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VIABILITY, ATTACHMENT EFFICIENCY, AND XENOBIOTIC METABOLIZING ENZYME ACTIVITIES ARE WELL MAINTAINED IN EDTA ISOLATED RAT LIVER PARENCHYMAL CELLS AFTER HYPOTHERMIC PRESERVATION FOR UP TO 3 DAYS IN UNIVERSITY OF WISCONSIN SOLUTION

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SUMMARY

Rat liver parenchymal cells were isolated by EDTA perfusion and were subsequently purified by Percoll centrifugation. The freshly isolated liver cells had a mean viability of 95% as judged by trypan blue exclusion. Isolated liver parenchymal cells were then stored at 0° C for up to 1 wk in University of Wisconsin solution (UW). During this hypothermic preservation, the viability was only slightly reduced to 92% after 1 d and to 85% after 3 d at 0° C. Thereafter, the viability decreased rapidly. After cold storage for up to 3 d, it was possible to use the parenchymal liver cells either in short-term suspension or in cell culture. The attachment efficiency in cell culture was the same for freshly isolated liver cells (84%) and after 2 d cold preservation (81%). The cytochrome P450 content and the enzyme activities of soluble epoxide hydrolase, UDP-glucuronosyl transferase, phenol sulfotransferase, and glutathione S-transferase were not significantly different between freshly isolated cells and cells after 3 d of hypothermic preservation. Furthermore, freshly isolated and intact liver cells stored for 3 d were used in the cell-mediated Salmonella mutagenicity test as a metabolizing system. Both fresh and stored liver parenchymal cells metabolized benzo(a)pyrene, 2-aminoanthracene, and cyclophosphamide to their ultimate mutagens. Thus, it was clearly demonstrated that EDTA-isolated liver parenchymal cells retain their xenobiotic metabolizing capacity after short-term hypothermic preservation for up to several days and, therefore, may help to maximize the usefulness of rarely available liver parenchymal cells such as those from humans and help to reduce the number of experimental animals required for pharmacological and toxicological *in vitro* investigations.

Key words: hepatocytes; cold storage; University of Wisconsin solution; xenobiotic metabolizing enzymes.

INTRODUCTION

Isolated liver parenchymal cells (PC) are extensively used in *in vitro* systems in biomedical research. PC can be isolated from the livers of various species either by enzymatic (Berry and Friend, 1969) or nonenzymatic (Wang et al., 1985) perfusion. The yield of viable cells from one rat liver is usually more than can be used in one experiment. Under conventional conditions, surplus PC cannot be stored and are discarded; therefore, attempts have been made to store isolated PC. Cryopreservation of isolated PC is possible but for a successful preservation a computer-controlled freezer is necessary (Diener et al., 1993). For short-term preservation of hours up to several days, hypothermic storage at temperatures between 0° to 5° C in special media have been suggested (Guyomard et al., 1990; Poullain et al., 1992; Sandker et al., 1992; Hammond and Fry, 1993). With respect to liver cells, the use of University of Wisconsin solution (UW) seems to be beneficial (Fuller and Cheetham, 1990; Rivas et al., 1993). UW is an organ preservation solution that is used to preserve livers, pancreata, and kidneys (D'Alessandro et al., 1991). In this study, we investigated whether ethylenedinitrilotetraacetic acid (EDTA) isolated rat PC maintain their xenobiotic metabolizing enzyme activities and whether they could be used for pharmacological and toxicological *in vitro* studies after hypothermic storage in UW.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 240–300 g were obtained from the Versuchstieranstalt (Tuttlingen, Germany). The rats were fed a standard diet (Altromin, Lage, Germany) and tap water *ad libitum*. Animals were handled in compliance with German regulations.

