Biochemical Pharmacology, Vol. 46, No. 1, pp. 111–116, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00
Pergamon Press Ltd

PRESERVATION OF THE RATE AND PROFILE OF XENOBIOTIC METABOLISM IN RAT HEPATOCYTES STORED IN LIQUID NITROGEN

JAN ZALESKI,* JOHN RICHBURG and FREDERICK C. KAUFFMAN
Laboratory for Cellular and Biochemical Toxicology, Rutgers University, Piscataway, NJ 08854, U.S.A.

(*Received* 14 *December* 1992; *accepted* 5 *March* 1993)

Abstract—A simple procedure for cryopreservation of rat hepatocytes that allows recovery of viable cells retaining activities of phase I and phase II drug metabolism equivalent to freshly isolated cells is described. The cooling process was initiated 30 min after incubation of freshly isolated hepatocytes at 37° in Krebs–Ringer bicarbonate buffer containing 15 mM glucose to allow for metabolic equilibration. At the end of this period, hepatocyte suspensions were supplemented with 1.7% albumin, 13.3% dimethyl sulfoxide, and the synthetic buffers, 3-[*N*-morpholino]propanesulfonic acid (MOPS) and *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid] (HEPES). Hepatocytes were cooled in a stepwise manner to −196° by holding the cells for 1 hr at −20° and then for 1 hr at −70° before transfer into liquid nitrogen. After thawing and removal of damaged cells by centrifugation in Percoll, the total recovery of viable hepatocytes subjected to freezing was about 42%. The contents of ATP, ADP, and AMP were not altered significantly in cells stored in liquid nitrogen. The metabolic competence of cryopreserved hepatocytes was further confirmed by their ability to synthesize urea from NH_4Cl and ornithine at the same high rate that was observed in freshly isolated cells (693 ± 68 and 740 ± 68 nmol · mg dry $wt^{-1} \cdot hr^{-1}$, respectively). Similarly, cryopreservation did not affect drug-metabolizing systems as indicated by the metabolism of benzo[*a*]pyrene and 7-ethoxycoumarin, two model substrates. In both freshly isolated and cryopreserved hepatocytes, 7-ethoxycoumarin was O-deethylated to 7-hydroxycoumarin at essentially the same rates (8.66 ± 0.75 and 8.25 ± 0.53 nmol · mg dry $wt^{-1} \cdot hr^{-1}$, respectively) and 7-hydroxycoumarin accumulated in hepatocyte suspensions almost exclusively in the conjugated form. The storage of hepatocytes in liquid nitrogen also did not affect the complex metabolism of benzo[*a*]pyrene to total oxygenated metabolites and, more importantly, to metabolites conjugated with glutathione, glucuronic acid, and sulfuric acid. Thus, cryopreserved hepatocytes represent a valid and convenient model to study drug biotransformation in intact cells.

The importance and usefulness of isolated rat liver parenchymal cells obtained by the method described two decades ago by Berry and Friend [1] are unquestionable. Because of their versatility and high metabolic activity, hepatocytes have been used in a large number of studies on the regulation of intermediary metabolism, cellular physiology, and metabolism of xenobiotics. The use of isolated hepatocytes has accelerated research and greatly reduced the number of animals required, when compared with those required for studies *in vivo* and with isolated perfused livers. A major disadvantage of isolated hepatocytes, however, is that they can only maintain their structural and metabolic integrity for a few hours. This is a particularly significant problem when hepatocytes are isolated from large experimental animals such as dogs, rabbits or monkeys, and scarcely available human liver. Recently, progress has been made in the cold-preservation of hepatocytes for 24–48 hr using University of Wisconsin solution [2, 3]. Cell culture represents another method for long-term storage of hepatocytes but, despite constant improvements in culture media, the activities of some drug-metabolizing enzymes in these cells are changed significantly *in vitro* [4, 5].

* Corresponding author. Tel. (908) 932-6900; FAX (908) 932-6905.

Although several procedures for hepatocyte cryopreservation using various modifications in the rate of cooling and composition of freezing media have been reported, a fully satisfactory method for preserving vital biochemical functions has not been described [6–13]. Drug-metabolizing systems are especially sensitive to disruption in hepatocytes stored in liquid nitrogen [9, 10, 13]. A recent report [13] published in this journal argued that cryopreserved rat hepatocytes were of limited use because of relatively low recovery of cells after thawing. Moreover, these cells metabolized testosterone and benzo[*a*]pyrene (B[*a*]P) at lower rates than freshly isolated cells. The present study describes a simple procedure for cryopreserving rat hepatocytes that maintains energy status and rates and patterns of 7-ethoxycoumarin and B[*a*]P metabolism. The two substrates were selected because both of these model drugs are converted to conjugated metabolites at relatively high rates in hepatocytes [14]. Thus, to support conjugation processes, constant energy-dependent regeneration of UDP-glucuronic acid, 3'-phosphoadenosine-5'-phosphosulfate, and glutathione is needed, which, in turn, depends on the maintenance of morphological and metabolic integrity of hepatocytes. Furthermore, deethylation of 7-ethoxycoumarin may be a good index of the total status of cytochrome P450, since several P450 isozymes catalyze this reaction [15].

