*Biochemical Pharmacology*, Vol. 41, No. 11, pp. 1751–1757, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00


# DRUG METABOLIZING ENZYME ACTIVITIES IN RAT LIVER EPITHELIAL CELL LINES, HEPATOCYTES AND BILE DUCT CELLS

DIETER SCHRENK,* IRIS EISENMANN-TAPPE,† ROLF GEBHARDT,† DORIS MAYER,‡ MOHAMED EL MOUELHI,* ELKE RÖHRDANZ,* PETER MÜNZEL* and KARL WALTER BOCK*§

*Institute of Toxicology, University of Tübingen, Wilhelmstrasse 56, D-7400 Tübingen; †Institute of Physiological Chemistry, University of Tübingen, Hoppe-Seyler-Str. 4, D-7400 Tübingen; and ‡Division of Cytopathology, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-6900 Heidelberg, Germany

(*Received* 26 *October* 1990; *accepted* 21 *January* 1991)

**Abstract**—P450-dependent mono-oxygenase and conjugating enzyme activities were studied in rat liver epithelial cells (RLEs) and compared to those in hepatocytes and bile duct cells. Various RLE cell lines were investigated since (a) they are suspected to be derived from cells in the lineage from putative pluripotent stem cells to either hepatocytes or bile duct cells, and (b) they may represent targets of chemical carcinogens. Despite considerable variation between lines, common features were recognized. P450-dependent monooxygenase activities (7-ethoxyresorufin *O*-deethylase and 7-ethoxycoumarin *O*-deethylase) were undetectable in all RLEs and bile duct cells, and were uninducible by benz(*a*)anthracene. In contrast, glucuronosyltransferase (GT), sulfotransferase and GSH transferase activities were clearly detectable. Conjugating enzyme activities increased until confluency of the cell cultures was reached. Under the latter conditions, GT activities towards 4-methylumbelliferone or benzo(*a*)pyrene-3,6-quinol (substrates of a 3-methylcholanthrene-inducible phenol GT) were similar to those found in hepatocytes or bile duct cells. Using a selective cDNA probe, phenol GT mRNA was clearly detectable in RLE1. In contrast, GT activity towards 4-hydroxybiphenyl was much lower than in hepatocytes or bile duct cells (0.04- and 0.03-fold). Sulfotransferase and GSH transferase activities were also roughly comparable to those found in hepatocytes and in bile duct cells. The results suggest that RLEs and bile duct cells exhibit both high conjugating enzyme activities and a lack of P450-dependent mono-oxygenase activities, a pattern resembling the 'toxin-resistance phenotype' found in putative preneoplastic hepatocyte foci and nodules.

A number of laboratories reported the isolation of permanently growing, epithelial-like cells isolated from neonatal [1, 2], juvenile [3] or adult liver [4]. This heterogenous group of continuously growing liver cells have been called rat liver epithelial cells (RLEs‖). Tsao *et al.* noted similarities between RLEs and oval cells [5] which are accumulating in liver after treatment with various carcinogens [6–10]. Oval cells have been suggested to have the capacity to differentiate into bile duct cells or hepatocytes [11–13]. These observations revived the stem cell hypothesis of the origin of both hepatocytes and bile duct cells. According to this hypothesis RLEs may be derived from cell lineages originating from pluripotent stem cells which later differentiate into either hepatocytes or bile duct cells [14]. It cannot be excluded, however, that some RLE cell lines are derived from hepatocytes which have undergone some steps of transformation leading to immortalization. Since RLEs easily dedifferentiate in culture, their heterogeneity is understandable.

RLEs may be targets of chemical carcinogens. They can be transformed *in vitro* [2]. It has even been proposed that hepatocellular carcinomas or cholangiocarcinomas originate from pluripotent precursor cells [15]. Since most carcinogens have to be converted to their ultimate reactants by drug metabolizing enzymes it was of interest to study phase I and phase II enzymes in comparison with differentiated hepatocytes and bile duct cells. A variety of RLE cell clones have been investigated which were isolated from neonatal liver (RLE1) [1], juvenile liver (RLE2) [16] and adult liver (RLE3) [4].