Chemicals. Trans-2,3-[2,3-^{3}H]diphenyloxiran was from Amersham Buchler (Braunschweig, Germany). Collagen IV, dimethyl sulfoxide, benzo(a)pyrene (BP), and 1-chloro-2,4-dinitrobenzene were from Sigma Chemical Co. (Deisenhofen, Germany). Cyclophosphamide (CP), 2-aminoanthracene (2-AA), lactobionic acid, raffinose, adenosine, and glutathione were from Aldrich (Steinheim, Germany). 1-Naphthol was from Merck (Darmstadt, Germany) and 2-naphthol was from Fluka (Neu-Ulm, Germany). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were from Biochrom (Berlin, Germany). University of Wisconsin solution (UW) (Via Span) was from Du Pont (Bad Homburg, Germany). Hydroxyethyl starch with a mean MW of 200 000 and a substitutional grade of 0.5, containing 10 mmol NaCl per liter, was kindly donated by Fresenius AG (Oberursel, Germany). All other chemicals were of reagent grade.

Liver cell isolation. The PC were isolated by the method of Wang et al. (1985) with slight modifications. The rats were anesthetized with an i.p. injection of pentobarbital (60 mg/kg) and the vena portae was cannulated. The livers were perfused *in situ* with 2 liters (flow rate = 35 ml/min) of 37° C

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warm EDTA buffer (140 m*M* NaCl, 5 m*M* KCl, 0.8 m*M* $MgSO_4$, 1.6 m*M* Na_2HPO_4, 0.4 m*M* KH_2PO_4, 2 m*M* EDTA, and 25 m*M* $NaHCO_3$, adjusted to pH 7.4). After perfusion, the liver was removed, dissociated, and filtered through gauze. The cell suspension was washed three times by centrifugation (5 min, 15 g, 4° C) in suspension buffer. The suspension buffer was similar to the perfusion buffer except that EDTA was omitted and 1 m*M* $CaCl_2$ and 2 g/liter bovine serum albumin (BSA) were added. After perfusion, the suspended cells had a mean viability of about 50%; therefore, a Percoll centrifugation step was applied. One volume of the resulting cell suspension was mixed with one volume of Percoll buffer (consisting of nine volumes Percoll and one volume of a 10 times concentrated suspension buffer, without BSA). Cells were centrifuged for 20 min at 175 g at 4° C. The pelleted cells were washed three times at 15 g. Thereafter, the viability was determined by trypan blue exclusion (final concentration of 0.1% trypan blue).

Storage in UW. Either the commercially available UW from Du Pont or a prepared UW-like solution (35.83 g/liter lactobionic acid, 50 g/liter hydroxyethyl starch, 3.4 g/liter KH_2PO_4, 1.23 g $MgSO_4 \times 7\ H_2O$, 17.83 g/liter raffinose $\times 7\ H_2O$, 1.34 g/liter adenosine, 0.136 g/liter Allopurinol, 0.9 g/liter glutathione, 16 mg/liter dexamethasone, 40 U/liter insulin) were used. No differences were observed between the UW and the UW-like solution in the investigated parameters and, therefore, no differentiation is made in the text. The freshly isolated PC were centrifuged for 5 min at 15 g, the supernatant was aspirated, and the cell pellet was suspended in ice cold UW with a final concentration of 4×10^6 PC/ml. The cells were stored on ice for at least 7 d. After 4, 24, 48, 72, 96, 120, 144, or 168 h the sedimented cells were resuspended and an aliquot was taken. These cells were washed three times at 15 g with a modified Krebs-Henseleit buffer (25 m*M* 4[2-hyroxyethyl]-1-piperazineethane-sulfonic acid, 0.5% D-glucose, 1 m*M* $CaCl_2$, 0.4 m*M* $MgSO_4$, 0.2% BSA, and the following L-amino acids: 40 mg/liter alanine, 20 mg/liter aspartate, 60 mg/liter asparagine, 40 mg/liter citrulline, 20 mg/liter cysteine, 150 mg/liter histidine, 150 mg/liter glutamate, 150 mg/liter glycine, 60 mg/liter isoleucine, 120 mg/liter leucine, 190 mg/liter lysine, 80 mg/liter methionine, 100 mg/liter ornithine, 80 mg/liter phenylalanine, 80 mg/liter proline, 100 mg/liter serine, 200 mg/liter threonine, 100 mg/liter tryptophane, 80 mg/liter tyrosine, and 120 mg/liter valine. Thereafter, the viability was determined.

