BIOMARKERS, 1999, VOL. 4, NO. 3, 188–202

Use of precision-cut human liver slices for studying the metabolism and genotoxic potential of xenobiotics by means of the ^{32}P-postlabelling technique: steps towards method validation using testosterone and 2-aminofluorene

ANDREAS BAUMANN[1,*], WERNER FESER[1], PETER CRAMER[1], RASOUL S. KERDAR[1], HARTMUT BLODE[1], JÜRGEN KÖRBER[2] and WILHELM KUHNZ[1]

[1] Research Laboratories, Schering AG, 13342 Berlin, Germany
[2] Virchow Hospital, Humboldt-University 13353 Berlin, Germany

Received 4 September 1998, revised form accepted 28 November 1998

In the present study, a new *in vitro* model combining the short-term incubation of precision-cut human liver slices with DNA-adduct analysis by the ^{32}P-postlabelling technique is proposed for investigation of the genotoxic potential of xenobiotics. For method validation, the metabolic turnover of testosterone (TES) and the DNA-adduct inducing potential of 2-aminofluorene (2-AF) were used. Precision-cut human liver slices were prepared from a total of 12 human liver samples which were freshly obtained as parts of resectates from liver surgery. The slices were incubated as submersion cultures with TES and 2-AF for up to 6 h in 12-well tissue culture plates at concentrations of 10–50 and 0.06–28 μM, respectively. Slices recovered from the slicing procedure in the 4 °C cold Krebs–Henseleit buffer as indicated by intracellular potassium concentrations which increased for 2 h and then remained stable until the end of the incubation. TES was extensively metabolized by human liver slices with a similar metabolite pattern as observed *in vivo*. Almost 90 % of the metabolites were conjugates. Major phase-I metabolites were androstendione, 6β-OH-androstendione, 6β-OH-TES, and 15β-OH-TES. After incubation with 2-AF, substance related DNA-adducts were detected which increased dose-dependently from 12 to 1146 adducts per 10^9 nucleotides. The adduct pattern consisted of one major adduct spot, A, representing 80–90 % of the total adduct level and up to four minor adduct spots, B–E. In summary, the present data demonstrate that precision-cut liver slices are a valuable alternative *in vitro* system for DNA-adduct determination to screen chemicals for potential genotoxicity in humans.

Keywords: human liver slice, genotoxicity, DNA-adduct, ^{32}P-postlabelling assay, metabolism.

Abbreviations: 2-AF, 2-aminofluorene; AND, Androstenedione; CYP, cytochrome P450; DHT, 5β-dihydro-TES; f, female; HL, human liver sample; m, male; TES, testosterone; UDS, unscheduled DNA-synthesis.

Introduction

The liver is the major site of xenobiotic biotransformation and therefore also a target of metabolically induced toxicity. Most genotoxic chemicals form covalently bound adducts with DNA and/or proteins because they are either highly reactive *per se*, or because they are converted metabolically to electrophilic reactive species (Miller and Miller 1966, 1981). Liver tissue has been used to establish several *in vitro* models for metabolism and toxicity studies. Human tissues represent a good

* Author for correspondence.

Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 1999 Taylor & Francis Ltd
http://www.tandf.co.uk/JNLS/bmk.htm
http://www.taylorandfrancis.com/JNLS/bmk.htm

way to bridge the gap when trying to extrapolate from results obtained in animal studies *in vitro* and *in vivo* to the human situation.

Monolayer cultures of hepatocytes have been widely used to determine xenobiotic-induced toxicity in general (Butterworth *et al.* 1989; Swierenga *et al.* 1991, Guillouzo 1992, Blaauboer *et al.* 1994) as well as the xenobiotic-induced genotoxicity employing the unscheduled DNA-synthesis (UDS) technique (Williams 1977) and DNA-adduct measurement (Topinka *et al.* 1993; Werner *et al.* 1996). Two major problems are encountered when working on human liver cells: first scarcity and unpredictability of human samples and second, functional variations from batch to batch (Guillouzo *et al.* 1993; Bach *et al.* 1996). Small biopsies, which are obtained from patients undergoing partial hepatectomy, are more frequently available than whole livers from organ donors. However, they are often not usable for isolation of hepatocytes because of their size, irregular surface and resulting difficulties in cannulating vessels for collagenase perfusion. To overcome these problems, tissue slices represent a useful alternative. Compared with hepatocytes as well as other *in vitro* models liver slices offer the advantage of conservation of tissue architecture and cell to cell communication (Krumdieck *et al.* 1980, Brendel *et al.* 1987, Sipes *et al.* 1987, Smith *et al.* 1989). Precision-cut liver slices have been widely employed to study xenobiotic metabolism and xenobiotic-induced toxicity (Smith *et al.* 1985, 1987, Conners *et al.* 1990, Fisher *et al.* 1993, Drahushuk *et al.* 1996 and Bach *et al.* 1996). They may also be useful to investigate the xenobiotic-induced genotoxicity employing the UDS-technique (Beamand *et al.* 1994, Lake *et al.* 1996). Another method indicating DNA-damage on a very early stage of cytotoxic lesions is the ^{32}P-postlabelling technique. This method can detect very sensitively covalently bound adducts of xenobiotics with DNA. Recent studies in our laboratory have demonstrated that, like hepatocytes (Topinka *et al.* 1993, Werner *et al.* 1996), precision-cut liver slices may also be used to measure the DNA-adduct inducing potential of selected steroid hormones (Baumann *et al.* 1996, Feser *et al.* 1998). In order to further characterize the model, the standard substrates testosterone (TES) and 2-aminofluorene (2-AF) were used in the present study for the assessment of metabolic turnover and adduct formation, respectively.

Due to the regio- and stereospecific hydroxylation, TES is a sensitive fingerprint for the intact metabolic capacity of selected cytochrome P450 isoenzymes and therefore a widely used marker substrate to check the metabolic activity of *in vitro* systems (Wood *et al.* 1983; Waxman 1988). 2-AF is an environmental genotoxic aromatic amine carcinogen which is well known for its marked ability to form DNA-adducts *in vivo* and *in vitro* following metabolic activation (Beland and Kadlubar 1990). Therefore, 2-AF was used in the present study as model compound not only because of its covalent binding to DNA but also because it has to be metabolically activated prior to adduct formation.

Material and methods

Chemicals

Testosterone (17β-hydroxyandrost-4-en-3-one, TES) was synthesized at Schering AG. The purity was examined by HPLC and was found to be > 99 %. [1,2,6,7-^{3}H]-TES, specific activity: 3.7 TBq was purchased from Amersham Buchler GmbH, Braunschweig, Germany. 2-Aminofluorene (2-AF) was purchased from Aldrich (Steinheim, Germany). The following reference substances were synthesized at Schering AG: androstendione (AND), 6β-OH-androstendione, 6β-OH-TES, 7α-OH-TES, 11β-OH-

Table 1. Description of used liver tissue and measurements made with the slices of the individual samples.

Sample No.	Patient Age	Patient Sex	Reason for surgery medication	Incubation TES	Incubation 2AF	Metabolism TES	Metabolism 2AF	DNA-adducts TES	DNA-adducts 2AF	K^+-levels
1	72	m	Liver tumour/n.d.	–	–	–	–	–	–	x
12	56	f	haemangioma/ estradiol-patch, ranitidin, salbutamol, methylprednisolone	–	x	–	–	–	x	–
15	35	m	n.d./n.d.	x	x	x	–	x	x	–
17	60	m	Hepatic metastasis/n.d.	x	x	x	–	x	x	x
19	71	m	Hepatic metastasis/n.d.	x	x	–	–	x	x	–
21	54	f	Liver cell adenoma/n.d.	x	x	x	x	x	x	–
22	46	f	Hepatic metastasis/n.d.	-	x	–	x	–	x	–
23	46	f	Liver cell adenoma/n.d.	x	x	x	x	–	x	–
24	51	m	Hepatic metastasis, ranitidin	–	x	–	x	–	x	x
25	35	m	Liver tumour/n.d.	–	x	–	x	–	x	–
27	40	m	n.d./n.d.	–	x	–	x	–	x	–
29	72	m	Liver tumour/Ranitidin, Omeprazol	x	–	x	–	–	–	x

x, Measurement made; –, no measurement made; n.d., no data available.

