Drug transport across the hepatocyte plasma membranes is a key factor in hepatic clearance. Hepatobiliary transport of endogenous and exogenous compounds is mediated by the co-ordinated action of multiple transport systems present at the sinusoidal (basolateral) and canalicular (apical) membrane domains of hepatocytes. The lipophilic properties of drugs enable them to cross the sinusoidal membrane by passive diffusion. However, for others, entry into the hepatocyte can be facilitated via a variety of sinusoidal transporters that augment passive diffusion. In this study, we optimized our previous methodology for cryopreservation and evaluated the metabolism and transport of thawed hepatocytes. Half of the freshly, isolated rat hepatocytes prepared by collagenase perfusion were immediately used for studies of transport of $[^{14}C]$taurocholate, $[^{3}H]$estrone sulfate and $[^{3}H]$estradiol 17$\beta$-o-glucuronide (1 $\mu$M) and metabolism of 7-hydroxy-4-(trifluoromethyl)-coumarin (100 $\mu$M), (3,4-difluorobenzoyl)-5,5-dimethyl-4-(4-methylsulfonylphenyl)-(5$\beta$)-furan-2-one; UGT, serum albumin; 7-HFC, 7-hydroxy-4-(trifluoromethyl)-coumarin; DFB, (3,4-difluorobenzyloxy)-5,5-dimethyl-4-(4-methylsulfonylphenyl)-(5$\beta$)-furan-2-one; UGT, UDP-glucuronol transferase.

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2 Abbreviations used are: NTCP, sodium-dependent taurocholate cotransporting polypeptide; OATP, organic anion-transporting polypeptide; MRP2, multi-drug resistance-associated transporter; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; 7-HFC, 7-hydroxy-4-(trifluoromethyl)-coumarin; DFB, (3,4-difluorobenzyloxy)-5,5-dimethyl-4-(4-methylsulfonylphenyl)-(5$\beta$)-furan-2-one; UGT, UDP-glucuronol transferase.

Drug transport across the hepatocyte plasma membranes is a key factor in hepatic clearance. Hepatobiliary transport of endogenous and exogenous compounds is mediated by the co-ordinated action of multiple transport systems present at the sinusoidal (basolateral) and canalicular (apical) membrane domains of hepatocytes. The lipophilic properties of drugs enable them to cross the sinusoidal membrane by passive diffusion. However, for others, entry into the hepatocyte can be facilitated via a variety of sinusoidal transporters that augment passive diffusion. Such drugs with molecular weights $>400$ tend to be hydrophilic since they usually contain polar groups and are ionized (either anionic or cationic) (Meijer et al., 1990; Ayrton and Morgan, 2001). These drugs enter the liver by sinusoidal drug transporters, including the sodium-dependent taurocholate cotransporting polypeptide (NTCP) (Hagenbuch and Meier, 1994), members of the organic anion-transporting polypeptide (OATP) (Bossuyt et al., 1996; Cattori et al., 2001), and the organic cationic transporter (Gründemann et al., 1994). Biliary elimination of drugs is mediated by different ATP-binding cassette-transporters as exemplified by the multidrug-resistance P-glycoprotein (MDR1, MDR2) for neutral and cationic compounds (Meijer et al., 1997), and the canalicular multi-drug resistance-associated transporter (MRP2) (Payen et al., 2000) for anionic and conjugated drugs.

In our laboratory, rat and human hepatocytes have been routinely used for studies related to hepatic metabolism and transport of xenobiotics. For transport studies, freshly isolated hepatocytes have been used to investigate drug uptake to determine the role of transporters in the overall drug disposition by the liver (Tan et al., 1999; Abuzahra et al., 2000; Kusuhara and Sugiyama, 2002; Meng et al., 2002). To this effect, freshly isolated rat hepatocytes have been commonly used since several reports have demonstrated good correlation between results obtained with rat hepatocytes with those from perfused liver and whole animal studies (Tan et al., 1999; Abuzahra et al., 2000; Kusuhara and Sugiyama, 2002). One of the limitations of this technique is the requirement for isolation of cells from fresh tissue every time an experiment is to be conducted. Another major hurdle is the scarcity of liver tissue available for some species, especially human.