Biochemical assays. Freshly isolated or preserved PC were homogenized by sonification. Protein determination was carried out as described by Lowry et al. (1951) with BSA as standard. Cytochrome P450 was measured spectrophotometrically by the method of Omura and Sato (1964). The activity of the major broad spectrum soluble epoxide hydrolase (sEH) was measured according to Schladt et al. (1986) with trans-2,3-[2,3-^{3}H]diphenyloxiran (trans-stilbene oxide). The activity of glutathione S-transferase (GST) was determined photometrically with the broad spectrum substrate 1-chloro-2,4-dinitrobenzene by the method of Habig et al. (1974). The activities of phenol sulfotransferase (ST) with 2-naphthol (Arand et al., 1987) and UDP-glucuronosyl transferase (UGT) with 1-naphthol (Bock and White, 1974) were measured fluorometrically.

Salmonella mutagenicity assay. The mutagenicity was determined using the Ames assay as described by Utesch et al. (1987). In brief, Salmonella typhimurium his$^-$ strain TA100 was grown overnight in Oxoid nutrient broth no. 2. The bacteria were centrifuged and resuspended to a titer of 10^8 to 10^9 bacteria per ml suspension medium (1.6 g Bacto nutrient broth and 5 g/liter KCl). Bacterial content was determined by nephelometry. The test compound, 1×10^6 viable PC in 500 µl modified Krebs-Henseleit buffer (without histidine), and 100 µl of the bacterial suspension were incubated in a shaking water bath (100 r/min) at 37° C for 1 h. After preincubation, 2 ml of 45° C warm top agar, which contained 0.55% agar, 0.55% NaCl, 50 µ*M* histidine, 50 µ*M* biotin, 50 µ*M* tryptophan, and 25 m*M* sodium phosphate buffer, adjusted to pH 7.4, was then added to each test tube and poured onto a petri dish with minimal agar consisting of 1.5% agar and Vogel-Bonner E medium with 2% glucose. After incubation in the dark for 2 d colonies (his$^+$ revertants) were counted. BP, dissolved in 10 µl dimethyl sulfoxide, was tested at 5, 10, 25, and 50 µg/plate. 2-AA, dissolved in 10 µl dimethyl sulfoxide, was tested at 0.5, 1, 2, and 5 µg/plate. CP was dissolved in water (100 µl) and tested at 50, 250, 500, and 1000 µg/plate. The results were expressed as revertants per nmol test compound derived from the linear portion of the mutagenicity versus mutagen concentration (specific mutagenicity).

Monolayer culture. Freshly isolated and preserved PC were seeded in DMEM including 10% FCS and 1 µ*M* dexamethasone at 7×10^6 PC per 10 cm cell culture dishes coated with collagen type IV and cultured at 37° C in a humidified atmosphere with 5% CO_2. The medium was changed to remove unattached cells 2 h after initiation of the cell culture; 20 h later, the cells were harvested with trypsin. Cells were counted in a hemocytometer and the attachment efficiency was determined.

Cell suspension. Freshly isolated or hypothermically preserved PC were suspended in a 1:1 mixture of modified Krebs-Henseleit buffer and Medium 199 at a cell density of 1×10^6 PC/ml. The suspension was incubated in a 37° C water bath at 100 r/min for up to 7 h. Viability was determined hourly.

Results

After EDTA isolation and subsequent Percoll density centrifugation, a PC suspension with a mean viability of 95 ± 2%, as judged by trypan blue exclusion, was obtained. An average of about 140×10^6 viable PC could be isolated from each rat liver. The cell

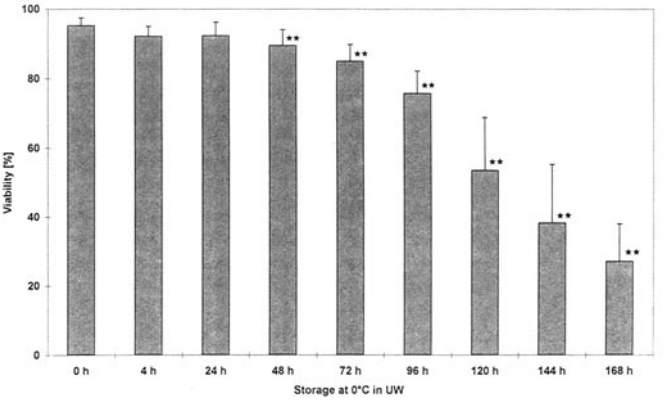

FIG. 1. Viability of freshly isolated rat liver parenchymal cells and of rat liver parenchymal cells after cold storage in UW at 0° C for up to 8 d. The viability was determined by trypan blue exclusion. Mean values ± standard deviations of at least four independent isolations. Dunnett's test was used for statistical analysis ($P < 0.01$**).