TES, 15β-OH-TES, 16α/β-OH-TES, androsterone, epi-androsterone, ethiocholanolone and 5β-dihydro-TES (DHT). The enzymes β-glucuronidase and arylsulphatase were obtained from Boehringer Mannheim GmbH (Germany).

Krebs–Henseleit buffer contained (per litre): 118 mM NaCl, 5.4 mM KCl, 1.0 mM $MgSO_4 \cdot 7\ H_2O$, 0.96 mM KH_2PO_4, 26 mM $NaHCO_3$, 2.3 mM $CaCl_2$, and 25 mM glucose $\cdot\ H_2O$. All chemicals were of analytical grade quality and were purchased from E. Merck (Darmstadt, Germany). Deionized water was used in all cases. Hanks medium was obtained from Fresenius Diagnostika (Bad Homburg, Germany). Micrococcal-nuclease and nuclease P1 were purchased from Sigma (Deisenhofen, Germany), spleen phosphodiesterase from Boehringer Mannheim (Mannheim, Germany), T4-polynucleotide kinase from Pharmacia Biotech GmbH (Freiburg, Germany), Polyethyleneimine (PEI)-cellulose plates from Macheray & Nagel (Düren, Germany) and [γ-^{32}P]ATP (specific activity: 3000 Ci mmol^{-1}) from Amersham (Braunschweig, Germany).

Human liver tissue

Fresh human liver tissue (surgical waste) was obtained as excess material removed during surgery in the Virchow Hospital, Berlin from altogether 12 patients (four females, and eight males). The use of the human liver for the studies had the approval of the ethical committee of the clinic. Prior to surgery, the patients were informed that parts of their removed liver tissue might be used for *in vitro* research.

Macroscopically normal liver was separated from damaged or diseased tissue by the surgical pathologist after macroscopic examination. The liver tissue was placed in ice-cold Hanks medium for transportation. The preparation of liver slices started at about 60 min after removal of the liver tissue from the patient. A medical record including the patient's age, sex, concurrent medication, pathology of the liver disease and date of surgery was obtained for each liver tissue donor.

Because only small liver tissue samples were available not all the analysis described below (TES and 2-AF-metabolism and DNA-adduct formation, intracellular K^+-levels) could be carried out for each liver sample. Therefore, the slices of individual liver samples were used for selected measurements, as indicated in table 1.

Preparation of liver slices

Cores were produced from fresh human liver tissue using a stainless steel tube (i.d. 8 mm) attached to a drilling machine. The machine was connected to a variable resistor to allow for a fine tuning of the rotation speed of the punch. Cores were immediately brought into ice-cold, oxygenated Krebs–Henseleit buffer and used directly for slicing.

Slices with a thickness of 270 μM (corresponding wet weight: 16.6 ± 2.0 mg, mean ± SD, n = 6) were made using a Krumdieck tissue slicer (Alabama Research and Development Corporation, Munford, USA). The entire slicing process was performed with ice-cold, oxygenated Krebs–Henseleit buffer.

Slices were removed from the collecting chamber and stored in buffer for a maximum of 15 min until incubation.

Incubation of slices

Slices were incubated in a submersion culture according to the method described by Dogterom (1993). For that purpose, the freshly prepared liver slices were placed individually or as pairs in 12-well plastic tissue culture plates with an incubation volume of 1.5 ml per well. The culture plates were placed on a gyratory shaker (90 rpm) which was placed in a temperature-controlled (37 °C) incubator (Hereaus, Hanau, Germany), with saturated humidity and an atmosphere of 95 % air/5 % CO_2. Slices floated and moved smoothly in the medium during rotation. The culture plates were pre-incubated with the buffer for 1 h in the incubator and then the slices were added.

For incubation with the test substances, solutions of TES and 2-AF in Krebs–Henseleit buffer containing 1 % (v/v) DMSO were prepared at concentrations of 10, 17 and 50 µM (TES) and 0.06, 0.55, 5.5 and 27.6 µM (2-AF). Each concentration was tested with four to eight human liver slices per liver sample.

Liver slices incubated with cell culture medium alone served as control samples.

After incubation, the slices were removed from the buffer, carefully rinsed with water and touched slightly on filter paper. Thereafter, they were weighed and analysed immediately or kept frozen at about –80 °C until analysis.

Viability of slices

The viability of the slices before and during incubation was checked by measurement of the intracellular K^+-content of individual slices. For this purpose, liver slices from four human liver samples (see above) were prepared and incubated as described above without the test substances for 0, 2, 4 and 6 h.

The carefully washed slices (fresh or stored frozen at about –80 °C) were placed into 1.0 ml water and homogenized by sonication. After diluting the homogenate 1:50 with water and adding 1 % CsCl as a spectral buffer, the K^+-content was determined by means of atomic absorption spectrometry (Perkin-Elmer 5000, Norwalk, USA) against a K^+-calibration curve. The results were expressed as nmol K^+ per mg liver tissue (wet weight). The protein content of the liver slices was determined spectrophotometrically from the homogenates by means of the BCA-method (Pierce and Suelter 1977). The average protein content of the human liver tissue of about 17 % of the wet weight was similar for all slices and remained unchanged during incubation.

Metabolic activity of slices

To determine the metabolic activity, liver slices of five liver samples were incubated with the model substrate TES. A 50 µM solution of the substance in Krebs–Henseleit-buffer was prepared and incubated with the liver slices as described above. After 2, 4 and 6 h of incubation, the slices were placed into 1.0 ml water and homogenized by sonication. For further preparation (see below), the homogenate and supernatant were stored at about –20 °C.

The concentrations of TES and its metabolites 6β-OH-TES, AND and 6β-OH-AND in the incubation medium were determined by means of HPLC directly after incubation and subsequent to enzymatic cleavage of conjugates. One ml samples of cell culture medium were diluted with 1 ml methanol and centrifuged. The supernatant was evaporated to dryness under a stream of nitrogen (45 °C) and directly analysed by HPLC after dissolution of the residue in a 250 µl aliquot of mobile phase A. The HPLC-system used consisted of a controlling unit, two pumps (Waters, Milford, USA), an auto sampler (Perkin Elmer, Norwalk, USA), a UV-detector (Spectroflow, Ramsey, USA) and a radiometric detector (Packard Instrument, Meriden, USA). The system was connected via an interface to a mainframe computer for data acquisition and evaluation.

Column:	Supelcosil (Merck, Darmstadt, Germany) RP 18, particle size: 5 µM, 250 × 4.6 mm
Mobile phase:	A: water/tetrahydrofuran (92.5/7.5; v/v)
	B: methanol/tetrahydrofuran (92.5/7.5; v/v)
Gradient:	73 % A/27 % B for 3 min
	27 % B ⇒ 60 % B within 32 min
	60 % B ⇒ 90 % B within 5 min
Detection:	UV absorption at 248 nm, and radiometric detection
Flow:	1.0 ml min^{-1}

For identification of the hydroxylated metabolites of TES, the corresponding reference standards were co-chromatographed with the samples using HPLC. For identification of the A-ring-reduced metabolites androsterone, epi-androsterone, ethiocholanolone and 5β-dihydro-TES, the corresponding reference standards were co-chromatographed on 20 × 20 cm TLC-plates coated with silicagel 60 F 254 (Merck, KGaA, Darmstadt, Germany) using three different mobile phases (chloroform/ethanol, 19/1, v/v; ethylacetate/*n*-hexane/acetic acid, 75/20/5, ethylacetate/cyclohexane, 50/50).

