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CHARACTERIZATION OF CRYOPRESERVED RAT LIVER PARENCHYMAL CELLS BY METABOLISM OF DIAGNOSTIC SUBSTRATES AND ACTIVITIES OF RELATED ENZYMES

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Abstract—The metabolism of testosterone and benzo(*a*)pyrene (BaP) which is mediated by diverse enzymes was determined in cryopreserved rat liver parenchymal cells and compared with that found in freshly isolated cells. In addition, the activities of single xenobiotic-metabolizing enzymes were measured by using specific substrates. The cytochrome P450 (P450)-mediated total metabolic conversion of testosterone was reduced to 55% in cryopreserved cells. The metabolite profile, i.e. the formation of single metabolites compared with total metabolic conversion, was however unchanged when compared with freshly isolated cells. A concomitant reduction in the activities of the involved P450 isoenzymes can therefore be postulated. The amount of detected phase I-metabolites of BaP was unaffected by the cryopreservation method. The formation of phase II-metabolites and total metabolic conversion of BaP in cryopreserved cells was however reduced to about 50–60%. The reduced glutathione *S*-transferase and more obviously phenol sulfotransferase activities measured in cryopreserved cells, may explain the impaired conjugation of BaP. The ratio between phase I- and phase II-metabolites was thus changed by cryopreservation. Density separation on Percoll yielded cryopreserved cells with a viability and metabolic capacity not measurably different from freshly isolated cells. To this extent, cryopreserved, Percoll-purified liver parenchymal cells are a useful *in vitro* system for drug metabolism studies. However due to the extensive loss in cell number during this procedure (recovery = 22% of freshly isolated cells) the application of this system is limited.

In vitro systems are increasingly used as a substitute for, or at least as a means of reducing the number of animal experiments *in vivo*. Freshly isolated, cultured or cryopreserved liver parenchymal cells (PC‡) are possible *in vitro* models for the study of drug metabolism. The first step in reducing the number of animals for such experiments is reached when freshly isolated cells are used in metabolism studies. Various test compounds at different dose levels can then be tested with PC from a single animal. The number of PC isolated is, in most cases, so large that many cells are often discarded. Culturing or freezing PC are possible methods for conserving these cells for their later use which results in a reduction in the number of laboratory animals used. A second advantage of both cultured and cryopreserved cells is the availability of relevant biological material from species which are usually rarely available for cell isolation such as monkey or man. Both methods are however difficult to apply to PC since these dedifferentiate *in vitro* rather rapidly. The culture of PC has been improved significantly over the last few years. Investigations from our own institute [1, 2] have however shown that specific differentiated functions of the PC, such as the activities of some xenobiotic-metabolizing enzymes, are still lost even under improved culture conditions. In an attempt to overcome these disadvantages, drug metabolism was studied in cryopreserved PC. On consulting the literature it became clear that evaluation of such *in vitro* systems is, in most cases, insufficient in that either insensitive or a limited number of parameters have been measured.

The aim of the present investigation was to use an optimized cryopreservation protocol§ and characterize the cryopreserved PC by the use of substrates which are metabolized by a broad variety of enzymes. The possible impairment of a single step in such a multistep pathway would cause a visible shift in the profile of metabolites. The metabolism of an endogenous (testosterone) and a xenobiotic substrate (benzo(*a*)pyrene; BaP), was therefore investigated. Specific xenobiotic-metabolizing enzyme activities were additionally determined to explain possible differences in the metabolic profile created by cryopreserved PC in comparison with freshly isolated cells. Finally, a critical proposal for the use of cryopreserved PC as an *in vitro* drug-metabolizing system is presented.

MATERIALS AND METHODS

Chemicals. Collagenase was purchased from Biochrom (Berlin, Germany); dimethylsulfoxide, Percoll, 1-chloro-2,4-dinitrobenzene and unlabelled BaP from Sigma (Deisenhofen, Germany). [7-^{3}H]-Styrene oxide, [G-^{3}H]BaP and [7,10-^{14}C]BaP were

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‡ Abbreviations: BaP, benzo(*a*)pyrene; P450, cytochrome P450; PC, rat liver parenchymal cells.

