oligomeric structure may be advantageous in stabilizing the membrane topology of the enzyme and in giving the enzyme system evolutional and adaptational flexibility.

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REFERENCES

1. Burchell B and Coughtrie MWJ, UDP-glu-

curonosyltransferases. *Pharmacol Ther* **43**: 261–289, 1989.
2. Tephly TR and Burchell B, UDP-glucuronosyltransferases: a family of detoxifying enzymes. *Trends Pharmacol Sci* **11**: 276–279, 1990.
3. Bock KW, Roles of UDP-glucuronosyltransferases in chemical carcinogenesis. *Crit Rev Biochem Mol Biol* **26**: 129–150, 1991.
4. Burchell B, Nebert DW, Nelson DR, Bock KW, Iyanagi T, Jansen PLM, Lancet D, Mulder GJ, Chowdhury JR, Siest G, Tephly TR and Mackenzie PI, The UDP-glucuronosyltransferase gene superfamily: suggested nomenclature based on evolutionary divergence. *DNA Cell Biol* **10**: 487–494, 1991.
5. Iyanagi T, Molecular basis of the multiple UDP-glucuronosyl-transferase isoenzyme deficiencies in the hyperbilirubinemic rat (Gunn rat). *J Biol Chem* **266**: 24048–24052, 1991.
6. Ritter JK, Chen FS, Tran HM, Kimura S, Yeatman S and Owens IS, A novel complex locus UGT1 encodes human bilirubin, phenol and other UDP-glucuronosyltransferase isozymes with identical carboxyl termini. *J Biol Chem* **267**: 3257–3261, 1992.
7. Lilienblum W, Platt KL, Schirmer G, Oesch F and Bock KW, Regioselectivity of rat liver microsomal UDP-glucuronosyl-transferase activities toward phenols of benzo(*a*)pyrene and dibenz(*a*,*h*)anthracene. *Mol Pharmacol* **32**: 173–177, 1987.
8. Bock KW, Lilienblum W and Pfeil H, Conversion of benzo(*a*)pyrene-3,6-quinol glucuronides with rat liver microsomes or purified NADPH-cytochrome c reductase and UDP-glucuronyl-transferase. *FEBS Lett* **121**: 269–272, 1980.
9. Lilienblum W, Bock-Hennig BS and Bock KW, 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. *Mol Pharmacol* **27**: 451–458, 1985.
10. Bock KW, Schirmer G, Green MD and Tephly TR, Properties of a 3-methylcholanthrene-inducible phenol UDP-glucuronyltransferase from rat liver. *Biochem Pharmacol* **37**: 1439–1443, 1988.
11. Bock KW, Gschaidmeier H, Seidel A, Baird S and Burchell B, Mono- and diglucuronide formation from chrysene and benzo(*a*)pyrene phenols by 3-methylcholanthrene-inducible phenol UDP-glucuronosyltransferase (UGT1A1). *Mol Pharmacol* **42**: 613–618, 1992.
12. Bevan DR and Sadler VM, Quinol diglucuronides are predominant conjugated metabolites found in bile of rats following intratracheal instillation of benzo(*a*)pyrene. *Carcinogenesis* **13**: 403–407, 1992.
13. Glatt H, Seidel A, Bochnitschek W, Marquardt H, Marquardt H, Hodgson RM, Grover PL and Oesch F, Mutagenic and cell transforming activities of triol epoxides as compared to other chrysene metabolites. *Cancer Res* **46**: 4556–4565, 1986.
14. Kempner ES and Schlegel W, Size determination of enzymes by radiation inactivation. *Anal Biochem* **92**: 2–10, 1979.
15. Kepner GR and Macey RI, Membrane enzyme systems; Molecular size determinations by radiation inactivation. *Biochim Biophys Acta* **163**: 188–203, 1968.
16. Kempner ES and Haigler HT, The influence of low temperature on the radiation sensitivity of enzymes. *J Biol Chem* **257**: 13297–13299, 1982.
17. Kempner ES and Fleischer S, Radiation inactivation of membrane components and molecular mass determination by target analysis. *Methods Enzymol* **172**: 410–439, 1989.
18. Kempner ES, Novel predictions from radiation target analysis. *Trends Biochem Sci* **18**: 236–239, 1993.
19. Vessey DA and Kempner ES, *In situ* structural analysis of microsomal UDP-glucuronosyltransferases by radiation inactivation. *J Biol Chem* **264**: 6334–6338, 1988.
20. Peters HM, Jansen PLM and Nauta H, The molecular weights of UDP-glucuronosyltransferase determined with radiation-inactivation analysis: a molecular model of bilirubin UDP-glucuronosyltransferase. *J Biol Chem* **259**: 11701–11705, 1984.
21. Bock KW and White INH, UDP-glucuronyltransferase in perfused rat liver and in microsomes: influence of phenobarbital and 3-methylcholanthrene. *Eur J Biochem* **46**: 451–459, 1974.
22. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
23. Elsner RH and Ziegler K, Determination of the apparent functional molecular mass of the hepatocellular sodium-dependent taurocholate transporter by radiation inactivation. *Biochim Biophys Acta* **983**: 113–117, 1989.
24. Elsner RH and Ziegler K, Radiation inactivation of multi-specific transport systems for bile acids and xenobiotics in basolateral rat liver plasma membrane vesicles. *J Biol Chem* **267**: 9788–9792, 1992.
25. Illing JPA and Benford D, Observations on the accessibility of acceptor substrates to the active centre of UDP-glucuronosyltransferase *in vitro*. *Biochim Biophys Acta* **429**: 768–779, 1976.
26. Bock KW and Lilienblum W, Roles of uridine diphosphate glucuronosyltransferases in chemical carcinogenesis. In: *The Handbook of Experimental Pharmacology* Vol. 112. *Conjugation/Deconjugation Reactions in Drug Metabolism and Toxicity* (Ed. Kauffman F), pp. 391–428. Springer Verlag, Berlin, 1994.
27. Boyer TD, Zakim D and Vessey DA, Direct, rapid transfer of estrone for liposomes to microsomes. *J Biol Chem* **255**: 627–631, 1980.
28. Bock KW and Schirmer G, Species differences of glucuronidation and sulfation in relation to hepatocarcinogenesis, mouse liver tumors; relevance to human cancer risk. *Arch Toxicol Suppl* **10**: 125–135, 1987.
29. Sutherland L, bin Senafi D, Ebner T, Clarke DJ and Burchell B, Characterisation of a human bilirubin UDP-glucuronosyltransferase stably expressed in hamster lung fibroblast cell cultures. *FEBS Lett* **308**: 161–164, 1992.
30. Segura-Aguilar J, Barreiro V and Lind C, Dicoumarol-sensitive glucuronidation of benzo(*a*)pyrene metabolites in rat liver microsomes. *Arch Biochem Biophys* **15**: 266–275, 1986.