2-AF-concentrations were determined in the cell culture medium before and after incubation of liver

slices by an automated HPLC-method with direct injection of the samples on a pre-column, pre-column purification and analytical separation after column switching.

The HPLC-system 400 (Kontron, Neufahrn, Germany) consisted of a PC-controlling unit, two pumps (model 420 S), an autosampler (model 465), a UV-detector (model 430), and a column switching module SE-2 (Gynkotek, Germering, Germany). The system was connected via an interface to a mainframe computer for data acquisition and evaluation.

Pre-column enrichment:

Column switching:	6-way valve model 7000 (Rheodyne)
Column:	Lichroprep RP-18 (Merck, KGaA, Darmstadt, Germany) particle size: 15–25 μm, 20 × 4.6 mm
Injection volume:	20–200 μl
Mobile phase I:	Water
Flow rate:	2 ml min^{-1}
Purge time:	3 min

Analytical separation:

Column:	ODS-Hypersil (Hypersil, Runcorn, UK), particle size: 5 μm, 125 × 4.6 mm
Mobile phase II:	acetonitrile/water, 45/55 (w/w)
Flow rate:	1.6 ml min^{-1}
Temperature:	room temperature (22–25 °C)
Detection:	UV absorption at 288 nm

Isolation of DNA

Four liver slices (about 100 mg) were pooled and homogenized in 1 ml of extraction buffer (10 mM Tris/HCl pH 8.0, 0.1 M EDTA and 0.5 % SDS). The homogenate was incubated with 50 units RNase A plus 200 units RNase T_1 for 18 h and, thereafter, with 8.0 units proteinase K for 1 h at 37 °C. The DNA was extracted with phenol and sevag (chloroform/isoamyl alcohol 24:1), precipitated in 96 % ethanol and washed with 70 % ethanol. The precipitate was dissolved in 100 μl of 10 mM Tris/HCl pH 8.0 and the DNA concentration was measured spectrophotometrically.

DNA hydrolysis to individual nucleotides, described by Reddy and Randerath (1986) was employed with minor modifications. Ten μg of DNA were digested to deoxyribonucleoside 3'-monophosphates by a mixture of 2.0 units micrococcal nuclease and 0.01 units spleen phosphodiesterase in 10 μl of 10 mM sodium succinate and 6.5 mM $CaCl_2$, pH 6.0 at 37 °C for 3 h. The 2-AF– and TES–DNA-adducts were enriched using the butanol extraction (Gupta, 1985) and nuclease P1 method (Reddy and Randerath 1986), respectively.

^{32}P-postlabelling assay

Following the respective enrichment procedure, the solution was evaporated to dryness under vacuum and the residue was dissolved in 8 μl water. Subsequently, the adducts were labelled with ^{32}P using the methods described by Gupta (1985) and Reddy and Randerath (1986) with slight modifications. A volume of 8 μl labelling cocktail containing 2.5 μl kinase buffer (200 mM Bicine-NaOH, pH 9.0, 100 mM dithiothreitol, 100 mM spermidine, 100 mM $MgCl_2$), 1.0 μl (4U) T4-polynucleotide kinase (PNK) and 4.5 μl (*ca* 45 μCi) carrier free [γ-^{32}P]ATP were added. After incubation at 37 °C for 45 min, 8 μl apyrase (40 mU) were added and the mixture was incubated for another 30 min at 37 °C.

The separation and detection of DNA-adducts was carried out as described by Gupta *et al.* (1982) with a slight variation. The labelled adduct bisphosphates were separated by thin-layer chromatography on PEI–cellulose plates. The PEI–cellulose plates were equipped with a 10 × 20 cm Whatmann filter paper which was folded in the middle (5 × 20 cm) and attached to the PEI–cellulose plate at both sides and developed overnight in the D1-direction with 1.0 M sodium phosphate, pH 6.8. The D2-direction was omitted. The plates were developed in the D3-direction with 4.0 M lithium formiate – 6.8 M urea, pH 3.4, in the D4-direction with 0.61 M sodium phosphate – 6.53 M urea – 0.61 M Tris HCl, pH 8.2 and in the D5-direction with 1.7 M sodium phosphate. Subsequently, adducts were made visible as spots, using a Fujix Bio-imaging analyzer BAS 2000 (Raytest, Straubenhardt, Germany) after an exposure time of 10–20 min. The relative adduct labelling (RAL-value) was quantified based on the spectrophotometric measurement of DNA concentration and the specific activity of [γ-^{32}P]ATP (Reddy and Randerath 1986). RAL-values were expressed as adducts per 10^9 nucleotides.

Results

Viability and metabolic activity of the slices during incubation

Intracellular K^+-concentrations were used as an indicative measure of the functional cell viability. The time course of the intracellular K^+-content was

Table 2. Intracellular K^+-concentrations during incubation of human liver slices ($n = 4$ slices per liver sample obtained from male patiens 1, 17, 24, and 29).

Incubation time (h)	(nmol K^+ mg^{-1} wet weight)	Mean K^+-concentration	
		Intra-individual variation (%)	Inter-individual variation (%)
0	36.5	2.8	13.5
2	54.9	5.5	17.0
4	55.8	5.3	15.8
6	49.7	7.4	15.0

determined over the incubation period of 6 h in slices of four human liver samples. The potassium levels increased from a mean initial value of 37 to 55 nmol K^+ mg^{-1} tissue during the first 2 h of incubation, reflecting the cells recovering from the slicing procedure and temperature change. Thereafter, K^+-concentrations remained stable until the end of incubation. The intra- and inter-individual variations were determined to be about 5 and 15 %, respectively (table 2).

Following 2 h incubation of ^{3}H-TES with human liver slices, 87 % of total radioactivity was measured in the incubation medium. The relative portion of radioactivity remaining in the slices declined during further incubation to about 10 %. Therefore, concentrations of the parent compound and its metabolites were only determined in the incubation medium.

The major phase-I metabolites of TES were AND, 6β-OH-AND, and 6β-OH-TES. Additionally, some A-ring-reduced metabolites, such as androstandione DHT and androsterone as well as its isomers epi-androsterone and ethiocholanolone were identified in the present study, indicating the intact 5α/β-reductase and 3α/β-oxidoreductase activity (figures 1 and 2).

After 6 h of incubation, almost 90 % of the parent compound and its

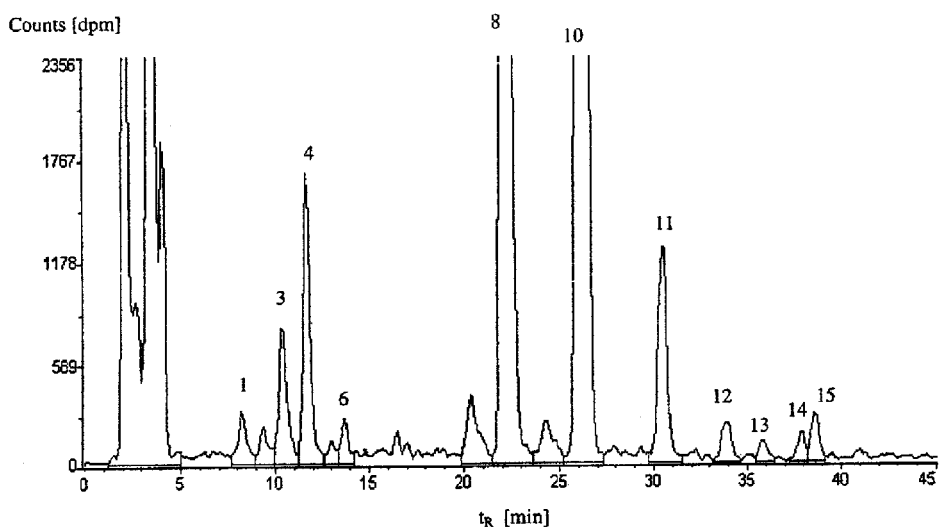

Figure 1. Separation of TES and its metabolites by reversed phase HPLC and radiometric detection after 2 h of incubation of human liver slices with 10 μM TES (1:7α-OH-TES, 3: 6β-OH-TES, 4: 6β-OH-AND, 6: 15β-OH-TES, 8: AND, 10: TES, 11: 5α/β-androstandione, 12: epi-androsterone 13: 5α-DHT, 14: 5β-DHT/ethiocholanolon, 15: androsterone).