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obtained from Amersham Buchler (Braunschweig, Germany); 1-naphthol and unlabelled styrene oxide from Merck (Darmstadt, Germany); and 2-naphthol from Fluka (Neu-Ulm, Germany). *trans*-[α,β-^{3}H]-Stilbene oxide was synthesized as described before [3]. Testosterone, 4-androsten-3,17-dione and corticosterone were supplied by Sigma; 6β-, 7α-, 16α- and 16β-hydroxytestosterone by Steraloids Inc./ Paesel (Frankfurt, Germany); 2α-, 2β- and 6α-hydroxytestosterone by the Steroid Reference Collection (D. N. Kirk, Dept. of Chemistry, Queen Mary College, London, U.K.); 15β-hydroxytestosterone from Schering AG (Berlin, Germany). 3-OH BaP was obtained from NCI Chemical Reference Standard Repository (Bethesda, MD, U.S.A.). BaP-1,6- and -3,6-quinones were prepared as described previously [4]; the sources of the other metabolites of BaP have been given elsewhere [5].

Buffer. Buffer for the isolation, suspension, cryopreservation and incubation of viable cells was a Krebs–Henseleit buffer containing 25 mM 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), D-glucose (0.5%), 1 mM $CaCl_2$, 0.4 mM $MgSO_4$, insulin (10 mg/L), bovine serum albumin (0.2%) and the amino acid mixture recommended by Seglen [6]. This buffer was supplemented when required with different additives, as described below.

Isolation and determination of the viability of PC. PC of adult male Sprague–Dawley rats (240–280 g, Süddeutsche Versuchstierfarm, Tuttlingen, Germany) were isolated according to Seglen [7] with, as previously described [8], slight modifications. The viability was determined by Trypan Blue exclusion.

Cryopreservation and thawing of isolated PC. Different parameters, such as cell number, cryopreservation buffers, type and concentration of cryoprotectant, cooling rate and time-dependent addition and removal of cryoprotectant, were varied. The cell yield and viability of PC cryopreserved by the different methods were investigated.* The following cryopreservation method was found to be optimal and was used in the experiments presented in this paper.

PC were suspended in a concentration of 5–7 $\times$ 10^6 cells/mL in the above described, ice-cold buffer with the addition of 4% dimethylsulfoxide. Five minutes later a solution of 16% dimethylsulfoxide was added to give a final concentration of 10%. One milliliter of this suspension was pipetted into 2-mL cryo tubes and immediately frozen in a computer-controlled freezing chamber (Cryoson BV-8; Schöllkrippen, Germany). The cooling rate (beginning with a temperature of 18°) was −1.8°/min for 10 min, 0°/min for 8 min, −2°/min for 4 min, shock-freezing from −8° to −28° within 0.1 min, −2.5°/min for 2 min, +2.5°/min for 2 min, −2°/min for 16 min, and −10°/min for 4 min (ending with a temperature of −100°). Afterwards the frozen samples were stored in liquid nitrogen.

The frozen cells were thawed by gentle shaking in a waterbath (37°). Dimethylsulfoxide was removed from the cells by gradual dilution of the 1-mL samples with 3, 6, and 10 mL of ice-cold buffer at 2-min intervals, and three times centrifugation (5 min at 15 *g*) and resuspension of the pelleted cells in ice-cold buffer. The cells were then counted, the viability determined by Trypan Blue exclusion and the cells either used directly for the different investigations or purified first by Percoll centrifugation.