It would be highly desirable if daily preparation could be avoided. This may be achieved by cryopreservation of surplus hepatocytes after isolation so that they can be stored for use when needed. We and others have already demonstrated that cryopreserved hepatocytes re-
tain metabolic capacities that are comparable with freshly prepared cells (Zaleski et al., 1993; Steinberg et al., 1999; Silva et al., 1999).

Although cryopreserved hepatocytes may be used and are suitable for drug metabolism studies, the transport of drugs within cryopreserved hepatocytes has not been reported (Kusuhara and Sugiyama, 2002). In this study, we further improved and optimized the cryopreservation methodology of rat hepatocytes to attain cells with drug transport capacities comparable with fresh cells. We furthered assessed whether a controlled freezing procedure would improve cell yield over a two-stage freezing protocol. Hengstler et al. (2000) have recently reported that a controlled slow-freezing protocol with a supercooling step minimized the release of latent heat and resulted in significant increases in viable cells after thawing. They reasoned that, as the temperature decreases and cell mixture began to freeze, crystallization would start, and the latent heat of fusion would be released resulting in the warming of the cells mixture. Since freezing and thawing are damaging to cells, these processes are major hindrances for successful cryopreservation. One way to minimize this phenomena is to supercool the freezing chamber at the moment when the cells are beginning to freeze to minimize the warming of the cell mixture as latent heat is being released. This process can be recorded by measuring the temperature of the cells in the cryovial as well as the temperature in the freezing chamber. Results from cryopreserved cells by this method clearly demonstrated that rat hepatocytes thus prepared were useful for the study of drug transport and metabolism.

Materials and Methods

Materials. [14C]-Dextran-carmoxy (6.5 mCi/ml), DMSO, polyvinyl-pyrrolidone, BSA (35%), trypsin inhibitor, L15 medium Leibovitz, and Krebs-Henseleit buffer were obtained from Sigma-Aldrich (St. Louis, MO). Tauro- [carbonyl-14C]-holic acid (sodium salt) 54.0 mCi/mmol and Percoll were purchased from Amersham Pharmacia Biotech Inc. (Baie d’Urfe, QC). Collagenase type 2 was procured from Worthington Biochemical Corp. (Freehold, NJ). [3H]Estrone sulfate (specific activity, 40 Ci/mmol) and [3H]estradiol [carbonyl-14C]cholic acid (sodium salt) 54.0 mCi/mmol and Percoll were purchased from Amersham Pharmacia Biotech Inc. (Baie d’Urfe, QC). [3H]Estradiol 17β-glucuronide (specific activity 40.5 Ci/mmol) were obtained from PerkinElmer Life Science Inc. (Boston, MA). n-Butyl phthalate was obtained from Fisher Scientific (Nepean, ON). Perchloric acid (70%) was purchased at B&K Scientific (Franklin Lakes, NJ).

Isolation of Rat Hepatocytes. All animal studies were approved by the institution’s animal care and use committee and were conducted in accordance with all applicable regulation. Rat hepatocytes were isolated from male Sprague-Dawley rats purchased from Charles River (200–250 g) by a two-step collagenase (70 U/ml) perfusion as described by Moldes et al. (1978). Viability of freshly isolated hepatocytes was assessed by trypsin blue uptake (0.2%). Hepatocyte preparations with a cell viability <85% were rejected.