Based on the comparison of drug and energy metabolism of cryopreserved and freshly isolated cells, the protocol for freezing, thawing, and recovery of cells stored in liquid nitrogen is validated.

MATERIALS AND METHODS

Chemicals. [G-^{3}H]B[*a*]P (80 Ci/mmol; Amersham, Arlington Heights, IL) was purified as follows. After evaporation of toluene, [G-^{3}H]B[*a*]P was diluted to about 2 μCi/μL with unlabeled 20 mM B[*a*]P (Sigma, St. Louis, MO) in dimethyl sulfoxide. Subsequently, KOH was added to achieve final concentrations of dimethyl sulfoxide and KOH equal to 56.6% (v/v) and 0.1 N, respectively. B[*a*]P was extracted from the dimethyl sulfoxide/KOH solution with *n*-hexane. Collagenase (type IV), Percoll, isotonic solution of trypan blue, pyruvate, 7-ethoxycoumarin, 7-hydroxycoumarin, β-glucuronidase (type H-3), and sulfatase (type H-2) were purchased from Sigma. All components of Krebs–Ringer bicarbonate buffers used were also obtained from Sigma. Dimethyl sulfoxide (HPLC grade) was purchased from Aldrich (Milwaukee, WI) and bovine serum albumin (fraction V, fatty acid-free) from ICN Biochemicals (Cleveland, OH).

Isolation of hepatocytes. Male rats of the Sprague–Dawley strain (Taconic Farms, Germantown, NY) were fed a standard Purina chow diet *ad lib.* Hepatocytes were isolated from rats weighing 200–400 g as described previously [16]. Krebs–Ringer bicarbonate buffer used for hepatocyte isolation was supplemented with 15 mM D-glucose. The density of hepatocyte suspensions was determined by counting in a Neubauer hemacytometer. The evaluation of hepatocyte viability was based on exclusion of a 0.1% isotonic solution of trypan blue (in albumin-free medium) and determination of a maximal rate of urea synthesis.

Cryopreservation of isolated hepatocytes. Immediately after isolation, hepatocytes (~20 mg dry wt/mL; 0.48 ± 0.01 ($\times 10^6$) cells/mg dry wt, N = 35) were incubated in a shaking water-bath for 30 min in Krebs–Ringer bicarbonate buffer containing 15 mM glucose under an atmosphere of 95% O_2 + 5% CO_2 at 37°. At the end of this incubation, 20% (w/v) albumin was added to achieve a final concentration of 1.7%, and the buffer was fortified by addition of 1 M 3-[*N*-morpholino]propanesulfonic acid (MOPS) and 1 M *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid] (HEPES) buffers, pH 7.4, to final concentrations of 17.3 and 8.6 mM, respectively. Finally, an appropriate volume of dimethyl sulfoxide was slowly added to hepatocyte suspensions to achieve a final concentration of 13.3% (v/v). At this stage the viability of the hepatocytes was determined after a 4-fold dilution as described above. Aliquots (4.5 mL) of hepatocytes were distributed quickly into 5-mL Nalgene cryogenic tubes (Nalge, Rochester, NY), and the tubes were placed at −20° for 1 hr followed by 1 hr at −70°. Subsequently, the tubes were immersed in liquid nitrogen for at least 11 days.