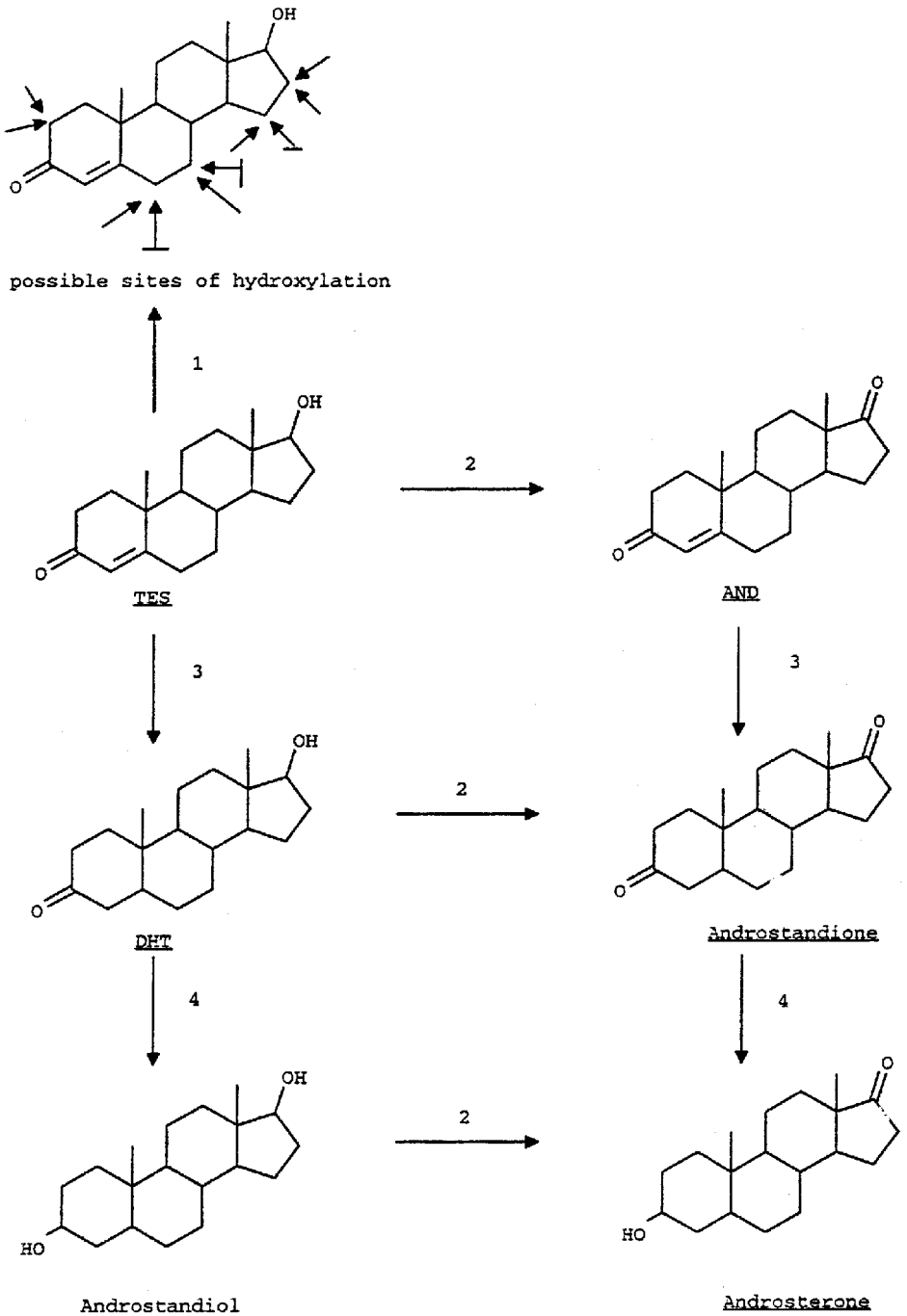

Figure 2. Major metabolic pathways of TES during incubation of human liver slices with 50 μM TES for 6 h; identified compounds (by co-chromatography) are underlined, enzymes involved in major metabolic reactions: 1: CYP isoenzymes for hydroxylations; 2: 17β-OH-oxidoreductase; 3: 5α-reductase; 4: 3α/β-OH-oxidoreductase.

metabolites were present as conjugates. About 70 % and 20 % of the conjugate fraction were glucuronides and sulphates, respectively. Following incubation of ^{3}H-TES with human liver slices from four male patients, on average 32 ± 4 % and 5 ± 3 % of the parent compound could be measured in the culture medium after 2 and 4 h of incubation, respectively. After enzymatic cleavage of conjugates, these values increased to 60 ± 9 % and 45 ± 12 %, respectively (figure 3).

2-AF concentrations were measured in the culture medium at the beginning and at the end of incubation. Within the incubation interval, they declined to 5.3 ± 3.4 % (n = 6) of the initial values. The compound was stable in control incubations without slices during the same time period.

DNA-adduct formation with 2-AF

In general, the adduct pattern of 2-AF consisted of a major adduct spot A and up to four minor adduct spots B–E (figure 4). The major adduct A was detectable even at the lowest 2-AF concentration of 0.06 μM (table 3). With increasing concentrations, all five adduct spots were detected and the total RAL-value increased dose-dependently from 12 to 1146 adducts per 10^{9} nucleotides. Considering the individual adduct spots A–E, 86 % of the total adduct level was contributed by the major adduct A which showed also a dose-dependent increase in intensity (tables 3 and 4). The relative contributions of the adducts B, C, D, and E were 6, 4, 3, and 2 %, respectively. At a 2-AF concentration of 28 μM, the total adduct levels determined in nine different human liver samples varied between about 300 and 1000 adducts per 10^{9} nucleotides (figure 5). The coefficients of intra-individual (between two slices of one tissue sample) and inter-interindividual (between nine liver samples) variation were 17 and 39 %, respectively. No sex-

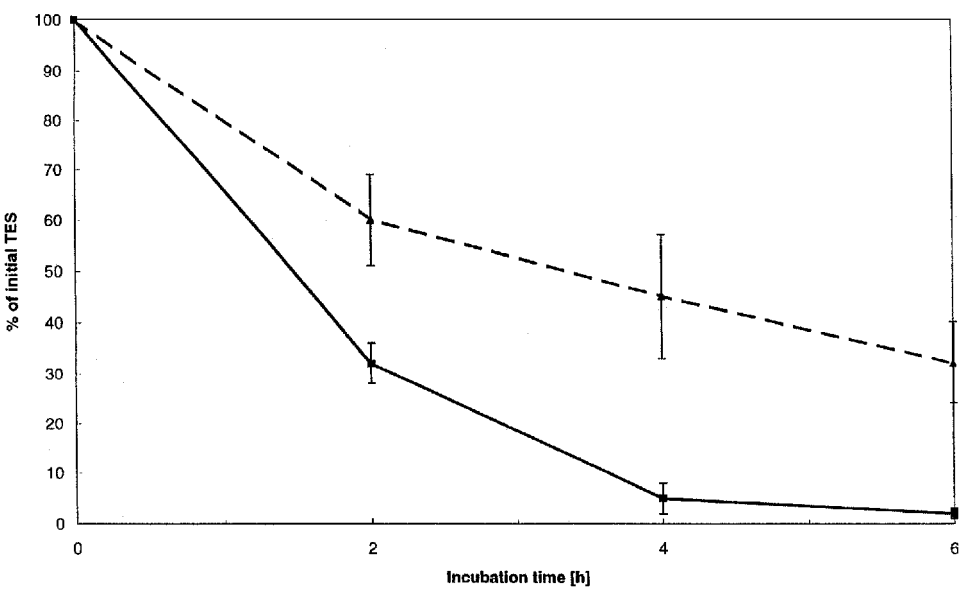

Figure 3. Time dependent decrease of TES-concentrations (mean ± SD of four liver samples) during incubation of human liver slices (initial concentration: 50 μM TES, four slices per tissue sample and time point were pooled for analysis), values generated before (solid line, ■) and after (dotted line, ▲) enzymatic cleavage of conjugates.