Cryopreservation and Thawing. Cryopreservation of rat hepatocytes was conducted by two methods. The first was one described previously (Silva et al., 1999) and involved a two-step freezing protocol. The second method employed a controlled freezing protocol with a programmable freezer (Cryomed system from Thermo Forma, Marietta, OH). In these methods, freshly isolated hepatocytes were incubated for 30 min in Krebs-Henseleit buffer containing 15 mM glucose and 12 mM HEPES, pH 7.4, at 5 × 10^6 cells/ml in a shaker-water bath at 37°C under an atmosphere of 95% O_2/5% CO_2. The cells were centrifuged and resuspended in cold L15-media containing 40% fetal bovine serum and 5% BSA, pH 7.4. Then a mixture containing an equal volume of cold L15-media containing 40% fetal bovine serum and 5% BSA, pH 7.4. Then a mixture containing an equal volume of cold L15-media containing 40% fetal bovine serum and 5% BSA, pH 7.4. Then a mixture containing an equal volume of cold L15-media containing 40% fetal bovine serum and 5% BSA, pH 7.4. Then a mixture containing an equal volume of cold L15-media containing 40% fetal bovine serum and 5% BSA, pH 7.4. Then a mixture containing an equal volume of cold L15-media containing 40% fetal bovine serum and 5% BSA, pH 7.4. Then a mixture containing an equal volume of cold L15-media containing 40% fetal bovine serum and 5% BSA, pH 7.4.

Transport Studies. Hepatocytes suspended in Krebs-Henseleit buffer at 2 × 10^6 cells/ml were preincubated in a shaker-water bath at 37°C for 30 min under an atmosphere of 95% O_2/5% CO_2. The uptake studies were initiated by the addition of radiolabeled substrate (1 µM final concentration) to the incubation mixture (1.4 ml final volume); sampling (125 µl) was performed in duplicates at specified times. The cells were rapidly separated from the incubation medium by a centrifugation method described by Faris et al., 1985. Briefly, each cell aliquot was added to an Eppendorf microtube consisting of dichloroacetic acid (400 µl) layered over 10% perchloric acid (250 µl) and was centrifuged (14,000 rpm) for 15 s. The supernatant was aspirated and the cells were washed with a solution of perchloric acid (100 µl) and resuspended in cold L15-media containing 40% fetal bovine serum and 5% BSA, pH 7.4.

Calculation and Statistics. The initial velocity was assessed by the linear portion of the plot of the amount accumulated into cells versus time. This occurred within 1 min of the uptake study. Hence, the data points (20, 40, and 60 s) were regressed to provide the initial uptake velocity.
Materials and Methods

Comparison of Methods 1 and 2. When the hepatocytes were precooled to 4°C and placed at −20°C for 1 h (Method 1), the release of latent heat occurred when the temperature in the cell mixture reached approximately −9°C with an increase of temperature of approximately 2°C, as shown in Fig. 1. It took 5 min for the freezing chamber to absorb the released heat. In contrast, under conditions in which the rate of freezing was controlled and the cells were supercooled prior to the freezing point (Method 2), the release of the latent heat was dramatically minimized (Fig. 1). These conditions were found to be the ones that resulted in the least increase in temperature during the freezing process.

Both methods for cryopreservation resulted in an initial high yield of cells with good viability as measured by trypan blue exclusion (Table 1). Upon further separation of viable cells from damaged ones by Percoll centrifugation, viabilities of the resulting cells increased albeit the yields decreased. This is to be expected since the Percoll centrifugation step will remove damaged cells (less dense than intact cells), but some viable cells will also be lost. Cells cryopreserved by Method 2 consistently gave a higher yield of viable cells after Percoll centrifugation compared with cells cryopreserved by Method 1 (Table 1). Furthermore, viability of the thawed hepatocytes after a 2-h incubation was not significantly different from that of freshly isolated cells incubated over the same time periods (Table 2). In contrast, thawed cells from cryopreservation with Method 1 had significantly lower viability at the end of the 2-h incubation period (Table 2). This has also been our experience with hepatocytes obtained from other species, including dog and human (results not shown). Since we were trying to obtain the highest yield of cells after thawing, we decided at this stage to favor the Method 2 over the Method 1 to characterize cryopreserved hepatocytes in transport studies.

Results

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Metabolic Studies.

