

# Influence of Preculture on the Prefreeze and Postthaw Characteristics of Hepatocytes

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**Abstract:** Recent studies performed in our laboratory have shown that a brief period of preculture prior to cryopreservation improves the postthaw viability of hepatocytes. The purpose of this investigation is to characterize specific metabolic and biochemical characteristics of the hepatocytes (both frozen and nonfrozen) to help elucidate the role of preculture on the postthaw viability. Fresh and thawed hepatocytes were cultured in a bioartificial liver (BAL) to determine albumin secretion as a function of time in culture. In addition, cell extracts were analyzed using nuclear magnetic resonance (NMR) spectroscopy to quantify changes in cell membrane composition and energetics as a function of time in culture prefreeze and postthaw. The results of these studies showed an increase in albumin concentration in the culture medium with time in culture for the period tested for both fresh and frozen and thawed hepatocytes. NMR spectroscopy of lipid extracts indicates that in vitro culture of hepatocytes results in an increase in cholesterol relative to membrane phospholipid. Moreover, the NMR results also indicate phospholipid interconversion, via specific lipases in cultured hepatocytes, and these changes are consistent with water permeability measurements performed previously. Significant changes in phosphoenergetics were also observed, with the net energy charge for the cells increasing significantly with time in culture. In addition, NMR spectra show increased levels of 6-phosphogluconate, another indicator of the cellular response to the stresses of isolation and ex vivo culture. These results suggest that energetic considerations may be a significant factor in the ability of hepatocytes to survive the stresses of freezing and thawing. Significant shifts in membrane phospholipids may also influence membrane permeability and postthaw survival. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* 71: 173–183, 2000/2001.

**Keywords:** hepatocyte; cryopreservation; in vitro culture

## INTRODUCTION

The ability to cryopreserve isolated hepatocytes is an important element to the development of artificial livers, toxicology testing of new drugs, and hepatocyte transplantation.

Human therapeutic applications of hepatocytes may require the pooling of cells to reach a therapeutic dose, coordination of cell supply with patient care regimes and the completion of safety and quality control testing. The screening of a variety of drugs under development is also facilitated by a consistent source of hepatocytes and the ability to test several drugs using cells from the same donor. Thus the ability to successfully cryopreserve isolated hepatocytes is important for a range of practical applications.

The majority of previous studies have looked at the postthaw viability of freshly isolated hepatocytes (for review, see Guillouzo et al., 1999). The relatively recent development of methods to culture hepatocytes in vitro while maintaining differentiated function (i.e., culture in a bioreactor or as spheroids) has led to an interest in the freezing characteristics of cultured cells. In a series of studies using hepatocytes cultured in a collagen sandwich matrix, the freezing characteristics of hepatocytes cultured in a collagen gel were studied. These studies have shown that the cryobiophysical characteristics of the hepatocytes are influenced by in vitro culture (Hubel et al., 1991; Yarmush et al., 1992). Specifically, the subzero water transport and intracellular ice formation characteristics of the cultured hepatocytes varied from those determined for freshly isolated cells. Assuming that water transport results from passive diffusion of water, the differences in water transport measured may reflect differences in membrane composition with time in culture.

Additional studies were performed to look at the influence of this culture configuration on the ability of the cells to survive freezing and thawing. Hepatocytes cultured in a collagen gel could be frozen to  $-80^{\circ}\text{C}$  and stored for times up to 30 days with the majority of cells surviving (Borel Rinkes, 1992; Kasai and Mito, 1993; Koebe et al., 1996). These studies indicate that methods of in vitro culture can alter the ability of cells to survive the stresses of freezing and thawing. Other investigators have examined the role of encapsulation on the ability of hepatocytes to survive freezing and thawing (Dixit et al., 1993; Guyomard et al., 1996).

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As with culture in a collagen gel, encapsulation of isolated hepatocytes prior to cryopreservation resulted in an increase in the postthaw viability. Recently, a method of cryopreserving isolated hepatocytes was developed (Darr and Hubel, manuscript submitted for publication). In this study, a brief period of culture in a spinner bioreactor (24 h) was shown to significantly improve the postthaw viability of isolated hepatocytes. These results indicate that *ex vivo* culture improves the postthaw viability of hepatocytes.

These studies do not provide the specific mechanism for the improvement in postthaw viability associated with *in vitro* culture. Previous studies have shown that the process of isolation results in damage to the cells. In a study by Dunn and colleagues (Dunn et al., 1992), the reduced levels of albumin secretion observed in freshly isolated cells was correlated with the loss in average polyribosome size. Another study showed that the oxygen consumption rate for freshly isolated cells was much higher than those observed for cultured cells (Yarmush et al., 1992). These studies imply that the isolation process has a significant influence on the metabolic function of the cells.

Thus, two potential hypotheses could be formulated to explain the mechanism behind the increase in postthaw survival with time in culture. First, the changes in biophysical characteristics of the cells (water transport) result in the improvement in postthaw viability. Second, the preculture period permits recovery from the stresses of isolation. A powerful method of testing these hypotheses was to use NMR spectroscopy to characterize membrane composition and phosphoenergetics in response to culture conditions. This methodology permits us to quantify a large variety of structural and metabolic characteristics of the cells from a single sample. We can then combine that information with the results of previous studies to look at potential mechanisms for the differences in postthaw viability observed and gain insight into cellular mechanisms for the typically poor postthaw viability of cryopreserved hepatocytes.

## METHODS

### Cell Isolation

Pig hepatocytes were harvested from 8 to 10 kg male pigs, using a two-step *in situ* collagenase perfusion technique that was modified from the original method developed by Seglen (Seglen, 1976). The pig was initially anesthetized with ketamine and rompun to allow for intubation and mechanical ventilation and subsequently anesthetized with isoflurane and paralyzed with succinylcholine. The liver was first perfused *in vivo* with oxygenated perfusion solution I (Per I) at 300 mL/min for 20 to 40 min. Per I is a calcium-free solution with 143 mM sodium chloride, 6.7 mM potassium chloride, 10 mM HEPES (Gibco, Grand Island, NY), and 1 g/L EDTA (Sigma Chemical Co, St. Louis, MO) at pH 7.4. The liver was then perfused *ex vivo* at 300 mL/min with oxygenated perfusion solution II (Per II). Per II con-

sists of 100 mM HEPES, 67 mM sodium chloride, 6.7 mM potassium chloride, 4.8 mM calcium chloride, 1% (v/v) bovine albumin and 1 g/L collagenase-D (Sigma), pH 7.6. Once the liver was visually dissolved (after 20–30 min), it was broken up and irrigated with cold William's E medium (Gibco) supplemented with 15 mM HEPES, 0.2 U/mL insulin (Lilly), 2 mM L-glutamine (Gibco), 100 U/mL penicillin (Celox, Hopkins, MN) and 100 mg/mL streptomycin (Celox). The released cells were filtered through nylon mesh with 100  $\mu$ m openings and washed via three centrifugations (50g) and resuspensions in the William's E medium. Viability for the harvests, as determined using trypan blue exclusion, ranged from 89% to 98%.