## MATERIALS AND METHODS

### *Materials*

Ham's F 12 medium was from Seromed (Berlin, Germany), fetal calf serum from Gibco/BRL (Eggenstein, Germany), NADP and isocitrate dehydrogenase from Boehringer (Mannheim, Ger-

§ Address for correspondence: Dr K.W. Bock, Institute of Toxicology, University of Tübingen, Wilhelmstrasse 56, D-7400 Tübingen, Germany.

‖ Abbreviations used: BA, benz(*a*)anthracene; BP, benzo(*a*)pyrene; EROD, 7-ethoxyresorufin *O*-deethylase; GT, UDP-glucuronosyltransferase; PAH, polycyclic aromatic hydrocarbons; RLE, rat liver epithelial cell.

many) and collagenase from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

*Cell culture conditions, RLEs*

*RLE1.* (Passage 17–22) isolated from neonatal liver of Fischer 344 rats, and transformed RLE1 (TRLE), obtained by treatment of RLE1 with aflatoxin $B_1$, were kindly provided by Dr S. S. Thorgeirsson, National Institute of Health, Bethesda, MD, U.S.A. [2]. RLE1 showed a uniform epithelial morphology as seen by phase contrast. The cells had a low saturation density. Reportedly, they contained a diploid number of chromosomes and where not tumorigenic when injected into Fischer 344 rats [2]. TRLE1 showed morphological similarity to the parent cells. However, the saturation density was much higher (2–3-fold). They proliferated under anchorage-independent conditions and formed tumors when injected into syngeneic hosts. Gamma-glutamyltranspeptidase activity was detectable but low in all RLEs and hepatocytes (<0.8 mUnits/mg protein) whereas an activity of >10 mUnits/mg protein was found in freshly isolated bile duct cells.

*RLE2a and RLE2b.* These are identical to clone RL-ET-8 and RL-ET-14, respectively, which were established from the liver of 10-day-old Sprague–Dawley rats [16] according to the procedures of Williams *et al.* [3]. They were used at passages 31–40 and 28–35, respectively.

*RLE3a, RLE3b and RLE3c.* These are identical to $c_1I$ (passage 83), $c_2I$ (passage 269) and $c_1II$ (passage 462) established from adult liver [4]. RLE3a, RLE3b and RLE3c showed epithelial-like morphology and low saturation density. γ-Glutamyltranspeptidase activity was low. Since glucose-6-phosphatase was biochemically and histochemically detectable [4], RLE3a to RLE3c may be of hepatocellular origin. Furthermore, RLE3a stored glycogen in excess.

All RLEs ($4 \times 10^5$ cells) were seeded on 9-cm plates and cultured in 7 mL Ham's F 12 medium supplemented with 10% fetal calf serum and $10^{-6}$ M dexamethasone for a period of 8 days.

*Bile duct cells.* Bile duct cells were obtained as described [17] and modified by I. Eisenmann-Tappe, S. Wizigmann and R. Gebhardt. Briefly, livers were perfused with collagenase and hyaluronidase and the remaining portal tract residue was digested with trypsin. The resulting cell suspension was purified by centrifugation on a discontinuous Percoll gradient. Final contaminations by hepatocytes and Kupffer cells were removed by several steps of selective adherence. As revealed by histochemical staining for gamma-glutamyltranspeptidase and cytokeratin 19, bile duct cells were obtained in a purity of >95%. The cells were cultivated at an initial density of $4 \times 10^5$ per 6-cm plate in Dulbecco's modification of Eagle's medium containing 10% fetal calf serum and 2 mM glutamine.

*Hepatocytes.* Hepatocytes were obtained by sequential perfusion technique [18] and plated on collagen-coated Petri dishes at $10^6$ cells/9 cm plate. Cells were cultivated in supplemented Ham's F 12 similar to the conditions used for RLEs. Medium was replaced after 2 hr.

*H4IIE cells.* These were cultured as described [19].

*Induction experiments*

Cells were treated for 48 hr with benz(*a*)anthracene (5 μg/mL). The inducer was added 2 hr (primary hepatocytes) or 24 hr (RLEs, bile duct cells) after plating, dissolved in 7 μL dimethyl sulfoxide (DMSO). Controls were treated with DMSO only.