Percoll purification of viable cryopreserved PC. Percoll centrifugation was carried out according to a modified method of Kraemer *et al.* [9]. A Percoll–Hanks solution was prepared by diluting one part of a 10× concentrated Hanks balanced salt solution (1.34 M NaCl, 54 mM KCl, 8 mM $MgSO_4$, 4 mM KH_2PO_4, 4 mM Na_2HPO_4; pH 7.4) with nine parts of Percoll. A suspension of thawed PC adjusted to 4 $\times$ 10^6 cells/mL was gently mixed with the same volume of the Percoll–Hanks solution and centrifuged for 10 min at 50 *g*. Viable PC with a density higher than 1.05 g/mL pelleted and were suspended in the modified Krebs–Henseleit buffer. After centrifugation for three times 5 min at 15 *g* viability was determined by Trypan Blue exclusion.

Protein determination and enzyme assays. PC were counted, suspended in appropriate buffers used for the different enzyme assays at a concentration of 4 $\times$ 10^6 cells/mL and carefully homogenized by sonification using a Branson-Sonifier B15 (four times 30 sec at 4°). Time and protein linearity were checked separately for each of the assays.

Protein determination was carried out according to Lowry *et al.* [10] with bovine serum albumin as a standard. Cytochrome P450 (P450) was measured spectrophotometrically according to Omura and Stato [11]. Microsomal epoxide hydrolase activity was measured according to the method of Oesch *et al.* [12, 13] with styrene 7,8-oxide as substrate. Cytosolic epoxide hydrolase activity was measured according to the method of Schladt *et al.* [14] with *trans*-stilbene oxide as substrate. Glutathione *S*-transferase activities were determined photometrically according to the method of Habig *et al.* [15] using the broad spectrum substrate 1-chloro-2,4-dinitrobenzene. Phenol sulfotransferase and UDP-glucuronosyltransferase were measured fluorometrically using 2-naphthol [16] and 1-naphthol [17] as substrates, respectively.

Metabolism of testosterone and BaP. Testosterone and BaP metabolism were determined in intact cells.

The P450-dependent hydroxylation of testosterone was determined by incubating 1 $\times$ 10^6 suspended PC in 2 mL buffer for 10, 20 and 30 min with 1 mM testosterone in a shaking water bath at 37°. Extraction, HPLC separation and quantification of the testosterone metabolites were performed as described previously [18].

Total metabolic conversion of BaP (cf. Table 4) was determined according to the method of Ireland *et al.* [19] with slight modifications. In brief, 0.5–1.5 $\times$ 10^6 PC were incubated in 2 mL buffer with 160 nmol [G-^{3}H]BaP (sp. act. 1.48 TBq/mmol) in a shaking water bath (37°) for 30–90 min. After the incubation period, 1 mL 0.15 N NaOH in 85% dimethylsulfoxide was added and unmetabolized BaP was removed by extraction (three times) with 4 mL hexane. The total metabolic conversion of BaP was quantitated by determination of the radioactivity

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Table 1. Trypan Blue exclusion, protein content and activities of xenobiotic-metabolizing enzymes in freshly isolated and cryopreserved rat liver parenchymal cells

	Freshly isolated	Cryopreserved
Trypan Blue exclusion (%)	82 ± 7	69 ± 6¶
Protein (mg/10^6 cells)	1.10 ± 0.20	0.99 ± 0.16
P450 (nmol/mg protein)	0.21 ± 0.04	0.19 ± 0.04
Microsomal epoxide hydrolase (U/mg protein)*	1.62 ± 0.31	1.37 ± 0.37
Cytosolic epoxide hydrolase (U/mg protein)†	15.2 ± 4.4	16.2 ± 5.2
Glutathione *S*-transferase (U/mg protein)‡	0.445 ± 0.192	0.295 ± 0.079
Phenol sulfotransferase (U/mg protein)§	2.52 ± 0.25	1.67 ± 0.32¶
UDP-Glucuronosyl transferase (U/mg protein)‖	16.1 ± 3.0	15.5 ± 1.5

One unit (U) is the amount of enzyme which metabolizes *1 nmol styrene oxide, †1 pmol *trans*-stilbene oxide, ‡1 μmol 1-chloro-2,4-dinitrobenzene, §1 nmol 2-naphthol or ‖1 nmol 1-naphthol per min.