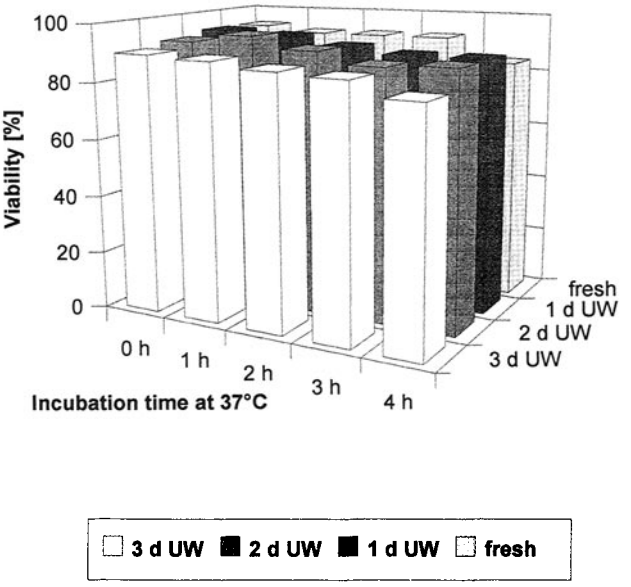

FIG. 2. Viability of freshly isolated rat liver parenchymal cells and of rat liver parenchymal cells after cold storage in UW at 0° C with subsequent suspension culture. The cells were suspended in modified medium 199 and incubated at 37° C for up to 4 h. The viability was determined by trypan blue exclusion.

TABLE 1

Storage at 0°C in UW	CYP P450 (nmol/mg)	sEH (pmol/min/mg)	UGT (nmol/min/mg)	ST (nmol/min/mg)	GST (nmol/min/mg)
0 h	0.22 ± 0.02	19.52 ± 14.48	13.10 ± 0.32	2.47 ± 1.53	477 ± 90
4 h	n.t.	20.21 ± 13.99	13.15 ± 0.66	2.60 ± 0.76	434 ± 5
24 h	0.20 ± 0.01	19.16 ± 12.76	13.84 ± 0.83	2.38 ± 0.79	462 ± 6
48 h	0.21 ± 0.01	18.86 ± 11.92	12.87 ± 2.31	2.30 ± 0.61	438 ± 58
72 h	0.20 ± 0.01	18.65 ± 12.65	13.81 ± 3.21	2.14 ± 0.36	403 ± 13

Content of cytochrome P450 (CYP P450) and enzyme activities of soluble epoxide hydrolase (sEH), UDP-glucuronosyl transferase (UGT), phenol sulfotransferase (ST), and glutathione S-transferase (GST) in freshly isolated rat liver parenchymal cells and in rat liver parenchymal cells after cold storage in UW at 0°C for up to 3 d was determined per mg protein. Mean values ± standard deviations of four independent isolations. No significant differences between freshly isolated and stored cells were seen (Dunnett's test $P<0.05$). n.t. = not tested.

suspension was free of nonparenchymal cells and the PC had almost no blebs.

When freshly isolated PC were stored for 1 d in UW at 0° C, viability was slightly but not significantly reduced to 92 ± 4% (Fig. 1); and, after 3 d cold storage in UW, the viability was still 85 ± 5%. Thereafter, cell viability decreased more rapidly (Fig. 1). However, 85% viability is satisfactory and thus the following investigations were performed on cells after 4 h and 72 h of hypothermic preservation.