Thawing and purification of cryopreserved hepatocytes. After removal from liquid nitrogen, tubes containing hepatocytes were placed immediately at 37°. The thawing process was completed within 4–5 min. In all subsequent steps, the temperature of Krebs–Ringer bicarbonate buffer used for washing and suspending of hepatocytes was approximately 37°. Hepatocyte suspensions were mixed with equal volumes of Krebs–Ringer bicarbonate buffer containing 15 mM glucose and 10 mM pyruvate, and hepatocytes were immediately sedimented by centrifugation at 50 *g* for 2 min. The pellet was washed twice with the same volume of the above solution, and hepatocytes were sedimented as above. Subsequently, the hepatocyte pellet was suspended in 10 vol. of Krebs–Ringer bicarbonate buffer containing 15 mM glucose and 10 mM pyruvate to achieve a cell density of about 20 mg dry wt/mL. Centrifugation in isotonic Percoll solution was used to remove damaged cells from the hepatocyte suspension [17]. Briefly, an equal volume of Percoll solution warmed to 37°, pH 7.4 [9 mL Percoll plus 1 mL of a solution containing 1.37 M NaCl, 0.054 M KCl, 0.008 M Mg_2SO_4 plus 0.2 mL of phosphate buffer (84 mM Na_2HPO_4 and 15 mM KH_2PO_4)], was mixed with the hepatocyte suspension. The suspension was centrifuged at 50 *g* for 5 min, and intact hepatocytes were collected at the bottom of the tubes. Hepatocytes were washed twice with Krebs–Ringer bicarbonate buffer containing 15 mM glucose (each time with about 1/2 of the initial volume of the hepatocyte suspension). Finally, hepatocyte pellets were suspended in 10 vol. of the same buffer. After determination of cell viability, hepatocytes were used for metabolic studies.

Incubation of isolated hepatocytes. Freshly isolated or cryopreserved hepatocytes (2–10 mg dry wt/mL) were incubated in 1 mL of Krebs–Ringer bicarbonate buffer containing 15 mM glucose and 1.5% (w/v) bovine serum albumin (fatty acid-free) under an atmosphere of 95% O_2 + 5% CO_2 in 10-mL Erlenmeyer flasks gently shaken at 37°. Urea synthesis was measured in cells incubated in the presence of 10 mM L-lactate, 2 mM L-ornithine, and 10 mM NH_4Cl. Drug metabolism was studied in hepatocytes (1.5 to 2.0 mg dry wt/mL) incubated with 200 μM 7-ethoxycoumarin or 40 μM [G-^{3}H]-B[*a*]P. 7-Ethoxycoumarin and [G-^{3}H]B[*a*]P were prepared as 100 and 20 mM stock solutions, respectively, in dimethyl sulfoxide. For study of B[*a*]P metabolism, the concentration of albumin in the incubation medium was raised to 2% (w/v). Albumin was omitted from the incubation medium when 7-ethoxycoumarin metabolism was studied. Incubations with B[*a*]P were terminated after 1 hr of incubation by adding 1 mL of ice-cold acetone. All other incubations were terminated on ice by adding 0.1 mL of 35% perchloric acid. Cell precipitates were removed by centrifugation, and perchloric acid supernatants were neutralized with 5 M KOH containing 0.05 M sodium acetate.

Metabolism of 7-ethoxycoumarin. The product of 7-ethoxycoumarin O-deethylation, 7-hydroxycoumarin, was determined fluorometrically using a Farrand filter fluorometer (excitation 340 nm, emission 450 nm) [14]. For determination of unconjugated 7-hydroxycoumarin, 50-μL aliquots of the supernatants were transferred into 1 mL of 0.02 M sodium carbonate buffer, pH 10.2, and

fluorescence was measured. The sum of 7-hydroxycoumarin glucuronide and sulfate production was measured as the difference between fluorescence of 7-hydroxycoumarin before and after hydrolysis with a mixture of β-glucuronidase and sulfatase. To achieve hydrolysis of the conjugates, 50-μL aliquots of the supernatants were mixed with 200 μL of 0.1 M sodium acetate buffer (pH 4.7) containing 0.02% albumin and 220 units of β-glucuronidase and 8 units of sulfatase. After a 1-hr incubation at 37°, 50-μL aliquots of the reaction mixture were transferred into 1 mL of 0.02 M sodium carbonate buffer, pH 10.2, and fluorescence was measured.