Values are means ± SD of at least three independent experiments; ¶significantly different ($P < 0.05$) from freshly isolated cells using Student's *t*-test.

in the aqueous phase in relation to that in the organic phase.

For the determination of phase I- and phase II-metabolism of BaP (cf. Table 3), 2 mL of a suspension of viable PC (1–2 × 10^6 PC/mL) in the buffer described above were incubated in 8-mL Erlenmeyer flasks with 80 μM [7, 10^{-14}C]BaP (sp. act. 440 MBq/mmol) dissolved in 20 μL dimethylsulfoxide. Incubation was performed in a rotating water bath at 37° for 30–120 min. The cells were then broken in an ultrasonic water bath (Sonorex RK-102H, Bandelin, Berlin, Germany) for 1 min, followed by the addition of 2 mL ethyl acetate. Extraction of the phase I-metabolites was performed as described [20]. The amount of phase II-metabolites (conjugates) was calculated from the radioactivity remaining in the aqueous phase. The phase I-metabolites of BaP were separated by reversed-phase HPLC using LiChrospher-100-CH-18 Super (4 μm; 4 × 250 mm; Merck, Darmstad, Germany) as the stationary phase. The mobile phase consisted of a linear gradient with a flow rate of 0.6 mL/min which went from 50% methanol in water to 100% methanol in 50 min, and remained at 100% methanol for 20 min. Detection and quantification was achieved using a radiomonitor (Ramona-LS, Isomess, Straubenhardt, Germany) equipped with a solid scintillator cell coupled to a UV monitor (280 nm; model D, LDC Milton Roy, Hasselroth, Germany). Identification of chromatographic peaks was performed by co-chromatography with synthetic derivatives of BaP.

RESULTS

Trypan Blue exclusion, protein content and enzyme activities

Using the described freezing protocol viability of the cryopreserved PC (69%) was slightly but significantly less than that of freshly isolated PC (82%; Table 1). No dependency of Trypan Blue exclusion from the time of storing the cells in liquid nitrogen (up to 1 year) existed. The other parameters presented in Table 1, i.e. protein and P450 content as well as the activities of several xenobiotic-metabolizing enzymes, were measured in cell homogenates of freshly isolated and cryopreserved PC. Cofactors for the different enzymes were added to the incubation mixtures. While these parameters do not give a direct measure of viability, they do however provide information about the stability of the measured enzymes during the freezing and thawing procedure, and about possible direct influences of remaining cryoprotectant upon the enzymes. As expected, most enzyme activities were not measurably different in cryopreserved PC as compared with freshly isolated PC. The activity of two conjugating phase II-enzymes were however different in the two systems. Glutathione *S*-transferase activity seemed to be reduced and that

Table 2. P450-dependent hydroxylation of testosterone by freshly isolated and cryopreserved rat liver parenchymal cells

Testosterone metabolites	Freshly isolated	Cryopreserved
	(% of total metabolite formation)	
6α-OH Testosterone	0.3 ± 0.2	0.4 ± 0.1
15β-OH Testosterone	0.4 ± 0.4	0.4 ± 0.3
7α-OH Testosterone	1.8 ± 0.7	2.6 ± 0.4
6β-OH Testosterone	9.5 ± 6.1	8.1 ± 5.5
16α-OH Testosterone	21.5 ± 5.2	17.4 ± 5.9
16β-OH Testosterone	0.3 ± 0.4	0.1 ± 0.2
2α-OH Testosterone	18.5 ± 3.6	15.2 ± 3.9
2β-OH Testosterone	1.3 ± 0.6	1.2 ± 0.8
4-Androsten-3,17-dione	46.4 ± 13.0	54.7 ± 15.8
Total metabolites	(nmol/min10^6 cells)	
(=100%)	4.68 ± 1.40	2.57 ± 0.08

Values are means ± SD ($N \geq 3$). No statistically significant differences were detected between the two groups of values.