In vitro studies can either be carried out in cell suspension or in cell culture. Here we showed that PC that were hypothermically preserved in UW for up to 3 d and then subsequently cultured in suspension at 37° C maintained a high viability for at least 4 h (Fig. 2). After 1 d cold storage, the viability of PC previously incubated for 7 h at 37° C still had a viability of 84%. Furthermore, the PC can be placed successfully into monolayer culture. The attachment efficiencies of freshly isolated and PC stored for 1 or 2 d were similar. A slight decrease in attachment efficiency, to 74% of seeded cells (as compared to 84% for freshly isolated PC), was observed after 3 d cold storage (Fig. 3) but, nevertheless, the PC formed a confluent monolayer after 1 d in culture.

When isolated PC are used for toxicological and pharmacological *in vitro* studies, the activities of diverse xenobiotic metabolizing enzymes must be well maintained. To assess this, the content of cytochrome P450 and some xenobiotic metabolizing enzyme activities were determined in freshly isolated PC and in PC after storage in UW at 0° C for up to 3 d. The cytochrome P450 content was only slightly reduced but this effect was not statistically significant (Table 1). The enzyme activities of sEH, UGT, ST, and GST (Table 1) were similar between freshly isolated PC and PC previously stored for 4, 24, 48, or 72 h in UW at 0° C.

In a first series of three independent experiments, freshly isolated and PC cold-stored for 1 d were used as a metabolizing system in the reverse mutation assay with Salmonella typhimurium TA100. Various concentrations of the promutagens BP, CP, and 2-AA were tested. Freshly isolated as well as cold-stored PC were able to metabolize these promutagens to their ultimate mutagens to the same extent as monitored by the induction of his^+ revertants of Salmonella typhimurium TA100 (Table 2). In a subsequent experiment, freshly isolated PC and PC that had been stored for 1, 2, or 3 d in UW at 0° C were used in the PC-mediated Salmonella mutagenicity test with 10 μg BP, 500 μg CP, and 2 μg 2-AA per plate. After metabolic activation by the fresh and cold-stored liver cells, BP and CP induced the same number of revertants (Fig. 4) and only with 2-AA was a slight reduction seen after 2 and 3 d of cold storage (Fig. 4).

Discussion

Biomedical research on liver functions increasingly makes use of isolated PC. The PC are used either in suspension or in cell culture; however, PC in suspension are only viable over a short period of time and PC in conventional monolayer culture rapidly lose their differentiated functions. Usually, not all of the PC isolated from one liver can be employed and the remaining PC are often discarded. A new cell isolation is then necessary for the next experiment and, therefore, it is highly desirable to store isolated superfluous PC for future experiments. This would help to reduce the number of laboratory animals used, reduce costs, and save time. In the case of rarely available livers from animals such as dog and monkey or from humans, a successful conservation protocol would be very beneficial. In particular, stored human PC could be used for metabolic studies or for the treatment of liver insufficiencies (Fuller et al., 1983).

One way of storing PC can be the computer-controlled cryopreservation of PC and their long-term conservation in liquid nitrogen (Utesch et al., 1992; Diener et al., 1993; 1994; 1995). The other possibility is hypothermic short-term preservation over several days in special media.

In the literature, only a few comparative investigations on the effect of storage solutions on isolated PC are available and the results are contradictory. Guyomard et al. (1990) and Poullain et al. (1992) reported that the cold storage of isolated rat liver PC at 4.5° C in Leibovitz medium was substantially better than in UW, in contrast to the findings of Fuller and Cheetham (1990). Previously, Sandker et al. (1992) and Hammond and Fry (1993) demonstrated the successful hypothermic conservation of rat liver PC for 1 d in UW.

TABLE 2

Promutagen	Specific Mutagenicity (revertants/nmol) Fresh PC	UW-Stored PC
Benzo(a)pyrene	2.30 ± 0.41	2.47 ± 0.39
2-Aminoanthracene	30.53 ± 2.32	28.89 ± 1.14
Cyclophosphamide	0.06 ± 0.02	0.06 ± 0.01

Specific mutagenicity (revertants/nmol) of benzo(a)pyrene, cyclophosphamide, and 2-aminoanthracene induced in the Salmonella typhimurium reverse mutation assay with TA100 in the presence of freshly isolated liver parenchymal cells (PC) or PC stored for 1 d in UW at 0°C, as metabolizing system. Mean values ± standard deviations of three independent isolations with three plates per concentration. No significant differences were observed between fresh and UW-stored PC using Student's *t* test ($P<0.05$).