Metabolism of B[a]P. The rate of total B[*a*]P metabolism was determined radiochemically by measuring the formation of oxygenated metabolites of B[*a*]P that were non-extractable with *n*-hexane from 56.6% (v/v) dimethyl sulfoxide in 0.1 N KOH as described earlier [14]. The method developed by Zaleski *et al.* [18] was used to determine the formation of conjugates of [^{3}H]B[*a*]P metabolites (glutathiones, glucuronides, and sulfates). Briefly, B[*a*]P metabolites present in 0.1-mL aliquots of supernatant from acetone-stopped incubations were separated by chromatography on silica gel thin-layer chromatography plates (1 mm, PLK5F from Whatman) using 1-butanol:0.01 M Tris:propionic acid (50:10:0.7) as the solvent system. The following areas of silica gel, designated by R_f values, were scraped from the plates: 0–0.21 (conjugates with glutathione), 0.21–0.32 (glucuronides I), 0.32–0.51 (glucuronides II), and 0.63–0.86 (sulfates). The radioactivity of the samples was measured by liquid scintillation counting after mixing with 1 mL of 25% hydrofluoric acid before addition of 10 mL of ScintiVerse II (Fischer, Fair Lawn, NJ). Locations of B[*a*]P and its metabolites on the silica gel were detected under long-wave UV light. R_f values for fluorescent spots were: 0.94 for B[*a*]P, 0.83 for sulfates, 0.39 to 0.43 for glucuronides, and 0.13 for glutathiones. These values were in the range of values found for corresponding standards [18, 19]. B[*a*]P metabolites that travel into the area of silica gel designated as glucuronides I are hydrolyzed by β-glucuronidase [18], but their structures are unknown. The sulfate fraction also potentially contained B[*a*]P-triols and B[*a*]P-tetrols and traces of B[*a*]P-7,8-dihydrodiol, B[*a*]P-9,10-dihydrodiol, and B[*a*]P-1,6-dione because of the proximity of their R_f values to R_f values of sulfated B[*a*]P metabolites [18–20].

Other determinations. Urea was determined colorimetrically using Sigma Procedure No. 535. Adenine nucleotides were determined spectrophotometrically as described previously [21].

Statistical evaluation of data. Throughout the report, mean values ± SEM are shown. Statistical significance of differences was calculated using a two-tailed Student's *t*-test for paired or unpaired data as applicable.

RESULTS

Metabolic viability of cryopreserved hepatocytes. Following the removal of damaged cells by centrifugation in Percoll, the mean viability of cryopreserved hepatocytes, tested by trypan blue exclusion, was 88.6 ± 1.3%, which was similar to that of eight corresponding preparations of hepatocytes isolated from different rats before freezing (85.2 ± 1.2%). The recovery of hepatocytes after thawing and purification was 41.6 ± 5.5% (N = 7; range of recovery: 27–68%).

The method for hepatocyte cryopreservation described in this report is based on the assumption that maintenance of cellular ATP content at its highest possible value before freezing is of utmost importance for the preservation of cellular structural and functional integrity after thawing. For this reason, hepatocytes were incubated immediately after isolation under an atmosphere of 95% O_2 + 5% CO_2 (see Materials and Methods for details) to allow for recovery from hypoxia that may have occurred during the isolation procedure, particularly during centrifugation. ATP and ADP contents were 9.7 ± 0.6 and 1.8 ± 0.2 nmol/mg dry wt (N = 7), respectively, after about 20 min of incubation. These values were essentially the same as those determined after a 1-hr incubation of hepatocytes (Table 1). Thus, a 30-min period of cell incubation before initiating the step-wise freezing was sufficient to achieve optimal status of adenine nucleotides.

The contents of adenine nucleotides, ATP/ADP ratios, and calculated energy charges in freshly isolated and cryopreserved hepatocytes are summarized in Table 1. There was no significant difference in the contents of ATP, ADP, AMP, and thus total adenine nucleotides between freshly isolated and cryopreserved hepatocytes. Consequently, the calculated energy charge for cryopreserved hepatocytes and unstored cells was identical. The ATP/ADP ratio remained high in cryopreserved cells. The preservation of energy-generating processes in cryopreserved hepatocytes was confirmed by determination of their ability to carry on the highly energy-dependent process of urea synthesis from NH_4Cl and ornithine. Rates of urea synthesis were the same in freshly isolated and cryopreserved cells (Table 1). Noteworthy, cryopreserved hepatocytes produced urea in a linear fashion over a 2-hr incubation period (the longest tested). In one experiment the amount of urea formed during a 1-hr incubation (639 nmol/mg dry wt) was doubled after 2 hr of incubation (1233 nmol/mg dry wt). For comparison, freshly isolated hepatocytes from the same rat produced 1274 nmol urea/mg dry wt over a 2-hr incubation period.