Table 3. BaP metabolism in freshly isolated and cryopreserved rat liver parenchymal cells

BaP metabolites	Freshly isolated	Cryopreserved
BaP 9,10-dihydrodiol	2.0 ± 0.4	2.3 ± 0.4
BaP 4,5-dihydrodiol	2.6 ± 0.6	1.4 ± 0.3
BaP 7,8-dihydrodiol	3.6 ± 1.1	3.7 ± 0.1
BaP 4,5-quinone	NM	0.9 ± 0.5
BaP 1,6-quinone	1.6 ± 0.9	1.6 ± 1.2
BaP 3,6-quinone	1.9 ± 1.3	2.5 ± 1.9
9-OH BaP	2.6 ± 1.8	1.9 ± 0.6
3-OH BaP	5.4 ± 1.2	7.5 ± 2.1
Total Phase I-metabolites	20.7 ± 9.3	24.7 ± 1.6
Total Phase II-metabolites	170.0 ± 43.9	85.8 ± 4.5
Total metabolites	197.6 ± 63.3	117.7 ± 6.5

Values represent the amounts of metabolites generated and are expressed in pmol/min/10^6 cells. They are means ± SD ($N \geq 3$). No statistical significant differences were detected between the two groups of values.
NM, not measurable.

of sulfotransferase was significantly lower in cryopreserved cells (Table 1).

Metabolism of testosterone

A more adequate viability criterium is the determination of the rate of metabolite formation by whole cells. We have shown in a previous publication [1] that testosterone is a suitable substrate for such purposes, since it gives valuable information about the activities of several distinct P450 isoenzymes [21, 22].

As shown in Table 2, 4-androsten-3,17-dione was the main metabolite in both metabolizing systems. The three major hydroxylated metabolites were 16α-, 2α- and 6β-OH testosterone. The metabolic profile of these major and also of the minor metabolites was very similar in cryopreserved and freshly isolated PC. The absolute amount of total metabolites seemed to be lower when testosterone was metabolized by cryopreserved PC. This may be because the activities of all P450 isoenzymes were reduced to a similar extent (i.e. about 55% of total metabolites formed in freshly isolated PC).

Metabolism of BaP

3-OH BaP and BaP 7,8-dihydrodiol were the major phase I-metabolites of BaP in freshly isolated and cryopreserved PC. BaP 4,5-quinone was hardly measurable, and all other phase I-metabolites were intermediate in the two systems (Table 3). The absolute amounts of all single and total number of phase I-metabolites were very similar in cryopreserved PC as compared with freshly isolated PC. Differences were however detected in the amounts of phase II- and of total metabolites of BaP formed which were about 50 and 40% lower in cryopreserved PC, respectively.

Influence of Percoll purification of cryopreserved PC

The viability and metabolic capacity of cryopreserved PC was shown to be improved by a density separation on Percoll [9]. We therefore reinvestigated those parameters which were reduced in the above described experiments. As shown in Table 4, sulfotransferase activity and the amount of total testosterone metabolites were elevated when the cells before and after Percoll centrifugation were compared. The metabolite profile of testosterone was not changed after Percoll centrifugation (data not shown). The fraction of Trypan Blue-excluding cells and the amount of total BaP metabolites were significantly higher in cryopreserved PC after Percoll centrifugation as compared with those before Percoll treatment. The levels of these parameters in the cryopreserved Percoll-PC were not measurably different from those in freshly isolated PC. The freezing procedure and purification step resulted however in a significant loss of cells. The final total number of viable cells was about 20% of the number of freshly isolated cells before freezing.

DISCUSSION

Different procedures for the cryopreservation of PC have been described in the literature [23–31]. Although many parameters, such as the cooling rate or the buffer systems, were varied, recovery and viability of the cells were reduced. However, the viability of PC could, in most instances, be increased by an additional Percoll centrifugation after thawing. In these respects, the present investigation is comparable with the most successful ones already described. Mean viability, measured as Trypan Blue exclusion, was 69% and 86% before and after Percoll centrifugation, respectively (Table 4).