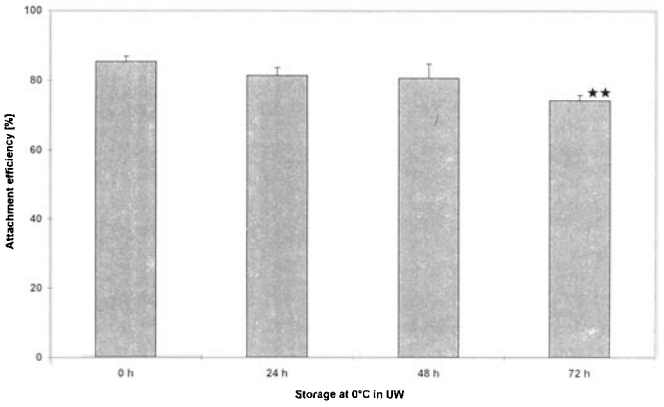

FIG. 3. Attachment efficiency of freshly isolated rat liver parenchymal cells and of rat liver parenchymal cells after cold storage in UW at 0° C for up to 3 d with subsequent monoculture. 7×10^6 cells were plated per 10-cm culture dish. After 2 h, the culture medium was changed to remove unattached cells. Twenty hours later, the cells were harvested with trypsin and the attachment efficiency was determined by cell counting. Mean values ± standard deviation of three independent isolations. Dunnett's test was used for statistical analysis ($P < 0.01$**).

However, in both cases a purification with Percoll centrifugation was necessary after cold storage to obtain good viability.

Results presented here show that isolated PC from the rat can be stored under hypothermic conditions in UW for up to 3 d without a substantial loss in the measured parameters. The ability of the cells to exclude trypan blue was only slightly reduced from 95% in freshly isolated PC to 92% after 1 d and 85% after 3 d in UW stored cells; these are viabilities that are acceptable for *in vitro* assays. Hypothermic preservation for more than 4 d is not recommended because after 3 d viability starts to decrease more rapidly (Fig. 1).

After cold storage for 1, 2, and 3 d, it was possible to use the PC either in suspension or in cell culture. During 4 h incubation at 37° C in suspension, they maintained a viability of more than 80%. For freshly isolated cells and cells cold-stored for 1 d, the 37° C incubation time was extended to 7 h and viability decreased from 97% to 84% at 37° C in suspension. Freshly isolated and suspended cells behaved similarly within this period, which is sufficient for short-term metabolism studies. Furthermore, the PC can be cultured in monoculture or in co-culture with a nonparenchymal epithelial cell line (data not shown) after cold storage in UW at 0° C. The attachment efficiencies of PC in monoculture after 1 and 2 d cold storage were the same when compared to freshly isolated PC, whereas, after 3 d of hypothermic conservation, the attachment efficiency was slightly reduced. However, both freshly isolated PC and preserved PC formed confluent monolayers within 1 d after cell seeding and there was no delayed attachment of the PC to culture dishes after cold storage, in contrast to results reported by Fuller and Cheetham (1990).

Our results on viability and cell culture of PC after hypothermic storage in UW were different to the results of Poullain et al. (1992), who found that PC lost their adhesion ability and had a pronounced decrease in viability after 48 h of cold storage. It is possible that the cell isolation procedure is responsible for this discrepancy, because Poullain et al. used the conventional two-step collagenase isolation technique and we isolated the PC by nonenzymatic EDTA perfusion. This assumption is supported by the experiments of Rivas et al. (1993), who also used the EDTA perfusion to isolate PC (not from rat but from rabbit) and they came to the conclusion that hepatocytes can be well preserved at 4° C for up to 72 h in UW solution based on morphologic and functional criteria. Furthermore, our own results on cryopreservation of collagenase-isolated PC (Diener et al., 1993) and EDTA-isolated PC (Diener et al., 1995) showed that survival and activities of xenobiotic metabolizing enzymes were better preserved in the nonenzymatically isolated PC after cryopreservation.

