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New Hepatocyte In Vitro Systems for Drug Metabolism: Metabolic Capacity and Recommendations for Application in Basic Research and Drug Development, Standard Operation Procedures

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ABSTRACT

Primary hepatocytes represent a well-accepted in vitro cell culture system for studies of drug metabolism, enzyme induction, transplantation, viral hepatitis, and hepatocyte regeneration. Recently, a multicentric research program has been initiated to optimize and standardize new in vitro systems with hepatocytes. In this article, we discuss five of these in vitro systems: hepatocytes in suspension, perifusion culture systems, liver slices, co-culture systems of hepatocytes with intestinal bacteria, and 96-well plate bioreactors. From a technical point of view, freshly isolated or cryopreserved hepatocytes in suspension represent a readily available and easy-to-handle in vitro system that can be used to characterize the metabolism of test substances. Hepatocytes in suspension correctly predict interspecies differences in drug metabolism, which is demonstrated with pantoprazole and propafenone. A limitation of the hepatocyte suspensions is the length of the incubation period, which should not exceed 4 hr. This incubation period is sufficiently long to determine the metabolic stability and to allow identification of the main metabolites of a test substance, but may be too short to allow generation of some minor, particularly phase II metabolites, that contribute less than 3% to total metabolism. To achieve longer incubation periods, hepatocyte culture systems or bioreactors are used. In this research program, two bioreactor systems have been optimized: the perifusion culture system and 96-well plate bioreactors. The perifusion culture system consists of collagen-coated slides allowing the continuous superfusion of a hepatocyte monolayer with culture medium as well as establishment of a constant atmosphere of 13% oxygen, 82% nitrogen, and 5% CO_2. This system is stable for at least 2 weeks and guarantees a remarkable sensitivity to enzyme induction, even if weak inducers are tested. A particular advantage of this system is that the same bioreactor can be perfused with different concentrations of a test substance in a sequential manner. The 96-well plate bioreactor runs 96 modules in parallel for pharmacokinetic testing under aerobic culture conditions. This system combines the advantages of a three-dimensional culture system in collagen gel, controlled oxygen supply, and constant culture medium conditions, with the possibility of high throughput and automatization. A newly developed co-culture system of hepatocytes with intestinal bacteria offers the possibility to study the metabolic interaction between liver and intestinal microflora. It consists of two chambers separated by a permeable polycarbonate membrane, where hepatocytes are cultured under aerobic and intestinal bacteria in anaerobic conditions. Test substances are added to the aerobic side to allow their initial metabolism by the hepatocytes, followed by the metabolism by intestinal bacteria at the anaerobic side. Precision-cut slices represent an alternative to isolated hepatocytes and have been used for the investigation of hepatic metabolism, hepatotoxicity, and enzyme induction. A specific advantage of liver slices is the possibility to study toxic effects on hepatocytes that are mediated or modified by nonparenchymal cells (e.g., by cytokine release from Kupffer cells) because the physiological liver microarchitecture is maintained in cultured slices. For all these in vitro systems, a prevalidation has been performed using standard assays for phase I and II enzymes. Representative results with test substances and recommendations for application of these in vitro systems, as well as standard operation procedures are given.

INTRODUCTION

The use of primary hepatocytes is well established for the study of drug metabolism and drug–drug interactions. In vitro systems with primary

hepatocytes are frequently applied for 1) prediction of in vivo metabolism of test substances, 2) identification of hepatotoxic and/or genotoxic substances, and 3) identification of substances that cause enzyme induction or inhibition (Hengstler et al., 1999; 2003; Osterod et al., 2001; 2002; Ringel et al., 2002). Good correlations have been observed between data obtained in vitro using hepatocytes and the in vivo situation (Hengstler et al., 2000a,b; Li et al., 1999).

A limitation of working with primary human hepatocytes is availability. Therefore, several groups initiated research projects to generate hepatocytes. Promising strategies include 1) transdifferentiation of human precursor cells to hepatocytes, 2) conditional (i.e., reversible) immortalization of primary hepatocytes, and 3) expansion of primary human hepatocytes in an adequate microenvironment by growth factors (Beerheide et al., 2002). However, although promising results have been achieved, these approaches have not yet resulted in generation of cells that express similar differentiated functions as primary human hepatocytes concerning metabolism and enzyme induction. Thus, primary hepatocytes cannot yet be replaced by any other cell type.

This article summarizes the results of a joint project comprising several groups from university and industry that has been initiated by the German Federal Ministry of Education and Research. Several hepatocyte in vitro systems have been optimized and standardized, and are discussed in this review, namely 1) hepatocytes in suspension, 2) perifusion culture systems, 3) liver slices, 4) co-culture systems of hepatocytes with intestinal bacteria, and 5) 96-well bioreactors. All these in vitro systems have been used with cryopreserved material and their properties were compared with those of the systems that used freshly isolated hepatocytes. For each of the studied in vitro systems, data characterizing their metabolic capacity, examples of application in drug development, and standard operation procedures (SOPs) are given.

HEPATOCYTES IN SUSPENSION

Introduction and Brief Technical Description

Freshly isolated and cryopreserved hepatocytes in suspension have been applied for short-term incubations up to 4 hr with test substances to predict their in vivo metabolism. From a technical point of view, hepatocytes in suspension are certainly the easiest to handle of all in vitro hepatocyte systems. Freshly isolated hepatocytes are readily available after isolation; cryopreserved hepatocytes are thawed and purified by Percoll centrifugation. Then the hepatocytes are incubated with test substances on a shaking water bath (see following "SOP" section). Usually a single incubation contains 1 million hepatocytes in 1 mL of suspension buffer in a glass vial together with the test substance. However, approximately 3 million initially cryopreserved hepatocytes are required for a single incubation due to the loss of cells during the thawing and Percoll purification procedures. Care should be taken that test substances are also incubated in suspension buffer, without hepatocytes to test their stability in the incubation buffer and their adhesion to the glass vial. When new analytical

procedures are employed, incubations of hepatocytes in suspension buffer without test substance should also be included.

Prevalidation with Standard Test Substances

The in vitro system of hepatocytes in suspension has been prevalidated by testing activities of several phase I and phase II metabolizing enzymes (Tables 1a–f) (Hengstler et al., 2000a,b; Steinberg et al., 1999). Data on lactate dehydrogenase release, albumin secretion, 7-ethoxyresorufin-O-deethylase (EROD) and 7-ethoxycoumarin-O-deethylase (ECOD) activities, testosterone hydroxylation, uridine 5′-diphosphate (UDP)-glucuronosyltransferase, and glutathione-S-transferase activities in freshly isolated and cryopreserved hepatocytes in suspension are summarized in Tables 1a–f. From these data, it is obvious that enzyme activities decrease during a 2-hr incubation period and that activities in cryopreserved hepatocytes are usually lower than in freshly isolated cells. Nevertheless, the decrease in phase I metabolizing activities—either during a 2-hr incubation period, or of freshly isolated compared with cryopreserved hepatocytes, or of cryopreserved hepatocytes compared with fresh liver tissue—was always less than 50% (Tables 1a–c). This should be acceptable for drug metabolism studies. Preservation of phase II enzymes is more problematic compared with phase I enzymes. Without Percoll purification, some activities in cryopreserved hepatocytes were lower than 50% of the activities in freshly isolated cells. However, after Percoll centrifugation, phase II enzyme activities were clearly improved—not completely to the level of freshly isolated cells, but to an extent that should be acceptable for metabolic studies. Therefore, Percoll purification was included in all studies with freshly isolated or cryopreserved hepatocytes in suspension.

Examples for Application

Pantoprazole

Today pantoprazole, 5-(difluormethoxy)-2-[3,4-demethoxy-2-pyridyl)methylsulfinyl]-benzimidazole, is one of the most frequently applied proton pump inhibitors. In Beagle dogs pantoprazole caused toxicity at higher doses. This toxicity was due to formation of the metabolite benzimidazo-2-thiol from pantoprazole in the liver of Beagle dogs. However, it has been shown that this toxicity in Beagle dogs is not relevant for humans, where benzimodazo-2-thiol was not detectable. We incubated cryopreserved hepatocytes of Beagle dogs in suspension with pantoprazole and observed a time dependent formation of benzimidazo-2-thiol (Fig. 1). Under similar conditions human hepatocytes did not form a detectable amount of benzimidazo-2-thiol. This example shows that cryopreserved hepatocytes in suspension can identify interspecies differences in drug metabolism that are responsible for interspecies differences in toxicity.

Table 1a. Human hepatocytes in suspension: viability and phase I metabolism of freshly isolated and cryopreserved cells.[a]

Time in suspension (min)	Freshly isolated hepatocytes	Cryopreserved hepatocytes
LDH[b] retention (%)		
0	89 ± 1.7	82 ± 4.2
30	85 ± 3.3	79 ± 3.0
60	84 ± 4.2	76 ± 3.3
120	80 ± 5.9	70 ± 5.0
Albumin secretion (ng/hr/10^6 hepatocytes)		
0	163 ± 41	256 ± 12
30	216 ± 35	262 ± 32
60	230 ± 47	265 ± 20
120	254 ± 64	262 ± 29
EROD[c] (pmol/min/mg protein)		
0	0.74 ± 0.19	0.46 ± 0.26
30	0.67 ± 0.26	0.44 ± 0.29
60	0.66 ± 0.19	0.54 ± 0.16
120	0.56 ± 0.19	0.44 ± 0.10
Liver homogenate	0.80 ± 0.21	
ECOD[d] (pmol/min/mg protein)		
0	49 ± 24	41 ± 18
30	39 ± 29	33 ± 21
60	36 ± 17	25 ± 17
120	17 ± 7	27 ± 17
Liver homogenate	56 ± 13	
Total TH[e] (pmol/min/mg protein)		
0	841 ± 156	592 ± 125
30	654 ± 171	561 ± 155
60	655 ± 101	514 ± 94
120	436 ± 47	512 ± 148
Liver homogenate	623 ± 452	

Total TH (pmol/min/mg protein)	Position of hydroxylation			
	6β-OHT	2β-OHT	6β-OHT	2β-OHT
0	626 ± 157	76 ± 18	468 ± 124	62 ± 19
30	517 ± 155	62 ± 15	442 ± 136	54 ± 14
60	525 ± 57	67 ± 11	398 ± 71	51 ± 12
120	335 ± 52	39 ± 5	405 ± 179	47 ± 24

[a] Data represent mean values and standard deviations of hepatocytes obtained from seven rats.
[b] LDH, Lactate dehydrogenase.
[c] EROD, 7-ethoxyresorufin-O-deethylase.
[d] ECOD, 7-ethoxycoumarin-O-deethylase.
[e] TH, testosterone hydroxylation.

Table 1b. Rat (Sprague Dawley, male) hepatocytes in suspension: viability and phase I metabolism of freshly isolated and cryopreserved cells.[a]

Time in suspension (min)	Freshly isolated hepatocytes	Cryopreserved hepatocytes
LDH[b] retention (%)		
0	85 ± 4.1	81 ± 12.8
30	83 ± 5.1	75 ± 15.3
60	82 ± 3.1	61 ± 4.1
120	80 ± 4.1	52 ± 2.6
EROD[c] (pmol/min/mg protein)		
0	0.94 ± 0.12	0.79 ± 0.33
30	1.07 ± 0.21	0.91 ± 0.19
60	0.66 ± 0.28	0.65 ± 0.26
120	0.76 ± 0.02	0.41 ± 0.21
Liver homogenate	1.31 ± 0.25	
ECOD[d] (pmol/min/mg protein)		
0	131 ± 28	115 ± 23
30	128 ± 16	109 ± 6
60	99 ± 15	83 ± 13
120	73 ± 12	53 ± 6
Liver homogenate	142 ± 31	
Total TH[e] (pmol/min/mg protein)		
0	1942 ± 335	1642 ± 318
30	1959 ± 724	1394 ± 512
60	1659 ± 53	1341 ± 230
120	1500 ± 18	988 ± 194
Liver homogenate	2542 ± 71	

Main individual TH products (pmol/min/mg protein)

	Position of hydroxylation					
	16α-OHT	2α-OHT	6β-OHT	2β-OHT	7α-OHT	15β-OHT
Freshly isolated hepatocytes						
0	582 ± 150	523 ± 166	226 ± 34	60 ± 15	57 ± 15	56 ± 12
30	522 ± 108	498 ± 150	177 ± 25	42 ± 18	42 ± 10	55 ± 9
60	530 ± 89	459 ± 125	167 ± 50	31 ± 7	40 ± 13	49 ± 8
120	505 ± 103	430 ± 129	128 ± 27	17 ± 3	38 ± 11	50 ± 10
Cryopreserved hepatocytes						
0	557 ± 158	421 ± 126	180 ± 38	41 ± 16	36 ± 11	53 ± 7
30	523 ± 133	352 ± 110	204 ± 36	31 ± 7	39 ± 10	55 ± 11
60	478 ± 104	350 ± 94	135 ± 22	22 ± 6	31 ± 7	49 ± 8
120	355 ± 97	238 ± 77	86 ± 18	14 ± 4	24 ± 7	41 ± 7

[a] Data represent mean values and standard deviations of hepatocytes obtained from four rats.
[b] LDH, lactate dehydrogenase.
[c] EROD, 7-ethoxyresorufin-O-deethylase.
[d] ECOD, 7-ethoxycoumarin-O-deethylase.
[e] TH, testosterone hydroxylation.

Table 1c. Mouse (NMRI, male) hepatocytes in suspension: viability and phase I metabolism of freshly isolated and cryopreserved cells.[a]

Time in suspension (min)	Freshly isolated hepatocytes	Cryopreserved hepatocytes
LDH[b] retention (%)		
0	88	94
30	77	83
60	74	82
120	68	82
EROD[c] (pmol/min/mg protein)		
0	6.3	6.1
30	5.6	6.6
60	5.3	5.9
120	5.4	5.3
Liver homogenate	3.6	
ECOD[d] (pmol/min/mg protein)		
0	243	167
30	191	153
60	156	118
120	125	76
Liver homogenate	215	
Total TH[e] (pmol/min/mg protein)		
0	1833	1541
30	1583	1250
60	1291	1166
120	1041	706
Liver homogenate	1375	

Main individual TH products (pmol/min/mg protein)

	Position of hydroxylation						
	6β-OHT	15α-OHT	6α-OHT	16α-OHT	7α-OHT	2α-OHT	6β-OHT
Freshly isolated hepatocytes							
0	136	61	58	42	42	38	25
30	120	50	58	38	40	33	22
60	96	45	54	26	56	36	17
120	69	41	39	23	50	30	16
Cryopreserved hepatocytes							
0	137	53	50	42	48	35	27
30	101	51	45	29	36	42	30
60	96	52	46	31	33	42	40
120	60	46	33	22	25	34	17

[a] Data represent mean values of hepatocytes pooled from six mice.

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Table 1d. Human hepatocytes in suspension: phase II metabolism of freshly isolated hepatocytes in suspension.

Time in suspension (min)	Activity (nmol HOBI/min/mg protein)
UDP-glucuronosyl transferase substrate: 4-hydroxybiphenyl (HOBI)	
0	39 ± 11
30	39 ± 12
60	33 ± 11
120	21 ± 8

Time in suspension (min)	Activity (nmol MUF/min/mg protein)
UDP-glucuronosyl transferase substrate: 4-methyl-umbelliferone (MUF)	
0	6.7 ± 2.0
30	6.4 ± 2.5
60	5.7 ± 0.7
120	4.9 ± 1.1

Time in suspension (min)	Activity (μmol CDNB/min/mg protein)
Glutathione S-transferase substrate: 1-chloro-2,4-dinitro-benzene (CDNB)	
0	0.60 ± 0.20
30	0.58 ± 0.18
60	0.62 ± 0.30
120	0.61 ± 0.34

Time in suspension (min)	Activity (nmol 2-NA/min/mg protein)
Sulfotransferase substrate: 2-naphthol (2-NA)	
0	0.21 ± 0.10
30	0.22 ± 0.02
60	0.20 ± 0.16
120	0.18 ± 0.08

Table 1e. Rat (Sprague Dawly, male) hepatocytes in suspension: phase II metabolism of freshly isolated cells.

Time in suspension (min)	Activity (nmol HOBI/min/mg protein)
UDP-glucuronosyl transferase substrate: 4-hydroxybiphenyl (HOBI)	
0	69 ± 26
30	78 ± 23
60	69 ± 27
120	66 ± 36

Time in suspension (min)	Activity (nmol MUF/min/mg protein)
UDP-glucuronosyl transferase substrate: 4-methyl-umbelliferone (MUF)	
0	18.0 ± 2.1
30	15.2 ± 2.8
60	16.2 ± 1.8
120	16.6 ± 1.4

Time in suspension (min)	Activity (μmol CDNB/min/mg protein)
Glutathione S-transferase substrate: 1-chloro-2,4-dinitro-benzene (CDNB)	
0	1.40 ± 0.28
30	1.24 ± 0.52
60	1.36 ± 0.60
120	1.20 ± 0.56

Time in suspension (min)	Activity (nmol 2-NA/min/mg protein)
Sulfotransferase substrate: 2-naphthol (2-NA)	
0	0.80 ± 0.30
30	0.60 ± 0.14
60	0.84 ± 0.50
120	0.45 ± 0.10

Table 1f. Mouse hepatocytes in suspension: phase II metabolism of freshly isolated cells.

Time in suspension (min)	Activity (nmol HOBI/min/mg protein)
UDP-glucuronosyl transferase substrate: 4-hydroxybiphenyl (HOBI)	
0	110 ± 39
30	107 ± 42
60	78 ± 36
120	84 ± 14

Time in suspension (min)	Activity (nmol MUF/min/mg protein)
UDP-glucuronosyl transferase substrate: 4-methyl-umbelliferone (MUF)	
0	26.1 ± 7.8
30	24.0 ± 9.9
60	28.2 ± 10.9
120	23.0 ± 12.7

Time in suspension (min)	Activity (μmol CDNB/min/mg protein)
Glutathione S-transferase substrate: 1-chloro-2,4-dinitro-benzene (CDNB)	
0	1.26 ± 0.30
30	1.24 ± 0.50
60	1.34 ± 0.60
120	1.16 ± 0.64

Time in suspension (min)	Activity (nmol 2-NA/min /mg protein)
Sulfotransferase substrate: 2-naphthol (2-NA)	
0	0.83 ± 0.20
30	0.58 ± 0.21
60	0.36 ± 0.22
120	0.37 ± 0.30

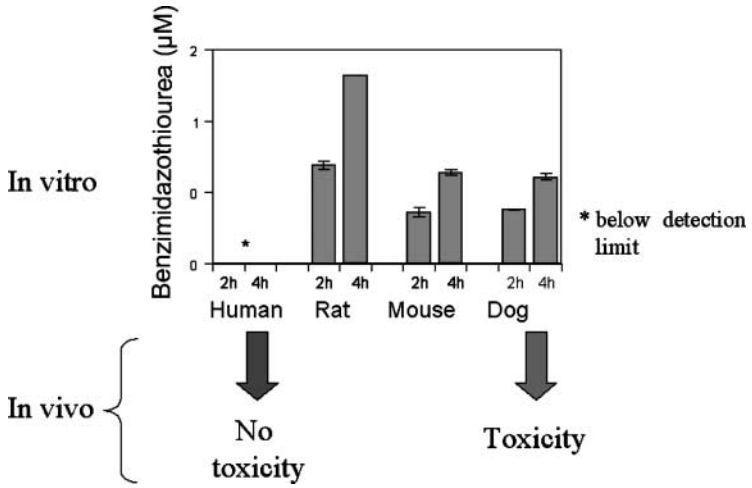

Figure 1. Formation of the toxic benzimidazothiourea from pantoprazole by cryopreserved hepatocytes in suspension.