### Preculture of Isolated Hepatocytes

The preculture protocol consisted of culturing freshly isolated hepatocytes for up to 24 h in a spinner vessel culture, using the technique described in more detail in Lazar et al. (1995a) and Peshwa et al. (1994). Briefly, isolated hepatocytes were inoculated into a spinner flask containing 100 mL of media to a final concentration of  $3 \times 10^5$  viable cells per mL. The media consisted of William's E media (Gibco, Grand Island, NY) containing 0.292 mg/mL L-glutamine (Gibco), 0.2 U/mL of porcine insulin (Lilly Research Laboratories, Indianapolis, IN), 25 ng/mL of epidermal growth factor, 5  $\mu$ g/mL linoleic acid, 500 mg/mL bovine serum albumin, 1 nM dexamethasone, 4 ng/mL glucagon, 6.25 mg/mL transferrin, 20 ng/mL of liver growth factor, 6.25 ng/mL of selenium, 0.1 mM  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 3 nM  $\text{H}_2\text{SeO}_3$ , 50  $\mu$ M  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 mM HEPES, 100 U/mL penicillin (Celox, Hopkins, MN) and 100 mg/mL of streptomycin (Celox). All components were from Sigma (St. Louis, MO) unless otherwise indicated. The flask was cultured in a 37°C incubator with 5%  $\text{CO}_2$  while being stirred using a magnetic stirring plate set at a speed of 90 rpm.

### Cryopreservation Protocol

#### Freezing Protocol

Hepatocytes were centrifuged at 100g for 10 min, resuspended at concentrations ranging from 6–25  $\times 10^6$  cells/mL in a cryopreservation solution containing William's E Medium supplemented with 10% dimethyl sulfoxide (DMSO), and transferred into Cryocyte freezing containers (50 mL bags, #4R-99-51, Baxter, Deerfield, IL). As per manufacturer instructions, total cell volume was approximately 10 mL. The samples were placed in aluminum bag presses. The final thickness of the solution in the bag after placing in the press was approximately 3–4 mm. The cell suspensions were frozen using a Kryo 10 Series III programmable freezer (Planer, Sunbury on Thames, England). The freezing protocol involved taking the samples from room temperature to 0°C at a cooling rate of 10°C/min. The sample was then held for 15 min at 0°C and cooled using the

cooling rate, B, to  $-8^{\circ}\text{C}$ . To induce nucleation of the extracellular solution, the sample was then cooled at  $50^{\circ}\text{C}/\text{min}$  to  $-45^{\circ}\text{C}$  and warmed at  $15^{\circ}\text{C}/\text{min}$  to  $-12^{\circ}\text{C}$ . The sample was then cooled at cooling rate, B, to a temperature of  $-60^{\circ}\text{C}$  and then a cooling rate of  $3^{\circ}\text{C}/\text{min}$  to  $-100^{\circ}\text{C}$ . At that temperature, the sample was removed from the controlled rate freezer and placed in liquid nitrogen storage. The frozen cells were stored in liquid nitrogen storage tanks in the vapor phase for a minimum of 24 h.

### Culture of Hepatocytes in Bioartificial Liver

To test the longterm viability of frozen and thawed hepatocytes, pig hepatocytes (both freshly isolated or precultured) were suspended in William's E media (Sigma) at a concentration of approximately  $100 \times 10^6$  cells/mL. Freshly isolated cells were obtained immediately after harvest and kept on ice until use. Under sterile conditions,  $225 \times 10^6$  cells were added to a collagen solution to a final volume to 20 mL. The collagen solution consisted of Vitrogen 100 (Cohesion, Palo Alto, CA) and  $4\times$  William's E mixed at a ratio of 3:1 and pH adjusted to 7.4. The cell suspension was injected into the lumen volume of a hollow-fiber bioreactor cartridge (Model H1P100-43 Diaflo, Amicon, Inc., Beverly, MA), which was immediately attached to the cultureware of a Liver X-2000 instrument (Regenerex, Inc., Minneapolis, MN).

Prior to cell inoculation, the hollow-fiber bioreactor (HFBR) cartridge and connecting tubing were conditioned using shell medium for 24 h prior to loading with the cells. This process permitted the shell medium to reach the appropriate temperature and pH ( $37^{\circ}\text{C}$  and a pH of 7.3). The media consisted of William's E media (Gibco) containing 0.292 mg/mL L-glutamine (Gibco), 0.2 U/mL of porcine insulin (Lilly Research Laboratories), 15 mM HEPES, 100 U/mL penicillin (Celox), and 100 mg/mL of streptomycin (Celox). After loading of the HFBR with the cells resuspended in a collagen gel, the cartridge was placed in-line in the cultureware, and shell-side circulation was immediately initiated at a flow rate of 4320 mL/h. The cartridge, cultureware, and shell media were all kept at  $37^{\circ}\text{C}$ . The lumen flow through the cartridge was initiated 24 h after the cells in the collagen gel were loaded using a flow rate of 9 mL/h.

Shell-side media was recirculated through the cultureware and cartridge throughout the experiment, with new media added to compensate for sampling losses. Conversely, the lumen media was passed through the cartridge once and collected in a waste reservoir. The pH of the shell media and the oxygen uptake of the cells in the cartridge were monitored by regular sampling of the cartridge inflow and outflow and measurements made using an external blood gas analyzer (Chiron Diagnostics, Essex, UK). Samples for the measurement of albumin secretion by the hepatocytes into the culture medium were obtained from a sampling port located at the outlet of the hollow-fiber bioreactor cartridge. The pH of the shell media was maintained at a range of 7.3–7.45 by means of the addition of  $\text{CO}_2$  and

$\text{O}_2$  gases, via the gas exchanger in the cultureware, which was adjusted manually, depending on the blood gas measurements. The lumen media was the same as the supplemented media used to preculture the cells. Experiments were run for 96 h, with samples taken of the shell media at 0, 1, 2, 4, 20, 24, 25, 28, 48, 52, 72, 76, and 96 h. Samples of the lumen media were taken at 25, 28, 48, 52, 72, 76, and 96 h.

### Albumin Concentration Measurement

The albumin production of the hepatocytes was determined using an enzyme-linked immunoassay (ELISA), as described in detail elsewhere (Lazar et al., 1995b). Briefly, 96-well plates (Nunc, Napierville, IL) were coated with rabbit antiserum to porcine albumin (Cappell, Durham, NC) in a ratio of 1:1000. The plates were incubated overnight at  $4^{\circ}\text{C}$ , washed with 0.05% Tween-20 (Bio-Rad, Hercules, CA) in phosphate buffered saline (PBS), blocked for 2 h at  $37^{\circ}\text{C}$  with 0.5% gelatin in PBS, and then rewashed with 0.05% Tween-20. Serial dilutions (1:4) of sample and porcine albumin standard (10  $\mu\text{g}/\text{mL}$ ) were added (100  $\mu\text{L}$ /well). The plates were incubated for 1 h at  $37^{\circ}\text{C}$  and washed. Peroxidase-conjugated goat anti-swine albumin was diluted 1:16,000 in Tween/PBS; then 100 mL of solution was added to each well, and the plates were incubated for 1 h at  $37^{\circ}\text{C}$  and washed. The plates were developed with 2,2' Azino-di(3-ethylbenzthiazoline-6-sulfonate) (Boehringer-Mannheim, Indianapolis, IN) for 1 h at room temperature. The optical density of the wells was determined using an ELISA reader (Bio-Tek, Woonski, VT).