Metabolism of 7-ethoxycoumarin. Metabolism of 7-ethoxycoumarin in hepatocytes was followed by determining the formation of free and conjugated 7-hydroxycoumarin. The latter metabolites were measured as a sum of glucuronides and sulfates. Metabolism of 7-ethoxycoumarin in freshly isolated and cryopreserved hepatocytes is compared in Table 2. Conjugates of 7-hydroxycoumarin were by far the main products of 7-ethoxycoumarin metabolism in both fresh and cryopreserved hepatocytes. Total rates of 7-ethoxycoumarin O-deethylation were the same in both preparations as were rates of 7-hydroxycoumarin conjugation. Similar and low amounts of unconjugated 7-hydroxycoumarin were found in the two types of hepatocyte suspensions

Table 1. Content of adenine nucleotides and the rates of urea formation from NH_4Cl in freshly isolated and cryopreserved rat hepatocytes

	Hepatocytes	
	Fresh	Cryopreserved
ATP content (nmol·mg dry wt^{-1})	9.0 ± 0.4	8.4 ± 0.7
ADP content (nmol·mg dry wt^{-1})	1.8 ± 0.1	2.0 ± 0.1
AMP content (nmol·mg dry wt^{-1})	0.3 ± 0.1	0.3 ± 0.1
Σ ATP, ADP and AMP (nmol·mg dry wt^{-1})	11.1 ± 0.4	10.7 ± 0.7
ATP/ADP	5.0 ± 0.5	4.2 ± 0.2
Energy charge	0.89 ± 0.01	0.89 ± 0.01
Urea formation (nmol·mg dry wt^{-1}·hr^{-1})	740 ± 68	693 ± 68

Values are means ± SEM calculated for four preparations of hepatocytes isolated from different rats. The content of adenine nucleotides was measured 1 hr after incubation of hepatocytes in the presence of 15 mM glucose. Energy charge is defined by the following equation: 1/2·{2·[ATP] + [ADP]/[ATP] + [ADP] + [AMP]}. For determination of the maximal rates of urea synthesis, hepatocytes were incubated for 1 hr in the incubation medium supplemented with 10 mM NH_4Cl, 2 mM ornithine, and 10 mM lactate. Cryopreserved hepatocytes were stored in liquid nitrogen from 14 to 114 days. Differences between the means were not statistically significant, as evaluated by Student's *t*-test for paired data.

Table 2. Comparison of the rates of 7-ethoxycoumarin metabolism in freshly isolated and cryopreserved rat hepatocytes

	Formation of 7-hydroxycoumarin (nmol·mg dry wt^{-1}·hr^{-1})		
Hepatocytes	Free	Conjugated	Total
Fresh	0.32 ± 0.07	8.34 ± 0.71	8.66 ± 0.75
Cryopreserved	0.50 ± 0.07	7.75 ± 0.57	8.25 ± 0.53

Values are means ± SEM calculated for four preparations of hepatocytes isolated from different rats. The rates of 7-ethoxycoumarin metabolism were determined immediately after cell isolation and after their storage in liquid nitrogen from 14 to 50 days. Metabolite formation was determined after 1 hr of incubation. Differences between the means were not statistically significant, as evaluated by Student's *t*-test for paired data.

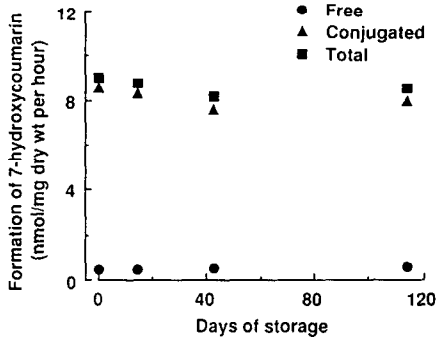

Fig. 1. Effect of long-term storage of rat hepatocytes in liquid nitrogen on their ability to metabolize 7-ethoxycoumarin. Rates of 7-ethoxycoumarin (200 μM) O-deethylation were measured as formation of free and conjugated 7-hydroxycoumarin (a sum of glucuronide and sulfate). Determinations were done in the same preparation of hepatocytes immediately after their isolation and after storage for 14, 43, and 114 days in liquid nitrogen. The experimental procedures are described in Materials and Methods.

after incubation. The ability of cryopreserved hepatocytes to metabolize 7-ethoxycoumarin to free and conjugated 7-hydroxycoumarin remained unchanged after about 4 months of storage in liquid nitrogen (Fig. 1).

Metabolism of B[a]P. Metabolism of [G-^{3}H]-B[*a*]P by isolated rat hepatocytes was studied in the presence of albumin to assure the highest possible rate of B[*a*]P oxygenation and the highest demand for factors involved in conjugation of hydroxylated metabolites [22]. Production of all three types of conjugated B[*a*]P metabolites (glutathiones, glucuronides, and sulfates) as well as total oxygenated metabolites was compared in freshly isolated and cryopreserved hepatocytes. As illustrated by the data presented in Fig. 2, there was no statistically significant difference between freshly isolated and cryopreserved hepatocytes in the formation of total and conjugated metabolites of B[*a*]P.