Propafenone

The class IC antiarrhythmic drug propafenone, 2′-[2-hydroxy-3-(propylamino)-propoxy]-3-phenylpropiophenone, represents another example for interspecies differences in metabolism. The largest differences have been observed for the metabolic formation of 5- vs. 4′-hydroxy propafenone. In humans 5-hydroxy propafenone is the main metabolite, whereas 4′-hydroxy propafenone is not detectable (Table 2). A similar result was obtained in vitro after incubation of propafenone with cryopreserved human hepatocytes in suspension for 2 hr. A completely different metabolism was observed for rats and mice; in vivo, formation of 4′-hydroxy propafenone strongly dominated. No 5-hydroxy propafenone was detected (Table 2). Again, this constellation was correctly "predicted" by cryopreserved rat and mice hepatocytes in suspension. Thus, rats (Sprague Dawley, male) and mice (NMRI, male) are not adequate species to extrapolate to the human propafenone metabolism; thus, by treatment of these rodents with propafenone, the toxicity of 5-hydroxy propafenone, the major human metabolite, cannot be evaluated in rats and mice. The ratio of 5- to 4′-hydroxy propafenone in male Beagle dogs is more similar to the human situation (Table 2). Studies with cryopreserved male cynomolgous monkey hepatocytes in vitro predict that the in vivo metabolism of this species closely resembles the metabolism in humans. However, data on the in vivo metabolism of propafenone in cynomologous monkeys are not available. The example of propafenone demonstrates that studies with the nonrodent species dog or monkey are required to evaluate the toxicity of this drug to accommodate for species differences in the drug metabolism.

However, it should be mentioned that such large interspecies differences in metabolism as described for propafenone or pantoprazole are not representative for the majority of compounds. For instance, interspecies differences between humans, male Sprague Dawley rats, and male NMRI mice in the metabolism of the calcium channel blocker verapamil are relatively small (data not shown).

Table 2. Propafenone metabolism in vitro in cryopreserved hepatocytes in suspension compared with the in vivo situation.

Metabolite (%)	Human		Rat (Sprague Dawley, male)		Mouse (NMRI, male)		Dog (Beagle, male)		Monkey (cynomolgus, male)
	In vivo	In vitro	In vivo	In vitro	In vivo	In vitro	In vivo	In vitro	In vitro
5-OH-propafenone	100	96	nd[a]	8	nd	6	60	58	88
4′-OH-propafenone	nd	4	100	92	100	94	40	42	12

[a] nd, not detectable.

Validation Studies

As described for pantoprazole and propafenone, the in vitro metabolism of 14 drugs was examined with cryopreserved hepatocytes in suspension. For these drugs, the in vivo metabolism (metabolites in blood, urine, and feces) in humans and at least one of the species rat, mouse, dog, or monkey is known. In these in vivo studies that have been performed by the cooperating pharmaceutical companies, 177 major metabolites have been identified. Metabolites contributing less than 3% to total metabolites were not considered. Of the 177 metabolites, 165 (93%) were formed in vitro by cryopreserved hepatocytes in suspension. Thus, this in vitro system exhibits a remarkable metabolic capacity, and quite a good predictivity for the major metabolites formed in vivo is observed. Metabolites not formed in vitro by the cryopreserved hepatocytes in suspension were phase II metabolites, predominantly glucuronides.

To examine the potential of the in vitro system with hepatocytes in suspension with respect to minor metabolites (contributing less than 3% to total metabolism), the metabolism of eight [^{14}C]-radiolabeled substances was examined using three in vitro systems with rat hepatocytes: 1) freshly isolated hepatocytes in suspension with 1×10^6 cells/mL for 2 hr, 2) cryopreserved hepatocytes in suspension with 1×10^6 cells/mL for 2 hr, and 3) freshly isolated hepatocytes in culture on collagen-coated dishes incubated for 24 hr. For comparison, the substances were also incubated with S9 rat liver fractions for 2 hr. The concentration of all eight test substances in the incubation was 25 μM. In contrast to the validation study described above, complete metabolic profiles were obtained in which all metabolites were analyzed, including minor metabolites that were formed only in amounts slightly above the detection limit (0.1% to 0.2% of the total radioactivity in the incubation). The results are summarized in Table 3.

The analysis of the metabolite profiles of the [^{14}C]-radiolabeled substances in the four in vitro metabolism systems shows that the incubation of the substances in freshly isolated hepatocytes in suspension results in substantial metabolism and the formation of numerous metabolites. In comparison to the freshly isolated hepatocytes, the cryopreserved cells in suspension show a somewhat attenuated metabolic capacity in the overall metabolism rate as well as in the number of formed metabolites. Some metabolites formed by the freshly isolated hepatocytes appear in a reduced amount, and some metabolites are totally absent after incubation with cryopreserved hepatocytes in suspension. The cryopreserved hepatocytes in suspension formed no additional metabolites compared with freshly isolated cells (Table 3). For a more detailed analysis of the differences between freshly isolated and cryopreserved hepatocytes, we differentiated between major and minor metabolites. Metabolites with more than 3% of the total radioactivity were considered to be major metabolites. This analysis showed that, from 18 major metabolites formed by freshly isolated cells, only 14 metabolites (78%) were formed by cryopreserved cells. From the 41 minor metabolites formed by the freshly isolated cells, only 16 minor metabolites (39%) were formed by the cryopreserved cells.

Freshly isolated hepatocytes in culture over 24 hr show a more extensive metabolism than either freshly isolated or cryopreserved hepatocytes in suspension over 2 hr. Differences in the metabolite profiles were also observed. Some metabolites obtained with suspended cells were not found with cells in culture, whereas vice versa the cultured cells formed some metabolites that were not obtained with the cells in suspension. With

Table 3. Metabolism of eight [^{14}C]-radiolabelled substances by freshly isolated and cryopreserved rat hepatocytes in suspension after 2 hr of incubation, by freshly isolated rat hepatocytes in culture after 24 hr of incubation and by S9 rat liver fractions after 2 hr of incubation.[a]

	Test substances[b]							
	[^{14}C]2	[^{14}C]03	[^{14}C]34	[^{14}C]42	[^{14}C]66	[^{14}C]69	[^{14}C]71	[^{14}C]80
Freshly isolated hepatocytes in suspension								
Metabolic stability[c] (%)	88.6	51.6	67.1	49.4	90.5	92.4	59.1	37.8
Total number of metabolites	7	16	6	7	2	5	12	4
Number of major metabolites[d]	2	5	2	1	2	1	1	4
Number of minor metabolites[d]	5	11	4	6	0	4	11	0
Formation of acyl glucuronide (%)	—	—	22.8	46.3	—	—	—	—
Cryopreserved hepatocytes in suspension								
Metabolic stability[c] (%)	96.1	28.4	94.1	92.0	96.3	95.6	86.6	67.7
Total number of metabolites	6	9	3	1	1	3	5	3
Number of major metabolites[d]	0	6	1	1	1	0	1	3
Number of minor metabolites[d]	6	3	2	0	0	3	4	0
Formation of acyl glucuronide (%)	—	—	3.5	8.0	—	—	—	—
Freshly isolated hepatocytes in culture								
Metabolic stability[c] (%)	63.5	35.6	14.8	5.2	100	86.9	78.8	87.7
Total number of metabolites	7	14	4	11	0	5	3	2
Number of major metabolites[d]	3	6	2	8	0	1	1	2
Number of minor metabolites[d]	4	8	2	3	0	4	2	0
Formation of acyl glucuronide (%)	—	—	71.8	54.9	—	—	—	—
S9 liver fractions								
Metabolic stability[c] (%)	95.7	40.8	95.6	85.6	87.2	83.9	71.7	77.8
Total number of metabolites	3	10	5	6	1	5	3	3
Number of major metabolites[d]	1	5	0	2	1	2	1	1
Number of minor metabolites[d]	2	5	5	4	0	3	2	2
Formation of acyl glucuronide (%)	—	—	1.5	4.9	—	—	—	—

[a] Concentration of test compounds in all systems was 25 μmol/L.

[b] All test substances are still under development and, thus, chemical structures cannot yet be published.

[c] Percent of parent compound after 2 hr of incubation compared with the initial concentration.

[d] Metabolites with $> 3\%$ of total radioactivity are considered major metabolites and those with $< 3\%$ are considered minor metabolites.

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hepatocytes in culture, 23 major metabolites were formed; 19 of these metabolites (83%) were also formed by freshly isolated hepatocytes in suspension. In contrast, of the 18 major metabolites formed by the freshly isolated hepatocytes in suspension, only 11 metabolites (61%) were formed by the hepatocytes in culture. With hepatocytes in culture, 23 minor metabolites were formed; 13 of these metabolites (56%) were also formed by freshly isolated hepatocytes in suspension. Of the 41 minor metabolites formed by the freshly isolated hepatocytes in suspension, 19 metabolites (46%) were formed by the hepatocytes in culture. In particular, an extensive formation of the acyl glucuronides of [^{14}C]34 and [^{14}C]42 is observed with the cultured hepatocytes. Freshly isolated cells in suspension also formed the acyl glucuronides to a considerable extent (32% or 84% for [^{14}C]34 and [^{14}C]42 relative to the cultured hepatocytes, respectively), but the formation of the acyl glucuronides by cryopreserved cells in suspension (4.9% or 14.6%, respectively) and by S9 liver fractions (2.1% or 8.9%, respectively) is strongly reduced. Altogether, the majority of the metabolites is formed by both freshly isolated hepatocytes in culture and in suspension. However, some metabolites are only formed by one of the in vitro systems.

In comparison to the in vitro hepatocyte systems, S9 liver fractions (without cofactor supplementation) show an attenuated metabolic capacity, the number of formed metabolites is smaller and, in particular, the formation of the phase II metabolites (e.g., the acyl glucuronides) is strongly reduced.

Overall, in the majority of the investigated cases, hepatocytes in culture show the most extensive metabolism followed by freshly isolated cells in suspension. The cryopreserved cells in suspension have a strongly reduced metabolic capacity and their metabolism is similar to that of the S9 liver fractions. In particular, the phase II metabolism—as demonstrated by the formation of the acyl glucuronides of two of the test substances—is extensive in freshly isolated cells in culture and in suspension, but strongly reduced in the cryopreserved cells in suspension and in S9 liver fractions.

The lack in metabolism by cryopreserved hepatocytes was not due to loss of viability of certain batches of cryopreserved hepatocytes. Trypan blue exclusion of all cryopreserved batches used for this study was higher than 90% after Percoll purification. In cryopreserved cells, EROD, pentoxyresorufin O-depentylation (PROD), ECOD, and 6β-hydroxylation of testosterone were 93%, 93%, 77%, and 92% of the respective activities of freshly isolated hepatocytes. However, the above-mentioned activities were performed with hepatocyte homogenate after external addition of the cofactor nicotinamide adenine dinucleotide phosphate (NADPH). These assays determine enzyme activities of the hepatocytes, but do not consider hepatocyte cofactor concentrations. Thus, a likely explanation for the reduced metabolism of the cryopreserved hepatocytes in suspension might be loss of cofactors not added to the assay system (cofactors for phase II enzymes), not compromised enzyme activities.

Interindividual Differences of Human Hepatocytes

Interindividual differences in drug metabolizing capacity of human hepatocytes are large, especially concerning enzymes for which functional polymorphisms are known (Arand et al., 1996; Hengstler et al., 1998; Strolin-Benedetti et al., 1999). Thus, studies on

drug metabolism should not rely on hepatocytes obtained from only one individual. Routinely, two strategies with cryopreserved human hepatocytes are applied: 1) "Ten in one": when cryopreserved hepatocytes are needed, one vial containing pooled hepatocytes from 10 different donors can be thawed to produce a mixture of cells from 10 donors, resulting in an "average phenotype." This strategy is adequate for quantitative identification of metabolites and for interspecies comparison. 2) If information about interindividual differences in metabolism is needed, several batches of characterized donors must be tested, including poor and extensive metabolizers for the most relevant enzymes. This strategy is possible because most commercially available human hepatocyte batches have been characterized for several drug metabolizing enzymes. However, this strategy is laborious and expensive.

Conclusions and Recommendations

Hepatocytes in suspension are recommended for short-term incubations with test substances for up to 2 hr. Hepatocytes in suspension represent a technically less demanding test system that can easily be used for screening purposes. Hepatocytes in suspension allow identification of the major metabolites, and usually interspecies differences are correctly predicted in a quantitative manner. Thus, hepatocytes in suspension are recommended for analyzing metabolic stability and metabolic profiles of test substances. In general, the metabolism by freshly isolated hepatocytes in suspension resembles more that by cultured hepatocytes, whereas the cryopreserved hepatocytes in suspension exhibit a metabolism that is somewhat more similar to that by S9 liver fractions. Freshly isolated hepatocytes in suspension are superior to S9 liver fractions in that they have a higher metabolic capacity and, in particular, a much more pronounced phase II metabolism. Freshly isolated hepatocytes in culture are suitable for long-term incubations for 24 hr or longer. This extended incubation period allows the accumulation of large amounts of metabolites, also of phase II metabolites. These large amounts of formed metabolites allow the isolation of metabolites in larger quantities, which are required for structure elucidation by nuclear magnetic resonance (NMR). The disadvantage of the medium-term hepatocyte cultures is that substances with a cytotoxic potential may damage the cells. In addition, in medium-term cultures the cell culture conditions may affect the expression of the drug metabolizing enzymes, in particular, with rodent hepatocytes. These variations may alter the metabolic profiles of the test substances and compromise interspecies comparison. Thus, interspecies comparisons seem to be more reliable when based on short-term in vitro systems such as hepatocytes in suspensions.

At present, none of the tested in vitro hepatocyte systems produced all the observed metabolites (e.g., some metabolites were only formed by the cells in suspension and others by the cultured cells). For hepatocytes in suspension, incubation periods are restricted to 2 or maximally 4 hr, thereafter, activities of some drug metabolizing enzymes decrease strongly. A limitation of this in vitro system is that minor metabolic pathways that contribute less than 3% to total metabolism, especially minor phase II metabolites, may be missed by cryopreserved hepatocytes in suspension.

SOPs

SOP for Isolation of Rat Hepatocytes

The basic two-step collagenase perfusion technique has been described by Seglen (1976). Thus, the basic preparation techniques are not repeated here.

- All buffers that come into contact with hepatocytes should be prewarmed to 37°C, and should be carbogen equilibrated before and during preparation.
- Anesthetize the rat (e.g., a male Sprague Dawley rat, 180–280 g) with an i.p. injection of pentobarbital (60 mg/kg).
- Perfuse the liver in situ via the vena portae for 15 min with ethylene glycol bis-(β-aminoethyl-ether)-N,N,N′,N′-tetraacetic acid (EGTA) (Merck, Darmstadt, Germany) buffer at 37°C.

EGTA buffer:

- 124 mL glucose solution (9 g D-glucose/L)
- 20 mL KH buffer (60 g NaCl/L, 1.75 g KCl/L, and 1.6 g KH_2PO_4/L; adjusted to pH 7.6 with NaOH)
- 20 mL HEPES buffer (60 g HEPES/L; adjusted to pH 7.6 with NaOH)
- 30 mL amino acid solution (0.27 g/L L-alanine, 0.14 g/L L-aspartic acid, 0.4 g/L L-asparagine, 0.27 g/L L-citrulline, 0.14 g/L L-cysteine, 1.0 g/L L-histidine, 1.0 g/L L-glutamic acid, 1.0 g/L L-glycin, 0.40 g/L L-isoleucine, 0.8 g/L L-leucine, 1.30 g/L L-lysine, 0.55 g/L L-methionine, 0.65 g/L L-ornithine, 0.55 g/L L-phenylalanine, 0.55 g/L L-proline, 0.65 g/L L-serine, 1.35 g/L L-threonine, 0.65 g/L L-tryptophan, 0.55 g/L L-tyrosine, 0.80 g/L L-valine; dissolve amino acids that cannot be dissolved at neutral pH by addition of 10 N NaOH and thereafter adjust to pH 7.6 by 37% HCl)
- 2 mL glutamine solution (7 g L-glutamine/L; freshly prepared)
- 1.0 mL insulin solution (2 g insulin/L dissolved in 1 N NaOH, adjusted to pH 7.6 by 1 N HCl; freshly prepared)
- 0.8 mL EGTA-solution (47.5 g EGTA/L; dissolved by addition of NaOH, adjusted to pH 7.6 by HCl)
- Continue perfusion for 30 min with the collagenase buffer (37°C).

Collagenase buffer:

- 124 mL glucose solution
- 25 mL KH buffer
- 25 mL HEPES buffer
- 38 mL amino acid solution
- 1.2 mL insulin solution
- 10 mL $CaCl_2$ solution (19 g $CaCl_2 \times 2H_2O$/L)
- 2.5 mL glutamine solution

- 125 mg collagenase [e.g., type CLSII; 255 U/mg; Biochrom (Berlin, Germany); dissolve collagenase in the prewarmed mixture of the above-mentioned solutions immediately before use]

The flow for the EGTA and collagenase buffers should be ~10 mL/min, and the pressure of perfusion should not exceed a 20 cm water column.

– After perfusion remove the liver from the animal, remove the liver capsula and dissociate carefully in suspension buffer.

Suspension buffer:
- 620 mL glucose solution
- 100 mL KH buffer
- 100 mL HEPES buffer
- 150 mL amino acid solution
- 10 mL glutamine solution
- 5 mL insulin solution
- 8 mL $CaCl_2$ solution
- 4 mL $MgSO_4$ solution (24.6 g/L $MgSO_4 \times 7H_2O$)
- 2 g BSA/L (dissolve in the mixture of the above-mentioned solutions)

– Filter the liver cell suspension through gauze.

– Centrifuge for 5 min at 50 × g.

– Wash twice with suspension buffer, centrifuge again, and resuspend in 30 mL suspension buffer.

– Determine trypan blue exclusion rate after 1:1 dilution of the hepatocyte suspension with a trypan blue solution (4 g/L trypan blue).

SOP for Isolation of Human Hepatocytes

The procedure described here is a modification of the technique described by Hengstler et al. (2000b).

– Human liver samples can be obtained from patients that undergo liver resection due to, for example, liver metastasis.

– The resected tissue should be immediately transferred into ice-cold suspension buffer (composition: described above).

– The resected liver tissue can be stored in ice-cold suspension buffer for at least 4 hr. The ischemia phase in situ (before resection of the tissue) was ≤30 min for all tissue specimens we have examined.

– Whenever possible, liver samples of approximately 100 g should be cut off in such a way that they only present one cut surface.

– Perfuse with buffer A for 20 min at 37°C. Perfusion should be performed by several blunt-end cannulae inserted into vessels of the cut surface. The number of cannulae depends on the number of large vessels available on the cut surface. The flow should be adjusted to approximately 65 mL/min that leave the perfused tissue.

Buffer A:
- 498 mL washing buffer (8.3 g/L NaCl; 0.5 g/L KCl; 2.4 g/L HEPES; adjusted to pH 7.4 with 4 N NaOH)
- 2 mL of EGTA solution

– Thereafter, perfuse with collagenase buffer for 30 min in a recirculating way at 37°C.

Collagenase buffer:
- Buffer C: 3.9 g/L NaCl, 0.5 g/L KCl, 2.4 g/l HEPES, 0.7 g/L $CaCl_2 \times 2H_2O$; adjust to pH 7.4 by 4 N NaOH and prewarm to 37°C before use.
- Dissolve 100 mg of collagenase (e.g., Sigma, C5138) in 200 mL of buffer C immediately before perfusion. Selection of an adequate batch of collagenase is the key critical step for successful isolation of human hepatocytes. Whereas the majority of all collagenases allows successful isolation of rat hepatocytes, selection of a good batch for human liver is more critical. The collagenase concentration also has to be optimized for an individual batch of collagenase.

– Transfer the tissue into a large Petri dish with suspension buffer. Scrape liver cells gently out with a spatula.

– Filter the liver cell suspension through gauze.

– Centrifuge for 5 min at 50 × g.

– Wash twice with suspension buffer, centrifuge again, and resuspend in 30 mL suspension buffer.

– Determine trypan blue exclusion rate after 1:1 dilution of the hepatocyte suspension with a trypan blue solution (4 g/L trypan blue).

SOP for Cryopreservation and Thawing of Hepatocytes

Cryopreservation

– Adjust hepatocytes to 3 Mio cells/mL in ice-cold suspension buffer (composition described above).