### NMR Spectroscopy

Proton and phosphorus NMR spectra of the hydrophobic and hydrophilic fractions were acquired on a Varian INOVA 4-channel spectrometer (Palo Alto, Ca) operating at a frequency of 600 MHz for  $^1\text{H}$ , and 242.8 MHz for  $^{31}\text{P}$ .

### Sample Preparation

Cell extracts for NMR analysis were prepared using a modification of the two-phase methanol-water/chloroform procedure developed by Tyagi et al. (1996). The entire extraction procedure was conducted in the cold room at  $4^{\circ}\text{C}$  using ice-cold solvents. Briefly, freshly isolated or cultured hepatocytes were collected ( $\sim 5 \times 10^8$ ), centrifuged, and washed three times with 10 mL aliquots of physiologic saline. The resulting cell pellet was combined with 10 mL of methanol (Sigma, St. Louis, MO) and 10 mL chloroform (Sigma, St. Louis, MO) in a 50 mL conical centrifuge tube (Corning, Inc., Corning, NY) and vortexed. Finally, 10 mL of water was added, giving a final solution volume of 30 mL. The mixture was vortexed and allowed to settle for approximately 60 min, centrifuged at 850 g for 15 min, and filtered through a fritted glass funnel. The clarified solution was allowed to stand over night at  $4^{\circ}\text{C}$  for phase separation.

The water-methanol fraction (upper layer) was collected and treated with 1 mg/mL Chelex (Biorad) ion exchange resin to remove divalent metal ions, then poured into 5 cm tissue culture dishes, frozen at  $-80^{\circ}\text{C}$  and lyophilized.

Cellular phospholipids in the chloroform fraction (lower layer) were converted into their potassium salts in the manner described by Moesgaard et al. (1999). Briefly, the chloroform samples were washed thoroughly with two 2 mL portions of 100 mM  $\text{K}^+$ -EDTA that had been adjusted to pH 6.0 with KOH. Samples were then evaporated to dryness at room temperature using a rotary evaporator and stored at  $-80^{\circ}\text{C}$ .

### *<sup>1</sup>H Spectroscopy of Cellular Lipids*

Lipid extracts were dissolved in 800  $\mu\text{L}$  of [<sup>2</sup>H] chloroform and transferred into a 5 mm NMR tube for <sup>1</sup>H spectral analysis. Proton spectra were acquired using a 45-degree pulse (5.5  $\mu\text{s}$ ) and a 10-s relaxation delay. The spectral sweep width was 8 kHz, 32k complex points were acquired, and 0.5 Hz line broadening was applied prior to Fourier transformation. Chemical shifts were referenced internally to the prominent peak appearing at 3.2 ppm attributed to the methyl head groups of phosphatidylcholine (Chi and Gupta, 1998). Metabolite peak areas were computed using the instrument software. The relevant peak assignments were made based on literature reports (Chi and Gupta, 1998; Pollesello et al., 1993; Pulkkinen et al., 1997).

### *<sup>31</sup>P Spectroscopy of Cellular Lipids*

Lipid samples containing phospholipid potassium salts were dissolved in 800  $\mu\text{l}$  of a 65:30:5 mixture (v:v:v) of  $\text{CDCl}_3$ ,  $\text{CH}_3\text{OH}$ , and  $\text{H}_2\text{O}$ , 0.1 M TRIS (pH 6.0), 0.2 M  $\text{K}^+$ -EDTA (Moesgaard et al., 1999) and transferred to a 5 mm NMR tube for <sup>31</sup>P spectral analysis. Phosphorous spectra were acquired using a 30-degree pulse (5.0  $\mu\text{s}$ ), a 5-s relaxation delay, and were the sum of 4000 transients. The spectral sweep width was 10 kHz, 8k complex points were acquired, and 1 Hz line broadening was applied prior to Fourier transformation. WALTZ proton decoupling was applied during the acquisition period (0.4 s), and the sample temperature was maintained at  $30^{\circ}\text{C}$ . Two spectra acquired with and without proton decoupling and using a 10-s relaxation delay showed that the phospholipids were fully relaxed using a 5-s delay, and that nuclear Overhauser enhancement of the phosphorus resonances due to proton decoupling were minimal in the 0.4-s acquisition period. Chemical shifts were referenced to phosphatidylcholine at  $-0.84$  ppm (Moesgaard et al., 1999), and the tentative peak assignments were made on the basis of literature reports (Greiner et al., 1998; Merchant et al., 1999; Moesgaard et al., 1999).

### *<sup>31</sup>P Spectroscopy of Hydrophilic Metabolites*

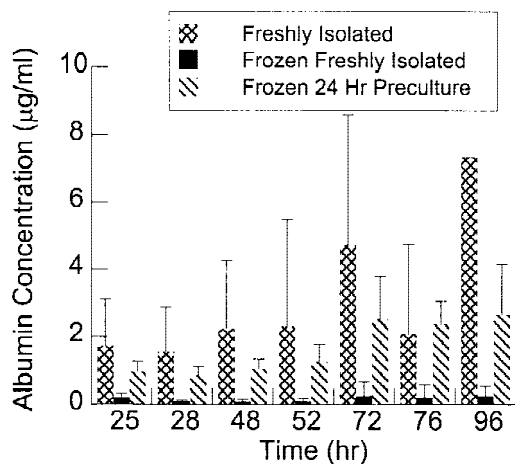
Lyophilized samples of the water-soluble fraction of the cell extracts were dissolved in 800  $\mu\text{L}$  of 50 mM TRIS buffer

(pH 8.4) containing 10 mM EDTA and 20% (v/v)  $^2\text{H}_2\text{O}$  for field-frequency lock. The solution was centrifuged, then filtered through a micro syringe filter (0.47  $\mu\text{m}$ ) to remove particulates, and loaded into a 5 mm NMR tube for <sup>31</sup>P spectral analysis. Phosphorous spectra were acquired using a 30-degree pulse (5.0  $\mu\text{s}$ ), a 5-s relaxation delay, and were the sum of 6000 transients. The spectral sweep width was 10 kHz, 8k complex points were acquired, and 0.5 Hz line broadening was applied prior to Fourier transformation. WALTZ proton decoupling was applied during the acquisition period (0.4s), and the sample temperature was maintained at  $30^{\circ}\text{C}$ . Spectra acquired using a 10-s relaxation delay indicated that the phosphorous metabolites were fully relaxed using a 5-s delay, and that nuclear Overhauser enhancement of the phosphorus resonances were minimal. Chemical shifts were referenced to glycerolphosphocholine at  $-0.13$  ppm (Merchant et al., 1999), and the tentative peak assignments were made based on previously published reports (Bell et al., 1993; Changani et al., 1999; Changani et al., 1996; Harvey et al., 1999; Lu et al., 1994; Gillham and Brindle, 1996; Williams et al., 1998). Metabolite peak areas were computed using the instrument software.