PC play an extraordinary role in the metabolism of endogenous and exogenous compounds. When efforts are made to prolong the limited life span of isolated PC, they rapidly lose most of their xenobiotic metabolizing enzymes. For example, when PC are cultured ST and most of the cytochrome P450 isoenzymes are drastically reduced (Utesch et al., 1991; Utesch and Oesch, 1992). GST and ST seem to be sensitive to freezing-thawing damage in cryopreserved PC of the rat (Diener et al., 1993).

Here, we clearly demonstrate that it is possible to maintain xenobiotic metabolizing enzyme activities over a period of 3 d at 0° C in UW. The cytochrome P450 content was unchanged and the enzyme activities of GST, UGT, ST, and sEH, all conjugating enzymes, were similar in freshly isolated and hypothermically preserved PC. The variability of sEH activities was due to the interindividual variations in Sprague-Dawley rats (Schladt et al., 1986), and the sEH activities of PC isolated from one animal were constant between freshly isolated and hypothermically preserved PC (data not shown). The activity of GST was also well maintained in isolated human PC after 1 d of hypothermic storage in UW, compared to freshly isolated human PC (data not shown).

The Salmonella reverse mutation assay is normally used to detect mutagens but this very sensitive test can also be used to determine the activities of enzyme preparations (Czich et al., 1994) or the enzymatic capacity of exogenous cellular metabolizing systems. In the present report, we used three well-known promutagens and compared the ability of fresh and stored PC to metabolize and activate them to ultimate mutagens. All three promutagens require the activation by different cytochrome P450 isoenzymes: BP is a polycyclic aromatic hydrocarbon that is predominantly activated by cytochrome P450 1A1 (Robertson et al., 1983), whereas, conjugation by UGT, ST, and

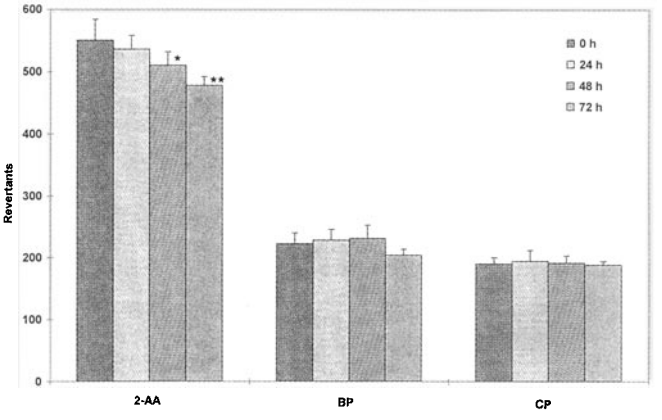

FIG. 4. Revertants of Salmonella typhimurium TA100 induced by 10 μg benzo(a)pyrene, 2 μg 2-aminoanthracene, or 500 μg cyclophosphamide per plate in the presence of freshly isolated liver parenchymal cells (PC) or in the presence of PC, cold stored for 1, 2, or 3 d in UW at 0° C. Mean values ± standard deviations of one isolation with six plates per concentration. Dunnett's test was used for statistical analysis ($P < 0.05$*, $P < 0.01$**).

GST leads to inactivation (Glatt et al., 1981); the aromatic amine 2-AA is mainly metabolized by cytochrome P450 isoenzyme 1A2 to the ultimate mutagen; the activation of CP to an alkylating compound is mainly catalyzed by cytochrome P450 2B1, 2C6, and 2C11 (Clarke and Waxman, 1989). Freshly isolated PC, as well as PC that were stored for up to 3 d at 0° C in UW, must have metabolized the test compounds in the same way because the frequencies of induced mutations in Salmonella TA100 were similar. This is especially interesting for BP because here a complex metabolic pathway involving toxication and detoxication has been described.

In summary, we have demonstrated that EDTA-isolated PC from rat livers can be stored successfully at 0° C in UW for up to 3 d. Viability and all investigated functions were nearly identical between freshly isolated PC and PC preserved hypothermically for up to 3 d. Therefore, it may be possible to use hypothermically preserved PC, isolated without the addition of collagenase but by EDTA perfusion, to ultimately reduce the number of experimental animals required. This method might also be used to provide isolated human PC, stored for short periods prior to experimental use.

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