– Determine the volume (original volume) and centrifuge the suspension for 5 min, 50 × g, 4°C.

– Discard a volume equal to two-thirds of the original volume from the supernatant and resuspend the cell pellet in the remaining suspension buffer (one-third of the original volume) by shaking gently.

- Add ice-cold suspension buffer containing 12% (v/v) of dimethyl sulfoxide (DMSO) to the cell suspension up to 50% of the original volume, resulting in a DMSO concentration of 4% and ~8 Mio/mL hepatocytes.
- After 5 min on ice, add suspension buffer containing 16% (v/v) of DMSO up to the original volume of the cell suspension, resulting in a DMSO concentration of 10% and 4 Mio hepatocytes/mL.
- After 5 min on ice, transfer the hepatocyte suspension to cold cryovials with 1.5 mL per vial.
- Start the freezing program within 5 min. The time period between the second addition of DMSO and initiation of cryopreservation should not exceed 10 min.
- The freezing procedure can be performed by any computer-controlled freezer (e.g., BV-8, Consarctic, Schöllgrippen, Germany). The freezing protocol should be performed as follows:

- Cooling in 10 min down to 0°C
- 8 min at 0°C
- In 4 min down to −8°C
- In 0.1 min down to −28°C
- In 2 min down to −33°C
- In 2 min up to −28°C
- In 16 min down to −60°C
- In 4 min down to −100°C

The temperature in the chamber and in one cryovial should be monitored by a chart record to control whether crystallization heat was sufficiently compensated.

- Transfer the cryovials into liquid nitrogen immediately after the freezing program has been finished.

This SOP has been optimized for primary hepatocytes, but has also been applied successfully for pancreatic islet cells (Von Mach, 2002; Von Mach et al., 2003).

Thawing

- Thaw the frozen hepatocytes quickly by gentle shaking in a 37°C water bath.
- Transfer the hepatocyte suspension into ice-cold erlenmeyer flasks immediately after thawing and dilute DMSO gradually by addition of cold suspension buffer, 0.5-, 1-, 2-, 3-, and 6-fold the volume of the thawed hepatocyte suspension. Suspension buffer should be added dropwise, and hepatocytes should be 3 min on ice before the next dilution step takes place.
- After centrifugation (4°C, 50 × g, 5 min) and resuspension in 10 mL suspension buffer, the hepatocytes can be purified by Percoll centrifugation.

Percoll Centrifugation

- Add into an ice-cold 50 mL Falcon tube:
  a. 12.5 mL of hepatocytes in suspension buffer (containing maximally 20 Mio hepatocytes)
  b. 8.55 mL of Percoll solution (Sigma)
  c. 950 μL 10 × HBSS (Pan, Germany)
- Mix gently and centrifuge at 70 × g, 4°C, for 7 min.
- After centrifugation, the cell pellet contains the intact hepatocytes. Resuspend the cell pellet in suspension buffer and wash once in suspension buffer (centrifugation at 50 × g, 3 min, 4°C).

Incubation with Test Substances in Suspension

- Transfer 1 mL of suspension buffer containing 1 Mio hepatocytes into a glass vial. The diameter of the hepatocyte suspension in the glass vial should be at least twice its height.
- Transfer the glass vials into a shaking water bath or into an air-conditioned rotation incubator (36–37°C; ~40 rpm).

Incubate for up to 4 hr. However, activities of xenobiotic metabolizing enzymes ≥60% of freshly isolated hepatocytes are guaranteed only up to 2 hr.

PERIFUSION CULTURE SYSTEMS

Introduction and Brief Technical Description

Usually, hepatocytes are maintained in culture flasks, Petri dishes, or multiwell plates during cultivation and with specific drugs for studies on biotransformation. This results in non-steady-state conditions due to diminishing substrate concentrations, product accumulation, and other deteriorating influences, such as low but continuous evaporation over time. Perifusion culture allowing the continuous superfusion of the cell monolayer with culture medium has been found to avoid these disadvantages and to generally improve viability, life-span, and metabolic performance of cultured primary hepatocytes (Gebhardt and Mecke, 1979a).

Comparable findings were made with considerably different perifusion systems (De Bartolo and Bader, 2001a; Dich and Grunnet, 1992; Gebhardt et al., 1996; Takeshita et al., 1998). In particular, it could be shown that perifused hepatocytes regained their normal hormonal sensitivity under conditions where the same cells in the usual stationary cultures did not respond to hormones at all (Gebhardt and Mecke, 1979b). Likewise, perifused hepatocytes showed an enhanced sensitivity toward mitogens, together with a more

adequate response to the growth-promoting effects of several carcinogens (Gebhardt and Fischer, 1995; Klein and Gebhardt, 1997). Moreover, while rat hepatocytes in stationary culture remained silent, growth stimulation by several tumor promoters could be demonstrated in the perifusion system (Klein and Gebhardt, 1997), indicating that only the latter approach rendered the hepatocytes sensitive enough for weak influences on cell proliferation. With regard to biotransformation, cytochrome P450 (CYP) levels and EROD activity were stabilized or could be easily induced in the perifused hepatocytes (Gebhardt and Mecke, 1979b), suggesting that these cells are suited for studies on drug metabolism.

A detailed description of the perifusion system is given by Gebhardt (1998) and Gebhardt et al. (1996). The cell supports consisted of collagen-coated siliconized glass or polystyrene plates in the size of microscopic slides. The chamber containing five such supports is gas-tightly sealed, temperature controlled, and subject to surface oxygenation. The proper supply of oxygen is further established by a separate oxygenator consisting of parallel ultrathin silicon tubing. The whole system is run with an atmosphere of 13% oxygen, 82% nitrogen, and 5% carbon dioxide as this oxygen concentration appeared to be optimal. Oxidation-sensitive drugs or biomolecules such as hormones can be infused directly in front of the culture chamber harboring the hepatocytes (Gebhardt, 1998; Gebhardt and Mecke, 1979b). The composition of the culture media is published (Gebhardt, 1998) or provided below.

Prevalidation with Standard Test Substances

The perifusion system was validated with several test substances being metabolized by selected phase I and phase II enzymes. Among these were ethoxyresorufin, ethoxycoumarin, testosterone, tolbutamide, dextromethorphan, midazolam, and p-nitrophenol. These substrates were tested with primary rat and human hepatocytes as well as with cryopreserved cells. In addition, co-cultures between rat hepatocytes and RL-ET-14 cells were used. One major finding was that stabilization and particularly induction by inducers such as phenobarbital (PB) or 3-methylcholanthrene (MC) of the respective enzyme activities was differentially influenced by perifusion with culture media designed to support phase I or phase II enzymes. This is illustrated for the metabolism of ethoxyresorufin (Fig. 2a and b), dextromethorphan (Table 4), and p-nitrophenol (Fig. 2). As shown in Fig. 2, EROD activity in normal rat hepatocytes was best stabilized in medium SI, where induction by PB and MC was also highest. Likewise, in human hepatocytes, dextrometorphan-O-demethylase was much better stabilized in medium SI than in medium SII, whereas induction seemed to take place only in medium SII (Table 4). Results obtained for testosterone metabolism (6β- and 16-alpha-hydroxylation) and tolbutamide (4′-hydroxylation) under basal and induced conditions show similar effects of the media and inducers with the exception of tolbutamide, which was equally well induced in media SI and SII (not shown). In contrast, the spontaneous increase of UDP-GT activity probed with p-nitrophenol was higher in medium SII compared with medium SI (Fig. 3). In this case, induction by MC was also higher in medium SII. These examples, however, show that these P450 isozymes are reasonably

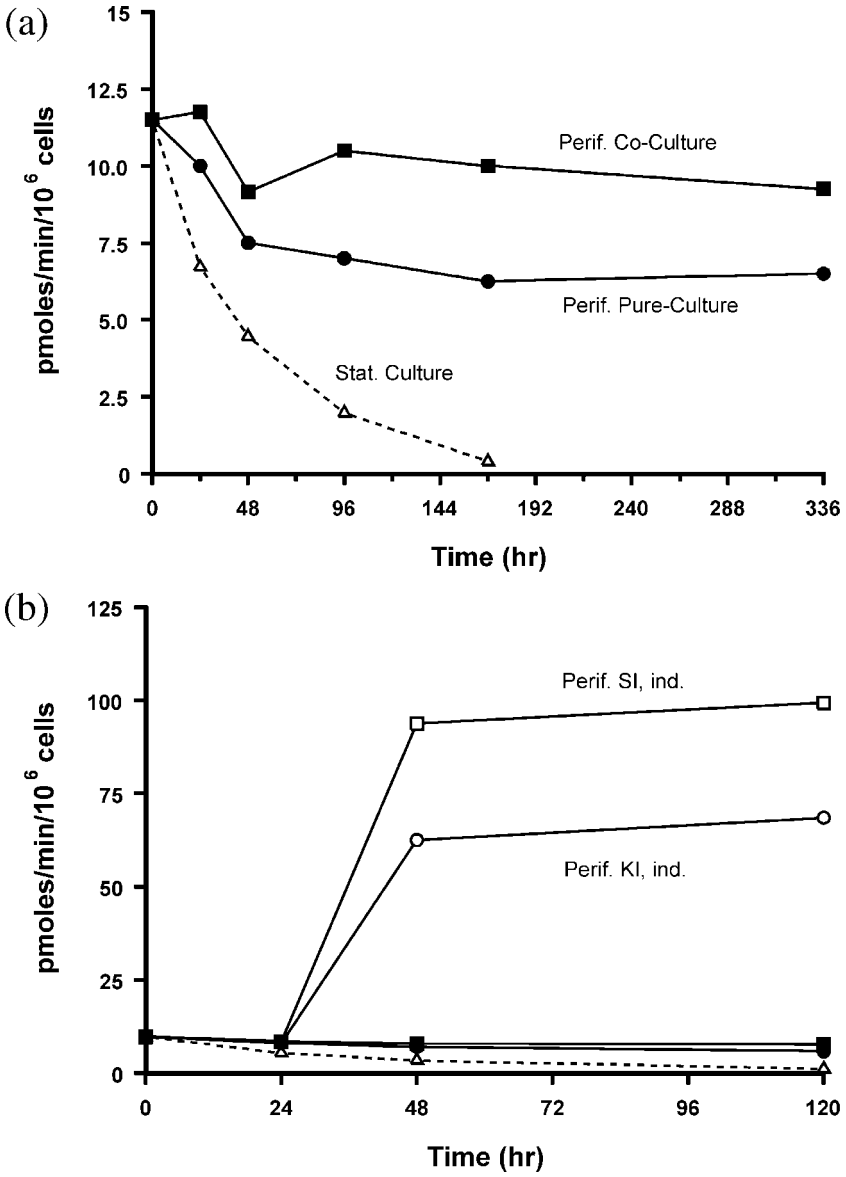

Figure 2. Time course of basal and induced EROD activity in primary rat hepatocytes. (a) Basal EROD activity in stationary culture (open triangles) or perifusion culture (closed symbols) as pure cell population (circles) and co-culture with RL-ET-14 cells (squares). (b) Induced activity in perifused hepatocytes with (open circles, squares) and without (closed circles, squares) MC and PB in medium SI (circles) and SII (squares). Inducers were added after 24 hr. Open triangles with dotted lines represent EROD activity of stationary cultures. Activity was determined using cell homogenates.

Table 4. Influence of medium and MC on dextromethorphan-O-demethylase in primary human hepatocytes maintained in stationary culture or perifusion.

Type of culture	Medium	Inducer	Dextromethorphan-O-demetylase[a] (pmoles/min/10^6 cells)
Stationary culture	KI	—	122.7 ± 63.6
	SI	—	134.2 ± 28.9
Perifusion	KI	—	378.8 ± 48.5
	SI	—	547.8 ± 41.1
	SI	MC	524.8 ± 43.6
	SII	—	385.7 ± 39.9
	SII	MC	458.6 ± 56.3

[a] Activity was measured after 5 days in culture.

stabilized by perifusion in both human and rat hepatocytes, and respond well to inducers provided through the proper culture medium.

Co-cultivation with an epitheloid cell line, the RL-ET-14 cells (Gebhardt, 2002), further influenced these features in a positive manner suggesting that cell–cell interactions play an important role. For instance, ECOD activity after induction with MC/PB for 48 hr amounted to 141 and 205 pmoles/min/10^6 cells in pure culture and to 181 and 220 pmoles/min/10^6 cells in co-culture in medium KI and SI, respectively. Similar results were found for EROD activity (Fig. 1).

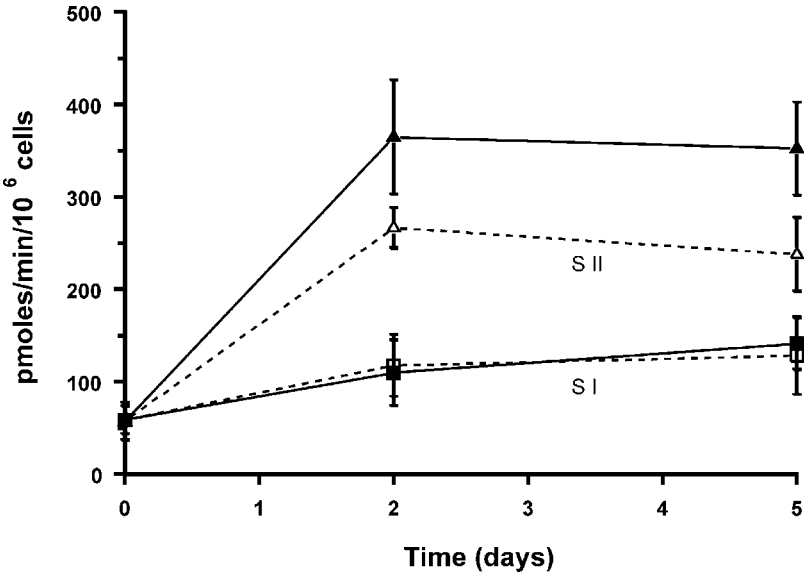

Figure 3. Comparison of UDP-GT activity in primary rat hepatocytes maintained in stationary culture (open symbols) or perifusion culture (closed symbols) in medium SI (squares) and SII (triangles). Activity against p-nitrophenol was determined using cell homogenates.

Another point concerns cryopreservation. As we have demonstrated, hepatocytes can easily be frozen after attachment to the cell supports, stored in liquid nitrogen, and afterward thawed directly in the perifusion apparatus. The last step minimizes cell damage during the thawing process and makes it easy to wash the cells free of any cryopreservative compound such as DMSO. An example of the excellent viability and functional performance of frozen cells compared with their fresh counterparts is given in Fig. 4 for EROD activity and Table 5 for UDP-GT activity. Cryopreservation as cell monolayer is possible with hepatocytes from rat or human origin and can also be performed when cells were kept as co-cultures with RL-ET-14 cells. These features render it possible to successfully compare cells from different frozen batches prepared from one piece of (human) liver.

Examples for Application

A major application of perifused hepatocytes concerns enzyme induction experiments. The long life span of perifused hepatocytes, combined with the possibility to continuously infuse low concentrations of an inducer, creates optimal conditions for such testing. Figure 5 illustrates the stable expression of CYP3A1 during cultivation.

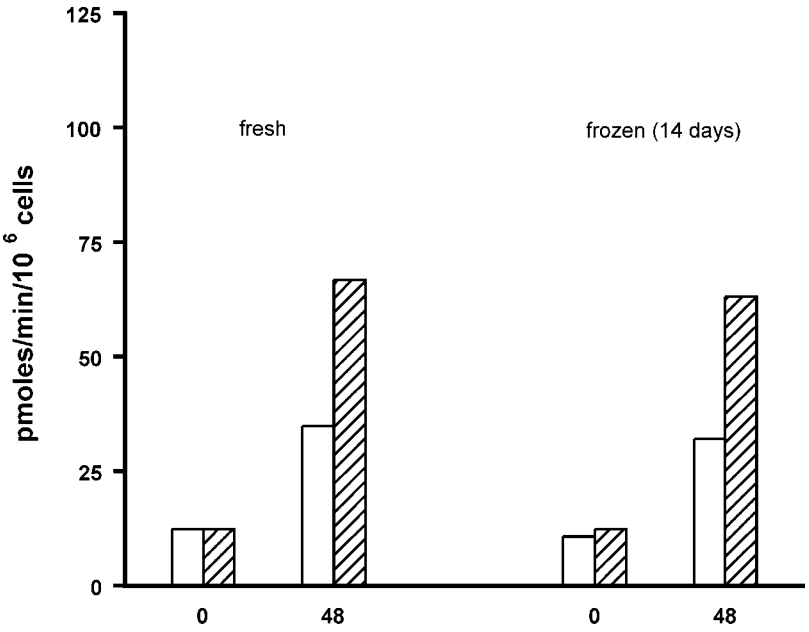

Figure 4. Comparison of EROD activity in freshly prepared primary rat hepatocytes and in cells cryopreserved for 24 days before and after induction with MC and PB for 48 hr. Stationary culture (open bars); perifusion (closed bars). Cryopreservation was performed with whole hepatocyte monolayers on perifusion cell supports.

Table 5. Influence of cryopreservation of primary rat hepatocytes on perifusion cell supports on UDP-GT activity in stationary culture and perifusion.

		UDP-GT[a] activity (pmoles/min/10^6 cells)	
		Cultivation time (hr)	
Type of culture	Type of cells	0	48
Stationary culture	Fresh	46.1 ± 13.6	200.8 ± 51.3
	Frozen[b]	37.7 ± 20.5	177.2 ± 53.6
Perifusion	Fresh	35.5 ± 19.8	231.8 ± 26.0
	Frozen	38.7 ± 21.1	238.5 ± 30.1

[a] UDP-GT, Uridine 5'-diphosphate-glucuronge transferase.
[b] Cryopreservation for 14 days in liquid nitrogen.

In Fig. 6, the influence of PCN and dexamethasone on CYP3A1 is depicted showing a nice induction by both compounds.

Another application concerns the perifusion of cells different from hepatocytes. For instance, genetically engineered V79 cells carrying human CYP species represent attractive model systems for investigating individual steps of biotransformation. When such cells are cultured in the perifusion system, it is possible to determine many enzyme parameters, such as kinetic constants, with great accuracy (Gebhardt et al., 1999). A particular advantage of the perifusion system is the fact that one and the same cell preparation can be perifused with different concentrations of the substrates in a sequential manner. An example of the resulting response (i.e., different metabolic rates that lead to different steady state concentrations) is depicted in Fig. 7. From such data, various kinetic constants can be derived (Gebhardt et al., 1999).

Conclusions and Recommendations

Perifused hepatocytes demonstrate a phenotype resembling hepatocytes in situ or in the freshly isolated state. This phenotype is relatively stable for a prolonged cultivation period in the order of at least 2 weeks and is able to readily respond to appropriate enzyme

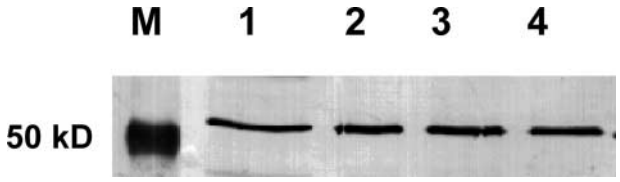

Figure 5. Time course of CYP3A1 protein in perifused primary rat hepatocytes analyzed by Western blot. Lines represent M: marker (50 kD); 1: fresh hepatocytes; 2: 24-hr perifusion; 3: 48-hr perifusion; 4: 120-hr perifusion.