So far, in our opinion, the most thorough investigation of drug metabolism in cryopreserved PC was that carried out by Powis *et al.* [31]. In that study, viability and P450-dependent dealkylation of 7-ethoxycoumarin (mainly by P450 IA1) were reduced by cryopreservation to 67% and 33%, respectively. Percoll centrifugation increased both the dye exclusion to 85% and the P450-dependent reaction to 69%. Furthermore, metabolism of biphenyl and predominantly its sulfate conjugation was clearly reduced. Glucuronide formation was less

Table 4. Influence of Percoll purification on Trypan Blue exclusion, cell yield and enzyme activities of cryopreserved rat liver parenchymal cells

	Freshly isolated − Percoll	Cryopreserved − Percoll	Cryopreserved + Percoll
Trypan Blue exclusion (%)	82 ± 7	69 ± 6	86 ± 3*
Number of viable cells ($\times 10^6$)	454 ± 141	354 ± 50	100 ± 77*
Phenol sulfotransferase (U/mg protein)	2.52 ± 0.25	1.67 ± 0.32	2.06 ± 0.69
BaP metabolites (pmol/min/10^6 cells)	267 ± 60	143 ± 47	268 ± 47*
Testosterone metabolites (nmol/min/10^6 cells)	4.68 ± 1.40	2.57 ± 0.08	3.67 ± 1.80

Values are means ± SD ($N \geq 3$); *significantly different ($P < 0.05$) from cryopreserved PC before Percoll purification using Student's *t*-test.

affected in cryopreserved PC as compared with freshly isolated cells.

The present investigation provides further information about the metabolic capacity of cryopreserved PC and, more importantly, yielded cryopreserved cells with higher metabolic rates. The advantage of using diagnostic substrates which are metabolized by different enzymes to complex metabolite profiles becomes obvious. Testosterone, for example, is metabolized in intact PC to 4-androstene-3,17-dione and to hydroxylated metabolites, mainly 16α-, 2α-, and 6β-OH testosterone (Table 2). These metabolites are predominantly formed, under the selected experimental conditions, by the main cytochrome P450 isoenzyme in untreated male rat, namely P450 IIC11. Further isoenzymes are however also involved. Androstendione is formed by rat microsomal P450s IIB1 and IIB2, and additionally by a cytosolic steroid dehydrogenase [32] when intact cells are used [1]. 16α-OH Testosterone is additionally formed by P450s IIB1, IIB2, IIC7 and IIC13, and 6β-OH testosterone by P450s IA1, IA2, IIC13 and IIIA1/2. Formation of the minor metabolites of testosterone additionally provides information about the activities of P450s IIA1–3. Using the above described optimized freezing protocol, total testosterone metabolite formation was reduced to about 50% (Table 2). The metabolic profile, i.e. the formation of single metabolites compared with total metabolic conversion was however very similar to that obtained with freshly isolated PC. No inductive or inhibitory effect of the formation of specific testosterone metabolites by possibly remaining dimethylsulfoxide in the cell preparations was measured. This means that the activities of all involved P450 isoenzymes were similarly reduced. In contrast, the P450 content, which was measured spectrophotometrically, was not significantly changed. A possible reduction in NADPH/P450 reductase activity or, probably more likely, a loss in NADPH may explain the reduced P450-mediated testosterone metabolism in cryopreserved PC.