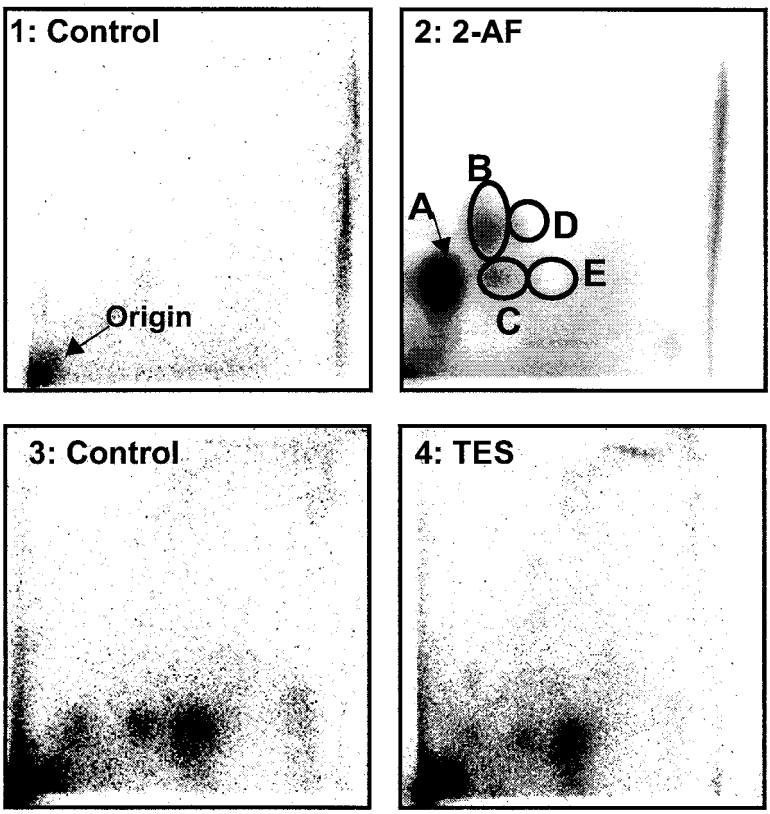

Figure 4. Representative DNA-adduct patterns of 2-AF (top) and TES (bottom) in human liver slices. 1: Control sample 2: incubation with 28 μM 2-AF for 6 h and enrichment with butanol extraction, because the major adduct of 2-AF is not resistent to nuclease P_1-treatment (Gupta and Earley 1988, Gallagher *et al.* 1989), exposure time: 20 min. Relative intensities of different adduct spots: adduct A: 86 %, adduct B: 6 %, adduct C: 4 %, adduct D: 3 %, adduct E: 2 %. 3: control sample, 4: incubation with 17 μM (5 μg ml^{-1}) TES for 6 h; enrichment with nuclease P_1 procedure; exposure time: 150 min.

specific differences could be observed for the DNA-adduct patterns and spot intensities.

DNA-adduct formation with TES

No DNA-adduct formation could be observed after incubation of TES with liver slices from donors 15, 17 and 19 at a concentration of 17 μM (5 μg ml^{-1}) (figure 4).

DNA-adduct formation in control liver samples

Liver slices incubated with drug-free culture medium served as controls. After 6 h of incubation, different DNA-adduct spots of low intensity were observed in control liver slices. The adduct patterns were quite heterogeneous and donor-specific. In general, these adduct patterns (background spots) were also detected after incubation with the test substances (figure 4).

Table 3. DNA-adduct levels (mean (SD)[a], $n = 2$) after incubation of human liver slices from female patients with 2-AF.

	2-AF		RAL-values (adducts per 10^9 nucleotides)					
HL	(μg ml^{-1})	(μM)	Adduct A	Adduct B	Adduct C	Adduct D	Adduct E	Total RAL-values
12	0.01	0.06	9.2 (1.2)	–	–	–	2.6 (0.5)	12 (2)
	0.1	0.55	67 (15)	2.7 (0.4)	3.0 (0.1)	1.6 (0.3)	4.0 (0.9)	78 (14)
	1	5.5	445 (43)	21.1 (3.1)	16 (7)	4.9 (1.0)	11.5 (0.3)	498 (52)
	5	27.6	983 (98)	67.7 (0.6)	41.4 (6.5)	30.5 (2.3)	23.8 (4.2)	1146 (112)
21	5	27.6	205 (52)	–	–	–	n.q.	205 (52)
22	1	5.5	410 (114)	15.0 (3.5)	19.7 (3.8)	–	6.8 (0.8)	452 (122)
23	1	5.5	871 (168)	–	–	–	–	871 (168)

n.q., Not quantifiable; –, not detectable.
[a] SD values are only given as rough estimates of the intra-sample variation.

Table 4. DNA-adduct levels (mean (SD)[a], $n = 2$) after 6 h incubation with human liver slices from male patients with 2-AF.

	2-AF		RAL-values (adducts per 10^9 nucleotides)					
HL	(μg ml^{-1})	(μM)	Adduct A	Adduct B	Adduct C	Adduct D	Adduct E	Total RAL-values
15	1	5.5	972 (9)	16.9 (2.6)	28.5 (0.9)	–	5.7[b]	1023 (3)
17	1	5.5	501 (121)	3.6 (1.0)	5.1 (0.1)	2.1 (0.3)	–	512 (120)
19	1	5.5	911 (94)	–	–	–	–	911 (94)
24	1	5.5	1040	–	–	–	–	1040*
25	1	5.5	724 (188)	–	–	–	–	724 (188)
27	1	5.5	304 (50)	n.q.	n.q.	–	–	304 (50)

n.q., Not quantifiable; –, not detectable.
[a] SD values are only given as rough estimates of the intra-sample variation.
[b] Single determination.

Discussion

Previous investigations have demonstrated that precision-cut liver slices are a valuable *in vitro* model for studying xenobiotic metabolism and toxicity (Sipes *et al.* 1987). They are the most efficient and economic way to obtain viable cells for *in vitro* investigations from small tissue samples, while at the same time providing mixed cell types representative of the intact organ. Even small human liver biopsies can be sliced very quickly without further precautions when the clinic and the laboratory are located in close vicinity. Under these circumstances no previous perfusion of the material is necessary. We obtained the human liver tissue about 1 h after removal from the patient and achieved optimum results when it was transported in ice-cold Hanks medium which is a balanced salt medium and more similar to the Krebs–Henseleit buffer used for slicing and incubation than any other 'physiological' buffer system.