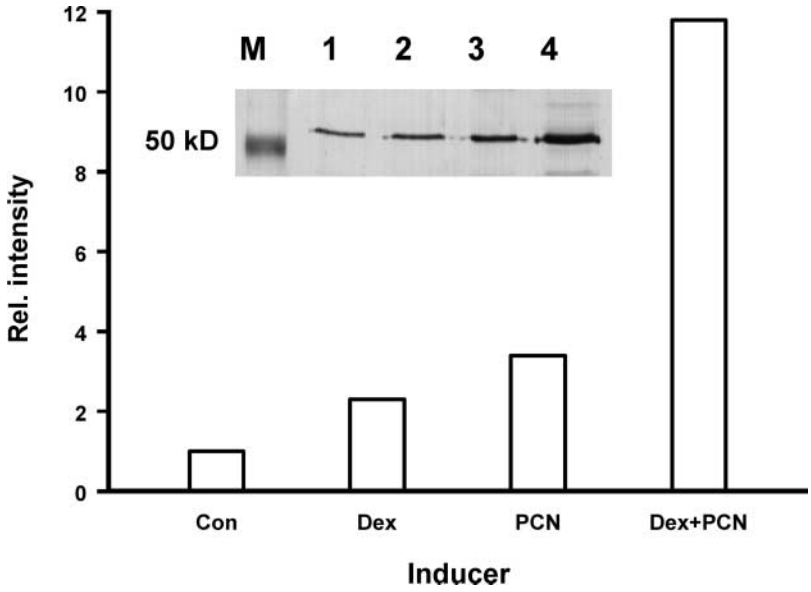

Figure 6. Influence of Dexa and PCN on CYP3A1 protein in primary rat hepatocytes perifused for 48 hr. CYP3A1 protein content was analyzed by Western blot, measured densitometrically, and depicted as relative intensities referring to control (Con) levels as 1. Induction during the last 24 hr was performed with Dexa (0.1 μM), PCN (10 μM), or both in combination. The inset shows the corresponding Western blot. Lines represent M: marker (50 kD); 1: control hepatocytes; 2: Dex; 3: PCN, and 4: Dex + PCN.

inducers. The main advantage of the perifusion system is brought forward, when weak inducers or low concentrations of inducers need to be tested, either because they are not soluble enough or because higher concentration may cause cellular damage. Likewise, few cell numbers as often occurs, if they are isolated from human liver samples, need to be no problem even for demanding investigations as perifusion allows sequential usage as well as easy cryopreservation and thawing.

SOPs

The SOP for hepatocyte isolation is provided in "SOP for isolation of rat hepatocytes" and "SOP for isolation of human hepatocytes." A thorough description of the perifusion system, together with a detailed SOP was described recently (Gebhardt, 1998). Therefore, only additional information is provided herein.

Basic Perifusion Conditions

Modifications compared with reported conditions (Gebhardt, 1998) were made with respect to the usage of special plastic cell supports that allow easy

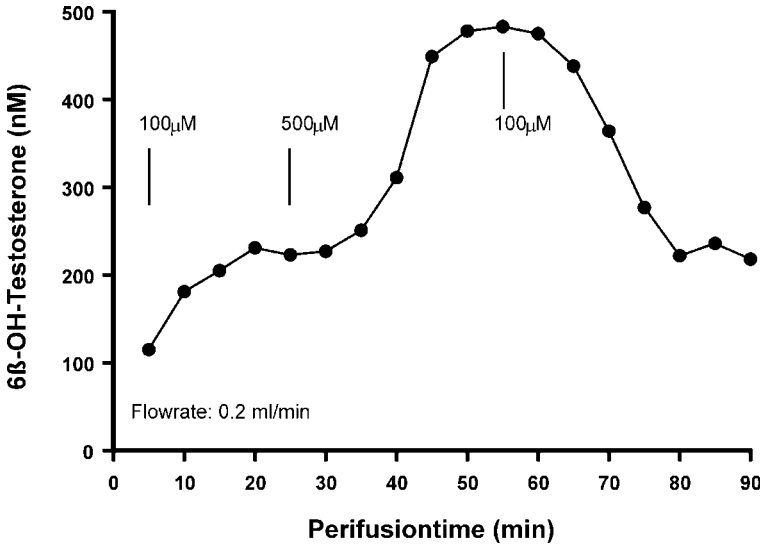

Figure 7. Production of 6β-hydroxytestosterone by perifused V79Mzh3A4hOR-1 cells at different concentrations of testosterone in the culture medium. Vertical lines indicate the time of switch between different testosterone concentrations.

cryopreservation of attached hepatocytes. These supports were obtained from InViSys Tübingen (Tübingen, Germany) and can be coated with either collagen type I as described (Gebhardt, 1998) or any other extracellular matrix, including sandwich configuration. Furthermore, the culture medium used for long-term perifusion was modified to medium SI and SII, depending on the aspect under study (see text above):

Long-term perifusion medium:

- Williams' medium E (WME) supplemented with 2.5% newborn calf serum, 2 mM L-glutamine, penicillin (50 U/mL), streptomycin (50 µg/mL), 0.1 µM insulin, 0.1 µM glucagon, and 0.1 µM dexamethasone.

Medium SI: Long-term perifusion medium supplemented with 1 mM sodium pyruvate, 0.2 mM glutathione, 10 ng/mL growth hormone, and 0.05% DMSO

Medium SII: Long-term perifusion medium supplemented with 0.5 mM sodium pyruvate, 0.3 mM glutathione, 2 ng/mL growth hormone, 2 ng/mL hepatocyte growth factor, and 0.05% DMSO

SOP for Cryopreservation and Thawing of Hepatocytes in Perifusion System

Cryopreservation

– Let hepatocytes attach on the cell support (InViSys Tübingen) as described (Gebhardt, 1998) or use already perifused hepatocytes at any stage.

- Retreive cell support with a monolayer of hepatocytes on it from culture chambers, place support into appropriate freezing container, and add ice-cold WME containing 4% DMSO.
- After 5 min on ice, replace medium by WME containing 10% DMSO.
- After 5 min on ice, transfer to freezing machine and continue with freezing according to the protocol mentioned in "Cryopreservation."

Thawing

- Place cell support with frozen hepatocytes immediately into the perifusion apparatus.
- Perifuse with short-term culture medium in the open perifusion mode at normal perifusion conditions [temperature, gasing etc., see Gebhardt (1998)] for 20 min. This will result in immediate exchange and removal of DMSO.
- Continue perifusion in any mode desired and with any appropriate perifusion medium.

LIVER SLICES

Introduction and Brief Technical Description

The use of hand-cut tissue slices was started by Warburg (1923). The disadvantage of such slices was the poor reproducibility and their thickness, which did not allow sufficient oxygen and substrate supply. After the development of rat hepatocyte isolation (Berry and Friend, 1969), isolated hepatocytes became the in vitro model of choice for pharmacological, toxicological, metabolism, and transport studies (Skett, 1994). Since the development of the Krumdieck slicer (Krumdieck et al., 1980), which allows the preparation of thin slices with reproducible thickness and a minimal trauma to the tissue, these precision-cut slices have become a suitable in vitro model for the investigation of hepatic xenobiotic metabolism, hepatotoxicity, transport, and other functions. In other reviews (De Kanter et al., 2002; Ekins, 1996a; Lerche-Langrand and Toutain, 2000; Olinga et al., 1997a; Parrish et al., 1995), detailed information on the use of precision-cut liver slices can be found, in part including comparisons of drug metabolism between slices and isolated hepatocytes as well as between the in vivo situation and in vitro systems.

In our own experiments fresh and cryopreserved rat and human liver slices were used. Rat livers were obtained from Han:WIST rats (33–40 days old), which were housed under standardized conditions and sacrificed by decapitation in ether anesthesia as described by Müller et al. (1998). Generally, male rats were used (exception: induction of CYP3A1 activity by dexamethasone also with female livers, see "Examples for Application"). Human liver tissue was obtained from macroscopically healthy surgical waste after partial hepatectomy. The current and optimized protocols for the preparation, cryopreservation, and incubation of precision-cut liver slices are given below

(see the following "SOPs" section). Prerequisite of our investigations on biotransformation (see the next section) was a sufficient slice viability.

The viability of rat liver slices was tested by unspecific parameters such as protein, potassium, DNA, and growth-stimulating hormone content, leakage of lactate dehydrogenase, and release of thiobarbituric acid reactive substances, showing stable conditions until 48 hr with fresh slices (Drobner et al., 2000; Müller et al., 1998). Potassium content in the incubated slices of about 80 nmol/mg wet weight has been reproduced in numerous experiments. A relative stability of potassium has also been shown with cryopreserved slices until 24 hr, although at lower levels (about 30–50 nmol/mg wet weight) and with higher variability than with fresh rat liver slices (Glöckner et al., 2001a). Growth-stimulating hormone amounting mostly to 1–1.5 and about 0.5 mg/g wet weight in fresh and cryopreserved slices, respectively, correlated well with potassium (Glöckner et al., 2001a). In contrast, histopathological characterization of fresh slices by means of light and electron microscopy indicates more or less time-dependent loss of glycogen and increasing vacuolization, followed by damages such as coagulative necrosis, especially in the central cell layers (Lupp et al., 2001; Neupert et al., 2003). In cryopreserved slices, the same qualitative changes occur faster than in fresh slices (Lupp et al., 2002). Nevertheless, differentiated liver functions such as biotransformation rates (see "Prevalidation with Standard Test Substances" and "Examples for Application") were fully or partially maintained in fresh and cryopreserved slices until 48 and 24 hr, respectively. Also albumin secretion, a liver-specific function, was partially maintained in fresh slices until 48 hr, decreasing from ≈ 2 μg/hr/mg protein after short-term incubation (2nd–5th hr) to 70% to 75% and 50% within 24 and 48 hr, respectively (Drobner et al., 2000; Glöckner et al., 2003b; Müller et al., 1998). In cryopreserved slices, this parameter was determined only during short-term incubation (2nd–4th hr) with half the rate of fresh slices (Glöckner et al., 1998). Our results are in accordance with the experiences of others considering slice histology to be a very sensitive parameter of viability that need not coincide with less sensitive biochemical or functional parameters [for review, see Lerche-Langrand and Toutain (2000)].

The viability of human liver slices has been estimated by potassium content and albumin secretion (Glöckner et al., 1999; 2003a). Potassium content (≈ 60 and 40 nmol/mg wet weight in fresh and cryopreserved slices, respectively) was stable in fresh and cryopreserved slices until 48 and 24 hr, respectively (i.e. it was somewhat lower than in fresh rat liver slices). Albumin was secreted by fresh human slices at a rate of ≈ 1.5 μg/hr/mg protein. The time course of secretion rate was similar to fresh rat liver slices, but after cryopreservation it was more markedly diminished to 25% to 30%. GSH content in fresh slices was stable between 2 and 24 hr incubation (2.32 ± 0.84 and 2.32 ± 0.33 mg/g wet weight, $n = 6$). Generally, human material may be very heterogeneous, and samples drawn under unsuitable conditions cannot be considered representative [Fisher et al. (2001); for review, also see Lerche-Langrand and Toutain (2000) and Olinga et al. (1997a)].

Our main interest was focused on biotransformation reactions and their inducibility in vitro. For this purpose, the metabolism of standard substrates was determined in intact liver slices. After various incubation times, slices were transferred to fresh medium, and the reaction was started by addition of the substrate only (no addition of any cofactor). The incubation time in the presence of substrates was limited to the initial linear formation of

metabolites. But in principle, a longer incubation with substrates is also possible to evaluate the total metabolic profile, including phase I and II metabolites. For the determination of a single metabolic step, slice homogenate can also be used, especially if the respective metabolite disappears in slices very rapidly by a subsequent metabolization. Such reactions were performed under optimal conditions (addition of cofactors). In either case, biotransformation rates were related to the protein content of slices or slice homogenate. Some details of biotransformation reactions are given below (see the following "SOPs" section). In induction experiments, the slices were exposed to several model inducers during an incubation for 24 hr before performing biotransformation reactions. A more sensitive and earlier marker of induction is the increase in specific mRNA, which was determined by competitive reverse transcriptase-polymerase chain reaction (RT-PCR).

Prevalidation with Standard Test Substances

Rat Liver Slices

To record different phase I reactions, the substrates testosterone and 7-ethoxycoumarin were mainly used. Several products of testosterone hydroxylation (TH) were measured by gas chromatography–mass spectrometry (GC–MS) or high-performance liquid chromatography (HPLC). In principle, results obtained after incubation in rollers (Müller et al., 1998) and in flasks (Glöckner et al., 2003b) are comparable. The suitability of incubation in shaken flasks for testosterone metabolism has also been reported by Olinga et al. (1997b). Summarized results of fresh rat liver slices after incubation in flasks till 24 hr are given in Table 6. 6β-, 16α-, and 2α-OH-testosterone were the main metabolites, the formation of which decreased by 25% to 50% within 24 hr. Minor metabolites like 2β-, 16β-, and 6α-OH-testosterone were detected with relatively large variability (no significant change within 24 hr). In some cases, further minor metabolites (e.g., 15β-OH-testosterone) have also been measured (not shown). In principle, our values were similar to (or slightly higher than) those of Ekins (1996b), de Graaf et al. (2000), and Olinga et al. (1997b)

Table 6. TH (pmol/min/mg protein) in intact fresh liver slices of male rats after 2 and 24 hr incubation.

Position of hydroxylation	2 hr activity		24 hr activity	
6β	274 ± 25	(17)[a]	200 ± 44	(10)
16α	148 ± 20	(17)	103 ± 17	(10)
2α	91 ± 11	(17)	61 ± 13	(10)
2β	30 ± 2.8	(17)	28 ± 8.4	(10)
16β	15 ± 2.9	(17)	7.0 ± 1.3	(10)
6α	4.0 ± 1.2	(17)	7.4 ± 6.0	(10)

[a]Arithmetic means and SEM are given, with *n* in parentheses.

(the latter limited to 2α-OH-testosterone) but higher than those of de Kanter et al. (1999) and Sohlenius-Sternbeck et al. (2000). In contrast to Ekins (1996b), de Kanter et al. (1999), and Sohlenius-Sternbeck et al. (2000), who found 16β-OH-testosterone to be the main metabolite, we measured the highest TH rates at 6β-position, which is in accordance with Olinga et al. (1997b). Cryopreservation of rat liver slices had no influence on metabolic profile (Table 7). The rates in fresh and cryopreserved slices of the same livers did not differ significantly in several single experiments (Glöckner et al., 2001a). In the summarized results obtained from different series of experiments (Tables 6 and 7), TH at 16α-, 2β-, and 16β-positions were significantly lower in cryopreserved than in fresh rat liver slices. Comparable absolute TH rates at 2α-position in cryopreserved slices were given by de Graaf et al. (2000).

Similar to TH, the rate of ECOD, a reaction catalyzed by several CYP forms, including CYP1A1/2, decreased by about 50% within 24 hr in fresh and cryopreserved rat liver slices (Tables 8 and 9). Absolute ECOD rates might be enhanced in the presence of DMSO (0.2%) without increasing CYP1A1 apoprotein (Müller et al., 1996).

The hydroxylation of midazolam at 4- and 1-position (M-4-H and M-1-H), preferentially catalyzed by CYP3A forms, was measured in fresh rat liver slices until 24 hr (Table 8). Again, the formation of both metabolites decreased within 24 hr by approximately 50%. The rate of M-4-H was considerably higher than M-1-H. This has also been described by Rekka et al. (2002), although in their study 10-fold lower rates were detected, decreasing to 20% to 25% within 20 hr. Perloff et al. (2000) reported on M-4-H in microsomes of mouse liver being seemingly twice as high as our rates in intact liver slices, but the rate was related to microsomal and not to total cell protein like in slices.

The metabolism of tolbutamide performed with fresh rat liver slices (Table 8) resulted in two main metabolites: hydroxy-tolbutamide (HTB) and carboxy-tolbutamide (CTB) (ratio about 10:1). For HTB formation no saturation conditions were reached with substrate concentrations of up to 3 mM, in contrast to CTB formation, which did not differ with substrate concentrations of 0.5–3 mM. In rat liver microsomes, Belanger and St-Hilaire (1991) described similar rates of HTB formation, but Ashforth et al. (1995) found 10-fold higher hydroxylation rates.

In addition to biochemical determinations of CYP functions, isoforms expression has been demonstrated immunohistochemically in fresh and cryopreserved rat liver slices

Table 7. TH (pmol/min/mg protein) in intact cryopreserved liver slices of male rats after 2 and 24 hr incubation.[a]

Position of hydroxylation	2 hr activity		24 hr activity	
6β	189 ± 32	(9)	168 ± 22	(10)
16α	86 ± 15	(9)	66 ± 8	(10)
2α	64 ± 15	(9)	36 ± 5	(10)
2β	13 ± 2.2	(9)	20 ± 3.6	(10)
16β	5.4 ± 1.6	(6)	2.2 ± 1.1	(6)
6α	1.3 ± 0.3	(9)	3.7 ± 1.4	(10)

[a]Arithmetic means and SEM, are given with *n* in parentheses.

Table 8. Phase I and II biotransformation reactions (pmol/min/mg protein) in intact fresh liver slices of male rats after 2, 24, and 48 hr incubation: ECOD, M-4-H and M-1-H, HTB and CTB, PNP-G and PNP-S, MUG, HBG.[a]

Reaction	2 hr activity		24 hr activity		48 hr activity	
ECOD[b]	116 ± 19	(14)	55 ± 10	(14)	39 ± 8	(8)
M-4-H[c]	243 ± 33	(5)	123 ± 18	(5)	—	
M-1-H[d]	37 ± 4	(5)	23 ± 2	(5)	—	
HTB[e]	405 ± 28	(5)	206 ± 27	(5)	—	
CTB[f]	46 ± 13	(5)	22 ± 4	(5)	—	
PNP-G[g]	682 ± 48	(11)	774 ± 83	(11)	512 ± 60	(6)
PNP-S[h]	110 ± 8	(11)	103 ± 13	(11)	49 ± 6	(6)
MUG[i]	641 ± 100	(9)	708 ± 44	(6)	547 ± 67	(6)
HBG[j]	244 ± 29	(6)	245 ± 28	(6)	123 ± 29	(6)

[a] Arithmetic means and SEM are given, with *n* in parentheses.
[b] ECOD, 7-ethoxycoumarin-O-deethylase.
[c] MC-4-H, midazolam 4-hydroxylation.
[d] MC-1-H, midazolam 1-hydroxylation.
[e] HTB, hydroxy-tolbutamide.
[f] CTB, carboxy-tolbutamide.
[g] PNP-G, p-nitrophenol glucuronidation.
[h] PNP-S, p-nitrophenol sulfation.
[i] MUG, 4-methylumbelliferone glucuronidation.
[j] HBG, 4-hydroxybiphenyl glucuronidation.

Table 9. Phase I and II biotransformation reactions (pmol/min/mg protein) in intact cryopreserved liver slices of male rats after 2 and 24 hr incubation.[a]

Reaction	2 hr activity		24 hr activity	
ECOD[b]	89 ± 8	(10)	39 ± 6	(10)
PNP-G[c]	358 ± 61	(6)	165 ± 50	(6)
PNP-S[d]	63 ± 11	(6)	31 ± 10	(6)
MUG[e]	452 ± 43	(7)	407 ± 66	(9)

[a] For further explanation, see Table 3.
[b] ECOD, 7-ethoxycoumarin-O-deethylase.
[c] PNP-G, p-nitrophenol glucuronidation.
[d] PNP-S, p-nitrophenol sulfation.
[e] MUG, 4-methylumbelliferone glucuronidation.

(Lupp et al., 2001; 2002). The liver-specific distribution was well preserved. With increasing incubation time overall, immunostaining of CYP2B1 and 3A2 decreased. As expected, CYP1A1 expression was very low in untreated liver slices.

To characterize phase II biotransformation, we determined the metabolism of three different substrates: sulfation and glucuronidation of p-nitrophenol (PNP-S, PNP-G) as well as 4-methylumbelliferone glucuronidation (MUG) and 4-hydroxybiphenyl glucuronidation (HBG). With fresh rat liver slices (Table 8), a stable function with all substrates until 24 hr and a slight or moderate decrease until 48 hr was found. Results concerning PNP conjugation are described in detail by Kuhn et al. (2001), indicating preferential PNP-G with higher substrate concentration. Details on MUG and HBG are given by Pissowotzki et al. (2003). In cryopreserved rat liver slices (Glöckner et al., 2001a), conjugation reactions reached rates somewhat lower than with fresh slices, but even fully or partly maintained until 24 hr (Table 9).