As was the case with testosterone metabolism, total BaP metabolism was reduced to about 50–60% by the cryopreservation procedure. The formation of phase I-metabolites of BaP was not quantitatively influenced (Table 3) although P450 isoenzyme activities, measured as testosterone hydroxylation, seemed to be reduced (Table 2). To explain this apparent discrepancy one has to keep in mind that formation of phase I-metabolites is an essential step in the formation of phase II-metabolites. In freshly isolated PC conjugating enzyme activities were high with the consequence that many formed phase I-metabolites are simultaneously conjugated. Glutathione and sulfate conjugates were in fact shown to contribute up to 30–40% of total phase II-products of BaP in freshly isolated PC under these experimental conditions (unpublished results). In cryopreserved PC both phase I- and II-metabolizing enzyme activities (glutathione transferase and, more pronouncedly, sulfotransferase, cf. Table 1) were reduced. Less phase I-metabolites should have been produced but, more relevantly, much less were conjugated at the same time. The ratio of total phase I- to total phase II-metabolites was therefore different in cryopreserved (1:3.5) as compared with freshly isolated PC (1:8; cf. Table 3). The activity of the third conjugating enzyme which was measured, i.e. glucuronosyltransferase, was unchanged by the cryopreservation method. This may explain why glucuronide formation of biphenyl was much less affected as compared with sulfate conjugation in the study of Powis *et al.* [31]. The interpretation of the presented data is however difficult because a possible loss of the respective cofactors for the metabolizing enzymes may have occurred as well. Furthermore, it is not absolutely clear which enzymatic step is rate limiting and, finally, which P450 isoenzymes were responsible for BaP oxidation under the selected experimental conditions. Purified P450 IA1 has the highest catalytic activity towards BaP [21]. This isoenzyme is however present only in small amounts (about 3% of total P450) in the liver of untreated rats [33]. In contrast, the main form in male rat liver (P450 IIC11) has a much lower catalytic activity towards BaP. Presumably both isoenzymes are involved in the initial BaP metabolism in isolated PC from untreated, male rat.

Testosterone metabolism and the enzyme activities listed in Table 1 were determined in previous studies [1, 2] when the quality of PC was being evaluated in culture. Similarities and differences exist between the stabilities of certain activities in these two *in vitro* systems. The activity of glucuronosyltransferase, for

example, was stable in both cryopreserved PC and 7-day-old PC in co-culture with other cell lines. In contrast, sulfotransferase activity was reduced to about 50–60% in both systems. P450 isoenzymes were differentially stabilized in co-cultured PC. P450 IIIA1/2 and P450 IA1 activities were well stabilized over the entire culture period while P450 IIC11 activity was totally lost after 7 days. In contrast to these results, the activity of all P450 isoenzymes was similarly reduced in cryopreserved PC. Overall, the pattern of xenobiotic-metabolizing enzyme activities was better retained in cryopreserved PC than in cultured cells. The survival of PC in suspension is however limited to periods of about 4 hr. These facts have to be taken into account when either of these two *in vitro* systems is used for different purposes.

As shown, the disadvantages of the cryopreservation model can be overcome when Percoll-purified cryopreserved PC are used. Trypan Blue exclusion, sulfotransferase activity and total metabolic conversion of testosterone and BaP were not measurably different in cryopreserved PC after Percoll centrifugation when compared with freshly isolated cells (Table 4). The main disadvantage of this procedure however is the loss of cells during Percoll centrifugation. For reasons as yet unknown, a great variation in the final recovery of cells in the different experiments was observed. A hypothetical selection of subpopulations of PC seemed not to occur because all biochemical parameters measured in the cryopreserved PC after Percoll purification were not significantly different from those of the freshly isolated cells. In this respect the described procedure seemed to select exclusively for viability (Trypan Blue exclusion) of the cells. Possible differences concerning other parameters which have not so far been measured may however exist.

In conclusion, cryopreservation of PC is indeed a useful *in vitro* system for the study of xenobiotic metabolism. Percoll separation of viable cells is however essential. When using unpurified cell populations one cannot exclude the fact that single metabolic pathways are reduced and possible shifts in the metabolite profile of a drug under study may occur. Due to the significant loss of cells this method of cryopreservation is limited to animal species from which either large amounts of cells can be isolated or which are rarely available for cell isolation. Extrapolation of such sophisticated methods from one aminal species to another is always a problem. However, using the above described freezing protocol, a successful cryopreservation of PC derived from the guinea pig, beagle dog and rhesus monkey could be achieved in our laboratory. Therefore, a general application of this procedure seems to be possible provided that PC of high quality are isolated initially.

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