Based on our first results using liver slices to investigate the DNA-adduct inducing potential of steroid hormones (Baumann *et al.* 1996 Feser *et al.* 1998), the present study extends and further supports the suitability of the liver slice model for studying the metabolism and genotoxic potential of xenobiotics using human tissue. TES and 2-AF served as markers for the metabolic capacity of the slices. In addition, the well known DNA-adduct inducing potential of 2-AF was used to assess the DNA-adduct formation potential in the present model. Additionally, it is

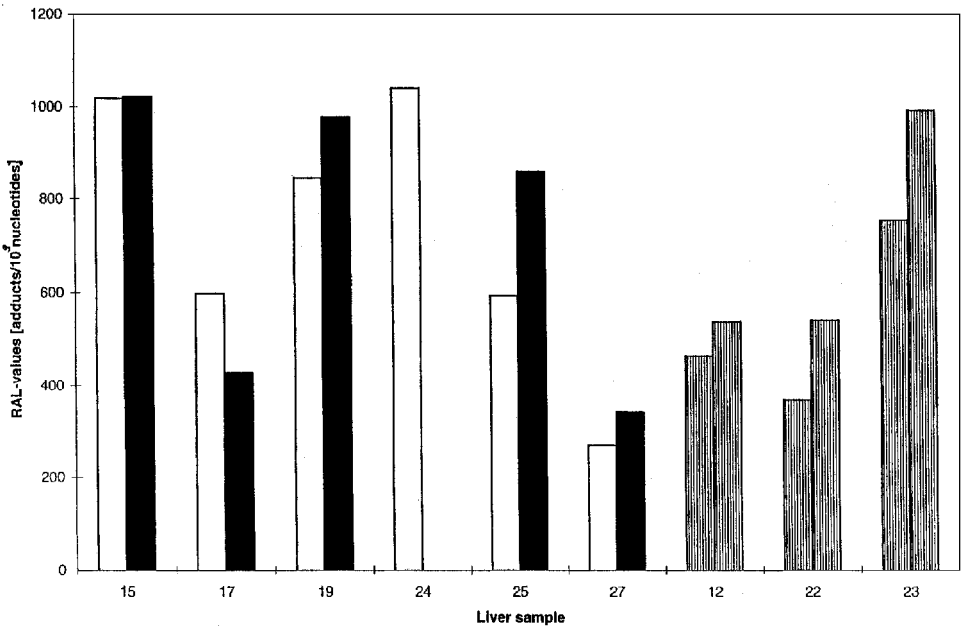

Figure 5. DNA-adduct levels after incubation of human liver slices from six male and three female (hatched columns) patients with 27.6 μM (5 μg ml^{-1}) 2-AF for 6 h ($n=2$ per liver sample). The two columns per sample represent two individual values (only one value for sample 24).

important to ascertain the viability of the liver tissue throughout the process of generation, and incubation of slices. This is of particular interest when validating a method and/or using less well defined human material. The criteria used for assessing viability are diverse and include histological investigations, markers of membrane permeability (LDH, AST, ALT, intracellular K^+) and functionality (ATP-content, protein synthesis, urea synthesis, metabolic marker substrates). We used the intracellular K^+-levels as an indicative marker of membrane permeability and/or TES as a metabolic marker substrate. The maintenance of an ion gradient between intra- and extracellular space is a sensitive indicator of the integrity of the plasma membrane. Intracellular K^+-levels are widely used for viability testing of liver slices and can be simply measured (Smith *et al.* 1989, Fisher *et al.* 1995, Bach *et al.* 1996). Slices recovered from the slicing procedure in the 4 °C cold Krebs–Henseleit buffer within 2 h. Thereafter, intracellular K^+-concentrations reached with about 50 nmol mg^{-1} wet weight the expected values as reported previously (Fisher *et al.* 1995). They remained stable during the incubation procedure, indicating an almost complete maintenance of cell viability.

TES is a widely used marker substrate due to its extensive metabolism by a variety of enzymes *in vivo* as well as *in vitro*. The major metabolic conversions are oxidoreductions at C3 and C17 as well as various C-hydroxylations. Thus, a variety of metabolites can be formed including their stereoisomers (Kochakian 1990). The main metabolite observed in humans *in vivo* is AND (Maser 1995) which was also observed in several *in vitro* models such as isolated perfused livers (Wortmann *et al.* 1971), liver homogenates (Stylianou *et al.* 1961; Lisbao *et al.* 1965; Engelhard 1971) and liver slices (Engelhard 1974) and also in the present study. Using microsomes, only minor amounts of AND could be found (Waxman 1988,

Mäenpää *et al.* 1993) which is probably due to the fact that the cytosolic 17β-hydroxysteroid-dehydrogenases which catalyse this reaction are mainly lost during the preparation of microsomes. After 6 h of incubation with human liver slices, at least 10 metabolites were detected besides the parent compound, indicating the presence of the major pathways of TES metabolism. Hydroxylated steroids are the major metabolites formed from TES and AND by the livers of several rodent species as well as by human liver (Kochakian 1990). The formation of the metabolite 6β-OH-TES is mainly catalysed by CYP 3A4, in part also by CYP 3A5 and 3A7 (Waxman *et al.* 1991, Oguri *et al.* 1994). The radiometric detection of the A-ring reduced (UV-inactive) metabolites indicates intact 5α/β-reductase and 3α/β-oxidoreductase activities in the liver slices.

The metabolite pattern of TES indicated an intact phase-I as well as phase-II-capacity during the incubation time of 6 h. Additional evidence of the maintenance of phase-I and -II metabolism in the liver slices was provided by the fact that the pro-mutagen and pro-carcinogen 2-AF was able to induce drug-specific DNA-adducts, because it is known that this substance requires metabolic activation prior to DNA-adduct formation (Beland and Kadlubar 1990). N-oxidation by hepatic CYP 1A2 and subsequent glucuronidation or O-acetylation of the N-hydroxy metabolite are crucial steps (Butler *et al.* 1989). Eventually, electrophilic arylnitrenium ions are generated which bind covalently to DNA and may cause mutagenic and carcinogenic lesions (Kirlin *et al.* 1989, Badawi *et al.* 1995). Rapid disappearance of the compound from the cell culture medium indicated its rapid metabolic conversion, which was confirmed by the appearance of DNA-adducts.

DNA-adducts are regarded as primary cytotoxic lesions and represent therefore an important biomarker for mutagenic and carcinogenic risk assessment (Purchase 1994). There are four principally different methods to determine DNA-adducts, such as radioimmuno-, fluorescence, labelling (^{3}H) and post-labelling assays (^{3}H, ^{32}P,^{33}P) which differ in their sensitivity, specificity, ruggedness and inter- and intra-laboratory variance. Of those, the ^{32}P-postlabelling assay is the only widely used and very sensitive method for the detection of DNA-adducts (Phillips and Castegnaro 1993, Randerath and Randerath 1994, Gupta and Spence-Beach 1996). The DNA-adduct formation by 2-AF in human liver slices resulted in four adduct spots with a similar pattern as observed after the *in vitro* reaction of N-hydroxy-2-aminofluorene with calf thymus DNA (Gupta *et al.* 1982). The major DNA-adduct formed *in vivo* after administration of 2-AF in experimental animals is N-(deoxyguanosin-8-yl)-2-AF (Beland and Kadlubar 1990, Culp *et al.* 1993, Feng *et al.* 1996). DNA-adducts had also been decribed after incubation of human hepatocyte cultures with 2-acetyl-2-aminofluoren (2-AAF) (Montheith and Gupta 1992). 2-AF and 2-AAF show similar activation pathways as indicated by the fact that O-acetylation of N-hydroxy-2-aminofluorene is probably an essential factor in 2-AF-DNA adduct formation (Feng *et al.* 1996).

In the present study, the adduct formation followed a dose–response relationship of the total adduct levels as well as of the individual adducts A–C, indicating that quantitative results can also be obtained from this *in vitro* model. However, one has to take into account that a high inter-individual variation of about 40 % was observed for the total adduct levels. This also holds true for the inter-individual variation of the metabolite formation after incubation with TES. Therefore, one has to consider that quantitative results from this *in vitro* model should be generated from at least three different liver samples. If one wants to

qualitatively rank different compounds for their genotoxic potential one should compare them in slices generated from the same liver sample.