To insure that cryopreserved hepatocytes retained their metabolic capacity, substrates known to be metabolized by several cytochromes P450 (bufuralol, tolbutamide and DFB and UGT (7-HFC) were incubated with hepatocytes before and after cryopreservation. The parent remaining after a 2-h incubation period with each probe was determined in fresh hepatocytes and compared with that observed in cryopreserved cells. Although not shown, the metabolite profiles of the probes in fresh and cryopreserved cells were identical. As shown in Fig. 2, there was no significant difference in the relative metabolism of most of these probes when incubated with fresh versus thawed cryopreserved hepatocytes. Metabolism of 7-HFC was significantly decreased (p < 0.05) in cryopreserved hepatocyte compare to freshly isolated cells. About 50% of the UGT activity was lost following cryopreservation. Reports by other groups have also

Effect of cryopreservation on viability of rat hepatocytes after 2 h of incubation

<table>
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<tr>
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<th>Freshly Isolated Cells</th>
<th>Method 1</th>
<th>Method 2</th>
</tr>
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<tbody>
<tr>
<td>Viability (%)</td>
<td>70.3 ± 0.3</td>
<td>57.5 ± 7.6</td>
<td>70.5 ± 3.2</td>
</tr>
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</table>

*p Value was assessed by trypan blue exclusion after 2 h of incubation in Krebs-Henseleit buffer as described under Materials and Methods. Data are expressed in percentage of viable cells ± S.E.M.

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demonstrated that hepatocytes lose a part of conjugative pathways after cryopreservation (Diener et al., 1995; Madan et al., 1999; Steinberg et al., 1999).

**Transport Studies.** Upon the admixture of the substrate and cells to result in 1 µM of substrate (radiolabeled and unlabelled) in 1.4 ml of 2 × 10⁶ cells, aliquots (125 µl) were quickly removed, and the intracellular concentration determined by a centrifugation technique. As shown in Fig. 3A, uptake of [³H]taurocholate into freshly isolated hepatocytes was rapid and linear within the first minute. The rate of accumulation was indistinguishable from that obtained with the cryopreserved hepatocytes obtained upon thawing (Fig. 3A; Table 3). Similarly, the initial rates of uptake for [³H]estosterone sulfate and [³H]estradiol 17β-d-glucuronide were also not significantly different in fresh cells versus the thawed cells (Fig. 3, B and C; Table 3). This limited set of results clearly suggests, for the first time, that cryopreserved hepatocytes retain their capacity to transport compounds across the plasma membrane. Furthermore, the transport of these compounds was temperature dependent and dose-saturating (results not shown), consistent with the mechanism of an active-transport mechanism. As an example, the $K_m$ and $V_{max}$ of taurocholate uptake were 36 µM and 12.3 nmol/10⁶ cells/min in freshly isolated cells, and 42 µM and 11.3 nmol/10⁶ cells/min in thawed cryopreserved (Method 2) cells.

**Discussion**

The isolated hepatocyte system that contains intact membranes serves as an important, physiologically relevant experimental tool for uptake studies. Some work has been carried out with human hepatocytes (Olinga et al., 1998a,b) whereas rat hepatocytes are used more routinely. The preparation is facile (Moldeus et al., 1978) and the intact cells contain the entire complement of transporters, including passive permeation and transporter-assisted uptake. The incubation of drug with freshly isolated suspended hepatocytes is usually conducted over a short time-frame (usually 1 min) such that the net uptake rate is mostly influx with little efflux and is time-independent. Conventional cultured cells tend to rapidly lose hepatic transport activity (Liang et al., 1993). Recently, it has been reported that hepatocytes cultured in collagen-sandwich environment regain polarity and form bile canalicular networks (Liu et al., 1998). In this model the biliary efflux transporters such as MRPI are maintained, however, uptake transporters such as NTCP are significantly down-regulated (Liu et al., 1999). In contrast, freshly isolated hepatocytes are reported to reflect the relative uptake rates found in vivo (Sandker et al., 1994; Zhou et al., 1994). Thus, cryopreservation poses as a viable alternate procedure for the storage of human and animal hepatocytes. Cryopreserved hepatocytes have been shown to retain most of the drug-metabolizing activities (Li et al., 1999) as well as inducibility of the drug-metabolizing enzymes (Silva et al., 1999; Hengstler et al., 2000). However, use of cryopreserved hepatocytes for uptake studies has not been attempted.