## RESULTS

### Albumin Secretion

An initial phase of the investigation involved characterizing the concentration of albumin in the culture medium for fresh and frozen and thawed cells over approximately 4 days in culture. Hepatocytes were cultured in a bioartificial liver device (BAL), described in more detail in Nyberg et al. (1993). Samples of the circulating medium were taken on a regular basis and tested for albumin concentration for the 96 h of operation. There was a steady increase in the albumin concentration in the culture medium with time for all the experimental conditions tested (Fig. 1). As a control,  $200 \times 10^6$  cells were taken immediately after isolation and cultured in the BAL. The postthaw function of two different preculture protocols was tested. For one condition, the cells ( $200 \times 10^6$ ) were frozen immediately postisolation and seeded into the BAL postthaw. The other protocol involved preculturing  $200 \times 10^6$  cells for 24 h, as described previously, freezing the cells and then seeding them into the BAL postthaw. The cryopreservation protocol used for both non-processed and precultured cells involved resuspending the cells into a cryopreservation solution containing 10% DMSO and freezing the cells using a controlled rate freezer at a cooling rate of  $1^{\circ}\text{C}/\text{min}$ . The albumin concentration in the culture medium for cells precultured for 24 h prior to cryopreservation is consistently higher than that observed for freshly isolated cells that have been frozen and thawed ( $p < 0.01$ ). An increase in albumin concentration with time in culture is also observed for the fresh, nonfrozen hepatocytes cultured in the BAL.



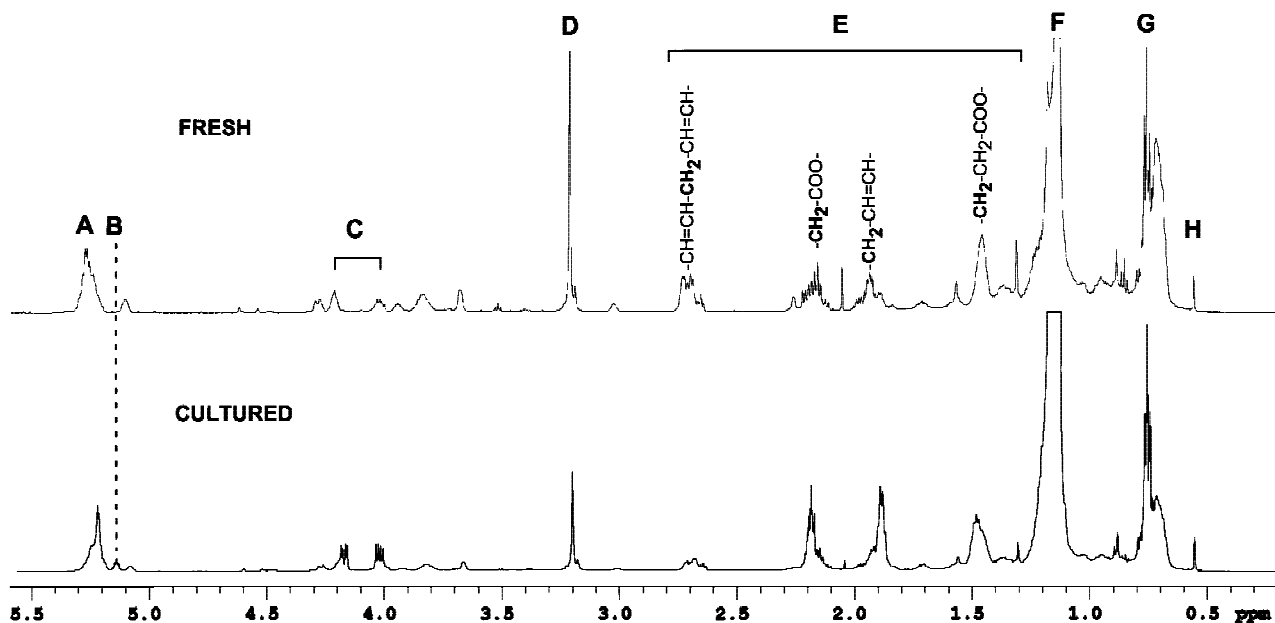
**Figure 1.** Albumin concentration in the lumen media as a function of time for hepatocytes cultured in a bioreactor. The bioreactor was inoculated with freshly isolated cells or frozen and thawed cells (fresh frozen cells or cells frozen after 24 h in culture) were used. The cells were cryopreserved in a solution containing 10% DMSO and using a cooling rate of 1°C/min. A minimum of three different experiments was used per experimental condition.

## NMR Spectroscopy

### Membrane Composition

The previous studies were important in establishing the differentiated function of both the fresh and frozen and thawed hepatocytes over a longer period in culture. The next phase of the investigation involved the use of NMR spectroscopy

to provide insight into the manner in which the biochemical and biophysical characteristics of hepatocytes change with time in culture. A culture period of 5 days was selected to permit determination of clear trends for observed shifts with time in culture. High-resolution  $^1\text{H}$  NMR spectra of lipid extracts are given in Figure 2. Relevant peak assignments were made based on literature reports (Chi and Gupta, 1998; Pollesello et al., 1993; Pulkkinen et al., 1998). Region A (5.25 ppm) corresponds to protons bound to double-bonded carbons ( $-\text{CH}=\text{CH}-$ ). Region B (5.14 ppm) corresponds to hydroxyl protons of glycerol and shows a distinct increase following 5 days of in vitro culture. The pair of multiplets centered at approximately 4.1 ppm (region C), along with the broad resonance at 5.08 ppm, are associated with the protons on the glycerol backbone of acylated glycerols. The splitting in the pair of resonances in region C following culture is attributed to the appearance of a free hydroxyl group on the glycerol backbone of lipids following deacylation. The singlet in region D (3.20 ppm) corresponds to the methyl protons of the choline moiety of choline-containing phospholipids (primarily phosphatidylcholine). Region E (1.4–2.8 ppm) is comprised of resonances attributed to methylene protons at the  $\alpha$  or  $\beta$  positions, with respect to carbon-carbon double bonds or carboxyl groups (see Fig. 2), and region F (1.15 ppm) is the result of aliphatic methylene protons. The complex group of coinciding resonances forming region G is due to methyl protons bound to the terminal carbon of fatty acids. The narrow singlet at 0.55 ppm is assigned to the C-18 methyl group of cholesterol.



**Figure 2.**  $^1\text{H}$  NMR spectra of lipid extracts from freshly isolated pig hepatocytes (upper spectrum) and cultured hepatocytes (lower spectrum). The cultured cells were grown as spheroids for 5 days (see text for cell culture details). The spectral peaks are scaled relative to the intensity of the terminal methyl proton resonance (region G) at 0.75 ppm. Peak assignments are: protons attached to double-bonded carbons (A), protons on acylated glycerol (B) and (C), methyl protons on the choline head-group of phosphatidylcholine (D), methylene protons on fatty acid chains (E) and (F), terminal methyl groups of fatty acid chains (G), and the methyl group of the  $\text{C}_{18}$  carbon of cholesterol (H).

Changes in hepatocyte lipid composition in response to in vitro culture were quantified by computing lipid composition parameters from the integrated proton NMR spectra (Chi and Gupta, 1998; Pollesello et al., 1993; Pulkkinen et al., 1998) and are summarized in Table I. All resonance intensities used to compute the lipid structural parameters were integrated using the spectrometer software, then adjusted to account for the number of protons in each resonance and finally normalized, with respect to the terminal methyl group intensity (region G, 0.75 ppm).