Human Liver Slices

Our own results with human liver slices have been published by Glöckner et al. (1999; 2003a), indicating suitability for investigating xenobiotic metabolism. Summarized results are given in Tables 10 and 11. Testosterone hydroxylation was highest at the 6β-position. Although decreasing, it remained measurable until 48 hr in several but not all livers. Absolute rates with huge interindividual variance were similar to those reported by Ekins et al. (1996), but higher than those reported by de Kanter et al. (1999) and Olinga et al. (1998). Further TH products have been found inconsistently and were not analyzed completely in all series of experiments. ECOD rates were considerably lower than in rat liver slices, decreasing similarly by 50% within 24 hr. This decline could be prevented by 0.1% and 0.2% DMSO. This effect of DMSO was limited to ECOD; it did not occur for TH and for several phase II reactions (Glöckner et al., 2003b). Other authors [e.g., Ekins et al. (1996) and Olinga et al. (1998)] measured complete metabolism of 7-ethoxycoumarin including 7-hydroxycoumarin conjugation. The total amount of metabolites reached values comparable to our ECOD rates. Phase II metabolism performed with three different substrates seemed to be more stable than phase I

Table 10. TH (pmol/min/mg protein) in intact fresh human liver slices after 2 and 24 hr incubation.[a]

Position of hydroxylation	2 hr activity		24 hr activity	
6β	325 ± 299	(6)	209 ± 43	(10)
2α	—		48 ± 11	(4)
2β	—		67 ± 21	(4)
16α	12 ± 10	(6)	8.9 ± 4.4	(10)
16β	20 ± 16	(6)	5.1 ± 3.5	(10)
6α	—		17 ± 3.8	(4)

[a] Arithmetic means and SEM are given, with *n* in parentheses.

Table 11. Phase I and II biotransformation reactions (pmol/min/mg protein) in intact fresh human liver slices after 2, 24, and 48 hr incubation.[a]

Reaction	2 hr activity		24 hr activity		48 hr activity	
ECOD[b]	34.1 ± 5.4	(18)	16.6 ± 3.7	(18)	4.7 ± 1.6	(8)
PNP-G[c]	377 ± 56	(5)	255 ± 66	(5)	113 ± 46	(5)
PNP-S[d]	21 ± 4	(5)	16 ± 6	(5)	15 ± 5	(5)
MUG[e]	518 ± 52	(6)	267 ± 58	(6)	106 ± 44	(6)
HBG[f]	150 ± 27	(6)	74 ± 23	(6)	24 ± 8	(6)

[a] For further explanation, see Table 3.
[b] ECOD, 7-ethoxycoumarin-O-deethylase.
[c] PNP-G, p-nitrophenol glucuronidation.
[d] PNP-S, p-nitrophenol sulfation.
[e] MUG, 4-methylumbelliferone glucuronidation.
[f] HBG, 4-hydroxybiphenyl glucuronidation.

metabolism; it remained well measurable even after 48 hr incubation in most samples. In cryopreserved human liver slices, only ECOD was determined. It was initially as high as in fresh slices and was maintained until 6 hr (34.3 ± 8.2 and 34.4 ± 13.0 pmol/min/mg protein, respectively, $n = 8$ and 7), but it was no longer detectable in most cases after 24 hr.

Examples for Application

An important factor of pharmacotoxicological effects of xenobiotics is the activity of drug metabolizing enzymes, which can be changed by inducing substances. Induction is a complex process needing well-differentiated intact cells with suitable viability. In the liver, the influence of several nonparenchymal cells on gene expression in hepatocytes is possible, which is included if slices are used as an in vitro model. Therefore, as the first step, we intended to prove specific enzyme induction in liver slices in vitro by known model inducers.

CYP1A1, a CYP isoform not or scarcely expressed in untreated liver, can be easily induced in vivo by xenobiotics like β-naphthoflavone (BNF). We could demonstrate this type of induction in vitro at mRNA level by exposure of fresh (Müller et al., 1998) and cryopreserved (Glöckner et al., 1998) rat liver slices to 25 μM BNF, indicating fast (within 6 and 24 hr) reproducible formation of CYP1A1-mRNA. Moreover, induction at protein level has been shown immunohistochemically (Lupp et al., 2001; 2002) and by increased ECOD activity (Glöckner et al., 2001a) in fresh and cryopreserved intact slices. One typical example of ECOD induction by BNF in fresh rat liver slices is shown in Fig. 8. The in vitro effect of BNF was detectable in fresh liver slices of untreated as well as of pretreated (50 mg/kg BNF p.o., preparation of slices 24 hr later) rats, (i.e., it was not substantially influenced by the expression state of the donor liver) (Glöckner et al., 2001b). This type of induction has also been proved in our experiments with fresh and cryopreserved human liver slices at mRNA level (Glöckner et al., 1999). Our results

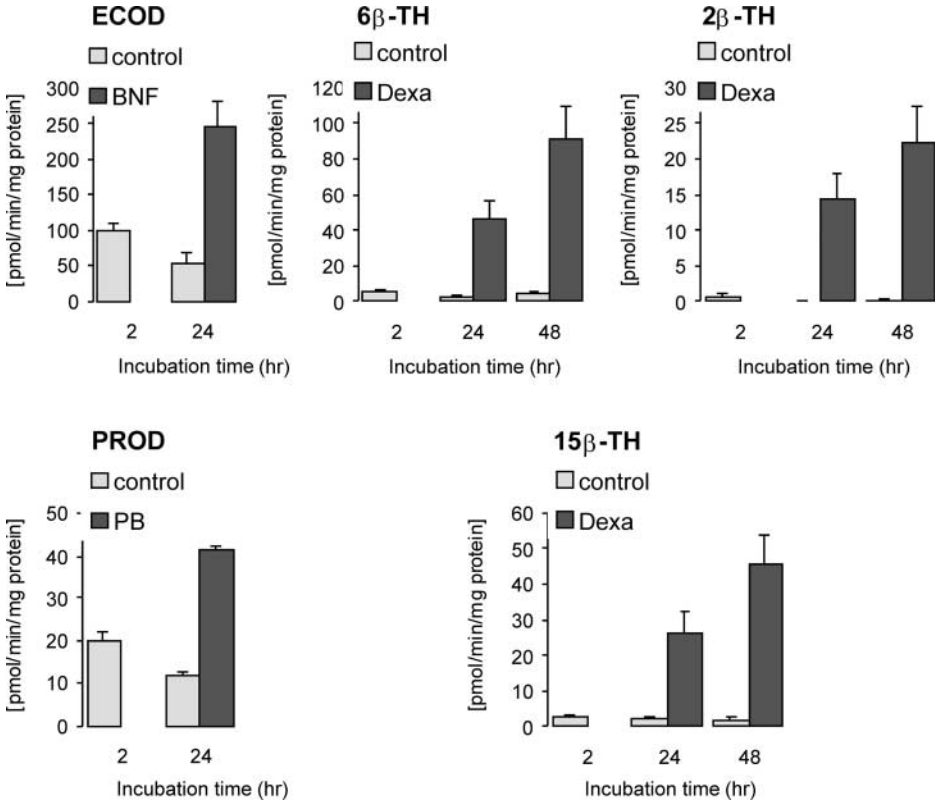

Figure 8. In vitro effect of the model inducers BNF (25 μM), PB (10^{-4} M), and Dexa (10^{-6} M) on biotransformation rates of rat liver slices. ECOD ($n = 6$, male livers) and TH (6β-, 2β-, and 15β-TH, $n = 6$, female livers) were performed with intact slices, PROD ($n = 13$, male livers) was performed with slice homogenate. Arithmetic means and SEM are given.

obtained from fresh rat and human liver slices correspond to those of other groups [for review, see Lerche-Langrand and Toutain (2000)] and are completed by results of Pan et al. (2002). With cryopreserved slices, no comparable literature data are available.

Phenobarbital is known to be an inducer of several CYP forms, including CYP2B1. In fresh rat liver slices, CYP2B1-mRNA is expressed constitutively as expected. Exposure to 10^{-4} M PB enhanced this mRNA species, beginning within 6 hr and progressing until 24 hr. In parallel, PROD activity was enhanced by PB within 24 hr (Müller et al., 2000). Summarized results of several experiments are given in Figs. 8 and 9. In contrast to BNF, the in vitro induction effect of PB on enzyme activity has been decreased by preceded strong in vivo induction because this in vivo effect of PB on enzyme activity did not decline until 3–6 days. Thus, in the case of high CYP2B1 expression of donor livers, enzyme induction in vitro was detectable only at mRNA level (Lerch et al., 2002). Induction of CYP2B1 by PB could also be shown in cryopreserved rat liver slices at mRNA level (Glöckner et al., 2002). At apoprotein level, immunohistochemical proof of

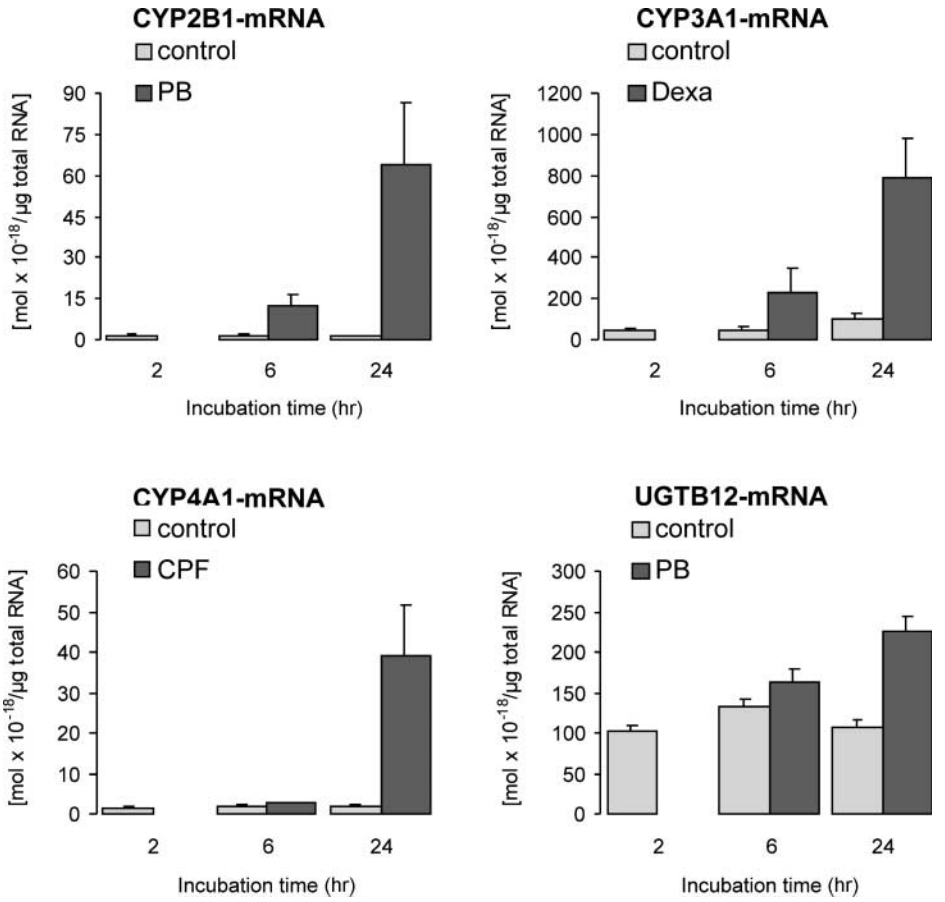

Figure 9. In vitro effect of the model inducers PB (10^{-4} M), Dexa (10^{-6} M), and ciprofibrate (CPF, 10^{-4} M) on the concentration of specific mRNAs in liver slices of male rats. Determination of specific mRNA concentrations was performed by competitive RT-PCR. Arithmetic means and SEM are given ($n = 4–9$ for CYP2B1-mRNA, $n = 4$ for CYP3A1-mRNA, $n = 6$ for CYP4A1- and UGT2B12-mRNA).

induction has been demonstrated by Lupp et al. (2001; 2002). Comparable literature data are rare. Lake et al. (1993; 1996) reported on induction of CYP2B1 concentration and ECOD activity 72 hr after exposure of fresh rat liver slices to PB. Pan et al. (2002) demonstrated CYP2B1-mRNA induction by exposure of rat liver slices to 5×10^{-4} M PB.

Besides CYP forms PB may also induce enzymes of phase II metabolism [e.g., UDP-glucuronosyltransferases (UGT)]. In contrast to CYP and other UGT-mRNAs, hepatic UGT2B12-mRNA is scarcely induced (induction factor about 2) after in vivo PB treatment of rats (Green et al., 1995). The question arose as to whether such a weak in vivo induction can be detected in vitro on liver slices. Indeed, after exposure of rat liver slices to PB (10^{-4} M) for 24 hr a 2-fold increase of UGT2B12-mRNA could be measured

(Pissowotzki et al., 2003) (i.e., liver slices are suitable even for the detection of poor induction effects). A typical example is given in Fig. 9.

In further experiments, the effect of inducers on CYP3A1, one of the main CYPs in rat liver, was checked. Known inducers of CYP3A1 are dexamethasone (Dexa) and pregnenolone 16α-carbonitril (PCN). Both inducers enhanced CYP3A1-mRNA moderately within 6 hr, but more intensively within 24 hr (effective concentration 1 μM). No difference of basal and induced CYP3A1-mRNA expression seems to exist between slices of male and female rats (Glöckner et al., 2003c). The effect of PCN could be demonstrated in both fresh and cryopreserved rat liver slices (Glöckner et al., 2002). The effect of Dexa on fresh slices is given in Fig. 9. In addition, Lupp et al. (2001; 2002) demonstrated CYP3A induction immunohistochemically in fresh and cryopreserved rat liver slices. With fresh male rat liver slices, a moderate increase of TH at 6β-position was measured 24 hr after Dexa exposure (Glöckner et al., 2003c; Steinmetzer et al., 2000), which was lower than the increase at mRNA level. With fresh female rat liver slices, however, the relative induction effect on TH was considerably more pronounced because the basal hydroxylation rate is lower than with male rat liver slices (Glöckner et al., 2003c). An example of TH induction by Dexa within 24 and 48 hr at 6β-, 2β-, and 15β-positions is given in Fig. 8. Enhanced expression by induction in vivo decreased rapidly in vitro in the subsequently prepared liver slices, so that the Dexa effect in vitro could also be proved with liver slices of organ donor rats with high CYP3A1 expression state (Lieder et al., 2002). Again, comparable literature data with liver slices are rare. CYP3A induction at enzyme level by PB in rat liver slices and by rifampicin in human liver slices has been described by Lake et al. (1996; 1997).

CYP4A1 can be induced by peroxisome proliferators as shown by Lake et al. (1994; 1996) at apoprotein level. In our experiments, this type of induction has been investigated by mRNA quantification after exposure of rat liver slices to ciprofibrate (Fig. 9). Pan et al. (2002) demonstrated CYP4A1-mRNA induction by exposure of rat liver slices to the peroxisome proliferator Wy-14,643.

Conclusions and Recommendations

Precision-cut liver slices have become a well-accepted in vitro tool for pharmacological and toxicological investigations. The preparation, incubation, and handling of tissue slices are simple and inexpensive. Proteases, which can alter cell functions and disturb cell–cell interactions, are not necessary for preparation. All experiments can be started without a preceding culturing of the slices (apart from a 1–2 hr preincubation period); that is, compared with cell cultures the time between the removal of the tissue from the organism and the beginning of the in vitro investigation is very short, which could partly explain the good maintenance of many functions. The complexity of liver structure is maintained (i.e., like in vivo interactions of parenchymal and nonparenchymal cells are possible), and the bipolarity of hepatocytes is not lost. Dedifferentiation of hepatic functions is diminished under these conditions. Co-cultures are unnecessary. Liver slices can be cryopreserved with sufficient function after thawing. Cryopreservation is particularly important for the optimal use of rare human tissue.

Slices cannot be used for chronic experiments. The use until 72 hr is possible, but for many purposes incubation times of 24 hr or less are sufficient.

A well-proved field of application of fresh and cryopreserved slices is the determination of xenobiotic metabolism, especially if the whole metabolic profile is of interest. The in vivo situation concerning phase I and II drug metabolism is reflected very well in liver slices. Factors that can qualitatively and quantitatively alter the metabolic pattern can be easily studied.

Fresh and cryopreserved liver slices are suitable for a very sensitive detection of inducing effects of xenobiotics on drug metabolism. Even a very poor in vivo induction can be predicted in vitro with liver slices. To get the highest possible induction factor, the determination of the respective mRNA after 6–24 hr incubation of the slices in the presence of the inducer is recommended. The changes in enzyme activities are not that marked, but in some cases the induction factor can be distinctly increased if liver slices from female or neonatal rats with low basal activity are used.

Liver slices can also be used for testing xenobiotics for acute hepatotoxicity. This is also the case for substances that need a toxification. Liver slices are particularly suitable if toxic effects on hepatocytes are mediated or modified by nonparenchymal cells (e.g., by cytokine release from Kupffer cells). In contrast to other in vitro models, morphological changes in the liver tissue after exposure to xenobiotics can also be studied.

SOPs

Preparation and Handling of Liver Slices

Fresh Rat Liver Slices

(I) Storage of liver immediately after excision and preparation of slices in Krebs-Henseleit-HEPES buffer (KHB), supplemented with 50 mg/L gentamicin, at room temperature, slicing of tissue cores (diameter 8 mm) with a Krumdieck slicer (thickness of slices 250 μm).

Modifications tested:

(i) Storage and preparation in KHB at room temperature in comparison with ice-cold KHB was without disadvantage for potassium and GSH content, albumin secretion, and CYP-dependent biotransformation until 48 hr.

(ii) Perfusion of rat livers before slice preparation had no advantage compared with nonperfused liver.

(II) Incubation medium: WME, supplemented with glutamin 2 mM, gentamicin 50 mg/L, insulin 1 μM, change of medium after 2 and 24 hr.

Modifications tested:

(i) Supplementation with Dexa (10^{-7} and 10^{-6} M) and DMSO (0.2%) had no advantage for albumin secretion until 48 hr.

(ii) Comparable results were observed with insulin concentrations of 0.1 and 1.0 μM concerning potassium and DNA content, albumin secretion, TH, and

ECOD until 24 hr, although ECOD rate in intact slices is higher with 1 μM insulin during 10 min reaction time (Glöckner et al., 2003b).

(III) Incubation at 37°C in Erlenmeyer flasks (up to 4 slices/5 mL in 25-mL flasks or up to 8 slices/10 mL in 50-mL flasks) under permanent gassing with carbogen (95% oxygen, 5% carbon dioxide).

Modifications tested:

(i) Results did not significantly differ after incubation rollers or flasks for potassium and GSH content, albumin secretion, and CYP-dependent biotransformation at least until 24 hr.
(ii) Results were similar with oxygen tensions of 40% to 95%, but decreased albumin secretion was observed with 20% oxygen (Drobner et al., 2000).

Fresh Human Liver Slices

Suitability of the same protocol as for rat liver slices.

Modifications tested:

(i) Medium supplementation with dexamethasone (10^{-7} M) had no significant effect on K^+ content, albumin secretion, TH, and ECOD until 48 hr.
(ii) Medium supplementation with DMSO (0.1 or 0.2%) had no significant effect on K^+ content, albumin secretion, TH, ECOD, conjugation of PNP, hydroxybiphenyl, and methylumbelliferone (Glöckner et al., 2003a).

Cryopreserved Rat Liver Slices

(I) Preparation like fresh rat liver slices.

Modifications tested:

(i) Sack's solution as storage and preparation solution had no advantage in comparison to KHB for potassium content, GSH content, and CYP-dependent biotransformation (Glöckner et al., 1998).
(ii) (II) Immediate slice exposure to 30% DMSO in Sack's solution at room temperature for 15 min.

Modifications tested:

(i) 30% DMSO were superior to 10% DMSO (Glöckner et al., 2001a).
(ii) Cooling during DMSO exposure had no advantage.
(iii) Results did not significantly differ after exposure to DMSO for 5–30 min.

(III) Separation of slices into plastic cryovials without any medium (4 slices/vial) and immediate freezing of closed vials in liquid nitrogen at the laboratory desk.