Investigation of the complete metabolic profile of a xenobiotic and any associated metabolism-mediated toxicity requires a system which reflects the *in vivo* situation as closely as possible. This seems to be possible with the presently described *in vitro* system with short-term incubation of liver slices in a simple submersion culture. The preparation of human liver slices is straightforward as compared with isolation of hepatocytes. Since the potential of a substance to induce DNA-adducts in man can not be routinely assessed *in vivo*, liver slices are of great value to examine the possible genotoxic potential of xenobiotics (*hazard identification*). Therefore, the combination of human liver slices and ^{32}P-postlabelling provides an important tool for *risk assessment* in humans.

Acknowledgements

The expert technical help of Ch. Gansau, K. Böttger, T. Jung, E. Schmid and A. Salomon is gratefully acknowledged.

References

BACH, P. H., VICKERS, A. E. M., FISHER, R., BAUMANN, A., BRITTEBO, E., CARLILE, D.J., KOSTER, H. J., LAKE, B. G., SALMON, F., SAWYER, T. W. and SKIBINKSI, G. 1996, The use of tissue slices for pharmacotoxicology studies. *ATLA – Alternative to Laboratory Animals*, **24**, 893–923.

BADAWI, A. F., HIRVONEN, A., BELL, D. A., LANG, N. P. and KADLUBAR, F. F. 1995, Role of aromatic amine acetyltransferases, NAT1 and NAT2, in carcinogen–DNA adduct formation in the human urinary bladder. *Cancer Research*, **55**, 5230–5237.

BAUMANN, A., KERDAR, R. S., CRAMER, P., FESER, W., BLODE, H., SALOMON, A. and KUHNZ, W. 1996, Use of rat and human liver slices for the detection of steroid hormone-induced DNA-adducts *in vitro* by means of the ^{32}P-postlabeling technique. *Pharmacology and Toxicology*, **78**, 214–223.

BEAMOND, J. A., PRICE, R. J., BLOWERS, S. D., WIELD, P. T., CUNNINGHAME, M. E. and LAKE, B. G. 1994, Use of precision-cut liver slices for studies of unscheduled DNA-synthesis. *Food and Chemical Toxicology*, **32**, 819–829.

BELAND, F. A. and KADLUBAR, F. F. 1990, Metabolic activation and DNA adducts of aromatic amines and nitroaromatic hydrocarbons. In *Chemical Carcinogenesis and Mutagenesis*, C.S. Cooper and P.L. Grover, eds (Berlin: Springer-Verlag), pp. 267–325.

BLAAUBOER, B. J., BOOBIS, A. R., CASTELL, J. V., COECKE, S., GROOTHUIS, G. M. M., GUILLOUZO, A., HALL, T. J., HAWKSWORTH, G. M., LORENZON, G., MILTENBURGER, H. G., ROGIERS, V., SKETT, P., VILLA, P. and WIEBEL, F. J. 1994, The practical applicability of hepatocyte cultures in routine testing. *ATLA – Alternatives to Laboratory Animals*, **22**, 231–241.

BRENDEL, K., GANDOLFI, A. J., KRUMDIECK, C. L. and SMITH, P. F. 1987, Tissue slicing and culturing revisited. *Trends in Pharmacological Sciences*, **8**, 11–15.

BUTLER, M. A., IWASAKI, M., GUENGERICH, F. P. and KADLUBAR, F. F. 1989, Human cytochrome P-450$_{PA}$ (P-450IA2), the phenacetin O-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines. *Proceedings of the National Academy of Sciences, USA*, **86**, 7696–7700.

BUTTERWORTH, B. E., SMITH-OLIVER, T., EARLE, L., LOURRY, D. J., WHITE, R. D., DOOLITTLE, D. J., WORKING, P. K., CATTLEY, R. C., JIRTLE, R., MICHALOPOLOS, G. and STROM, S. 1989, Use of primary cultures of human hepatocytes in toxicology studies. *Cancer Research*, **49**, 1075–1084.

CONNERS, S., RANKIN, D. R., GANDOLFI, J., KRUMDIECK, C. L., KOEP, L. J. and BRENDEL, K. 1990, Cocain hepatotoxicity in cultured liver slices: a species comparison. *Toxicology*, **61**, 171–183.

CULP, S. J., POIRIER, M. C. and BELAND, F. A. 1993, Biphasic removal of DNA-adducts in a repetitive DNA sequence after dietary administration of 2-acetylaminofluorene. *Environmental Health Perspectives*, **99**, 273–275.

DOGTEROM, P. 1993, Development of a simple incubation system for metabolism studies with precision-cut liver slices. *Drug Metabolism and Disposition*, **21**, 699–704.

DRAHUSHUK, A. T., MCGARRIGLE, B. P., TAI, H. L., KITIREEWAN, S., GOLDSTEIN, J. A. and OLSON, J. A. 1996, Validation of precision-cut liver slices in dynamic organ culture as an *in vitro* model for studying CYP 1A1 and CYP1A2 induction. *Toxicology and Applied Pharmacology*, **140**, 393–403.

ENGELHARDT, D., EISENBURGH, J., UNTERBURGER, P. and KARL, H. J. 1971, Untersuchungen über den Stoffwechsel von Testosteron und Δ4-Anrostendion in der Leber des Menschen. *Klinische Wochenschrift*, **49**, 439–440.

ENGELHARDT, D., UNTERBURGER, P. and KARL, H. J. 1974, Studies on testosterone metabolism in liver and extrahepatic tissues of man. *Journal of Steroid Biochemistry*, **5**, 318–319.

FENG, YI, JIANG, W. and HEIN, D. W. 1996, 2-aminofluorene – DNA adduct levels in tumor-target and nontarget organs of rapid and slow acetylator syrian hamsters congenic at the NAT2 locus. *Toxicology and Applied Pharmacology*, **141**, 248–255.

FESER, W., KERDAR, R. S., BAUMANN, A., KÖRBER, J., BLODE, H. and KUHNZ, W. 1998, DNA adduct formation of selected sex steroids in human liver slices *in vitro*. *Toxicology in vitro*, **12**, 353–364.

FISHER, R. L., BRENDEL, K. and HANZLIK, R. P. 1993, Correlation of metabolism, covalent binding and toxicity for a series of brombenzene derivatives using rat liver slices *in vitro*. *Chemico-Biological Interactions*, **88**, 191–208.

FISHER, R. L., HASAL, S. J., SIPES, I. G., GANDOLFI, A. J. and BRENDEL, K. 1995, Comparative metabolism and toxicity of dichlorobenzenes in Sprague–Dawley, Fischer-344 and human liver slices. *Human and Experimental Toxicology*, **14**, 414–421.

GALLAGHER, J. E., JACKSON, M. A., GEORGE, M. H., LEWTAS, J. and ROBERTSON, I. G. C. 1989, Differences in detection of DNA adducts in the ^{32}P-postlabeling assay after either L-butanol extraction or nuclease P1 treatment. *Cancer Letters*, **45**, 7–12.

GUILLOUZO, A. 1992, Hepatotoxicity. In *In vitro Toxicity Testing. Applications to Safety Evaluation*, J. M. Frazier, ed. (New York, Marcel Dekker), pp. 45–83.

GUILLOUZO, A., MOREL, F., FARDEL, O. and MEUNIER, B. 1993, Use of human hepatocyte cultures for drug metabolism studies. *Toxicology*, **82**, 209–219.

GUPTA, R. C., REDDY, M. V. and RANDERATH, K. 1982, ^{32}P-postlabeling analysis of nonradioactive aromatic carcinogen–DNA adducts. *Carcinogenesis*, **3**, 1081–1092.

GUPTA, R. C. 1985, Enhanced sensitivity of ^{32}P-postlabeling analysis of aromatic carcinogen DNA adducts. *Cancer Research*, **45**, 5656–5662.

GUPTA, R. C. and EARLEY, K. 1988, ^{32}P-adduct assay: comparative recoveries of structurally diverse DNA adducts in the various enhancement procedures. *Carcinogenesis*, **9**, 1687–1693.