The cholesterol-to-phosphocholine ratio was significantly higher in the 5-d cultures, compared to freshly isolated hepatocytes ( $p = 0.013$ ). The increase in cholesterol-to-phosphocholine content with time in culture was also observed in NMR spectra obtained from nonfrozen hepatocytes cultured for 24 h and cells from the same populations that were frozen, thawed, and assayed at 2 h and 24 h postthaw (Table I). Five days of in vitro culture also resulted in a statistically significant elevation of the hydroxyl-to-phosphocholine ratio, compared to freshly isolated hepatocytes ( $p = 0.004$ ). An increase in hydroxyl-to-phosphocholine ratio is also observed postthaw for cryopreserved samples, compared to nonfrozen cells cultured for 24 h. No statistically significant alterations in the ratios of cholesterol to fatty acid chains, hydroxyl groups to fatty acid chains, phosphocholine to fatty acid chains, unsaturation ratio, or average chain length were detected in any of the samples.

High-resolution proton decoupled  $^{31}\text{P}$  NMR spectra of the phospholipids (K+ salts) extracted from freshly isolated

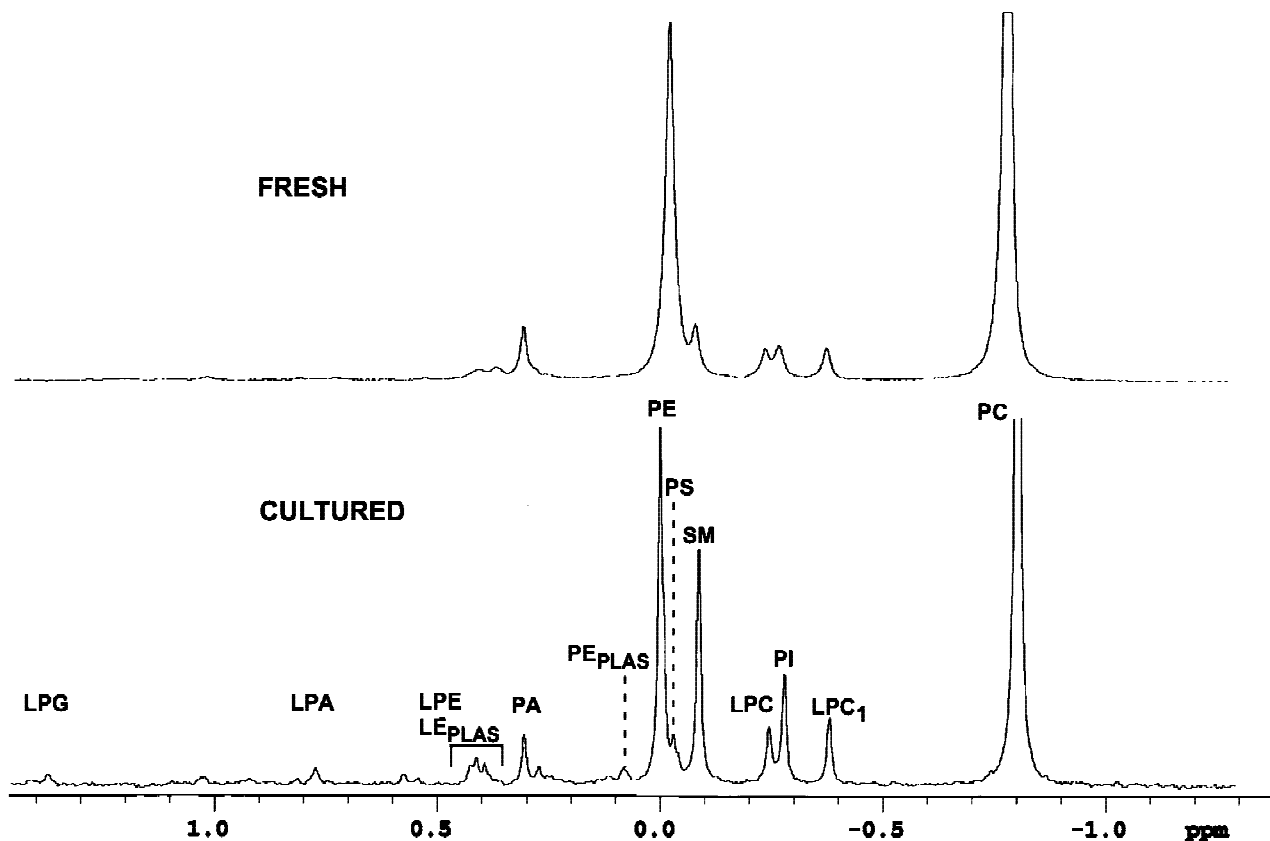
porcine hepatocytes and 5-d hepatocyte cultures are shown in Figure 3. Tentative peak assignments were made based on literature reports (Greiner et al., 1998; Merchant et al., 1995; Moesgaard et al., 1999). The  $^{31}\text{P}$  NMR chemical shifts of phospholipids has been shown to be dependent on the counter ion and solvent system used; therefore, peak assignment based on chemical shift data alone is unreliable. Our objective in these studies was simply to determine if, using a previously reported solvent system (Moesgaard et al., 1999), there are alterations in the phospholipid composition of cultured hepatocytes that can be detected using  $^{31}\text{P}$  NMR.

The upfield region of the  $^{31}\text{P}$  spectrum from 0.35–1.42 ppm contains signals predominantly from the lyso derivatives of phospholipids, including lysophosphatidylglycerol (LPG), 1.38 ppm; lysophosphatidic acid (LPA), 0.78 ppm; and lysophosphatidylethanolamine (LPE), and subtypes, 0.6 ppm. The other tentatively assigned resonances include phosphatidic acid (PA), 0.3 ppm; phosphatidylethanolamine plasmalogen ( $\text{PE}_{\text{plas}}$ ), 0.08 ppm; phosphatidylethanolamine (PE),  $-0.09$  ppm; phosphatidylserine (PS),  $-0.07$  ppm; sphingomyelin (PM),  $-0.95$  ppm; phosphatidylinositol (PI),  $-0.24$  ppm; lysophosphatidylcholine (LPC),  $-0.27$  ppm; 1-deacylated lysophosphatidylcholine (LPC1),  $-0.39$  ppm; and phosphatidylcholine (PC),  $-0.82$  ppm. The spectra obtained showed an increase with time in culture for three different quantities: (1) the ratio of lysophosphatidylcholine with respect to phosphatidylinositol ( $\approx -0.3$  ppm); (2) the ratio of sphingomyelin with respect to phosphatidylethanol-

**Table I.** Phosphorous-containing metabolite ratios in porcine hepatocytes computed from  $^{31}\text{P}$  NMR spectra of the water-soluble fraction of two-phase cell extracts. Metabolite ratios of freshly isolated hepatocytes (see text for the isolation procedure) are compared with cells cultured in vitro for 5 days and with cryopreserved hepatocytes at 2 and 24 h postthaw. Cryopreserved hepatocytes were subject to a 24-h preculture prior to freezing.