We have previously described a technique for cryopreserving rat and human hepatocytes (Silva et al., 1999). Our previous, two-step freezing method (Method 1) demonstrated the importance of allowing freshly isolated hepatocytes to recover their ATP levels by preincu-

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**TABLE 3**

Lack of difference in uptake of various radiolabeled substrates

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Uptake Rate (nmol/min/10⁶ cells)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Freshly Isolated</td>
</tr>
<tr>
<td>[¹⁴C]Taurocholate</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>[³H]Estrone Sulfate</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>[³H]Estradiol 17β-D-glucuronide</td>
<td>0.14 ± 0.03</td>
</tr>
</tbody>
</table>

* The rate of uptake was extrapolated from the slope of the linear portion of the curve (points of 0.15 to 1 min). No statistical difference, n = 3.

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**FIG. 3.** Effect of cryopreserving rat hepatocytes on the uptake transport of tracer concentrations of model compounds.

Cryopreserved or freshly isolated rat hepatocytes in Krebs-Henseleit buffer, pH 7.4, were incubated as described under Materials and Methods. [¹⁴C]taurocholate (A), [³H]estrone sulfate (B), and [³H]estradiol 17β-d-glucuronide (C) transport into hepatocytes was measured as described under Materials and Methods. Cells were incubated with 1 µM of compounds. At each time point an aliquot was spun through oil to pellet viable cells as described under Materials and Methods. Data are expressed as mean ± S.E.M. There is no significantly difference. n = 4 (A), n = 3 (B) and (C).
bating the cells for 30 min at 37°C prior to freezing (Silva et al., 1999). The present programmed freezing protocol (Method 2) showed improved recovery (Table 1) and viability (Table 2). Moreover, similar phase I metabolic activities were observed (Fig. 2). Although UGT lost 50% activity, phase II metabolic activity was still observed in cryopreserved rat hepatocytes (Fig. 2).

More importantly, we demonstrated, for the first time that cryopreserved hepatocytes retained their ability in drug transport. To assess the ability of thawed cryopreserved hepatocytes to transport compounds, we focused on three well known substrates of uptake transporters and compared their uptake in thawed cryopreserved hepatocytes to freshly prepared hepatocytes that are shown to retain functional uptake transporter activities (Kato et al., 1999). Moreover, uptake by freshly prepared hepatocytes appears to be predictive of drug uptake in vivo in the liver (Akhteruzzaman et al., 1999; Kato et al., 1999; Abu-Zahra et al., 2000). As a starting point for investigation, the compounds chosen, taurocholate, estrone sulfate, and estradiol 17β-d-glucuronide, are prototypic substrates of NTCP and members of the OATP family (Hagenbuch and Meier, 1994; Jacquemin et al., 1994; Eckhardt et al., 1999; Cattori et al., 2001; Sugiyama et al., 2002). No difference in initial transport rate was found (Table 3). However, the transporter functions of the organic anion transporter and organic cations by organic cationic transporter and those at the canalicular membrane, namely, P-glycoprotein and bile salt export pump, within cryopreserved hepatocytes remain unknown, although it is recognized that upon isolation, internalization of MRP2 occurs (Roelofs et al., 1995).

In conclusion, this study demonstrates optimization in the cryopreservation of hepatocytes with Method 2. Even though cryopreservation still resulted in loss of cells, the majority of cells that survived the freezing and thawing processes appeared metabolically similar, with respect to UGT, to those of the fresh cells. Furthermore, we also showed for the first time that cryopreserved rat hepatocytes retain drug transport activity after thawing. These preliminary observations rendered confidence for use of the cryopreserved cells in drug metabolism and transport studies. We are currently assessing this methodology to hepatocytes from other species, including human.

**References**