GUPTA, R. C. and SPENCE-BEACH, G. 1996, Natural and endogenous adducts as detected by ^{32}P-postlabeling. *Regulatory Toxicology and Pharmacology*, **23**, 14–21.

KIRLIN, W. G., TRINIDAD, A., YEROKUN, T., OGOLLA, F., FERGUSON, R. J., ANDREWS, A. F., BRADY, P. K. and HEIN D. W. 1989, Polymorphic expression of acetyl coenzyme A-dependent O-acetyltransferase-mediated activation of N-hydroxyarylamines by human bladder cytosols. *Cancer Research*, **49**, 2448–2454.

KOCHAKIAN, C. D. 1990, Metabolites of testosterone: significance in the vital economy. *Steroids*, **55**, 92–97.

KRUMDIECK, C. L., DOS SANTOS, J. E. and HO, K. J. 1980, A new instrument for the rapid preparation of tissue slices. *Analytical Biochemistry*, **104**, 118–123.

LAKE, B. G., BEAMOND, J. A., WIELD, P. T. and PRICE, R.J. 1996, Use of precision-cut liver slices to evaluate species differences in 2-acetylaminofluorene-induced unscheduled DNA-synthesis. *Toxicology and Applied Pharmacology*, **138**, 231–241.

LISBOA, B. P., DROSSÉ, J. and BREUER, H. 1965, Stoffwechsel vonTestosteron in Leberschnitten des Menschen. *Zeitschrift für Physikalische Chemie*, **342**, 123–131.

MÄENPÄÄ, J., PELKONEN, O., CRESTEIL, T. and RANE, A. 1993, The role of cytochrome P450 3A (CYP3A) isoform(s) in the oxidative metabolism of testosterone and benzphetamine in human adult and fetal liver. *Journal of Steroid Biochemistry and Molecular Biology*, **44**, 61–67.

MASER, E. 1995, Xenobiotic carbonyl reduction and physiological steroid oxidoreduction. *Biochemical Pharmacology*, **49**, 421–440.

MILLER, J. A. and MILLER, E. C. 1966, Mechanisms of chemical carcinogenesis: Naturate of proximate carcinogens and interactions with macromolecules. *Pharmacological Review*, **18**, 805–838.

MILLER, E. C. and MILLER, J. A. 1981, Searches for ultimate chemical carcinogens and their reactions with cellular macromolecules. *Cancer*, **47**, 2327–2345.

MILLER, G., BEYER, J., HALL, G. L., DE GRAFFENRIED, L. A. and ADAMS, P. E. 1993, Predictive value of liver slices for metabolism and toxicity *in vivo*: use of acetaminophen as a model hepatotoxicant. *Toxicology and Applied Pharmacology*, **104**, 108–116.

MONTHEITH, D. K. and GUPTA, R. C. 1992, Carcinogen–DNA-adducts in cultures of rat and human hepatocytes. *Cancer Letters*, **62**, 87–93.

OGURI, K., YAMADA, H. and YOSHIMURA, H. 1994, Regiochemistry of cytochrome P 450 isoenzyme. *Annual Review of Pharmacology and Toxicology*, **34**, 251–279.

PHILLIPS, D., CASTEGNARO, M. and BARTSCH, H. 1993. Postlabeling methods for detection of DNA adducts. Vol. 124 (Lyon: IARC).

PIERCE, J. and SUELTER, C. H. 1977, An evaluation of the coomassie brilliant blue G-250 dye-binding method for quantitative protein determination. *Analytical Biochemistry*, **81**, 478–480.

PURCHASE, I. F. H. 1994, Current knowledge of mechanisms of carcinogenicity: Genotoxicity versus non-genotoxins. *Human and Experimental Toxicology*, **13**, 17–28.

RANDERATH, K. and RANDERATH, E. 1994, ^{32}P-postlabeling methods for DNA adduct detection: Overview and critical evaluation. *Drug Metabolism Reviews*, **26**, 67–85.

REDDY, M. V. and RANDERATH, K. 1986, Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. *Carcinogenesis*, **7**, 1543–1551.

SIPES, I. G., FISHER, R. L., SMITH, P. F., STINE, E. R., GANDOLFI, A. J. and BRENDEL, K. 1987, A dynamic liver culture system: a tool for studying chemical biotransformation and toxicity. *Archives of Toxicology*, Suppl. 11, 20–33.

SMITH, P. F., FISHER, R., SHUBAT, P. J., GANDOLFI, A. J., KRUMDIECK, C. L., KOEP, L. J. and BRENDEL, K. 1987, In vitro cytotoxicity of allyl alcohol and bromobenzene in a novel organ culture system. *Toxicology and Applied Pharmacology*, **87**, 509–522.

SMITH, P. F., GANDOLFI, A. J., KRUMDIECK, C. L., PUTNAM, C. W., ZUKOSKI, C. F., DAVIS, W. M. and BRENDEL, K. 1985, Dynamic organ culture of precision liver slices for *in vitro* toxicology. *Life Sciences*, **36**, 1367–1375.

SMITH, P. F., MCKEE, R., GANDOLFI, P. A. J., KRUMDIECK, C. L., FISHER, R. and BRENDEL, K. 1989, Precision-cut liver slices: a new *in vitro* in toxicology. In *In vitro Toxicology: Model Systems and Methods*, C.A. McQueen, ed. (New Jersey: Telford Press), pp. 93–130.

STYLIANOU, M., FORCHIELLI, E., TUMILLO, M. and DORFMAN, R. I. 1961, Metabolism *in vitro* of 4-C^{14}-testosterone by a human liver homogenate. *Journal of Biological Chemistry*, **236**, 692–694.

SWIERENGA, S. H. H., BRADLAW, J. A., BRILLINGER, R. L., GILMAN, J. P. W., NESTMANN, E. R. and SAN, R. C. 1991, Recommended protocols based on survey of current practice in genotoxicity testing laboratories. I. Unscheduled DNA synthesis assay in rat hepatocyte culures. *Mutations Research*, **246**, 235–253.

TOPINKA, J., ANDRAE, U., SCHWARZ, L. R. and WOLFF, T. 1993, Cyproterone acetate generates DNA adducts in rat liver and in primary rat hepatocyte cultures. *Carcinogenesis*, **14**, 423–427

WAXMAN, D. J. 1988, Interaction of hepatic cytochromes P450 with steroid hormones. *Biochemical Pharmacology*, **37**, 48–71.

WAXMAN, D. J., LAPENSON, D. P., AOYAMA, T., GELBOIN, H. V., GONZALES, F. J. and KORZEKWA, K. 1991, Steroid hormone hydroxylase specificities of eleven cDNA-expressed human cytochrome P450s. *Archives of Biochemistry and Biophysics*, **290**, 160–166.

WERNER, S., TOPINKA, J., KUNZ, S., BECKERTS, T., HEIDECKE, C.-D., SCHWARZ, L. R. and WOLFF, T. 1996, Studies on the formation of hepatic DNA-adducts by the antiandrogenic and gestagenic drug, cyproterone acetate. In *Biological Reactive Intermediates V* (New York and London: Plenum Press), pp. 253–257.

WILLIAMS, G. M. 1977), Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cultures. *Cancer Research*, **37**, 1854–1851.

WOOD, A. W., RYAN, D. E., THOMAS, P. E. and LEVIN, W. 1983, Regio- and stereoselective metabolism of two C19 steroids by five highly purified and reconstituted rat hepatic cytochrome P450 isozymes. *Journal of Biological Chemistry*, **258**, 8839–8847.

WORTMANN, W., KNAPSTEIN, P., MAPPES, G., DICK, G. and OERTEL, G. W. 1971, *In vivo* perfusion of the human liver with steroids. *Acta Endocrinologica*, **68**, 561–566.