Lipid composition parameter	Parameter definition	Metabolic response to in vitro culture			24-hr Culture prior to cryopreservation		
		Freshly isolated ( $n = 7$ )	5-day culture ( $n = 3$ )	$p^a$	No freeze ( $n = 1$ )	2-hr Postthaw ( $n = 1$ )	24-h Postthaw ( $n = 1$ )
Cholesterol/phosphocholine	$\frac{\text{Cholesterol C18 methyl}}{-\text{N}(\text{CH}_3)_3}$	$0.342 \pm 0.134$	$0.977 \pm 0.308$	0.013	0.417	0.371	0.615
Hydroxyl/phosphocholine	$\frac{-\text{OH protons}}{-\text{N}(\text{CH}_3)_3}$	$0.339 \pm 0.129$	$2.529 \pm 0.867$	0.004	0.562	0.524	0.666
Cholesterol/fatty acids	$\frac{\text{Cholesterol C18 methyl}}{-\text{CH}_3 \text{ (Terminal)}}$	$0.029 \pm 0.008$	$0.035 \pm 0.013$	0.491	0.025	0.024	0.037
Hydroxyl/fatty acids	$\frac{-\text{OH groups}}{-\text{CH}_3 \text{ (Terminal)}}$	$0.037 \pm 0.036$	$0.087 \pm 0.010$	0.072	0.034	0.034	0.040
Phospholipid/fatty acids	$\frac{-\text{N}(\text{CH}_3)_3}{-\text{CH}_3 \text{ (Terminal)}}$	$0.104 \pm 0.065$	$0.037 \pm 0.012$	0.143	0.060	0.065	0.059
Unsaturation ratio	$\frac{-\text{CH}=\text{CH}-}{-\text{CH}_3 \text{ (Terminal)}}$	$0.683 \pm 0.366$	$0.348 \pm 0.112$	0.194	0.438	0.439	0.412
Average chain length	$\frac{(-\text{CH}_3 \text{ Tot.}) + -\text{CH}_2 + -\text{CH}=\text{CH}- + -\text{CO}_2}{-\text{CH}_3 \text{ (Terminal)}}$	$16.299 \pm 2.291$	$17.146 \pm 1.223$	0.591	15.665	15.749	15.576

<sup>a</sup>Non-paired  $t$ -means test,  $p \leq 0.05$  is considered significant. Lipid composition parameters are reported as the sample mean  $\pm$  standard error.



**Figure 3.**  $^{31}\text{P}$  NMR spectra of phospholipids ( $\text{K}^+$  salts) from freshly isolated pig hepatocytes (upper spectrum) and 5-d cultures (lower spectrum). Chemical shifts are reported with respect to phosphatidylcholine at  $-0.84$  ppm, and tentative peak assignments are based on literature values using the same solvent system and phospholipid ionic form. Peak assignments are: lysophosphatidylglycerol (LPG), lysophosphatidic acid (LPA), lysophosphatidylethanolamine (LPE), lysoethanolamine plasmalogen ( $\text{LE}_{\text{plas}}$ ), phosphatidic acid (PA), phosphatidylethanolamine plasmalogen ( $\text{PE}_{\text{plas}}$ ), phosphatidylethanolamine (PE), phosphatidylserine (PS) sphingomyelin (SM), lysophosphatidylcholine (LPC), phosphatidylinositol (PI), 1-deacylated lysophosphatidylcholine ( $\text{LPC}_1$ ), and phosphatidylcholine (PC).

amine ( $\approx -0.13$ – $0.05$  ppm); and (3) the weak signals in the upfield region ( $0.35$ – $1.42$  ppm) from lyso phospholipid derivatives.

### Phosphoenergetics

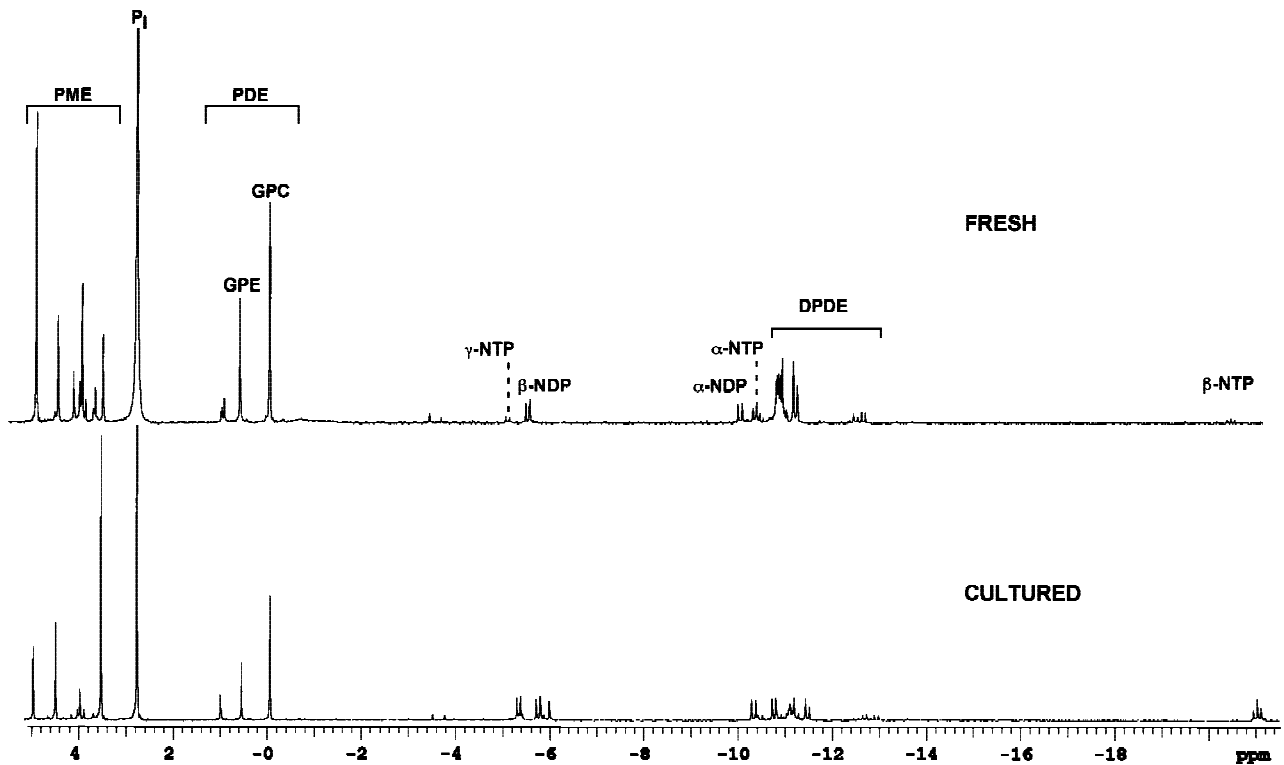
In Figure 4, the phosphorous NMR spectra for freshly isolated hepatocytes and for hepatocytes cultured for 5 days were obtained from the water-soluble fraction of the cell extracts. The complicated monoester region of the spectrum is expanded in Figure 5. Metabolites were identified based on chemical shift values taken from previous literature reports (Bell et al., 1993; Changani et al., 1999; Changani et al., 1996; Gillham and Brindle, 1996; Harvey et al., 1999; Lu et al., 1994; Williams et al., 1998). Metabolites quantified for this investigation are 6-phosphogluconate (6PG), 4.92 ppm; glucose-6-phosphate (G6P), 4.51 ppm; glycerol-3-phosphate (G3P), 4.44 ppm; phosphoglycerate (PG), 4.10 ppm; fructose-6-phosphate (F6P), 3.97 ppm; phosphoethanolamine (PE), 3.92 ppm; AMP, 3.84 ppm; NADP(H), 3.63 ppm; phosphocholine (PC), 3.47 ppm; inorganic phosphate (Pi), 2.74 ppm; glycerolphosphoethanolamine (GPE), 0.55 ppm; glycerolphosphocholine (GPC),  $-0.13$  ppm; nucleo-

side triphosphate (primarily ATP),  $-5.30$ ,  $-10.70$ , and  $-21.10$  ppm; and NDP (primarily ADP),  $-5.75$ , and  $-10.25$  ppm. Two principal alterations in phosphoenergetic metabolism were observed. First, the relative ATP content of the freshly isolated cells is depleted nearly to the limit of NMR detection, and an almost tenfold increase was observed after 5 days in culture (Table II). Phosphoenergetic recovery of the cultured hepatocytes is also reflected in the approximately 25% increase in the energy charge of the cultured, with respect to the freshly isolated hepatocytes. Second, elevated levels of 6-phosphogluconate (6PG), a product of the metabolism of glucose-3-phosphate (G3P) through the pentose phosphate pathway (PPP), were observed in both freshly isolated hepatocytes and hepatocytes cultured for 5 days.