*Enzyme assays*

Petri dishes were washed three times with PBS, and cell monolayers were harvested with a rubber policeman in 1 mL 0.1 M Tris/0.25 M sucrose, pH 7.4. Cells were homogenized with a Dounce homogenizer and the protein concentration was determined according to Lowry *et al.* [20]. 7-Ethoxyresorufin *O*-deethylase (EROD) was determined according to Burke and Mayer [21] and 7-ethoxycoumarin *O*-deethylase according to Ullrich and Weber [22]. GT activities were measured using 4-methylumbelliferone [23], 4-hydroxybiphenyl [24] and benzo(*a*)pyrene-3,6-quinol [19] as substrates. GSH transferase was measured with 1-chloro-2,4-dinitrobenzene as substrate [25] and sulfotransferase with 4-methylumbelliferone as substrate [26]. γ-Glutamyltranspeptidase was assayed by the method of Persijn and van der Slik [27].

*Preparation of a synthetic phenol GT cDNA probe*

Two oligonucleotide primers were prepared by automatic synthesis, corresponding to nucleotides 71–93 and to the complementary sequence of nucleotides 328–350 of the described phenol GT cDNA [28]. They were taken as primers for the polymerase chain reaction [29] using isolated DNAs from rat liver. The amplified DNAs corresponded to the expected 280-base pair fragment (nucleotides 71–350) of the variable part of DNA sequences of multiple GT isozymes. The synthetic probes were labeled with [$^{32}$P]dCTP using the multiprime DNA labeling system (Amersham Buchler, Braunschweig, Germany).

*Extraction of RNA and Northern-blot analysis*

Total RNA was isolated from rat hepatoma H4IIE cells, RLE and TRLE cells by the method of Chirgwin *et al.* [30]. RNA was denatured for electrophoresis in a 1.5% agarose gel containing 2.2 M formaldehyde, transferred to Hybond N (Amersham Buchler, Braunschweig, Germany) by capillary blotting and covalently bound to the membrane by UV irradiation. The membranes were hybridized with the $^{32}$P-labeled phenol GT DNA probe (nucleotides 71–350). Prehybridization was carried out (at 44° for 24 hr) in a solution containing 50% deionized formamide, 6 × SSC (0.9 M NaCl/0.09 M sodium citrate buffer, pH 7.0), 10 × Denhardt solution, 0.5% sodium dodecyl sulfate (SDS) and 0.4 mg/mL denaturated herring sperm DNA. It was followed by hybridization with the $^{32}$P-labeled phenol GT cDNA probe in the same solution for a minimum of 24 hr at 44°. After hybridization, membranes containing RNA were washed twice in 2 × SSC (0.3 M NaCl/0.03 M sodium citrate buffer, pH 7.0), 0.1% SDS for 20 min at 44°. Blots were exposed to

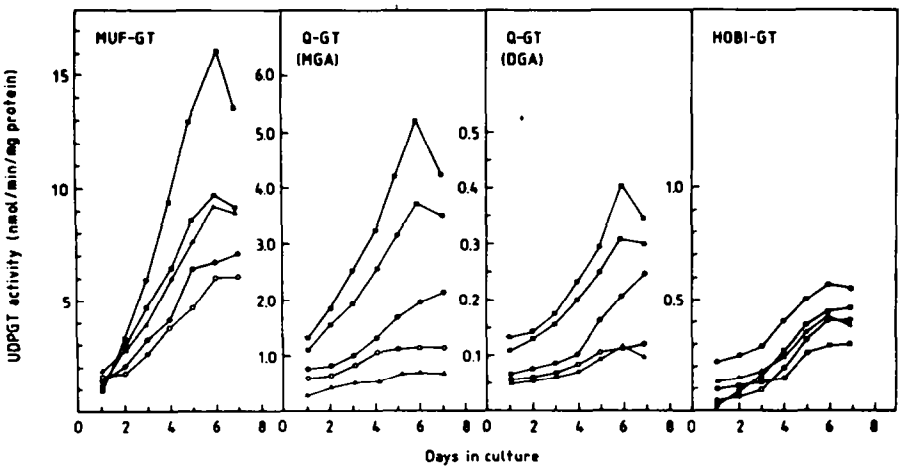


Fig. 1. Effects of cell density on GT activities in RLEs. GT activities were determined in homogenates from RLE1 (▲); RLE2a (□) and RLE2b (■); RLE3a (○) and RLE3b (●). MUF, 4-methylumbelliferone; Q, BP(benzo(*a*)pyrene)-3,6-quinol; MGA, BP-3,6-quinol 6-monoglucuronide; DGA, BP-3,6-quinol diglucuronide; HOBI, 4-hydroxybiphenyl. $4 \times 10^5$ cells/9 cm plate were seeded at zero time.