Modifications tested:

(i) Fast freezing is superior to slow computer-controlled freezing (Glöckner et al., 1996).
(ii) Preceding storage of loaded vials on ice before freezing enhances variability of results with thawed slices (Glöckner et al., 1996).

(IV) Rapid thawing of the slices by putting the cryovials into a 37–38°C water bath and by fast addition of 2 mL 37–38°C WME, and immediate transfer of the whole content to incubation flasks containing additional WME (ready for incubation, finally 4 slices/5 mL).

Modifications tested:

(i) WME as thawing medium instead of Sack's solution has no disadvantage.
(ii) Additional washing of slices after thawing is not necessary.
(iii) Medium supplementation with FCS has no advantage.

(V) Incubation-like fresh rat liver slices.

Cryopreserved Human Liver Slices

The protocol according to rat liver slices is used, but 10% DMSO is dissolved in WME instead of Sack's solution.

Modifications tested:

(i) In contrast to rat liver slices, no advantage of 20 or 30% over 10% DMSO on potassium content, albumin secretion, and ECOD was observed.
(ii) Cooling during DMSO exposure had no advantage for potassium content, GSH content, and ECOD.
(iii) WME as vehicle medium for DMSO and as thawing medium instead of Sack's solution has no disadvantage on potassium content and ECOD.
(iv) Preincubation of slices for 30 min at 37°C before exposure to DMSO does not improve potassium content, GSH content, and ECOD.
(v) Additional washing of slices after thawing is not necessary.

Determination of Biotransformation Rates

TH in intact liver slices: substrate concentration 0.5 mM, reaction time 20 min, determination of metabolites in the incubation medium after solid-phase extraction by GC–MS or by HPLC as described by Glöckner et al. (2003c) or Müller et al. (1998), respectively.

ECOD in intact liver slices: substrate concentration 0.8 mM, reaction time 10 min, determination according to Müller et al. (1998).

PROD, according to Müller (1990), with slice homogenate instead of 9000 g supernatant.

Midazolam 4- and 1-hydroxylation (M-4-H and M-1-H) in intact liver slices: substrate concentration 0.5 mM, reaction time 20 min, and determination of metabolites by GC–MS according to Martens and Banditt (1997).

HTB and CTB in intact liver slices: substrate concentration 3 mM, reaction time 40 min, and determination of metabolites by HPLC according to Hansen and Brosen (1999).

PNP-G and PNP-S in intact liver slices: substrate concentration 0.125 mM, reaction time 20 min, and determination according to Kuhn et al. (2001).

MUG in intact liver slices: substrate concentration 0.25 mM, reaction time 20 min, and determination according to Glöckner et al. (2001a).

HBG in intact liver slices: substrate concentration 0.25 mM, reaction time 90 min, and determination according to Lilienblum et al. (1982).

CO-CULTURE SYSTEMS FOR HEPATOCYTES AND INTESTINAL BACTERIA

Introduction and Brief Technical Description

For the toxicological and pharmacological assessment of xenobiotics, knowledge of the enterohepatic metabolism is of decisive importance. Our knowledge on the metabolic interaction of liver and intestinal microflora is derived almost entirely from studies using experimental animals. The two-chamber system for the co-culture of hepatocytes and fecal microflora under aerobic and anaerobic conditions, respectively, allows analysis of the sequential metabolism of chemicals by liver and microflora in vitro (Figs. 10(A), 10(B), and 11). The culture device consists of two compartments separated by a permeable polycarbonate membrane (diameter 5 cm). The chambers have a relatively low volume of 7 mL and can be independently gassed. In the aerobic compartment, hepatocytes are cultivated as a monolayer on the collagen-coated membrane; in the anaerobic compartment, fecal human microflora is maintained as a suspension. The exchange of metabolites of the cells takes place across the permeable membrane.

The membrane is clamped in two tight-fitting holding rings and can be easily replaced. A cannula projects into each chamber to perfuse the media with a gas mixture. The compartment with the hepatocytes is supplied with a mixture of 19% O_2, 71% N_2, and 10% CO_2, whereas the compartment with the microflora receives an anaerobic mixture of 90% N_2 and 10% CO_2 to create culture conditions similar to those predominating in the anaerobic intestine. The hepatocyte monolayer is protected against gas bubbles by a fine mesh consisting of high-grade steel. Prior to their use in the co-culture system, the liver cells have to be plated on the collagen-coated polycarbonate membranes, which were fixed between two holding rings and incubated in a conventional Petri dish. The cells attach rapidly to the collagen substrate and after 1 hr the membranes were inserted into the co-culture system. The hepatocytes form a monolayer on the membranes that is, however, not tight. The test compounds were added to the aerobic side of the chamber to allow their initial metabolism by the hepatocytes. Six culture units could be gassed simultaneously by the gassing facilities used (Figs. 10 and 11). During the experiments, the culture units were placed in a

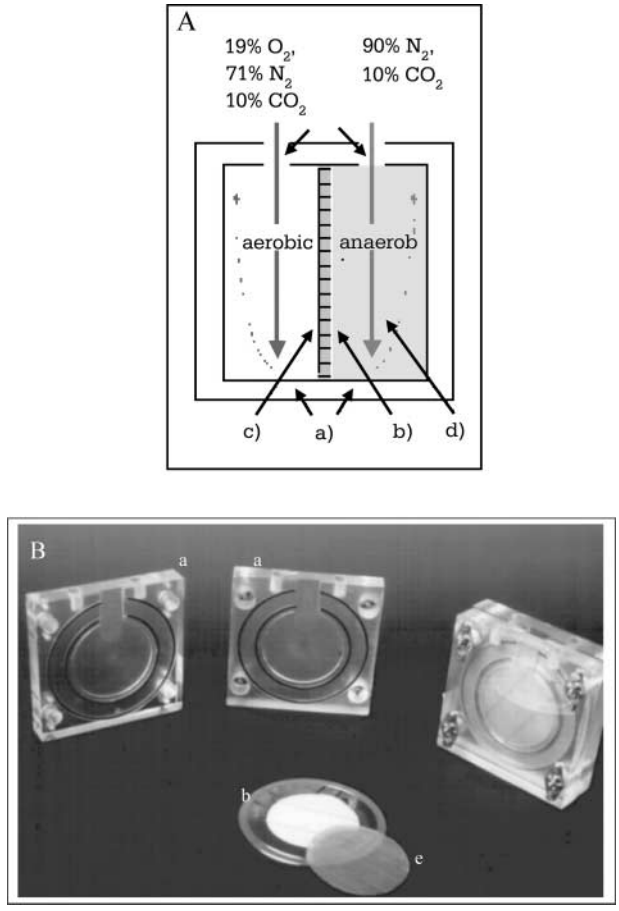

Figure 10. Schematic diagram (A) and photograph (B) of the two-chamber system: a) two halves of the device containing the culture compartments, b) polycarbonate membrane fixed between two tight-fitting rings, c) monolayer of hepatocytes, d) suspension of fecal microflora, e) a fine mesh for the protection of hepatocytes against gas bubbles, and f) cannulas for gassing the media project into each chamber.

small incubator in a humidified atmosphere at 37°C. The microflora used exhibited significant activities of drug metabolizing enzymes. The various enzymes tested (azoreductase, nitroductase, β-glucuronidase, β-glucosidase, and sulphatase) differed only moderately in the samples of three volunteers who differed significantly in their eating habits for years. The co-culture system should be used only for short-term incubations (4 hr) because of the rapid change of the composition of the intestinal microflora and the change of the pH in the suspension.

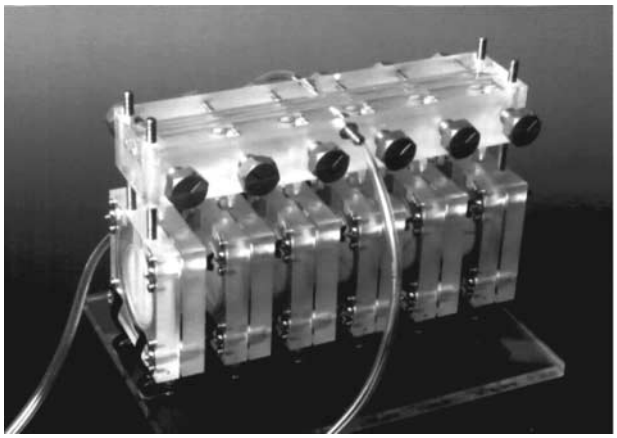

Figure 11. Six co-culture chambers, which are provided with aerobic and anaerobic gas mixtures via a joint supply system.

Prevalidation with Standard Test Substances

To characterize the co-culture system, we studied the activities of various drug metabolizing enzymes of the hepatocytes and the microflora.

Determination of Enzyme Activities of Hepatocytes in Co-cultures

For freshly isolated rat hepatocytes, the activities of various CYP forms and conjugating enzymes served as markers. The enzyme activities are tested in the co-culture system during a 4-hr culture period intended for the test protocol.

Ethoxycoumarin O-deethylase activity and TH were determined in intact monolayers according to Donato et al. (1994), with minor modifications. Monolayers of hepatocytes were washed twice with phosphate-buffered saline (PBS) (pH 7.4, containing 10 mM HEPES). For the determination of ECOD, the cells were incubated with 150 μM 7-ethoxycoumarin (EC) for 30 min. For the quantitation of the total amount of the primary metabolite 7-hydroxycoumarin (OH-C), it was necessary to hydrolyze the conjugates. Thus, aliquots of 300 μL were hydrolyzed for 2 hr by the addition of 100 U of β-glucuronidase and 800 U of arylsulphatase in 100 μL of 0.1 M sodium acetate buffer (pH 4.5), as described by Donato et al. (1994). The fluorescence of OH-C was determined in the samples before and after hydrolysis, at 368 nm excitation and 456 nm emission. Metabolites of testosterone were determined using HPLC according to Sonderfan et al. (1987). Corticosterone served as the internal standard for the calculation of the testosterone metabolites. At the retention time (RT) of 33 min of corticosterone, the testosterone metabolites showed the following RTs: RT 13 min: 7α-OH-T (hydroxytestosterone); RT 15 min: 6β-OH-T; and RT 18 min: 16α-OH-T; RT 28 min: 2α-OH-T.

For the following enzyme, assays cells were scraped off, washed in ice-cold PBS, and homogenized with 10 strokes of a Potter-Elvehjem homogenizer. Using 3-hydroxy-benzo(a)pyrene (50 μM) as substrate, UDP-glucuronosyltranferase activity (GT1) was assayed fluorometrically in cell homogenates (5 μg DNA/mL) in the presence of 0.01% Brij58, according to Singh and Wiebel (1979). The benzo(a)pyrene-glucuronide was extracted in methanol–chloroform (1:2, v/v) and the fluorescence determined at 378 nm excitation and 425 nm emission. 4-Hydroxy-biphenyl glucuronidation (GT2) were assayed fluorometrically in cell homogenates (2 μg DNA/mL) in the presence of 0.0625% Brij58 and 0.5 mM 4-hydroxy-biphenyl, according to Bock et al. (1980). The biphenyl-glucuronide was extracted in chloroform and the fluorescence determined at 290 nm excitation and 327 nm emission.

Glutathione S-transferase activity was determined spectrophotometrically in the 100.000 g supernatants [(1 mg protein/ml) of homogenized cells using 1-chloro-2,4-dinitrobenzene (1 mM) as substrate] (Habig and Jakoby, 1981). Phenol sulphotransferase activities were assayed fluorometrically in the 100.000 g supernatants (3 mg protein/mL) of homogenized cells with the substrate 3-hydroxy-benzo(a)pyrene (50 μM) (Wiebel et al., 1986). The benzo(a)pyrene-sulphate was extracted in propanol–hexane (1:3, v/v) and the fluorescence determined at 382 nm excitation and 415 nm emission. DNA and protein concentrations were determined by the method of Cesarone et al. (1979) and Peterson et al. (1977), respectively, with calf thymus DNA and albumin as standards.

Of the CYP-dependent monooxygenase activities studied, deethylation of EC is catalyzed by various CYP isoenzymes, including Cyp 1A1/2, 2B1/2, 2C6/7/11/12/13, and 2E1 (Ryan and Levin, 1990), whereas the hydroxylation of testosterone represents isoenzyme-specific reactions. 2α- and 16α-testosterone hydroxylations are preferentially catalyzed by Cyp 2C11, 6β-hydroxylation by Cyp 3A, and 7α-hydroxylation by Cyp 2A1 (Arlotto et al., 1991; Sonderfan et al., 1987; Waxman et al., 1983).

The stability of primary rat hepatocyte cultures in the two-chamber system was accompanied by some loss of the monooxygenase activities determined. Activity of ECOD decreased by about 30% in culture, as shown in Table 12. The resulting OH-C was conjugated by more than 95% with sulphate and/or glucuronic acid. The stability of the enzyme reactions of TH differed; 6β-hydroxylation showed the greatest loss, whereas the 7α-hydroxylation increased (Table 13). The expression of the enzymes catalyzing the deethylation and hydroxylation reactions was not changed after the co-culture of the hepatocytes with the fecal microflora for 4 hr. It has to be noted, however, that the remaining CYP enzyme activities are still sufficient to study drug metabolism in vitro. The decrease of CYP activities during the culture of rat hepatocytes is a well-known phenomenon. Since the 1990s, refined culture techniques have been developed to slow down the decrease of CYP enzymes (Schwarz and Wiebel, 1993; Schwarz et al., 1996). These approaches, however, are not readily applicable to the present co-culture system.

With respect to the conjugation reactions studied, the results show that they are well maintained in the in vitro system (Table 3). The activities of GT1, GT2, and glutathione-S-transferase (GST) remained stable or decreased insignificantly, whereas ST activity increased by about 50%. Similar to the phase I enzymes, the activities of the phase II enzymes were not changed after the co-culture of the hepatocytes with fecal microflora for 4 hr.

Table 12. Conjugating enzyme activities in rat hepatocytes cultured in absence and presence of human fecal microflora.[a]

	Enzyme activities[b] (% of freshly isolated cells)			
Microflora	GST[c]	ST[d]	GT1[e]	GT2[e]
−	95 ± 17	155 ± 22	106 ± 13	88 ± 29
+	87 ± 24	156 ± 39	104 ± 8	89 ± 19

[a] Hepatocytes were plated on a permeable polycarbonate membrane, which was fixed between two tight-fitting rings. After 1 hr, the membrane was inserted into the co-culture device. Culture of the cells was continued in the absence and presence of fecal microflora for 4 hr.
[b] After 4 hr, the metabolism of 1-chloro-2,4-dinitrobenzene by GST, 3-hydroxy-benzo(a)pyrene by ST and GT1, respectively, and 4-hydroxy-biphenyl by GT2 was determined. Data are expressed as percent of freshly isolated cells. Enzyme activities of the freshly isolated cells (100%) are GST = 781 ± 135 nmol/min × mg cytosolic protein; ST = 0.15 ± 0.02 nmol/min × mg cytosolic protein; GT1 = 216 ± 71 nmol/min × mg DNA; GT2 = 117 ± 62 nmol/min × mg DNA. Data represents the means ± SD of three experiments.
[c] GST, glutathione-S-transferase.
[d] ST, sulfotransferase.
[e] See text for descriptions of GT1 and GT2.

Determination of Microbial Enzyme Activities

To characterize the microflora, various reducing and hydrolytic enzyme activities were studied in anaerobic cultures. Nitroreductase activity was determined fluorometrically using 1-nitropyrene (40 μM) as a substrate. 1-Aminopyrene was extracted in ethyl acetate and the fluorescence determined at 362 nm excitation and 424 nm emission,

Table 13. CYP-dependent monooxygenase activities in rat hepatocytes cultured in absence and presence of human fecal microflora.[a]

	Enzyme activities[b] (% of freshly isolated cells)				
Microflora	ECOD[c]	6β-TH[d]	2-TH	7-TH	16-TH
−	68 ± 10	55 ± 4	74 ± 10	117 ± 14	80 ± 10
+	67 ± 5	60 ± 6	78 ± 11	128 ± 11	83 ± 11

[a] Hepatocytes were plated on a permeable polycarbonate membrane, which was fixed between two tight-fitting rings. After 1 hr, the membrane was inserted into the co-culture device. Culture of the cells was continued in the absence and presence of fecal microflora for 4 hr.
[b] After 4 hr, the metabolism of EC by ECOD and testosterone by TH was determined. Data are expressed as percent of freshly isolated cells. The CYP-dependent activities of the freshly isolated cells (100%) are in pmol/min × mg DNA: ECOD = 4940 ± 870; 6β − TH = 368 ± 87; 2α − TH = 744 ± 105; 7α − TH = 50 ± 10; 16α − TH = 805 ± 99. Data represent the means ± SD of three experiments.
[c] ECOD, 7-ethoxycoumarin-O-deethylase.
[d] TH, testosterone hydroxylation.

according to Kinouchi and Ohnishi (1983). Azoreduction was assayed spectrophotometrically with the substrate amaranth (0.5 mM), following the method of Wise et al. (1982). The enzyme activities of β-glucuronidase, β-glucosidase, and sulphatase were determined by assaying the hydrolysis of the p-nitrophenyl conjugates (8 mM p-nitrophenyl-β-D-glucuronide, p-nitrophenyl-β-D-glucopyranoside, and p-nitrophenyl-sulphate, respectively), according to Stahl and Fishman (1984). The enzyme activities were studied in three different cultures of fecal microflora, which were obtained from three volunteers who differed significantly in their eating habits for years. Volunteer 1 ate meat every day, volunteer 2 preferred a balanced diet, and volunteer 3 was a vegetarian. As shown in Table 14 the fecal microflora exhibited significant enzymes activities, and the activities of the various enzymes differed only maximally by a factor of three in the samples of the three volunteers.

In view of the fact that reducing and hydrolytic activities predominate the drug metabolism of the microflora azoreductase, nitroreductase, β-glucuronidase, β-glucosidase, and sulphatase has been determined in anaerobic cultures of the fecal microflora. The results obtained were in good agreement with data published by Ikeda et al. (1994) and Rowland et al. (1986): azoreductase 9 nmol/hr × mL, β-glucuronidase 2.2–3.5 μmol/hr × mL, and β-glucosidase 3.4–4.9 μmol/hr × mL. We are not aware of data on the hydrolysis of p-nitrophenyl-sulphate and the nitroreduction of 1-nitropyrene by suspension cultures of human feces.

It is remarkable that the activities of the reductases and hydrolases showed relatively small differences in the samples of the three volunteers despite their clearly different nutrition. With respect to the influence of the nutrition on the enzyme activity of the human fecal microflora, conflicting results have been published (Mallett and Rowland, 1988). Although several authors noticed a significant influence of the nutrition on the individual enzyme activities (Buddington et al., 1996; Ling and Hanninen, 1992), others did not (Bouhnik et al., 1996). However, several authors reported that the enzyme activities of the microflora of several experimentees differed (Ikeda et al., 1994; McBain and Macfarlane, 1998).

Table 14. Enzyme activities in suspension cultures of human fecal microflora.[a]

	Enzyme activities (nmol/hr × mL)[b]				
Volunteer	Azoreductase	Nitroreductase	β-Glucuronidase	β-Glucosidase	Sulphatase
1	79 ± 10	125 ± 74	2867 ± 115	1933 ± 153	750 ± 70
2	78 ± 10	106 ± 66	1867 ± 58	2200 ± 100	600 ± 20
3	76 ± 20	113 ± 50	970 ± 60	1767 ± 60	460 ± 40

[a] Enzyme activities were determined in anaerobic 1-hr cultures of fecal microflora (0.1 g feces/mL) from three volunteers: volunteer 1 (daily meat), volunteer 2 (mixed diet), and volunteer 3 (vegetarian).

[b] Cultures were incubated with 0.5 mM amaranth; 40 μM 1-nitropyrene; and 8 mM p-nitrophenyl-β-D-glucuronide, p-nitrophenyl-β-D-glucopyranoside, or p-nitrophenyl-sulphate, respectively, for 60 min. Data represent the means ± SD of three experiments.