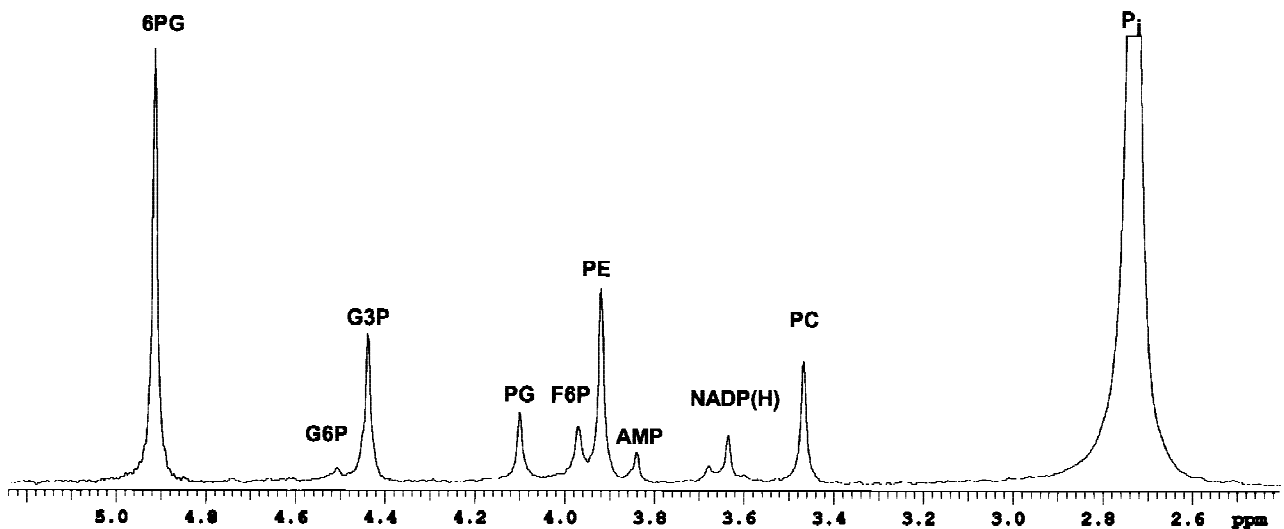
## DISCUSSION

### Albumin Secretion

The culture of the hepatocytes in the BAL showed that albumin concentration in the lumen medium was lower ini-



**Figure 4.**  $^{31}\text{P}$  NMR spectra of the water-soluble fraction from cell extracts from freshly isolated pig hepatocytes (upper spectrum) and hepatocytes cultured *in vitro* for 5 days. Chemical shifts are reported with respect to glycerophosphocholine at  $-0.13$  ppm. Metabolite peaks were normalized, with respect to total NMR visible phosphorous, or with respect to the integral of the monoester region (see Table I), and the peak assignments are based on chemical shift values from the literature (see text). The metabolite assignments are: phosphomonoester region (PME), predominantly glycolytic and phospholipid intermediates; inorganic phosphate ( $\text{P}_i$ ); phosphodiester region (PDE), primarily glycerophosphoethanolamine (GPE) and glycerophosphocholine (GPC);  $\gamma$ -nucleoside triphosphate ( $\gamma$ -NTP),  $\beta$ -nucleoside diphosphate ( $\beta$ -NDP),  $\alpha$ -nucleoside diphosphate ( $\alpha$ -NDP),  $\alpha$ -nucleoside triphosphate ( $\alpha$ -NTP), diphosphodiester region (DPDE), mainly NAD (H) and UDP-sugars, and  $\beta$ -nucleoside triphosphate ( $\beta$ -NTP).



**Figure 5.**  $^{31}\text{P}$  NMR spectra of the monoester region of the water-soluble fraction of cell extracts from freshly isolated pig hepatocytes. Chemical shifts are reported with respect to glycerophosphocholine at  $-0.13$  ppm. The metabolite assignments are: 6-phosphogluconate (6PG), glucose-6-phosphate (G6P), glycerol-3-phosphate (G3P), phosphoglycerate (PG), fructose-6-phosphate (F6P), phosphoethanolamine (PE), adenine monophosphate (AMP), nicotinamide adenine dinucleotide phosphate (NADP), phosphocholine (PC), and inorganic phosphate ( $\text{P}_i$ ).

**Table II.** Membrane lipid composition of porcine hepatocytes from  $^1\text{H}$  NMR spectra of the hydrophobic fraction of two-phase cell extracts. Lipid composition parameters of freshly isolated hepatocytes are compared with cells cultured in vitro for 5 days, and with cryopreserved hepatocytes at 2 and 24 h postthaw. Cryopreserved hepatocytes were subject to a 24-h preculture prior to freezing.

Metabolite parameter	Metabolic response to in vitro culture		$p^b$	24-h Culture prior to cryopreservation		
	Freshly isolated ( $n = 7$ )	5-Day culture ( $n = 3$ )		No freeze ( $n = 1$ )	2-h Postthaw ( $n = 1$ )	24-h Postthaw ( $n = 1$ )
<b>Nucleotide metabolites</b>						
Energy charge <sup>a</sup>	0.517 ± 0.054	0.756 ± 0.040	0.00015	0.645	0.530	0.640
NTP/Total phosphorous	0.012 ± 0.006	0.100 ± 0.071	0.00753	0.028	0.012	0.049
<b>Membrane intermediates</b>						
PC/Total phosphorous	0.165 ± 0.062	0.441 ± 0.037	0.0002	0.227	0.213	0.112
PE/Total phosphorous	0.093 ± 0.037	0.012 ± 0.006	0.4178	0.066	0.077	0.170
GPC/Total phosphorous	0.139 ± 0.022	0.068 ± 0.014	0.0009	0.13	0.08	0.08
GPE/Total phosphorous	0.056 ± 0.009	0.037 ± 0.007	0.012	0.08	0.02	0.05
PC/PE	2.046 ± 1.109	6.189 ± 2.017	0.0025	3.430	2.781	0.665
GPC/GPE	1.923 ± 0.513	1.831 ± 0.137	0.7721	1.627	4.715	1.639
<b>Glycolytic Metabolites</b>						
6PG/Total phosphomonoester	0.265 ± 0.147	0.219 ± 0.087	0.631	0.479	0.369	0.259
G3P/Total phosphomonoester	0.176 ± 0.034	0.175 ± 0.034	0.986	0.115	0.202	0.173
PG/Total phosphomonoester	0.044 ± 0.013	0.013 ± 0.004	0.005	0.023	0.026	0.036
F6P/Total phosphomonoester	0.041 ± 0.023	0.037 ± 0.020	0.806	0.014	0.013	0.029

<sup>a</sup>Energy charge =  $([\text{ATP}] + ([\text{ADP}/2])/([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$  (Lehninger, 1975).