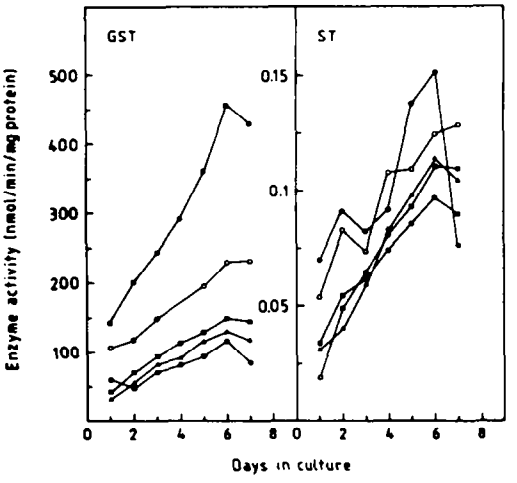


Fig. 2. Effects of cell density on GSH transferase and sulfotransferase. Symbols for the RLE cell lines are the same as in Fig. 1.

Kodak XAR-5 film at −70° with intensifying screens for seven days. The relative amounts of mRNA were estimated by densitometric scanning of autoradiograms of Northern blots.

## RESULTS

GT, GSH transferase and sulfotransferase activities increased in all RLEs investigated until confluency of the cell cultures was reached (Figs 1 and 2). The mechanisms responsible for the increase of enzyme activities are unknown. This behaviour of enzyme activities in low passage cultures was in contrast to that observed in a high passage culture which may have been spontaneously transformed (RLE3c) and in a chemically transformed RLE1 (TRLE1). In RLE3c and TRLE1 phenol GT activity decreased after an initial small increase (not shown). At confluency cell density was similar for all RLE cell lines including RLE3c. However, TRLE1 did not show the phenomenon of contact inhibition. Despite the similarities between RLE cell lines considerable differences in the levels of conjugating enzymes were also recognizable. For example, the PAH-inducible phenol GT activity was highest in RLE2b and lowest in RLE1. In contrast, 4-hydroxybiphenyl GT and sulfotransferase activities were very low in RLE2b.

Enzyme activities reached after 6 days in culture were compared to those found in hepatocytes, in bile duct cells and in liver (Tables 1 and 2). In Table 1, GT activity towards BP-3,6-quinol (a selective substrate of a PAH-inducible phenol GT which catalysed both BP-3,6-quinol mono-and diglucuronide formation [31]) was compared with 7-ethoxyresorufin *O*-deethylase (EROD) activity, catalysed by the PAH-inducible P450 isozyme, P450IA1. EROD activity was undetectable in all RLEs and bile duct cells. It was not inducible by benz[*a*]anthracene in these cells, in contrast to its marked inducibility in hepatocytes. Similarly, 7-ethoxycoumarin *O*-deethylase activity (an overlapping substrate for several P450 isozymes) was also not detectable in RLEs (not shown). Similar to P450 reactions, phenol GT activities were not inducible by benz(*a*)anthracene in RLEs. Phenol GT activities were roughly comparable in RLEs to those found in hepatocytes and bile duct cells. Phenol GT mRNA was clearly detectable in RLE1 after 6 days (Fig. 3A). Expression of phenol GT was higher in TRLE1 (the transformed RLE1 cell line). In TRLE1 phenol GT activity towards 4-methylumbelliferone was also highest at confluency of the cell culture which, however, was reached already at 3 days after seeding. Maximal GT activity was higher in TRLE1 than in RLE1 (about 2-fold; not shown). As shown in Fig. 3B, the phenol GT mRNA level declined after 5 days.