Examples for Application

The interaction of the drug metabolism of hepatocytes and fecal microflora has been demonstrated using the model compound EC and the developmental drug EMD 57003 (Merck KGaA), a thiadiazinon derivate. Both compounds were oxidized and conjugated by liver cells. In co-cultures of hepatocytes and fecal microflora, the resulting glucuronides and sulphoconjugates were split by hydrolytic enzymes of the intestinal microflora.

7-Ethoxycoumarin

As already mentioned, EC is deethylated and subsequently conjugated by liver cells. The resulting glucuronides and sulphoconjugates can be split by hydrolytic enzymes of the intestinal microflora. As shown in Fig. 12, monocultures of hepatocytes metabolized the coumarin derivative extensively to its conjugates in the two-chamber system (line a). The 100% value corresponds to the total amount of OH-C detectable after complete

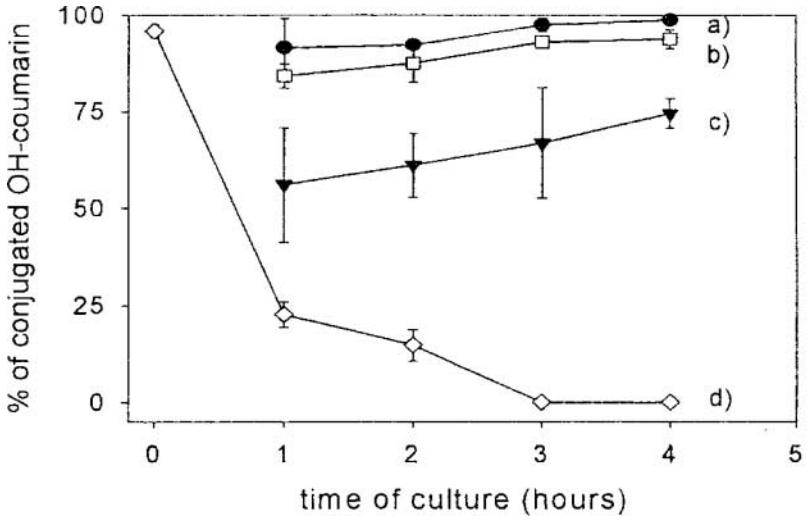

Figure 12. Formation and cleavage of OH-coumarin conjugates by hepatocytes and microflora in the co-culture system. Hepatocytes were incubated with 150 μM EC in the absence and presence of fecal microflora: a) 1.2×10^5 hepatocytes/cm^2, b) 1.2×10^5 hepatocytes/cm^2 and cultures of human feces (0.01 g feces/mL), c) 0.6×10^5 hepatocytes/cm^2 with 0.02 g feces/mL. The test compound EC was added to the aerobic compartment of the co-culture system. Monocultures of the microflora (0.01 g feces/mL) were incubated with medium containing conjugated OH-coumarin (0.5 ± 0.09 pmol/mL). This medium was obtained from hepatocyte cultures that had been incubated with 150 μM EC for 4 hr. The concentration of free and total OH-coumarin (free + conjugated OH-coumarin) was determined after different times of culturing. Data are expressed as percentage of the conjugated metabolites of the total OH-coumarin metabolites (free + conjugated OH-coumarin). Means ± SD of three different experiments.

hydrolysis of the conjugates. The extent of the biotransformation of EC and the almost complete conjugation of the metabolites formed (92% and 99% after 1 and 4 hr, respectively) was similar to that in hepatocytes cultured on collagen in Petri dishes (data not shown). Monocultures of fecal microflora are capable of completely hydrolyzing the OH-C conjugates (line d). About 80% and 100% of the OH-C-glucuronides and sulphoconjugates were split after 1 and 3 hr, respectively. Co-cultures of hepatocytes and fecal microflora showed different concentrations of the conjugated metabolites. In the presence of 1.2×10^5 hepatocytes/cm^2 and 0.01 g feces/mL, about 80% of the metabolites were conjugated (line b); this percentage decreased, however, to almost 50% using 0.6×10^5 hepatocytes/cm^2 and 0.02 g feces/mL (line c). A dynamic balance is established between the formation and hydrolysis of the conjugates because the "free" OH-C is reconjugated by the liver cells. Accordingly, the level of the unconjugated metabolites depended on the concentration of both the hepatocytes and microflora (line b and c).

EMD 57003

Similar to the findings obtained with EC, the metabolic interaction of hepatocytes and microflora could also be demonstrated studying the developmental drug EMD 57033 (Merck KGaA) in the co-culture system. The thiadiazinon derivate EMD 57033 is demethylated and/or hydroxylated by CYP-dependent metabolism to the metabolites M-I, II, and III (Fig. 13). The phenolic hydroxygroup can be subsequently conjugated (M-I and M-III conjugates). Monocultures of hepatocytes form the metabolites M-I, II, and III, as well as their conjugates, as shown by the HPLC-chromatograms in Figs. 14C and D. The concentration of the parent compound and its metabolites was similar in both compartments of the culture system. In contrast to the hepatocyte cultures, monocultures of fecal microflora did not metabolize EMD 57033 (Fig. 5). In co-cultures of hepatocytes and fecal microflora, the concentrations of the conjugates of M-I and M-III were significantly decreased at the expense of the respected unconjugated metabolites M-I and M-III (Figs. 14D and E).

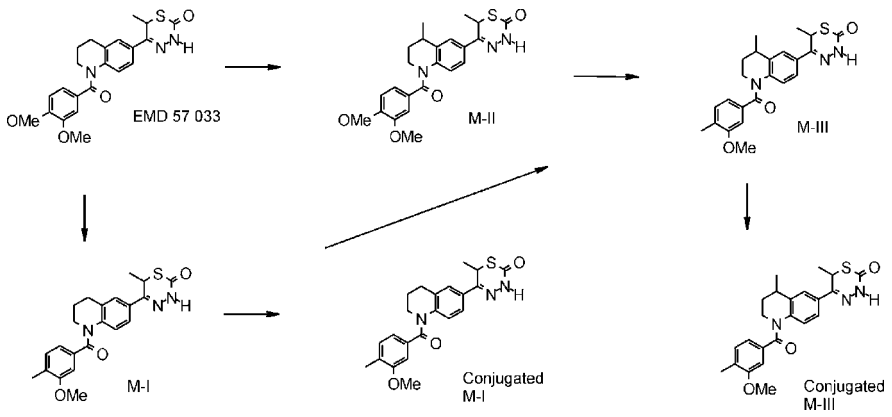

Figure 13. Proposed metabolism of EMD 57033 in rats.

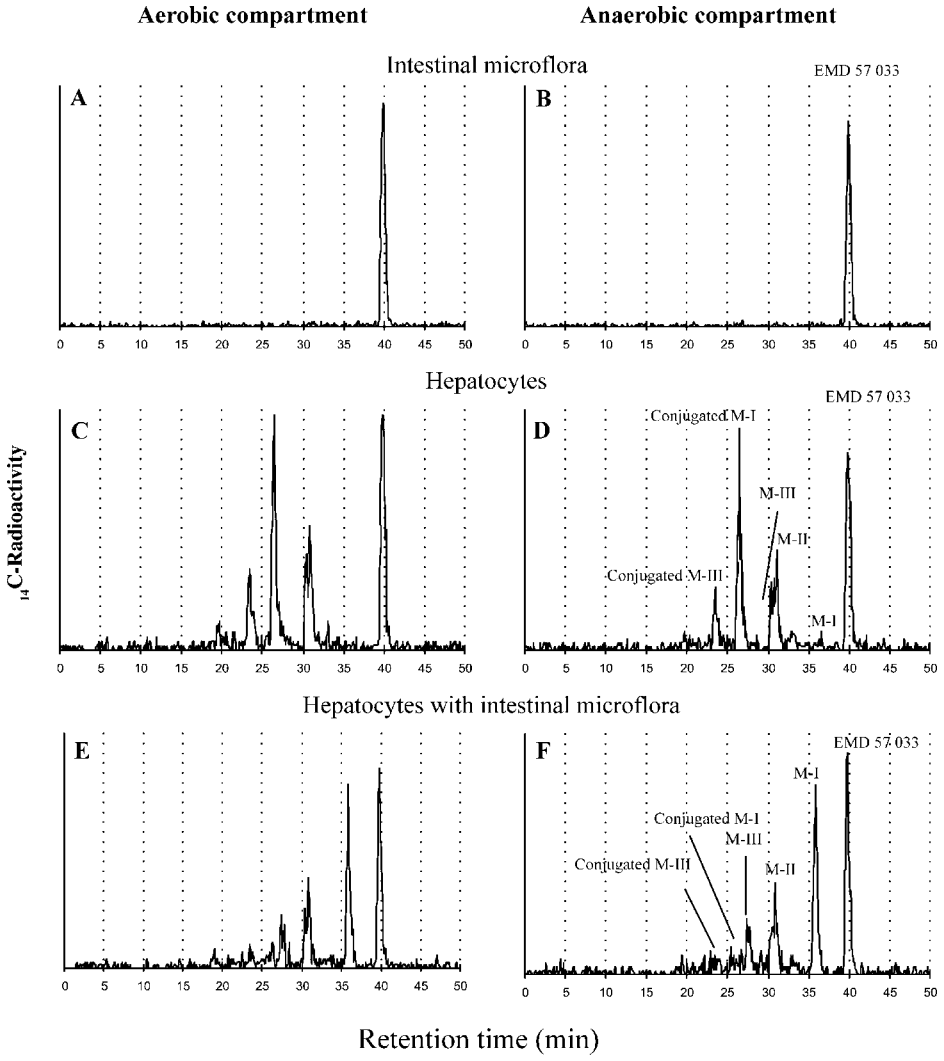

Figure 14. Metabolism of EMD 57033 by hepatocytes and intestinal microflora in the co-culture system. ^{14}C-Labeled EMD 57033 (1.4×10^6 dpm/culture) was added to the aerobic compartment of the co-culture system and incubated with the following cultures: 0.01 g human feces/mL (A and B), 1.2×10^5 hepatocytes/cm^2 (C and D), and 1.2×10^5 hepatocytes/cm^2 and 0.01 g human feces/mL (E and F). Aliquots of the aerobic (A, C, E) and the anaerobic (B, D, F) compartment were withdrawn, centrifuged, and frozen in liquid N_2. Samples (200 μl) were analyzed by HPLC (column: Purospher, Merck; absorption 320 nm). RT 40 min: EMD 57033; RT 36 min: M-I; RT 31 min: M-II; RT 27.5 min M-III; RT 26.5 min: conjugated M-I; RT 23.5 min: conjugated M-III.

Conclusions and Recommendations

The usability of the co-culture system for the study of the metabolic interaction of liver and microflora has been demonstrated using the model compounds EC and the developmental drug EMD 57003. This co-culture system allows not only the culture of the eukaryotic and prokaryotic cells in close vicinity under aerobic and anaerobic (i.e., very different physiological conditions), but also exchange of the metabolites formed. The polycarbonate membrane that separates the hepatic and the microbial compartment has been shown to be permeable not only for molecules of the size of the xenobiotics used in this study, but also for larger molecules, such as the proteins albumin and lactate dehydrogenase (data not shown). The hepatocytes cover the membranes; the monolayers formed, however, are not tight (i.e., the test compounds may also diffuse between the cells from one compartment to the other). Nevertheless, vectorial transport through the cells is likely to occur simultaneously. It was not possible to determine the oxygen concentration in the culture chamber due to technical difficulties. We assume, however, that anaerobic conditions predominate in the microbial compartment due to the constant gassing with 90% N_2/10% CO_2 and the fact that the separating membranes are almost completely covered by the hepatocyte monolayer. Minor amounts of oxygen that may reach the border region of the anaerobic compartment will be most likely eliminated due to the anaerobic gassing. This is suggested by preliminary experiments using a Clark electrode, which show the rapid elimination of oxygen from solutions by flowing them with an anaerobic gas mixture.

The newly developed co-culture system offers the possibility to study the metabolic interaction of liver and intestinal microflora. Drugs and chemicals that may come into contact with the intestinal microflora are those that are orally ingested but poorly absorbed in the upper intestinal tract, or those excreted in bile either unchanged or as a metabolite. The latter may undergo enterohepatic circulation (i.e., conjugated biliary metabolites may be split by bacterial hydrolases, reabsorbed and conjugated again in the liver). This process is a decisive factor in the disposition of many drugs, increasing their half-life in the body substantially (Larsen, 1988; Rubinstein, 1995). The in vitro system may be particulary useful in three areas. First, it may help to obtain initial results on hepatic and microbial metabolism of drugs during an early phase of drug development. This may be interesting in the development of prodrugs which are transformed to the active compound by microbial metabolism (Rubinstein, 1995). Second, the in vitro system may assist in the study of compounds of toxicological interest, enabling the simultaneous examination of metabolism and toxicity. Finally, several possibilities for a further development of the culture system are conceivable. For example, the use of human instead of rat hepatocytes would greatly increase the relevance of the in vitro data obtained. The hepatocytes may be also replaced by other mammalian cells, such as intestinal epithelial cells, which would make it possible to study the metabolic interaction of enterocytes and microflora.

There is currently no other in vitro system available designed for studying the direct metabolic interaction of liver and intestinal flora. The reasons for this are most likely the high demands to be met by such a co-culture system. Although hepatocytes have to be cultured in the presence of oxygen, the intestinal microflora requires anaerobic conditions.

We have solved these principal difficulties by developing a two-chamber culture system, in which both compartments can be gassed separately. The exchange of metabolites of the cells takes place across a permeable membrane.

SOPs

SOP for Isolation of Rat Hepatocytes

See SOPs for hepatocytes in suspension.

SOPs for Isolation and Cryopreservation of Human Fecal Microflora

- Samples of human feces should be obtained from volunteers.
- Feces should be immediately transferred into anaerobic conditions (85.5% N_2, 4.5% H_2, and 10% CO_2).
- Suspend aliquots of 1 g feces in 10 mL ice-cold modified WME:

Modified WME:
WME was supplemented with:

- 3.7 g $NaHCO_3$/L
- 20 mM HEPES
- 2 mM glutamine
- 2 KIE trasylol/mL
- 2.5 mU insulin/mL
- 0.1 μM Dexa
- 15 nM $NaSeO_3$

- Centrifuge 10 min at 500 × g and 4°C.
- Add 10% glycerol to the supernatant and transfer the suspension a 1.5 mL in cryovials.
- Cool down in a styropor box at − 80°C.
- Transfer the vials into liquid nitrogen after 12 hr.

SOPs for Culture of Rat Hepatocytes on Membranes

Prior to transfer the cells in the co-culture system, hepatocytes were cultured on polycarbonate membranes in 7 mL of modified WME at a density of 1.2×10^5 viable cells/cm^2 at 37°C in a humidified gas mixture of air balanced with 10% CO_2.

- Clamp and stretch the polycarbonate membrane (Whatman, Cyclopore Polycarbonat, pore size 0.4 μm, diameter 7 cm) between two tight-fitting rings (inner diameter, 5 cm) and transfer it in a 10-cm Petri dish.
- Coat the membrane with 125 μL with rat tail collagen/80% EtOH (1:1); dry 24 hr.
- Transfer 1.2×10^5 viable cells/cm^2 in 1 mL modified WME (as described above and fortified with 5% fetal calf serum) at the membrane.
- Add 7 mL modified WME after 30 min.
- After 1 hr, remove the medium and the unattached hepatocyte and insert the membranes framed with the holding rings into the co-culture system.
- Continue cultivation of the cells in the co-culture system in serum-free modified WME.

SOPs for Culture Rat Hepatocytes and Intestinal Microflora in the Co-culture System

Co-culture of Hepatocytes and Fecal Microflora

Figure 10 shows a schematic representation of the co-culture system that has been developed in the present project. Prior to the insertion of the membrane into the culture device, rat hepatocytes were cultured on the membrane. Put the compartments of the chamber together. The hepatocyte monolayer is protected against gas bubbles by the fine mesh consisting of high-grade steel. Fill each chamber with 7 mL of modified WME. The gassing facilities are designed so that six culture units arranged side by side can be gassed together (Fig. 11). Put the cannulas into each chamber to perfuse the media with a gas mixture.

Place the co-culture systems in a small incubator and keep in a humidified atmosphere at 37°C during the experiments. Start the perfsuion of the chambers with gas:

- Each compartment should be perfused with 8 mL gas/min.
- The "hepatocyte compartment" of the culture system should be gassed with an aerobic gas mixture: 19% O_2, 71% N_2, and 10% CO_2.
- The "microflora compartment" should be gassed with an anaerobic gas mixture: 90% N_2 and 10% CO_2.

The gas escapes through the openings at the top of the culture device, which also allow the collection of samples.

If not stated otherwise, after 15 min, intestinal microflora were added into the co-culture system.

- Thaw the frozen intestinal microflora quickly by gentle shaking into 37°C water bath.

– Transfer the feces suspension (0.01 g feces/mL) in the the anaerobic compartment of the co-culture system.

The test compounds were added to the aerobic side of the chamber to allow their initial metabolism by the hepatocytes.

24- AND 96-WELL BIOREACTORS

Introduction and Brief Technical Description

Studies on drug metabolism with its many possible interactions and side effects are usually performed in laboratory animals. Beside interspecies differences, there is the disadvantage of intraspecies variations in drug metabolism and, of course, the ethical problem of using living animal models. The use of a pharmacological in vitro screening bioreactor provided with primary hepatocytes presents an alternative solution, comprising not only the advantages of avoiding animal experiments, but also a more cost-effective, exact, and faster way of analysis.

Indispensable demands of such a kind of bioreactor are miniaturization for high throughput analyses and the maintenance of in vivo-like hepato-specific functions of primary hepatocytes. Therefore, we developed a mini bioreactor (G 106) for optimized cell culture and drug screening conditions. Based on the formerly developed flat membrane bioreactor (Bader et al., 1998; 1999; De Bartolo and Bader, 2001b; De Bartolo et al., 2000), the developed construction represents a scale down to conventional 24- and 96-well plate format to allow high throughput screening. By using a gas-permeable, biocompatible membrane, elevated oxygen supply, which is an inalienable requirement of primary hepatocyte culture, is guaranteed.

The mini bioreactor scaffold is composed of polycarbonat with 24 (1.77 cm^2) or 96 (0.32 cm^2) holes, respectively. Under the polycarbonat scaffold, the gas-permeable PTFE membrane of 25-μm thickness, a silicon seal, and a backing of metal, also with 24 or 96 holes, were screwed (Fig. 15). To ensure optimal microscopical observations, this

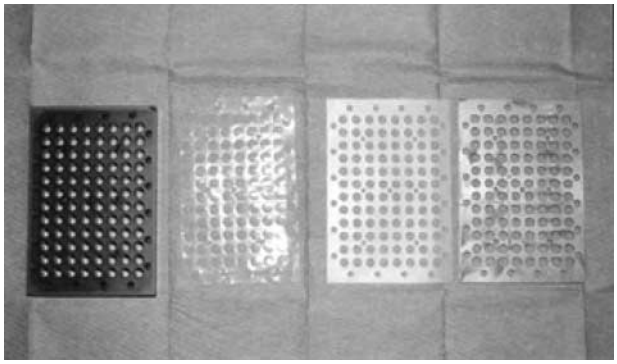

Figure 15. Scaffold, membrane, seal, and backing of metal as the components of 96-well mini bioreactor.

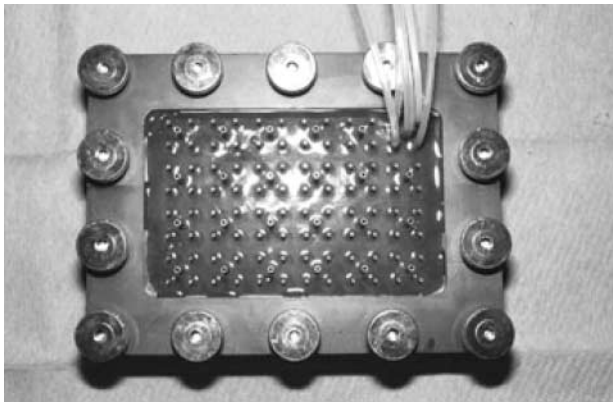

Figure 16. Mini bioreactor in 96-well format with perfusion system.

membrane is glassy. Hepatocytes or even other cell types can be cultured in direct contact with the gas-permeable membrane or on the extracellular protein-coated membrane. The oxygen permeability of the membrane is $11.6*10^3\,cm^3*m^{-2}\,d^{-1}$ (i.e., 90 μmol per well and day; example for 24-well bioreactor). A 24-well bioreactor with 250,000 hepatocytes per well, consumes 6.5 to 19.5 μmol O_2 per well and day (Hay et al., 2001). Thus, the mini bioreactor guarantees an oxygen supply that is 5 to 14 times higher than necessary. In addition, a constant perfusion with cell culture medium is possible (Fig. 16). The advantages of a continuous medium flow are standardized and stable culture conditions. Therefore accumulation of catabolites is prevention.