<sup>b</sup>Non-paired *t*-means test,  $p \leq 0.05$  is considered significant. Metabolite ratios are reported as the sample mean ± standard error.

tially and increased with time in culture. The trend in increasing albumin secretion with time in culture was true for both freshly isolated cells and frozen and thawed cells. The albumin concentrations measured at a specific time in culture were less for the cells that were frozen and thawed than freshly isolated cells. As indicated in the Methods section, the same population of cells ( $200 \times 10^6$ ) were followed through whatever steps in the process were required (isolation, preculture, freezing, and thawing). The differences in albumin concentration measured in the lumen media as a function of time may reflect the influence of preculture, freezing, and thawing on cell losses and/or metabolic function. The longterm functional studies described in this investigation are consistent with a previous study (Darr and Hubel, manuscript submitted for publication) in which preculturing cells prior to cryopreservation improved the post-thaw viability of the cells.

## NMR Spectroscopy

### Membrane Composition

As indicated in the Results section, there were changes in the amount or ratio of membrane components. Specifically, the cholesterol content of the hepatocyte membranes, estimated by computing the ratio of cholesterol to phosphatidylcholine from the  $^1\text{H}$  spectra of the lipid extracts, increased nearly threefold after 5 days in culture, compared with freshly isolated cells (Table II). These results are consistent with previous reports that show membrane fluidity and permeability decrease with increasing cholesterol content (Males and Herring, 1999; Nouri-Sorkhabi et al., 1994; Waldeck et al., 1995) and that cultured hepatocytes display

reduced water permeability, compared to freshly isolated cells (Yarmush et al., 1992). Ideally, performing permeability and NMR spectroscopy studies on the same population of cells would be needed to establish the clear link between the observed changes in permeability and membrane composition.

Proton NMR spectra of the lipid extracts also indicate that in vitro culture stimulates phospholipid interconversion, perhaps through the activation of specific lipases. Region C in the proton spectra (Fig. 2) contains protons from the glycerol backbone of phospholipids and triglycerides and shows an increased splitting with time in culture concurrent with an increase in the resonance at 5.13 ppm (Fig. 2, region B), corresponding to hydroxyl protons (Esclassan et al., 1994). Both of these spectral shifts are consistent with the appearance of a free hydroxyl group on the glycerol backbone resulting from cleavage of an acyl fatty acid group from the glycerol moiety (typically at the  $\text{C}_2$  position) of phospholipids and/or triglycerides. Increased membrane turnover and phospholipase activity in cultured hepatocytes is also indicated by the nearly threefold increase in phosphocholine observed in the  $^{31}\text{P}$  spectra of the water-soluble fraction of the cell extracts (Table I). This conclusion is further supported by the relative increase in lysophosphatidyl derivatives (i.e., phosphatidyl phospholipids containing only one fatty acid ester linkage and, hence, a free hydroxyl group) such as lysophatidylcholine ( $-0.27$  ppm) and other lysophospholipid derivatives (0.4 to 1.4 ppm) observed in the  $^{31}\text{P}$  spectra of the lipid extracts of cultured hepatocytes (Fig. 3). A predominant feature of these spectra is the relative increase in the peak at  $-1.1$  ppm, tentatively assigned to sphingomyelin. Sphingomyelin has been shown to be an important mediator of membrane stability and permeability.

Specifically, elevated levels of sphingomyelin have been linked to increased nuclear membrane permeability and a corresponding increase in mRNA transport in regenerating rat liver (Albi et al., 1999; Tomassoni et al., 1999). The identification of phospholipids based on  $^{31}\text{P}$  chemical shift information is complicated by the dependency of  $^{31}\text{P}$  chemical shifts on the phospholipid counter ions and solvent system used, making peak identification difficult, based on chemical shift alone (Menseses and Glonek, 1988; Moesgaard et al., 1999). However, the tentative peak assignments made for this study are consistent with previous reports using identical phospholipid salts ( $\text{K}^+$ ) and solvent system (Moesgaard et al., 1999).

Our results do not imply unequivocal characterization of phospholipid composition but rather indicate that in vitro culture of hepatocytes stimulates modification of cellular phospholipids that may regulate membrane permeability and require specific consideration for designing cryopreservation procedures.

### Phosphoenergetics

$^{31}\text{P}$  NMR spectra of the water-soluble fraction of cellular extracts reveal two principal alterations in phosphoenergetic metabolism that may impact freeze-thaw survivability of isolated hepatocytes. First, the relative ATP content of the freshly isolated cells is depleted nearly to the limit of NMR detection (Fig. 4). Following 5 days of culture, however, the total ATP level recovers, increasing nearly tenfold (Table I). Phosphoenergetic recovery of the cultured hepatocytes is also reflected in the approximately 25% increase in the energy charge of the cultured, with respect to the freshly isolated hepatocytes. As defined in Table I, the energy charge is roughly the ratio of the ATP over ATP and its breakdown products, ADP and AMP.

Studies to date have not correlated energy state of the cells with increased (or decreased) ability to survive the stresses of freezing and thawing. Toussaint and colleagues have hypothesized that nonlethal stresses generate damage to cells (Toussaint et al., 1994; Toussaint et al., 1995). In turn, the cell initiates defense and repair mechanisms that temporarily increase metabolic activity of the cells. The stresses also accelerate the accumulation of damage, decrease the ability of the cell to withstand the stresses, and reduce energy metabolism and repair mechanisms. Experimental studies have shown that cell metabolism can influence the ability of cells to survive non-freezing stresses (Toussaint et al., 1994, 1998). Specifically, the decoupling of the mitochondria resulted in a decrease in fibroblast survival when exposed to tert-butylhydroperoxide (TBHP). Further studies are needed to determine if the metabolic activity directly influenced the ability of cells to survive the stresses of freezing and thawing.

Second, the phosphorous spectra of the water-soluble fraction of the cell extracts may indicate that the combined stresses of the isolation process (hypoxia, chemical and mechanical stresses) results in activation of pentose phosphate

pathway (PPP). Specifically, the high levels of 6-phosphogluconate (6PG), a product of the metabolism of glucose-3-phosphate (G3P) through the PPP, and corresponding low levels of G3P are consistent with this observation (Fig. 5). Significant levels of 6PG have not been measured in extracts from homogenized fresh liver (Changani et al., 1996, 1999; Dagnelie et al., 1999; Harvey et al., 1999). The pentose phosphate pathway generates reducing power (in the form of NADPH) for the reduction of oxidized glutathione and is necessary for the prevention and repair of cellular oxidative damage (Voet and Voet, 1995). The activation of the PPP may indicate the response of the cells to oxidative damage incurred during the isolation procedure and illustrates the importance of suitable and well-designed preculture conditions to allow the cells to survive the added stress of freezing and thawing associated with cryopreservation.

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