DISCUSSION

Surprisingly, earlier studies on cryopreservation of hepatocytes do not provide much information on the preservation of general biochemical functions, particularly energy metabolism before freezing and after thawing. Since a constant supply of energy stored in the form of "energy rich" phosphate bonds is necessary to maintain cell structure and compartmentalized organization of metabolic processes, successful cryopreservation requires that general metabolic pathways be maintained. Loss of this structural–functional interrelationship leads to cell damage and death. Hepatocytes have very

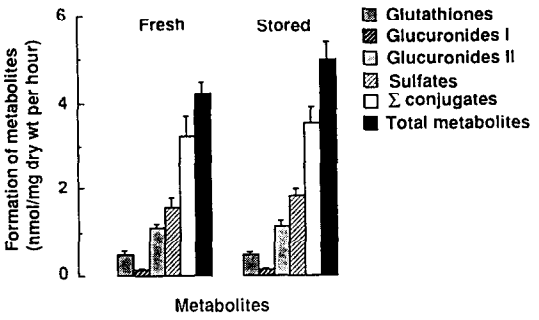

Fig. 2. Comparison of the rates of B[*a*]P metabolism in freshly isolated and cryopreserved rat hepatocytes. Values are means ± SEM calculated from three hepatocyte preparations in each group. The mean value for stored hepatocytes was calculated for different hepatocyte preparations maintained in liquid nitrogen for various periods of time ranging up to 114 days. Total metabolites represent formation of B[*a*]P metabolites that are not extractable into *n*-hexane. Σ conjugates were calculated as a sum of B[*a*]P metabolites conjugated with glutathione, glucuronic acid, and sulfuric acid. The experimental procedures are described in Materials and Methods. Differences between the means were not statistically significant, as evaluated by Student's *t*-test for unpaired data.

efficient mechanisms for relatively quick restoration of "energy stores" diminished by hypoxia and cooling, upon restoration of oxygen and elevation of temperature to the optimal [23, 24]. However, reaccumulation of ATP is possible only when the intracellular contents of ATP degradation products, such as ADP and AMP, are not critically diminished by further degradation to adenosine that can easily diffuse from cells or be metabolized further to xanthine. Generation of oxygen free radicals by xanthine oxidase upon restoration of the normal concentration of oxygen is considered to contribute to cellular damage [25]. Maintaining ATP/ADP ratios as high as possible during the process of hepatocyte cryopreservation and subsequent thawing and purification of cells after their removal from liquid nitrogen is critical for the development of a method of cryopreservation. This was achieved by minimizing as much as possible exposure of hepatocytes to hypoxic conditions and low temperatures. Instead of slowing down hepatocyte metabolism by lowering the temperature of cell suspensions before freezing, recovery from storage, and removal of damaged cells after thawing, hepatocytes were maintained at 37° in well-oxygenated medium containing glucose before placing them at −20° and immediately after removing from storage at −196°.

A simple method for the cryopreservation of hepatocytes described in this report provides means for long-term storage and a relatively high recovery of viable cells. Inasmuch as the basic biochemical capabilities of hepatocytes, particularly their ability to synthesize ATP, have profound effects on xenobiotic metabolism [26], considerable attention was devoted to evaluating the energy status of cryopreserved hepatocytes. It was shown that neither adenine nucleotide contents, ATP/ADP ratio nor calculated energy charge was changed significantly in cryopreserved hepatocytes (Table 1). More importantly, cryopreserved cells retained the capacity to carry on highly energy-demanding urea synthesis from NH_4Cl at the rate observed in freshly isolated cells (Table 1). Urea synthesis requires not only at least four ATP equivalents for synthesis of 1 mol of urea but also precisely organized movements of intermediates between the cytoplasmic and mitochondrial compartments. Thus, the metabolic viability of hepatocytes recovered from storage in liquid nitrogen was fully confirmed by measurements of urea synthesis. Conversely, significantly lower rates of urea synthesis and gluconeogenesis in cryopreserved hepatocytes in comparison with rates in control hepatocytes have been reported by others [6, 27]. It is noteworthy that the ATP/ADP ratios in freshly isolated and cryopreserved hepatocytes of fed rats (Table 1) were similar to those determined by others in the liver of fed rats *in vivo* [28] or isolated rat hepatocytes [17].