Prevalidation with Standard Test Substances

The mini bioreactor was prevalidated with several standard test substances (Langsch, 2002). We studied phase I metabolism by CYP by analyses of enzyme activities of EROD (Fig. 17) and ECOD (Fig. 4), as well as phase II metabolism by UGT (Fig. 19), sulfotransferase (ST) (Fig. 20), and GST (Fig. 21). Induction of phase I enzymes (Fig. 22) were performed using PB (1.5 mM), 3-methylcholanthrene (3-MC) (5 μM), clofibrate (Clof) (1 mM), and isoniazide (INH) (100 μM); induction of phase II enzymes were performed using PB and rifampicin (50 μM). Diazepam metabolism was analyzed by quantification of oxazepam, desmethyldiazepam, and temazepam, three major metabolites of diazepam. Furthermore, metabolic capacities were analyzed by measurement of albumin secretion (Fig. 23), urea production (Fig. 24), and glucose/lactate turnover. All studies were conducted in comparison to conventional well cultures, and analyses were carried out up to 12 days in culture. Examples of metabolic studies are shown in Figs. 17 to 24. Activities of phase I enzymes EROD and ECOD remained stable during the complete time of culture, but striking differences between conventional well cultures and mini bioreactors were observed. Both phase I enzyme activities were higher in mini bioreactor cultures. Also phase II UGT, ST, and GST expressed higher enzyme activities in hepatocytes cultured in mini bioreactors compared

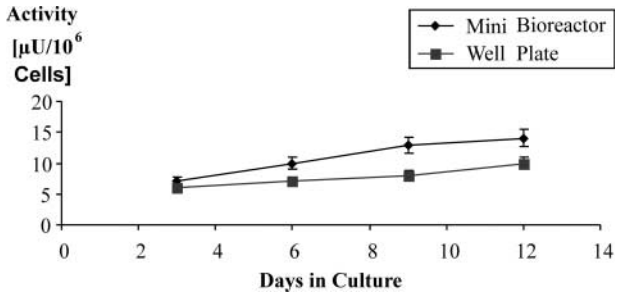

Figure 17. EROD activities of hepatocytes in mini bioreactor and conventional well plates.

with hepatocytes in conventional well culture systems. Induction of phase I (EROD, ECOD) and phase II (GST, UGT, ST) enzymes in mini bioreactor systems was examined by several standard inducers. Different degrees of enzyme induction [e.g., of EROD (Fig. 22)], demonstrate the capacity of mini bioreactor culture systems to respond and display in vivo like phase I and II metabolism. EROD activity was induced 35 times by 3-MC and 2.4 times by PB compared with controls; in contrast, no induction could be observed using Clof or INH. In addition, hepatocyte-specific functions such as albumin and urea production were expressed to a greater extent in mini bioreactor systems compared with conventional well cultures. Also, morphology of hepatocytes after 10 days in culture in mini bioreactor shows the expression of cell–cell contacts, formation of a confluent monolayer, and reorganization of bile ducts (Fig. 25).

Examples for Application

Cryopreservation

To provide a flexible use of hepatocytes cryopreservation is an extremely helpful tool for applications such as bioartificial liver support systems or drug screening models in high throughput analyses. Unfortunately, conventional cryopreservation of primary

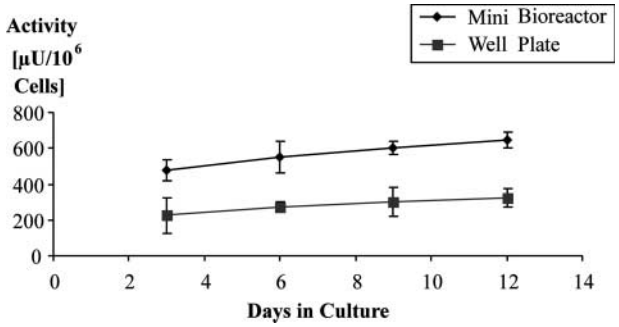

Figure 18. ECOD activities of hepatocytes in mini bioreactor and conventional well plates.

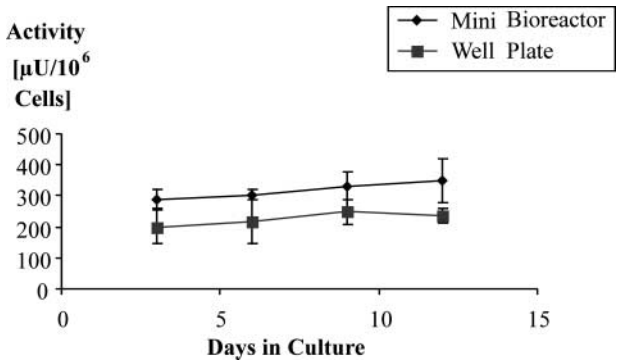

Figure 19. UGT activities of hepatocytes in mini bioreactor and conventional well plates.

hepatocytes results in strongly reduced viability and loss of function. Therefore, optimized cryopreservation models have been developed (for a detailed description, see "SOPs").

Figure 26 shows albumin synthesis of cryopreserved cells and nonfrozen controls. Initially, albumin production decreased compared with controls. However, after 14 days in culture albumin, production of frozen hepatocytes was similar to that of controls. Cryopreservation of hepatocytes in bioreactors lead to a decrease in urea production to approximately 60% of freshly isolated hepatocytes (Fig. 27). However, the rate of urea production by cryopreserved and thawed hepatocytes was constant for up to 14 days in culture (Fig. 27). Before cryopreservation, both cells in bioreactor produced nearly the same amounts of urea (Fig. 27). Production of urea of controls decreased slightly, whereas frozen hepatocytes produced a constant rate of 60%.

Pharmacological Application

Hypericum (plant) extracts have been recommended for some clinical applications, including depression. Extracts contain at least 10 components that may contribute to its

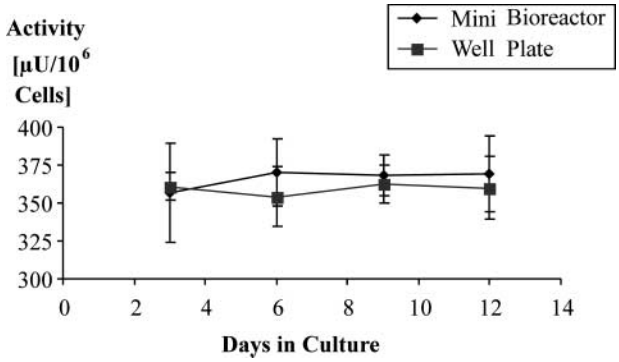

Figure 20. ST activities of hepatocytes in mini bioreactor and conventional well plates.

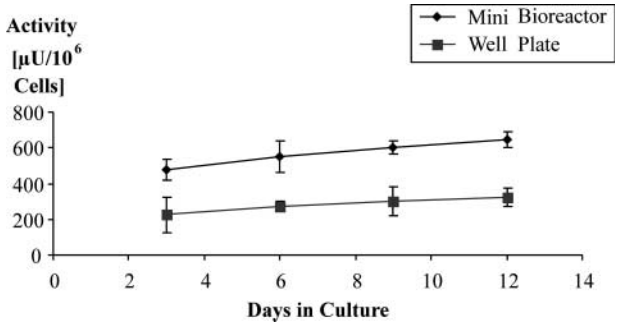

Figure 21. GST activities of hepatocytes in mini bioreactor and conventional well plates.

effects. *Hypericum* is known to interact with several drugs by induction of CYP isoenzymes. Therefore, several extractions of *Hypericum* were analyzed in mini bioreactor culture systems (Langsch, 2002). A methanol extraction (Jarsin™), powdered drug, tea, oil, hyperforin, and hypericin (both are components of the *Hypericum* extracts) were screened with respect to induction or inhibition of CYP enzymes. Alterations of EROD (CYP 1A) and ECOD (CYP 2B) activities, as well as changes in diazepam metabolism (CYP 2C/3A), were analyzed. Different extractions were dissolved in Cremophor RH 40 and added to cell culture supernatants in 1%, 0.1%, and 0.01% solutions onto liver cell cultures in mini bioreactors. Hyperforin and hypericin were solved in DMSO and added to liver cell cultures in 1%, 0.01%, and 0.0001% solutions. After incubation of 2, 3, and 4 days, cell culture supernatants were detached and media supplemented with substratum were incubated for 1 hour. In addition, negative controls without extractions (only solvents Cremophor RH 40 or DMSO) and positive controls with model inducers for CYP enzymes (3-methylcholanthrene, PB, and dexamethasone) were included. The addition of *Hypericum* extracts did not induce enzyme activities of EROD and ECOD

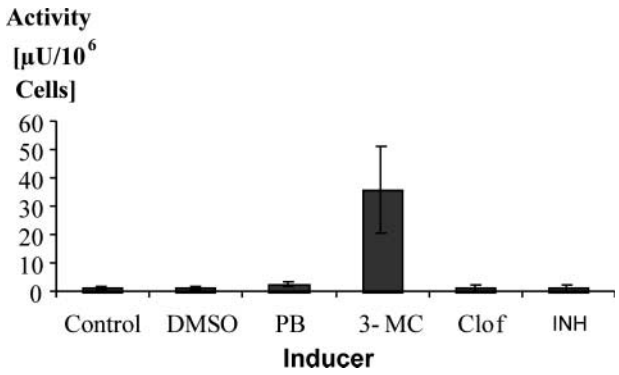

Figure 22. Induction of EROD activities by several inducers of hepatocytes in mini bioreactor (DMSO, dimethysulfoxide; PB, phenobarbital; 3-MC, 3-methylcholanthrene; Clof, clofibrat; INH, isoniazid).

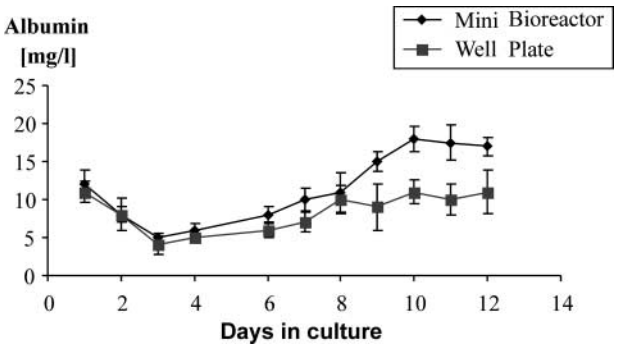

Figure 23. Albumin production of hepatocytes in mini bioreactor and conventional well plates.

(CYP 1A1/2, 2B6), As expected, positive controls (model inducers) lead to an increase in enzyme activities. In contrast, several preparations of *Hypericum* strongly induced diazepam metabolite formation, in particular, hyperforin and powder (Table 15). Therefore, the mini bioreactor has the capacity to predict drug–drug interactions.

Further Applications

The mini bioreactor was also tested with cell types other than hepatocytes. For example, endothelial cells, cardiomyocytes, fibroblasts, chondrocytes, and neurones were cultured for several days in the mini bioreactor. These cell types could be cultured successfully, forming a confluent monolayer and expressing differentiated cell type-specific characteristics proven by immunocytochemistry.

Conclusion and Recommendations

The mini bioreactor was developed to imitate the in vivo-like biotransformation of test substances. It guarantees a microenvironment similar to sandwich cultures

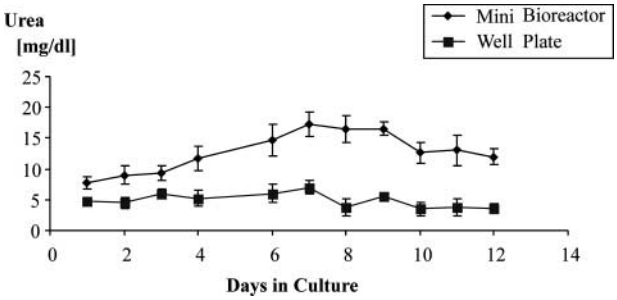

Figure 24. Urea production of hepatocytes in mini bioreactor and conventional well plates.

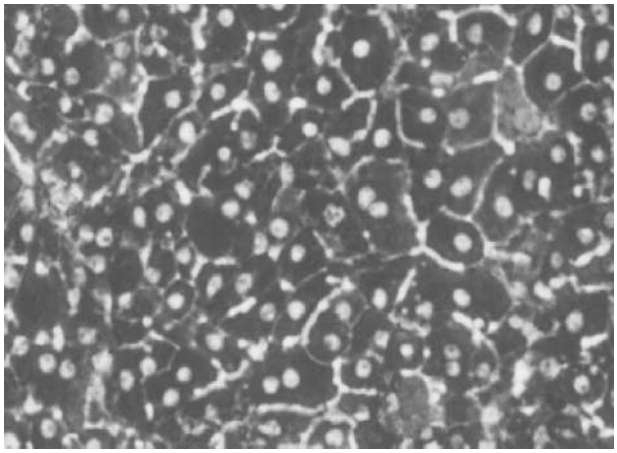

Figure 25. Hepatocytes after 10 days in culture in mini bioreactor.

(Fruehauf et al., 1996; Kern et al., 1997) and optimal oxygen supply by oxygenating PTFE membranes. The advantage of cultivation in direct contact to a gas-permeable membrane is the exactly definable gas exchange of oxygen and carbon dioxide. In addition, microscopic observation through a glassy membrane is possible. Miniaturization enables pharmacological screening in high throughput analyses.

Cryoconservation offers the possibility of short-term applications independent from primary cell sources. In addition, liver-specific functions and morphology remained stably expressed during the entire 12-day culture period. Therefore, culture conditions in oxygenating mini bioreactors allow studies for up to 12 days. In contrast to some other in vitro systems, such as hepatocytes in suspension, they lose many functions after incubation periods longer than 4 hr.

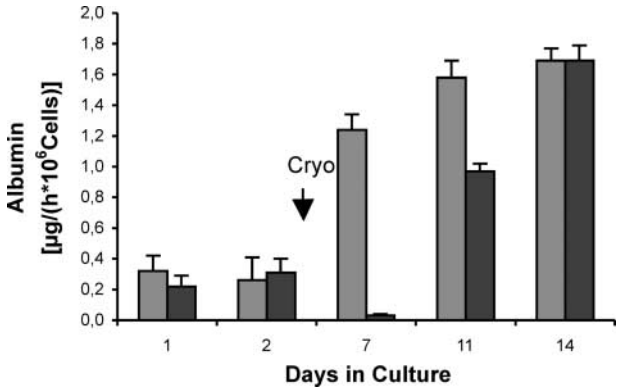

Figure 26. Albumin synthesis of cryopreserved (red) hepatocytes and nonfrozen controls (blue) (Cryo, cryopreservation).

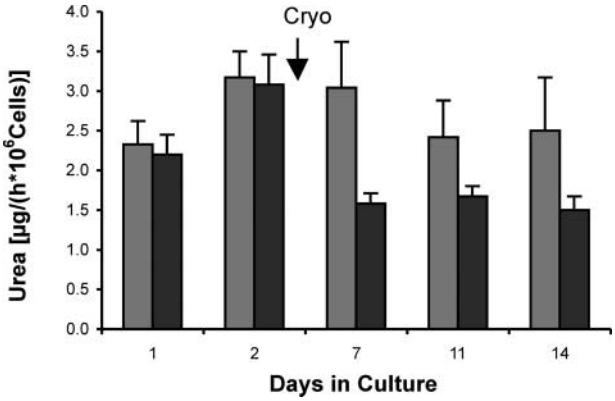

Figure 27. Urea production of cryopreserved (red) hepatocytes and nonfrozen controls (blue) (Cryo, cryopreservation).

SOPs

Cell Culture of Human and Porcine Hepatocytes

Cells were seeded on one layer of collagen I as monolayer and enclosed within two layers of collagen I:

Table 15. Effects of various preparations of *Hypericum* extracts on CYP enzyme activities EROD, ECOD, and diazepam metabolites desmethyldiazepam (DMD), temazepam (TEM), and oxazepam (OX) as relative expressions to individual controls (control = 100%).

Preparation	EROD[a]	ECOD[b]	DMD	TEM	OX
Hyperforin	n.d.[c]	n.d.	++[d]	+[e]	++
Hypericin	n.d.	n.d.	0[f]	−[g]	+
Extract	0	0	++	++	++
Powder 1	0	0	+	+	++
Powder 2	n.d.	n.d.	++	++	0
Powder 3	n.d.	n.d.	+++[h]	++	+
Tea	0	0	+	0	++
Oil	0	0	0	0	+

[a] EROD, 7-ethoxyresorufin-O-deethylase.
[b] ECOD, 7-ethoxycoumarin-O-deethylase.
[c] n.d., not determined.
[d] ++, 40%–60% induction.
[e] +, 20%–40% induction.
[f] 0, no effect (0 ± 20%)
[g] −, inhibition.
[h] +++, >60% induction.

- After cell isolation, hepatocytes were resuspended in WME, and supplemented with 5% FCS and 5.44% premix. Premix:
  - Penicillin 9070 kU/L
  - Streptomycin 9.1 g/L
  - Glutamin 26.5 g/L
  - Insulin 3628 U/L
  - Glukagon 317.5 μg/L
  - Prednisolon 18.14 mg/L
- Hepatocytes were seeded into wells coated with collagen I at a density of up to 140,000 cells/cm^2 in 280 μL medium/cm^2.
- The second layer of collagen was added 24 hr (human) or 48 hr (porcine) later:
  - Culture medium with nonadherent cells was removed, and a second layer of liquid and ice-cold collagen was added on top of the hepatocytes.
  - Thirty minutes following gelation culture, medium was placed on top.

To analyze drug metabolism and gene expression, the addition of test substances to culture media was performed starting 1 day after application of the second layer of collagen.

Preparation of Bioreactors for Storage

One day after hepatocyte isolation, cells cultured in single collagen gel were frozen, whereby cultures were at least 50% confluent. Ice-cold cryopreservation medium (FCS + 10% DMSO) was used. Cell culture medium was removed and replaced with cryopreservation medium. Cell were preincubated for 10 min at 4°C.

Cryopreservation

Cryopreservation was performed using a computer-controlled freezer (Consartic) that measures temperature using a 0.1 mm thin Pt-100 element. Thus, exact measurement of temperature is possible even at small layer thickness. The chamber of the freezer was precooled to 4°C; after 5 min, temperature was cooled down up to −40°C by −1°C/min, followed by freezing up to −60°C by −2.5°C/min and up to −100°C by −5°C/min. After 15 min freezing at −100°C for 15 min, bioreactors were stored at −150°C.

Defrosting

Bioreactors were wiped with ethanol (70%) to remove frozen condensed water. Cells were incubated with culture medium (37°C) for 10 min at 37°C. Thereafter, medium was removed and replaced by fresh medium.

Culture of Thawed Bioreactors

One day after defrosting, the second layer of collagen was applied. Cell culture medium was replaced daily by fresh medium.

ABBREVIATIONS

CYP	cytochrome P450
EC	7-ethoxycoumarin
ECOD	7-ethoxycoumarin O-deethylase
GST	glutathione S-transferase
ST	phenol sulphotransferase
GSH	glutathione
UGT	UDP-glucuronosyltransferases
GT1	UDP-glucuronosyltransferase activity with 3-hydroxy-benzo[*a*]pyrene as substrate
GT2	UDP-glucuronosyltransferase activity with 4-hydroxy biphenyl as substrate
OH-C	7-hydroxycoumarin
OH-T	hydroxytestosterone
PBS	phosphate buffered saline
RT	retention time
TH	testosterone hydroxylase.

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