In Table 2 several GT activities (including those towards 4-methylumbelliferone and towards 4-

Table 1. Induction by polycyclic aromatic hydrocarbons of P450IA1 and phenol GT activities in rat liver and in hepatocytes and lack of induction in bile duct cells and in RLEs*

| Cell type | P450IA1 (EROD) (pmol/min/mg protein) | Phenol GT BP-3,6-quinol (nmol/min/mg protein) | Phenol GT BP-3,6-quinol monoglucuronide (nmol/min/mg protein) |
|---|---|---|---|
| Liver | 11 ± 4 | 4.0 ± 1.0 | 0.3 ± 0.1 |
| Liver (MC-treated) | 2231 ± 286 (203) | 41.0 ± 10.0 (12) | 6.9 ± 0.2 (23) |
| Hepatocytes | 12 ± 6 | 5.0 ± 0.2 | 0.6 ± 0.1 |
| Hepatocytes + BA | 1012 ± 183 (84) | 7.3 ± 1.0 (1.5) | 1.3 ± 0.1 (2.2) |
| Bile duct cells ± BA | <2 | 2.4 ± 0.2 | 0.1 ± 0.2 |
| RLE1 ± BA | <2 | 0.5 ± 0.1 | 0.2 ± 0.1 |
| RLE2b ± BA | <2 | 5.1 ± 0.1 | 0.4 ± 0.1 |
| RLE3a ± BA | <2 | 1.0 ± 0.1 | 0.1 ± 0.1 |

* RLE1, RLE2b, RLE3a = selected RLE cell lines obtained from neonatal, juvenile and adult rat liver, respectively, which are described in detail in Materials and Methods; BA, benz[*a*]anthracene; MC, 3-methylcholanthrene. The induction factor is shown in parentheses.

hydroxybiphenyl), GSH transferase and sulfotransferase activities were compared. GT activity towards 4-methylumbelliferone is known to be catalysed by phenol GT (similar to GT activity towards BP-3,6-quinol) whereas 4-hydroxybiphenyl GT activity is catalysed by a phenobarbital-inducible GT isozyme. In contrast to phenol GT activities, 4-hydroxybiphenyl GT activity was much lower in RLEs and in bile duct cells. GSH transferase and sulfotransferase activities were roughly comparable in RLEs and hepatocytes, except that sulfotransferase appeared to be highest in bile duct cells.

## DISCUSSION

RLEs are widely used in studies of chemical hepatocarcinogenesis [2, 32–36] or as co-cultures to maintain the properties of primary hepatocyte cultures [16, 37]. Therefore the pattern of drug metabolizing enzymes in RLEs and their regulatory properties is of considerable interest.

Levels of conjugating enzymes varied considerably in the different lines of RLEs studied. Nevertheless, a common pattern could be recognized. PAH-inducible P450 activities and their inducibility by PAHs was not detectable. In contrast, phenol GT activities, GSH transferase and sulfotransferase activities were present at levels which were roughly comparable to those found in hepatocytes. Using a cDNA probe, expression of phenol GT mRNA could clearly be demonstrated. This pattern and lack of inducibility by PAH-type inducers are similar to properties found in differentiated bile duct cells (Table 1, Refs 38 and 39). In Table 1 inducers were added to growing RLEs whereas hepatocytes were not in the cell cycle under the experimental conditions. However, lack of induction was also observed when the inducers were added to confluent

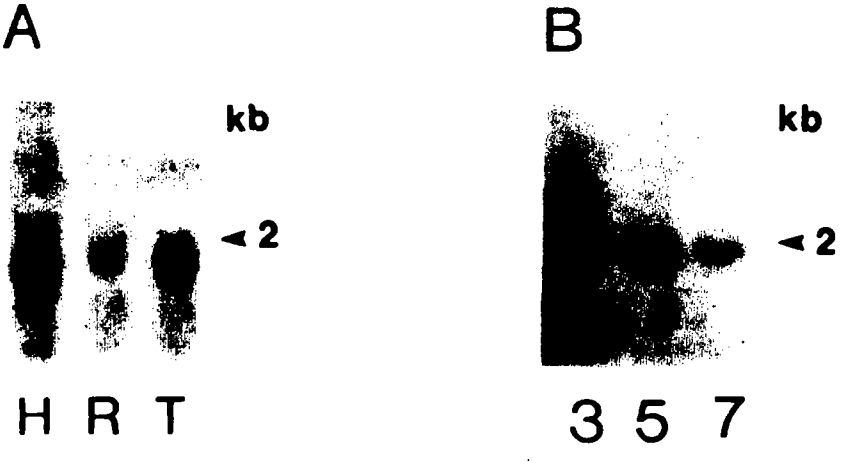


Fig. 3. (A) Blot analysis of phenol GT mRNA from RLE1 (R) and from TRLE1 (T). mRNA from rat hepatoma H4IIE cells (H) was used as a standard for phenol GT expression. Total RNA (20 μg) was subjected to blot analysis. (B) Time course of mRNA levels. Cells were analysed 3, 5 and 7 days after seeding of $4 \times 10^5$ cells/9 cm plate.