Full preservation of the capacity to generate ATP by hepatocytes subjected to storage in liquid nitrogen correlated well with the ability of these cells to metabolize 7-ethoxycoumarin via *O*-deethylase to free and conjugated 7-hydroxycoumarin at the rates observed in unstored cells (Table 2 and Fig. 1). Preservation of conjugation activity is especially important, since the phase II reactions of drug metabolism are particularly sensitive to freeze-induced alterations [9, 10, 13]. Intactness of drug-metabolizing systems in cryopreserved rat hepatocytes was confirmed by maintenance of the complex metabolism of B[*a*]P. Cryopreserved hepatocytes converted B[*a*]P to oxygenated metabolites and their conjugates with glutathione, glucuronic acid, and sulfuric acid at rates that were identical to those in freshly isolated hepatocytes (Fig. 2). Thus, both phases of drug metabolism were fully preserved after long-term storage of hepatocytes in liquid nitrogen.

Drug metabolism has been used frequently to validate methods used for hepatocyte cryopreservation [7–11, 13, 29, 30]. However, the ability of hepatocytes to form conjugates as an indicator of their metabolic integrity after cryopreservation has been used only sporadically. Fuller *et al.* [9] showed that conjugation of bilirubin is reduced 80% after hepatocyte cryopreservation. Powis *et al.* [10] reported that whereas total conversion of biphenyl to hydroxybiphenyl and formation of hydroxybiphenyl glucuronide are affected only marginally by freezing of hepatocytes, the sulfation of hydroxybiphenyl is diminished by 80% of the value determined in fresh hepatocytes. Recently, Utesch *et al.* [13] reported that the ratio between phase I- and phase II-metabolites of B[*a*]P is changed by cryopreservation of hepatocytes due to an impairment in conjugation of B[*a*]P metabolites by sulfotransferase and glutathione *S*-transferase. These earlier studies are in striking contrast to results presented above, indicating that hepatocytes cryopreserved and processed after removal from liquid nitrogen metabolized both B[*a*]P and 7-ethoxycoumarin in

the same fashion as did freshly isolated hepatocytes (Fig. 2 and Table 2).

In summary, the biochemical properties of rat hepatocytes cryopreserved according to the method described in this study compare favorably with those of freshly isolated cells. Additional studies to evaluate the metabolism of different classes of xenobiotics and natural compounds are needed to document that cryopreserved hepatocytes are indeed a valid model for the study of hepatic drug biotransformation. The general applicability of the procedure described in this report for cryopreservation of hepatocytes isolated from different animal species and humans remains to be established.

Acknowledgements—This study was supported in part by NIEHS Center Grant ES 05052 and a grant from Merck Research Laboratories.