Table 2. Phase II enzyme activities in rat liver, hepatocytes, bile duct cells and RLE cells

| Cell type | Glucuronosyltransferase (4-Methylumbelliferone) (nmol/min/mg protein) | Glucuronosyltransferase (4-Hydroxybiphenyl) (nmol/min/mg protein) | GSH transferase (1-Chloro-2,4-dinitrobenzene) (nmol/min/mg protein) | Sulfotransferase (4-Methylumbelliferone) (pmol/min/mg protien) |
|---|---|---|---|---|
| Liver | 10.1 ± 4.2 | 12.2 ± 2.0 | 96 ± 17 | 351 ± 29 |
| Liver + MC | 33.4 ± 7.2 (3.3) | 14.5 ± 2.4 (1.2) | ND | ND |
| Hepatocytes | 10.5 ± 1.2 | 11.6 ± 1.2 | 63 ± 7 | 276 ± 49 |
| Hepatocytes + BA | 20.5 ± 2.9 (2.0) | 13.6 ± 2.2 (1.2) | 156 ± 26 (2.5) | 304 ± 76 (1.1) |
| Bile duct cells ± BA | 11.4 ± 3.1 | 10.8 ± 2.5 | 385 ± 56 | 981 ± 230 |
| RLE1 ± BA | 9.0 ± 0.7 | 0.4 ± 0.1 | 126 ± 36 | 111 ± 41 |
| RLE2b ± BA | 16.1 ± 3.8 | 0.3 ± 0.2 | 465 ± 42 | 155 ± 26 |
| RLE3a ± BA | 6.0 ± 1.3 | 0.4 ± 0.1 | 220 ± 31 | 124 ± 52 |

Cell types and abbreviations are the same as those described in Table 1. ND, not detected.

RLE1 cells (not shown). Furthermore, the Ah receptor (determined as described [40]) which is controlling the induction of P450IA1 and of phenol GT by benz[*a*]anthracene was not detectable in confluent RLEs, in contrast to its presence in hepatocyte cultures (P. Cikryt and D. Schrenk, unpublished results). The pattern of drug metabolizing enzymes found in various RLEs and bile duct cells shows some resemblance to the 'toxin-resistance phenotype' observed at cancer prestages (putative preneoplastic hepatocyte foci and hepatocyte nodules [41–43]).

A considerable variability of conjugating enzyme activities in different lines of RLEs may be ascribed to several factors: (a) RLEs have been isolated from different rat strains and (b) from rats of different ages. (c) RLEs may be derived from cells at different developmental stages in the lineage from stem cells to differentiated cells. (d) The multiple RLE lines may reflect various stages of dedifferentiation, according to their different cell passage numbers. Despite this remarkable heterogeneity study of drug metabolizing enzymes in RLEs may help to interprete toxicity data obtained with these cells. RLEs can be transformed *in vitro* [2, 32]. They, or their counterparts *in vivo*, may even represent prime targets of carcinogens and may represent the origin of some hepatocellular carcinomas and cholangiocarcinomas [15]. It has been suggested that RLEs may resemble isolated oval cells [5]. Oval cells *in vivo* are known to proliferate after administration of various hepatocarcinogens [6–10]. Evidence has been obtained that oval cells may differentiate into hepatocytes or bile duct cells [11–13]. However, the origin of RLEs is still uncertain. RLE3a–RLE3c exhibit a number of properties which indicate a hepatocellular origin [4], whereas other clones used in coculture with hepatocytes have been suggested to be derived from bile duct cells [37]. Because of the possibility of dedifferentiation and spontaneous transformation of RLEs during culture, it may be important to find the conditions under which their original properties are maintained, and differentiation to either hepatocytes or bile duct cells can be achieved.

*Acknowledgements*—The authors wish to thank Dr P, Cikryt (Institute of Toxicology, University of Würzburg, Germany), Ms S. Beck, Mrs A. Hintzen, Mrs U. Jongepier and Mrs I. Letsch for expert technical assistance, Mrs A. von Bank and Mrs E. Schenk for typing the manuscript as well as the Deutsche Forschungsgemeinschaft and the Alexander-von-Humboldt-Stiftung for financial support.

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