REFERENCES

1. Berry MN and Friend DS, High-yield preparation of isolated liver parenchymal cells. *J Cell Biol* **43**: 506–520, 1969.
2. Sorrentino D, Van Ness K, Ribeiro I and Miller CM, Functional and morphological features of isolated hepatocytes preserved in University of Wisconsin solution. *Hepatology* **14**: 331–339, 1991.
3. Sandker GW, Sloof MJH and Groothuis GMM, Drug transport, viability and morphology of isolated rat hepatocytes preserved for 24 hours in University of Wisconsin solution. *Biochem Pharmacol* **43**: 1479–1485, 1992.
4. Utesch D, Molitor E, Platt KL and Oesch F, Differential stabilization of cytochrome P-450 isoenzymes in primary cultures of adult rat liver parenchymal cells. *In Vitro Cell Dev Biol* **27**: 858–863, 1991.
5. Utesch D and Oesch F, Dependency of the *in vitro*-stabilization of differentiated functions in liver parenchymal cells on the type of cell line used for co-culture. *In Vitro Cell Dev Biol* **28**: 193–198, 1992.
6. Le Cam A, Guillouzo A and Freychet P, Ultrastructural and biochemical studies of isolated adult rat hepatocytes prepared under hypoxic conditions. *Exp Cell Res* **98**: 382–395, 1976.
7. Karlberg I and Lindahl-Kiessling K, Preservation of freshly isolated liver cells in liquid nitrogen at −196°C. *Mutat Res* **85**: 411–416, 1981.
8. Novicki DL, Irons GP, Strom SC, Jirtle R and Michalopoulos G, Cryopreservation of isolated rat hepatocytes. *In Vitro* **18**: 393–399, 1982.
9. Fuller BJ, Grout BW and Woods RJ, Biochemical and ultrastructural examination of cryopreserved hepatocytes in rat. *Cryobiology* **19**: 493–502, 1982.
10. Powis G, Santone KS, Melder DC, Thomas L, Moore DJ and Wilke TJ, Cryopreservation of rat and dog hepatocytes for studies of xenobiotic metabolism and activation. *Drug Metab Dispos* **15**: 826–832, 1987.
11. Loretz LJ, Li AP, Flye MW and Wilson AGE, Optimization of cryopreservation procedures for rat and human hepatocytes. *Xenobiotica* **19**: 489–498, 1989.
12. De Loecker R, Fuller BJ, Gruwez J and De Loecker W, The effects of cryopreservation on membrane integrity, membrane transport, and protein synthesis in rat hepatocytes. *Cryobiology* **27**: 143–152, 1990.
13. Utesch D, Diener B, Molitor E, Oesch F and Platt K-L, Characterization of cryopreserved rat liver parenchymal cells by metabolism of diagnostic substrates and activities of related enzymes. *Biochem Pharmacol* **44**: 309–315, 1992.
14. Zaleski J, Kwei GY, Thurman RG and Kauffman FC, Suppression of benzo[*a*]pyrene metabolism by accumulation of triacylglycerols in rat hepatocytes: Effect of high-fat and food-restricted diet. *Carcinogenesis* **12**: 2073–2079, 1991.
15. Edwards AM, Glistak ML, Lucas CM and Wilson PA, 7-Ethoxycoumarin deethylase activity as a convenient measure of liver drug metabolizing enzymes: Regulation in cultured rat hepatocytes. *Biochem Pharmacol* **33**: 1537–1546, 1984.
16. Zaleski J and Ontko JA, Reciprocal effects of energy utilization on palmitate oxidation and esterification in hepatocytes of fed rats. *Biochim Biophys Acta* **836**: 134–142, 1985.
17. Berry MN, Edwards AM and Barritt GJ, *Isolated Hepatocytes: Preparation, Properties and Applications*. Elsevier, Amsterdam, 1991.
18. Zaleski J, Bansal SK and Gessner T, Formation of glucuronide, sulphate and glutathione conjugates of benzo[a]pyrene metabolites in hepatocytes isolated from inbred strains of mice. *Carcinogenesis* **4**: 1359–1366, 1983.
19. Zaleski J, Steward AR and Sikka HC, Metabolism of benzo[*a*]pyrene and (−)-*trans*-benzo[*a*]pyrene-7,8-dihydrodiol by freshly isolated hepatocytes from mirror carp. *Carcinogenesis* **12**: 167–174, 1991.
20. Steward AR, Zaleski J and Sikka MC, Metabolism of benzo[*a*]pyrene and (−)-*trans*-benzo[*a*]pyrene-7,8-dihydrodiol by freshly isolated hepatocytes of brown bullheads. *Chem Biol Interact* **74**: 119–138, 1990.
21. Zaleski J and Bryla J, Effects of oleate, palmitate, and octanoate on gluconeogenesis in isolated rabbit liver cells. *Arch Biochem Biophys* **183**: 553–562, 1977.
22. Zaleski J, Kwei G, Thurman RG and Kauffman FC, Benzo(a)pyrene metabolism in hepatocytes isolated from rats maintained on high-fat and calorie restricted diets. *Eur J Pharmacol* **183**: 1353, 1990.
23. Busza AL, Fuller BJ, Proctor E and Gadian D, The time course of changes in liver phosphorus metabolites during hypothermic preservation measured by ^{31}phosphorus nuclear magnetic resonance. *Cryo-Lett* **9**: 20–25, 1988.
24. Fuller BJ, Busza AL and Proctor E, Possible resuscitation of liver function by hypothermic reperfusion *in vitro* after prolonged (24-hour) cold preservation—A ^{31}P NMR study. *Transplantation* **50**: 511–515, 1990.
25. Fuller BJ, Gower JD and Green CJ, Free radical damage and organ preservation: Fact or fiction? *Cryobiology* **25**: 377–393, 1988.
26. Thurman RG and Kauffman FC, Factors regulating drug metabolism in intact hepatocytes. *Pharmacol Rev* **31**: 229–251, 1980.
27. Gomez-L MJ, Lopez P and Castell JV, Biochemical functionality and recovery of hepatocytes after deep freezing storage. *In Vitro* **20**: 826–832, 1984.
28. Tarnowski W and Seeman M, Konzentrationsanderungen von Effektoren gluconeogenetischer Schlusselenzyme in der Rattenleber unter gluconeogenetischen Bedingungen. *Hoppe-Seyler's Z Physiol Chem* **348**: 829–838, 1967.
29. Coundouris J, Hannah C and Hawksworth G, Cryopreservation of isolated hepatocytes. *Biochem Soc Trans* **14**: 692–693, 1986.
30. Lorentz L, Wilson AGE and Li AP, Promutagen activation by freshly isolated and cryopreserved rat hepatocytes. *Environ Mol Mutagen* **12**: 335–341, 1988.